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(71)出願人　500284580
ジェネンコー・インターナショナル・インク
アメリカ合衆国　カリフォルニア州　パロ・アルト、ページ・ミル・ロード　９２５
(74)代理人　100071010
弁理士　山崎　行造
(74)代理人　100104086
弁理士　岩橋　赳夫
(74)代理人　100121762
弁理士　杉山　直人
(74)代理人　100126767
弁理士　白銀　博

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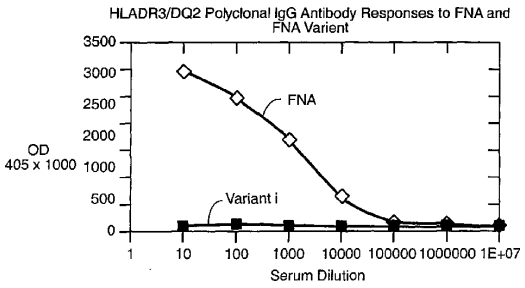
(54)【発明の名称】変化した免疫反応をもたらすタンパク質及びその作成または使用方法

(57)【要約】
本発明は、過剰アレルギー性及び低アレルギー性組成物を作成するための新規な方法及び組成物に関する。具体的には、本発明はエピトープを認識するＴ細胞の能力を中和または低下させ、従ってタンパク質に対するヒトの感作を防ぐことを含む。あるいは、強化した免疫反応を引き起こすためにＴ細胞エピトープを変異させる。
【選択図】図２１



【特許請求の範囲】
【請求項１】
以下のステップを含む、人工タンパク質のアレルギー潜在性を特定する方法、
ａ）第１の遺伝子導入マウスへの目的タンパク質を用いた免疫付与、及び第２の遺伝子導入マウスへの前記目的タンパク質の変異体である人工タンパク質を用いた免疫付与。さらに、前記目的タンパク質はＴ細胞エピトープを含み、前記変異体と前記目的タンパク質は変化したＴ細胞エピトープを有することにより異なる；
ｂ）前記第１及び前記第２の免疫性を与えられた遺伝子導入マウスの血清の回収；
ｃ）血清の抗原特異的免疫グロブリンの測定；及び
ｄ）前記遺伝子導入マウスにおいて異なる免疫反応を引き起こす前記目的タンパク質と前記その変異体における、該目的タンパク質と該その変異体との免疫反応の比較。
【請求項２】
前記目的タンパク質が酵素である、請求項１に記載の方法。
【請求項３】
前記酵素がプロテアーゼである、請求項２に記載の方法。
【請求項４】
抗原特異的免疫グロブリンが免疫グロブリンＧ（ＩｇＧ）である、請求項１に記載の方法。
【請求項５】
第１の遺伝子導入マウス及び第２の遺伝子導入マウスがＨＬＡ　ＤＲ３／ＤＱ２である、請求項１に記載の方法。
【請求項６】
ＨＬＡ　ＤＲ３／ＤＱ２遺伝子導入マウスが、内生的Ｉ－ＡクラスＩＩ分子の発現が欠如しているマウスと戻し交配されている、請求項５に記載の方法。
【請求項７】
前記Ｔ細胞エピトープをアミノ酸置換によって変化させる、請求項１に記載の方法。
【請求項８】
前記Ｔ細胞エピトープを、前記目的タンパク質の同族体の末端部分に対応する部分に置き換えられた前記Ｔ細胞エピトープを含有する前記目的タンパク質の末端部分を有することにより変化させる、請求項１に記載の方法であって、前記同族体は前記置換Ｔ細胞エピトープと同一のＴ細胞エピトープを含まない、請求項１に記載の方法。
【請求項９】
変異体によって引き起こされる免疫反応が、目的タンパク質によって引き起こされる免疫反応より少ない、請求項１に記載の方法。
【請求項１０】
変異体によって引き起こされる免疫反応が、目的タンパク質によって引き起こされる免疫反応より大きい、請求項１に記載の方法。
【請求項１１】
以下のステップを含む、人工タンパク質に対するヒトのアレルギー反応を予測するために、遺伝子導入マウスを使用する方法、
ａ）第１の遺伝子導入マウスへの目的タンパク質を用いた免疫付与、及び第２の遺伝子導入マウスへの前記目的タンパク質の変異体である人工タンパク質を用いた免疫付与。さらに、該目的タンパク質はＴ細胞エピトープを含み、該変異体と該目的タンパク質は変化したＴ細胞エピトープを有することにより異なる；
ｂ）前記第１及び前記第２の免疫性を与えられた遺伝子導入マウスの血清の回収；
ｃ）血清の抗原特異的免疫グロブリンの測定；及び
ｄ）前記遺伝子導入マウスにおいて異なる免疫反応を引き起こす該目的タンパク質と該その変異体における、該目的タンパク質と該その変異体との免疫反応を比較。前記免疫反応はヒトにおけるアレルギー反応を予測する。
【請求項１２】

前記目的タンパク質がプロテアーゼである、請求項１１に記載の方法。
【請求項１３】
Ｔ細胞エピトープを含む目的ポリペプチドの変異体であって、前記変異体と前記目的ポリペプチドがヒトにおいて異なる免疫反応を引き起こすようにＴ細胞エピトープを変化させたことにより、前記目的ポリペプチドと異なる前記変異体。
【請求項１４】
前記変異体によって引き起こされる免疫反応が前記目的タンパク質によって引き起こされる免疫反応よりも大きい、請求項１３に記載の変異体。
【請求項１５】
以下を含む、タンパク質によって引き起こされる免疫反応を特定する方法、
ａ）単一の血液を起源として樹状細胞液及びナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞液を得る；
ｂ）前記樹状細胞の増殖を促進させる；
ｃ）前記分化樹状細胞液及び前記ナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞を前記タンパク質と結合させる；及び
ｄ）ステップｃ）におけるＴ細胞の増殖を測定。
【請求項１６】
さらに、前記Ｔ細胞の増殖と第２のタンパク質の増殖とを比較することを含む、請求項１５に記載の方法。
【請求項１７】
目的タンパク質及び第２のタンパク質が互いに同族体である、請求項１６に記載の方法。
【請求項１８】
目的タンパク質及び第２のタンパク質がプロテアーゼである、請求項１７に記載の方法。
【請求項１９】
目的タンパク質及び第２のタンパク質がそれぞれ同じタンパク質の異なるペプチドである、請求項１８に記載の方法。
【請求項２０】
以下を含む、目的ポリペプチドの免疫原性を変化させる方法、ａ）前記ポリペプチドの免疫原性の特定；ｂ）前記ポリペプチドにおけるＴ細胞エピトープの同定；及びｃ）前記ポリペプチドの免疫原性を変化させるために前記Ｔ細胞エピトープを変化させる。
【請求項２１】
前記Ｔ細胞エピトープを、少なくとも１つのアミノ酸置換を有することにより変化させる、請求項２０に記載の方法。
【請求項２２】
前記Ｔ細胞エピトープを前記目的ポリペプチドの一部と置き換えることにより変化させる、請求項２０に記載の方法であって、その目的ポリペプチドの一部は、該目的ポリペプチドの同族体において前記Ｔ細胞エピトープを含まない部分との対応部分に前記Ｔ細胞エピトープを含む。
【発明の詳細な説明】
【０００１】
発明の背景
工業的、医薬的そして商業的に使用されるタンパク質は急速に普及している。その結果、このような普及によりそれらに暴露される機会が増え、特定の人々はこれらのペプチドに対して感作を引き起こし、そこにさらにさらされることで有害で致命的にさえなり得る過剰なアレルギー反応を引き起こすという安全上の問題の原因ともなっている。例えば、プロテアーゼは特定の個人にとっては危険な過敏症を引き起こすことが知られている。その結果、例えば洗濯用洗剤、化粧品、織物加工等の産業においてプロテアーゼが有用であり、例えばより効果的な染み抜き能を有する洗剤としての改良型プロテアーゼを提供するために、その分野において広範囲の研究が成されているにも関らず、産業上のプロテアーゼの使用は、特定の人々に過敏性アレルギー反応を生じさせる可能性があるため問題となっ

ている。

【０００２】

これらの問題を解決するために多くの研究が行われてきた。プロテアーゼによる免疫原性の可能性を減らすために研究されてきた方法の中で、空中でプロテアーゼを運ぶ粉塵やエアゾールの仕事場における濃度を制御したり最小限に抑えることで接触の可能性を減らすように改良された製造工程、実際にプロテアーゼ製品から生じる粉塵やエアゾールの量を減らすために改良された造粒工程、そして最終製品による潜在的なアレルギー性汚染物質のレベルを減らすために改良された回収工程が使用されてきた。しかしながら、プロテアーゼのアレルギー性を減らす試みは、それ自体は、比較的失敗している。代わりに、過敏症の人の免疫グロブリンＥ（ＩｇＥ）によって認識されるプロテアーゼのエピトープを隠す試みや（国際特許出願公開第ＷＯ９２／１０７５５号）、問題のプロテアーゼにポリマーやペプチド／タンパク質を付加することにより抗原決定基の性質を拡張させたり変化させたりする試みが成されてきた。

【０００３】

適応性免疫反応が過剰または不適切なかたちで起こると、それを体験する人は過敏症になっているという。過敏反応は、通常は有益である免疫反応が不適当に作用した結果であり、時には炎症反応や組織の損傷を引き起こす。それらの過敏反応は多くの抗原によって引き起こされる。そして過敏反応の原因は各個人によってさまざまである。過敏症は通常、抗原との最初の接触では生じないが、ほとんどの場合、その後接触を続けると生じる。過敏症の１つの形態は、花粉、チリダニまたは動物のふけのような無害な環境抗原に対してＩｇＧ反応が向けられた時に生じる。ＩｇＧ感作肥満細胞による薬理学的伝達物質の、結果的に生じる放出によって、喘息または鼻炎のような症状を有する急性炎症反応が引き起こされる。

【０００４】

それでもなお、ＩｇＧ部位を修飾する等の方法では最初の感作反応の発生を抑制することは一般的には成功しないだろう。従って、そのような方法は、続いて起こる過敏反応の重症度を中和したり低下させたりするかもしれないが，実際に感作される人の数は減少しない。例えば、ある人が特定の抗原に対して過敏症であることがわかっている場合、このような状況で取り得る一般的で唯一安全な方法は、その過敏症の人をできるだけ完全に抗原から隔離することである。実際に、他のいずれの方法を採っても過敏症の人の健康には悪い。従って、過敏症の人にとって特定のタンパク質の危険性を減少させることは重要であるが、産業上の目的のためには、タンパク質が最初の段階で過敏反応を開始できないようにすることが遥かに有益である。

【０００５】

Ｔリンパ球（Ｔ細胞）は免疫反応の誘発及び調節、並びに免疫エフェクター機能の遂行において中心的存在である。病原菌や腫瘍に対する特異的な免疫はこれらのＴ細胞に依存することは公知であり、さらにＴ細胞は傷の治癒に寄与していると信じられている。一方、これらの反応の制御に失敗すると、自己攻撃を引き起こす可能性がある。一般的に、抗原は、多様な細胞表面構造を介して、Ｔ細胞による抗原認識に適した形で抗原または部分抗原を捕獲し、提示している抗原提示細胞の形をとって、Ｔ細胞に提示される。Ｔ細胞（Ｔ細胞レセプター）の表面にあるレセプターによる特異的エピトープの認識に基づき、Ｔ細胞は細胞増殖を含む一連の複雑な相互作用を開始し、それが結果としてＢ細胞による抗体の産生となる。Ｔ細胞及びＢ細胞は共に、所定のタンパク質やペプチド上に存在する抗原エピトープによって活性化されるが、これらの単核細胞により認識される実際のエピトープは、一般的に同定されていない。実際に、免疫多様性を生じさせるＴ細胞を活性化させるエピトープは、免疫反応中にＢ細胞によって後になって認識されるエピトープと同じでないことが極めて多い。従って、過敏症に関して、Ｔ細胞と抗原間における特異的抗原相互作用は抗原暴露に対する免疫反応の開始の重要な要素ではあるが、その相互作用の詳細、すなわち認識されるエピトープは、その後に続いて起こる、完全なアレルギー反応への発展とはたいてい無関係である。

【０００６】
国際特許出願公開第ＷＯ９６／４０７９１号は、ポリアルキレン酸化物を出発物質として用いて、アレルギー性が低下したポリアルキレン酸化物－ポリペプチド結合体を作成する方法を開示している。
【０００７】
国際特許出願公開代ＷＯ９７／３０１４８号は、２つ以上のポリペプチド分子が共有結合している１つの高分子キャリアー分子を含む、アレルギー性が低下したポリペプチド結合体を開示している。
【０００８】
国際特許出願公開第ＷＯ９６／１７９２９号は、１～３０の多分子を前駆体ポリペプチドに結合させるステップを含む、アレルギー性が低下したポリペプチドを作成する方法を開示している。
【０００９】
国際特許出願公開第ＷＯ９２／１０７５５号は、動物において、低下した免疫反応を誘導する変異タンパク質を作成する方法を開示している。この出願では、目的タンパク質、すなわち一連のプロテアーゼ及びそれらの変異体を用いてラットに免疫性を与えている。そのラットの血清は、その後、免疫性を与えられた血清中に産生し、存在しているポリクローナル抗体の目的タンパク質やそれらの変異体に対する反応性を測定するために用いられた。これらの結果から、その血清中の抗体がそのタンパク質やそれらの変異体と比較的よく反応するか否かを決定することができ、従って、タンパク質中のどの変化が、Ｉｇ（免疫グロブリン）の結合能を中和もしくは低下させることができそうなのかを分析することが可能となる。これらのラットの試験から、１２７、１２８、１２９、１３０、１３１、１３６、１５１、１５２、１５３、１５４、１６１、１６２、１６３、１６７、１６８、１６９、１７０、１７１、１７２、１７３、１７４、１７５、１７６、１８６、１９３、１９４、１９５、１９６、１９７、２４７、２５１、２６１番残基に対応するいずれかのズブチリシン３０９残基を改変させると、結果として免疫原性が変化するであろうという結論に達した。
【００１０】
国際特許出願公開第ＷＯ９４／１０１９１号は、前駆体である単量体タンパク質のオリゴマーの形態を含む低アレルギー性タンパク質であり、そのオリゴマーは実質的にその活性を維持しているタンパク質を開示している。
【００１１】
これまでの研究により、特定のタンパク質のアレルギー性を低下させる方法や特定の人にアレルギー反応を引き起こすエピトープの同定方法が提供されてきたが、これらのエピトープを同定する検出方法は、一般的に、すでに抗原に暴露されている血清中のＩｇＥ及びＩｇＧの測定であることが必要である。しかしながら、いったんＩｇ反応が開始されてしまうと、すでに感作が生じてしまっている。従って、これらのエピトープを中和すれば、結果として感作が起こる可能性が明らかに低下し、最初の感作の可能性が減るであろうから、最初の段階で感作を引き起こすエピトープを特定する方法が必要とされている。さらに、増進された免疫反応を引き起こすタンパク質の作成や、低免疫反応を引き起こす天然タンパク質の同定もまた必要とされている。本発明はこれら及びその他の要求を満たすものである。
【００１２】
発明の概要
本発明は、望ましい免疫反応を引き起こすことができるタンパク質、かかるタンパク質を同定し、かつ作成する方法及びかかるタンパク質を使用する方法を提供する。例えば、下記の詳細な説明からも明らかになるように、提供される方法及び組成物は、過剰アレルギー性及び低アレルギー性組成物の形態として有用である。ここで用いられる、過剰及び低アレルギー性とはそれぞれ、その組成物が本発明のタンパク質を含まない同じ組成物よりも、多くのまたは少ない免疫反応を引き起こすことを意味する。かかる組成物として、洗

剤組成物、織物加工、コンタクトレンズ洗浄液や洗浄剤、ペプチド加水分解物、廃棄物処理製品、肌、髪及び口腔用を含む化粧品製剤、血栓除去剤のような薬剤、酵素のような調査薬並びにワクチンを含む治療剤を挙げることができる。

【００１３】

本発明の特徴の１つは、目的ポリペプチドが選択され、かつ提供されることである。目的ペプチドはＴ細胞エピトープを持つものが好ましく、下記に記載するように様々である。しかしながら、天然特性に基づいて目的ペプチドを選択することもでき、改変させなくてもよい。さらに、Ｔ細胞エピトープを持たない目的ペプチドを選択することもでき、Ｔ細胞エピトープを持つように改変させることができる。

【００１４】

ここで提供される本発明の特徴の１つとして、Ｔ細胞エピトープを含んだ目的ペプチドの変異体がある。この変異体は、変化させたＴ細胞エピトープを持つことにより前記目的ペプチドとは異なり、このような該変異体や該ポリペプチドは、ヒトによって異なる免疫反応を引き起こす。かかる変異体は、前記目的ペプチドよりも多くのまたは少ない免疫反応を誘導するために調製され、選択される。

【００１５】

目的ポリペプチドは任意の目的ポリペプチドでよい。特徴の１つとして、かかるポリペプチドは、酵素、ホルモン、因子、ワクチン及びサイトカインからなる群より選択される。実施形態の１つとしては、目的のポリペプチドは特定のヒトには、その特定のヒトに内生的なものとして認識されず、或いは自分自身として認識されない。ここで示されるように、目的ポリペプチドは酵素でもよい。また、実施形態の１つとして、その酵素はリパーゼ、セルラーゼ、エンド‐グルコシダーゼＨ、プロテアーゼ、カルボヒドラーゼ、レダクターゼ、酸化酵素、イソメラーゼ、転移酵素、キナーゼ及びホスファターゼからなる群より選択される。好ましい実施形態としては、目的ポリペプチド及びその変異体はそれぞれ、少なくともいくつかの同じ活性を含む。例えば、あるプロテアーゼの変異体が提供されると、前記変異体は免疫反応を変化させて引き起こす。しかし、その変異体は依然として検出可能であり、好ましくはプロテアーゼ活性が比較可能である。

【００１６】

目的ポリペプチド変異体が提供されると、Ｔ細胞エピトープはアミノ酸置換、欠失、付加及びそれらの組み合わせを含む多数の方法に改変され得る。好ましくは、Ｔ細胞エピトープはアミノ酸置換を有するように改変される。ここで１つの実施形態として、アミノ酸置換物は目的ポリペプチドの同族体のアミノ酸に対応するように作られ、ここでその同族体は目的ポリペプチドの対応する位置に同じＴ細胞エピトープを含まない。特徴の１つとして、少なくとも１つのＴ細胞エピトープを含む目的ポリペプチドの末端部分は、目的ポリペプチドの同族体の対応する末端部分に置換され、その置換物は前記の異なる免疫反応を引き起こす。

【００１７】

また、他の実施形態としては、望ましい免疫反応を引き起こすポリペプチドをコードする核酸がここで提供されている。さらに、本発明は、本発明で提供される核酸を含有する発現ベクター及び宿主細胞を含む。さらに、いったん本発明のポリペプチド及びそれらの変異体が同定されると、実質的に相同な配列やポリペプチドや変異体をハイブリダイズする配列を同定することができ、それらが本発明で提供される。相同性はさらに下記に定義され、好ましいとされる同一性と共に、類似性または同一性についても言及することができる。好ましくは、相同配列は、ここで提供されるポリペプチド及び変異体の活性を有するペプチドをコードするアミノ酸配列または核酸である。

【００１８】

本発明のさらにもう１つの特徴は、あるタンパク質によって引き起こされる免疫反応を特定する方法である。実施形態の１つは、以下のステップを含む方法である：（ａ）単一の血液供給源から樹状細胞液及びナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞液を調製する；（ｂ）前記樹状細胞液における分化を促進させる；（ｃ）前記分化樹状細胞液と

前記ナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞を前記タンパク質と混合させる；及び（ｄ）前記ステップ（ｃ）におけるＴ細胞の増殖を測定する。

【００１９】

対照となるタンパク質の免疫反応を同定するために、タンパク質によって引き起こされる免疫反応を特定する方法もまた使用され得る。従って、１つの特徴としては、タンパク質の免疫反応を特定する方法は、対照となる１つ以上のタンパク質の免疫反応をもさらに含む。同じタンパク質における変異体や同じタンパク質における別の型などのタンパク質、例えば同じタンパク質における異なるプロテアーゼまたは異なるペプチドは、それぞれが同族体になり得る。

【００２０】

本発明はさらに、目的ポリペプチドの免疫原性を変化させる方法を提供する。すなわち；前記ポリペプチドの免疫原性を特定し；前記ポリペプチド中のＴ細胞エピトープを同定し；そして前記ポリペプチドの免疫原性を変化させるためにＴ細胞エピトープを改変させる方法を提供する。ここで記載されるように、単一のアミノ酸を改変させたり、または目的ポリペプチドのある部分を同族体の対応する部分と交換させて、その交換部分が変化した免疫反応を引き起こすようにして、前記変化を起こすことができる。

【００２１】

本発明の別の特徴は、以下の記載により当業者に理解される。

【００２２】

発明の詳細な説明

本発明に従って、Ｔ細胞エピトープを同定する方法を提供する。さらに、本発明の方法に従って、比較的効果のない、または効果のあるＴ細胞エピトープを持つ天然タンパク質またはＴ細胞エピトープのない天然タンパク質を含んだタンパク質を同定することができる。従って、本発明により、適切な反応を引き起こすために変異させたタンパク質だけでなく天然タンパク質も含む、望ましい免疫反応を引き起こすタンパク質の同定及び作成が可能となる。当然のことながら、タンパク質、ポリペプチド及びペプチドの語は時に交換しての使用が可能である。ペプチドがタンパク質の一部分であることは、その用語が使われている分野において、当業者は当然に知っていることである。

【００２３】

実施形態の１つとして、本発明は、以下のようにエピトープ及びエピトープ以外のものを同定する測定法を提供する：分化樹状細胞をナイーブヒトＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞並びに目的ペプチドと混合させる。より詳細には、以下のステップを含むＴ細胞エピトープを認識する方法を提供する：（ａ）単一の血液供給源から樹状細胞液及びナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞液を調製する；（ｂ）前記樹状細胞液における分化を促進させる；（ｃ）前記分化樹状細胞液と前記ナイーブＣＤ４陽性及び／またはＣＤ８陽性Ｔ細胞を目的ペプチドと混合させる；（ｄ）前記ステップ（ｃ）におけるＴ細胞の増殖を測定する。

【００２４】

実施形態の１つとして、分析される目的ペプチドは、目的ポリペプチドから得られる。本発明の実施により、ヒトまたはヒトの採取試料において感作を引き起こし得るエピトープの位置を正確に同定することが可能となる。本発明の好ましい実施形態は、目的ポリペプチドの全部または一部に対応する一連のペプチドオリゴマーを調製することである。例えば、そのタンパク質の関連部分または全体を網羅するペプチドライブラリーが作成される。また、１つの実施形態として、ペプチドを作成するための方法は、ペプチドライブラリーに重複部分を取り入れることであり、例えば、１番目のペプチドは対象タンパク質の１－１０番アミノ酸配列に対応し、２番目のペプチドは対象タンパク質の４－１４番アミノ酸配列に対応し、３番目のペプチドは対象タンパク質の７－１７番アミノ酸配列に対応し、４番目のペプチドは対象タンパク質の１０－２０番アミノ酸配列に対応する等のようにして作成し、全分子に対応する代表的ペプチドを作成するまで続ける。ここで提供した測定法で、各ペプチドをそれぞれ分析することによって、Ｔ細胞によって認識されるエピト

ープの位置を正確に同定することが可能である。上記の実施例において、１つの特定のペプチドがその隣のペプチドよりも大きく反応すると、３つのアミノ酸内にエピトープ・アンカー部分を容易に同定できるだろう。これらのエピトープの位置を特定した後、そのペプチドが元のタンパク質と異なるＴ細胞反応を引き起こすまで、各エピトープ内のアミノ酸を改変させることが可能である。もう１つの方法として、例えば病原菌または腫瘍細胞のような標的に対する免疫反応を活性化するエピトープを原型で使用することである。さらに、天然の形態で用いることができ、望ましい高または低アレルギー潜在性のＴ細胞エピトープを有する、タンパク質が本発明において同定され得る。

【００２５】

ここで用いられている“抗原提示細胞”とは、その表面に、Ｔ細胞表面にあるレセプターによって認識される抗原を提示する免疫系の細胞を意味する。抗原提示細胞の例としては、樹状細胞、樹枝状細胞（ｉｎｔｅｒｄｉｇｉｔａｔｉｎｇ　ｃｅｌｌｓ）、活性化Ｂ細胞およびマクロファージが挙げられる。

【００２６】

ここで用いられている“Ｔ細胞増殖”とは、抗原存在下または抗原非存在下において、Ｔ細胞を抗原提示細胞とインキュベーションする間に生じるＴ細胞の数を意味する。

【００２７】

ここで用いられている“基準Ｔ細胞増殖”とは、ペプチドまたはタンパク質抗原が存在しない状態で、抗原提示細胞に対する暴露に反応して、ヒトにおいて通常認められるＴ細胞増殖を意味する。この目的のために、各ヒトの試料ごとの形で、抗原が存在しない状態における抗原提示細胞に反応するＴ細胞増殖として、基準Ｔ細胞増殖レベルを特定する。

【００２８】

“Ｔ細胞エピトープ”とは、その抗原を構成するペプチドに対する免疫反応の開始において、Ｔ細胞レセプターによって認識されるペプチドまたはタンパク質の特徴部分を意味する。Ｔ細胞によるＴ細胞エピトープの認識は、抗原提示細胞に発現されている主要組織適合分子（ＭＨＣ）クラスＩまたはクラスＩＩに結合している抗原のペプチド断片をＴ細胞が認識する作用機構を介すると一般的に考えられている（例えば、Ｍｏｅｌｌｅｒ、Ｇ．編集，“Ａｎｔｉｇｅｎｉｃ　Ｒｅｑｕｉｒｅｍｅｎｔｓ　ｆｏｒ　Ａｃｔｉｖａｔｉｏｎ　ｏｆ　ＭＨＣ－Ｒｅｓｔｒｉｃｔｅｄ　Ｒｅｓｐｏｎｓｅｓ，”Ｉｍｍｕｎｏｌｏｇｉｃａｌ　Ｒｅｖｉｅｗ，Ｖｏｌ．９８，Ｐ．１８７（Ｃｏｐｅｎｈａｇｅｎ；Ｍｕｎｋｓｇａａｒｄ）（１９８７））。

【００２９】

ここで用いられている“試料”とは、未刺激である（ｎａｉｖｅ）、すなわち問題の抗原に対して感作されていない単核細胞を含む。

【００３０】

ここで用いられている“同族体”とは、同様の触媒作用、構造及び／または目的タンパク質としての用途を有するタンパク質または酵素を意味する。本発明の目的として、例えば異なる種由来の同じ機能タンパク質のように、同族体と目的タンパク質は必ずしも進化論的に関連しない。目的タンパク質内のエピトープを、同族体由来類似断片で置換すると、その改変による混乱が減るであろうから、目的タンパク質と同様の三次及び／または一次構造を有する同族体を発見することが望ましい。従って、相同性の高い酵素は、エピトープ置換体の最も望ましい供給源を提供するであろう。あるいは、もし可能であれば、特定のタンパク質のヒト類似物に関心を向けることは都合のよいことである。例えば、細菌ズブチリシン内の特異的エピトープを、ズブチリシンに対するヒト類似物（すなわちヒトズブチリシン）由来配列で置換すると、結果的に、細菌タンパク質におけるアレルギー性が低下するであろう。

【００３１】

“類似”配列は、その置換アミノ酸が、目的タンパク質におけるエピトープまたはそれに近接する部分に対して、同様の機能、三次構造及び／または保存残基を示すことを確認することによって特定できる。従って、そのエピトープ領域が、例えばαヘリックスまたは

βシート構造を含む場合には、その置換アミノ酸は、その特異的構造を維持しなければならない。

【００３２】

本発明で提供される測定法に従って特定されるエピトープは、目的タンパク質の免疫潜在力を減少または増大させるために、その後修飾される。好ましい実施形態としては、修飾すべきエピトープが引き起こすＴ細胞増殖レベルが、試料における基準Ｔ細胞増殖よりも３倍多い。修飾した場合、そのエピトープが引き起こす基準増殖は、試料中の基準増殖の３倍未満であり、好ましくは２倍未満、最も好ましくは、実質的に試料中の基準増殖以下である。

【００３３】

好ましくは、そのエピトープは以下の方法の１つで修飾する：（ａ） エピトープのアミノ酸配列を、目的タンパク質に対するヒト同族体由来の類似配列で置換する；（ｂ）エピトープのアミノ酸配列を、目的タンパク質に対する非ヒト同族体由来であり、かつ目的タンパク質のアミノ酸配列よりもＴ細胞認識により生じる例えばアレルギーのような免疫反応が少ない類似配列で置換する；（ｃ） エピトープのアミノ酸配列を、実質的にエピトープの主要三次構造特性を模倣する配列であるが、目的タンパク質のアミノ酸配列よりもＴ細胞認識により生じる例えばアレルギーのような免疫反応が少ない配列で置換する；または（ｄ）目的タンパク質のアミノ酸配列よりもＴ細胞認識により生じる例えばアレルギーのような免疫反応が少ない任意の配列で置換する。

【００３４】

しかしながら、エピトープは望まれる結果次第で他の方法で修飾され得ることが、当業者によって容易に認識されるだろう。例えば、Ｔ細胞ワクチンが要求される場合、エピトープのアミノ酸配列は、強いＭＨＣ結合及び／またはＴ細胞認識を介してペプチドへの免疫反応を増大させるアミノ酸で置換されると考えられる。また、別の実施例において、自己抗原に対する自己免疫反応を変化させたい場合、エピトープのアミノ酸配列は、炎症またはその他の免疫反応の減少或いは変化を引き起こすアミノ酸で置換されると考えられる。

【００３５】

本発明は、免疫反応を調節することが望まれるすべてのタンパク質に適用される。例えば、Ｔ細胞ワクチンとして使われるペプチド、または癌、伝染病そして自己免疫疾患等に対する治療薬として使われるペプチドまたはタンパク質である。本発明のタンパク質及びペプチドは必ずしもナイーブなタンパク質及びペプチドではないことは当業者に容易に認識される。実際に、本発明の１つの実施形態において、ここに記載される測定法は、混ぜ合わせた遺伝子由来タンパク質の免疫反応を特定するために使用される。混ぜ合わせた遺伝子の記述及びそのような遺伝子の表示は以下を参照、Ｓｔｅｍｍｅｒ，Ｐｒｏｃ．Ｎａｔ’ｌ Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９１：１０７４７（１９９４）；Ｐａｔｔｅｎ，ｅｔ ａｌ．，Ｃｕｒｒｅｎｔ Ｏｐｉｎｉｏｎ ｉｎ Ｂｉｏｔｅｃｈｎｏｌ．８：７２４（１９９７）：Ｋｕｃｈｎｅｒ&Ａｒｎｏｌｄ，Ｔｒｅｎｄｓ Ｂｉｏｔｅｃｈｎｏｌ．１５：５２３（１９９７）；Ｍｏｏｒｅ，ｅｔ ａｌ．，Ｊ．Ｍｏｌ，Ｂｉｏｌ．２７２：３３６（１９９７）；Ｚｈａｏ，ｅｔ ａｌ．，Ｎａｔｕｒｅ Ｂｉｏｔｅｃｈｎｏｌ．１６：２５８（１９９８）；Ｇｉｖｅｒ，ｅｔ ａｌ．，Ｐｒｏｃ．Ｎａｔ’ｌ Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９５：１２８０９（１９９８）；Ｈａｒａｙａｍａ，Ｔｒｅｎｄｓ Ｂｉｏｔｅｃｈｎｏｌ．１６：７６（１９９８）；Ｌｉｎ，ｅｔ ａｌ．，Ｂｉｏｔｅｃｈｎｏｌ．，Ｐｒｏｇ．１５：４６７（１９９９）；及びＳｕｎ，Ｊ．Ｃｏｍｐｕｔ．Ｂｉｏｌ．６：７７（１９９９）。該測定法は混ぜ合わせた遺伝子がコードするタンパク質の免疫反応を予測するために使われる。タンパク質が特定されると、該タンパク質に対する免疫反応を調節するためにそのタンパク質を改変させることができる。

【００３６】

上記タンパク質及びペプチドに加えて、本発明はタンパク質のアレルギー性を減少させるために使用することができる。これらのタンパク質は、グルカナーゼ、リパーゼ、セルラーゼ、エンド‐グルコシダーゼＨ（ｅｎｄｏ‐Ｈ）、プロテアーゼ、カルボヒドラーゼ、

レダクダーゼ、酸化酵素、イソメラーゼ、転移酵素、キナーゼ、ホスファターゼ、アミラーゼ等を含むが、これらに限定されるものではない。天然アミノ酸配列の動物、例えばヒトに対するアレルギー性の減少に加えて、本発明は、例えばその機能的活動を変化させるために改変したタンパク質のような、ヒトタンパク質変異体のアレルギー性の減少を含む。多くの場合、例えば活動を増加させるヒトタンパク質の突然変異体は、結果として該変異タンパク質中の新しいＴ細胞エピトープが結合する。本発明の測定法は、該新しいＴ細胞エピトープの存在を特定し、該変異タンパク質のアレルギー性を減少させる置換アミノ酸を特定するために使用することができる。

【００３７】

本発明は上記タンパク質及び他の多くを含むが、便宜上、以下に本発明の特に好ましい実施形態であるプロテアーゼの修飾について記載する。プロテアーゼとは、一般的に、タンパク質またはペプチドのペプチド結合を切断するために作用するカルボニル加水分解酵素である。ここで用いられている“プロテアーゼ”とは、天然のプロテアーゼまたは組換えプロテアーゼを意味する。天然プロテアーゼとして、α－アミノアシルペプチド加水分解酵素、ペプチジルアミノ酸加水分解酵素、アシルアミノ酸加水分解酵素、セリンカルボキシペプチダーゼ、メタロカルボキシペプチダーゼ、チオールプロテイナーゼ、カルボキシルプロテイナーゼ及びメタロプロテイナーゼが挙げられる。エンド及びエキソプロテアーゼと同様、セリンプロテアーゼ、メタロプロテアーゼ、チオールプロテアーゼおよび酸性プロテアーゼも含まれる。

【００３８】

本発明の実施形態の１つとして、ハイブリッドポリペプチドが提供される。“ハイブリッドポリペプチド”とは、少なくとも２つの異なるタンパク質、好ましくは互いに同族体であるタンパク質に由来する改変タンパク質である。好ましい実施形態において、その２つの末端は完全長活性タンパク質に対応して結合させることができる。また、好ましい実施形態において、該同族体は実質的な類似点を共有するが、同一のＴ細胞エピトープは持たない。従って、実施形態の１つにおいて、例えば、Ｃ末端に１つ以上のＴ細胞エピトープを持つ目的ポリペプチドは、該Ｃ末端を影響力の少ないＴ細胞エピトープをＣ末端に持つ同族体のＣ末端、Ｔ細胞エピトープをＣ末端にほとんど持たない、またはＴ細胞エピトープをＣ末端に持たない同族体のＣ末端と置換することが可能である。このようにして、同族体間のＴ細胞エピトープを特定できることにより、種々の免疫反応を引き起こす変異体を多様なものにすることができることが当業者に理解される。さらに、本発明の変異体を作成するために、配列の内部や、２つ以上の同族体が使用できることは当然である。

【００３９】

より一般的には、ここで提供される変異体は、前駆体アミノ酸配列からなる１つ以上のアミノ酸における置換、欠失、挿入またはそれらの組み合わせによって、前駆体アミノ酸配列から得られる。このような修飾は、前駆体タンパク質の操作によるのではなく、むしろその前駆体酵素のアミノ酸配列をコードする“前駆体ＤＮＡ配列”の修飾である。このような前駆体ＤＮＡ配列の操作のための適当な方法として、当業者に公知の方法（例えば、欧州特許出願第０　３２８２９９号、国際特許出願第ＷＯ８９／０６２７９号、並びに、この中で既に参照されている米国特許および特許出願を参照）と同様に、この中に開示されている方法が挙げられる。

【００４０】

ズブチリシンは、通常タンパク質またはペプチドのペプチド結合を切断するために作用する細菌または真菌のプロテアーゼである。ここで用いられている“ズブチリシン”とは、天然ズブチリシンまたは組換えズブチリシンを意味する。一連の天然ズブチリシンは、各種細菌種によって産生され、かつしばしば分泌されることが知られている。この系列のメンバーのアミノ酸配列は、完全には相同でない。しかしながら、この系列のズブチリシンは、同じ、または類似の型のタンパク質分解活性を示す。このクラスのセリンプロテアーゼは、それらをキモトリプシン関連クラスのセリンプロテアーゼと区別する触媒三つ組残基を特徴づける共通のアミノ酸配列を共有する。ズブチリシンとキモトリプシン関連セリ

ンプロテアーゼは共に、アスパラギン酸塩、ヒスチジン及びセリンを含有する触媒三つ組残基を有する。ズブチリシン関連プロテアーゼにおいて、これらのアミノ酸の相対配列は、アミノ末端からカルボキシル末端への方向でみると、アスパラギン酸塩－ヒスチジン－セリンである。しかしながら、キモトリプシン関連プロテアーゼにおいては、相対配列は、ヒスチジン－アスパラギン酸塩－セリンである。従って、ここでズブチリシンとは、ズブチリシン関連プロテアーゼの触媒三つ組残基を有するセリンプロテアーゼをいう。実施例は、図３において特定されているズブチリシンを含むが、それらに限定されない。一般的に、本発明の目的のために、プロテアーゼ内のアミノ酸の番号付けは、図１に示されている成熟バシラス・アミロリケファシエンス・ズブチリシン配列に割り当てられている番号に対応する。

【００４１】

“組換え体”、“組換え型ズブチリシン”または“組換え型プロテアーゼ”とは、天然アミノ酸配列中の１つ以上のアミノ酸の置換、欠失または挿入をコードする変異体（または突然変異体）ＤＮＡ配列を作成することで、ズブチリシンまたはプロテアーゼをコードするＤＮＡ配列が修飾されているズブチリシンまたはプロテアーゼをいう。このような修飾を形成する適当な方法であって、この中に開示されている方法と組み合わせ得る方法として、米国特許第４，７６０，０２５号（ＲＥ３４，６０６）、米国特許第５，２０４，０１５号、及び米国特許第５，１８５，２５８号に開示されている方法が挙げられる。

【００４２】

“非ヒトズブチリシン”、及びそれらをコードするＤＮＡは、多くの原核生物及び真核生物から得ることができる。適当な原核生物の例として、大腸菌（Ｅ．ｃｏｌｉ）またはシュードモナスのようなグラム陰性菌、及びミクロコッカスまたはバシラスのようなグラム陽性バクテリアが挙げられる。ズブチリシン及びそれらの遺伝子を入手できる真核生物の例として、サッカロマイセス・セレヴィシエ（Ｓａｃｃｈａｒｏｍｙｃｅｓ ｃｅｒｅｖｉｓｉａｅ）のような酵母菌、アスペルギルス（Ａｓｐｅｒｇｉｌｌｕｓ）種のような真菌が挙げられる。

【００４３】

“ヒトズブチリシン”とは、例えばヒト由来プロテアーゼのケキシンファミリーのようなズブチリシン型の触媒活性を有するヒト由来タンパク質を意味する。かかるタンパク質の例が図７の配列に示されている。さらに、例えばペプチド結合を加水分解する能力等といったペプチドの本質的な活性を維持し、マウスまたはウサギのようなヒト以外の供給源に由来するものを含んだここで提供されるタンパク質の誘導体または同族体は、目的ポリペプチドに対して少なくとも５０％以上、好ましくは少なくとも６５％以上、そして最も好ましくは少なくとも８０％以上、さらに好ましくは少なくとも９０％以上、そして場合によっては９５または９８％程度の相同性を有することが好ましい。実施形態の１つとしては、前記図に目的ポリペプチドを示す。

【００４４】

ここで用いられているアミノ酸位置番号は、図１に示されている成熟バシラス・アミロリケエファシエンス・ズブチリシン配列に対して割り当てられている番号をいう。しかしながら、本発明は、この特定のズブチリシンの変異体に限定されるものではなく、バシラス・アミロリケファシエンス・ズブチリシンにおける特定の同定された残基に対して、“等価”の位置にあるアミノ酸残基を含む前駆体プロテアーゼにまで及ぶ。本発明の好ましい実施形態において、前駆体プロテアーゼはバシラス・レンタス・ズブチリシンであり、その置換、欠失または挿入は、上記のアミノ酸残基に対応するバシラス・レンタス（以下、「Ｂ．レンタス」と記すときもある）における等価なアミノ酸残基において作成される。

【００４５】

前駆体プロテアーゼの残基（アミノ酸）は、もしそれがバシラス・アミロリケファシエンス・ズブチリシンにおける特定の残基もしくはその残基の一部に対して、相同（すなわち、一次または三次構造での位置において対応する）または類似（すなわち、化学的に結合し、反応し、または相互作用する同じまたは類似の機能的能力を有する）であれば、バシ

ラス・アミロリケファシエンス・ズブチリシンの残基に対して等価である。ここで使われる“対応する”とは、一般的に、ペプチドにおける類似の位置をいう。

【００４６】

一次構造における相同性を評価するために、前駆体プロテアーゼのアミノ酸配列を、バシラス・アミロリケファシエンス・ズブチリシンの一次配列、及び特にその配列が公知であるズブチリシンの不変残基であることが知られている一連の残基と直接比較する。例えば、ここで図２は、Ｂ．アミロリケファシエンス・ズブチリシンとＢ．レンタス・ズブチリシン間の保存残基を示す。保存残基を並べ、配列を維持するために必要な挿入及び欠失を考慮に入れた後（すなわち、任意の欠失または挿入による保存残基の除去を避ける）、バシラス・アミロリケファシエンス・ズブチリシンの一次配列における特定のアミノ酸配列に等価である残基を明確にする。保存残基の配列は、好ましくは、そのような残基の１００％を保存するべきである。しかしながら、保存残基の７５％より多い、または５０％程度の配列が保存されていれば、同一残基配列を特定するのに十分である。触媒三つ組残基であるＡｓｐ３２／Ｈｉｓ６４／Ｓｅｒ２２１の保存は維持されているべきである。

【００４７】

例えば、バシラス・アミロリケファシエンス、枯草菌、バシラス・リケニフォルミス（ｃａｒｌｓｂｅｒｇｅｎｓｉｓ）及びバシラス・レンタス由来ズブチリシンのアミノ酸配列を、アミノ酸配列間に最大値の相同性を与えるように並べる。これらの配列を比較すると、各配列中に多くの保存残基が含まれていることが分かる。図２は、ＢＰＮ’とＢ．レンタス間の保存残基を特定する。

【００４８】

これらの保存残基は、上に述べたように、例えばバシラス・レンタス由来ズブチリシン（１９８９年７月１３日に公開された国際特許出願公開第ＷＯ８９／０６２７９号）、この明細書中の好ましいプロテアーゼ前駆体酵素、または好ましいバシラス・レンタス・ズブチリシンに対して相同性が高く、ＰＢ９２（欧州特許出願第０　３２８　２９９号）として言及されているズブチリシンのような、他のズブチリシンにおいて、バシラス・アミロリケファシエンス・ズブチリシンに対応する等価アミノ酸残基を特定するために使用できる。特定のこれらズブチリシンのアミノ酸配列を、保存残基の相同性が最大となるように、バシラス・アミロリケファシエンス・ズブチリシンの配列と共に、図３Ａ及び３Ｂに並べた。それらから分かるように、バシラス・アミロリケファシエンス・ズブチリシンと比較して、バシラス・レンタスの配列には多くの欠失がある。従って、例えば、他のズブチリシンにおけるバシラス・アミロリケファシエンス・ズブチリシンのＶａｌ１６５に対応する等価アミノ酸は、Ｂ．レンタス及びＢ．リケニフォルミスではイソロイシンである。

【００４９】

従って、例えば、＋１７０番位置におけるアミノ酸は、Ｂ．アミロリケファシエンスおよびＢ．リケニフォルミス・ズブチリシンではいずれもリジン（Ｋ）であり、サビナーゼではアルギニン（Ｒ）である。本発明における変異プロテアーゼの１つの実施形態において、しかしながら、バシラス・アミロリケファシエンス・ズブチリシンの＋１７０番に対する等価アミノ酸は、アスパラギン酸（Ｄ）である。本発明において、全てのアミノ酸の略語および１文字表記は、ｔｈｅ Ｐａｔｅｎｔｌｎ Ｕｓｅｒ Ｍａｎｕａｌ（ＧｅｎＢａｎｋ，Ｍｏｕｎｔａｉｎ Ｖｉｅｗ，ＣＡ）１９９０，ｐ．１０１の記載に従う。

【００５０】

相同配列は、“配列比較アルゴリズム”を用いることによっても特定できる。例えば、局所ホモロジーアルゴリズム（Ｓｍｉｔｈ ＆ Ｗａｔｅｒｍａｎ，Ａｄｖ．Ａｐｐｌ．Ｍａｔｈ．２：４８２（１９８１））、ホモロジー配列アルゴリズム（Ｎｅｅｄｌｅｍａｎ ＆ Ｗｕｎｓｃｈ，Ｊ．Ｍｏｌ．Ｂｉｏｌ．４８：４４３（１９７０））、類似検索（Ｐｅａｒｓｏｎ ＆ Ｌｉｐｍａｎ，Ｐｒｏｃ．Ｎａｔ’Ｌ Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：２４４４（１９８８））、これらアルゴリズムのコンピュータ処理の実行（ＧＡＰ，ＢＥＳＴＦＩＴ，ＦＡＳＴＡ，及びＴＦＡＳＴＡ ｉｎ ｔｈｅ Ｗｉｓｃｏｎｓｉｎ Ｇｅｎｅｔｉｃｓ Ｓｏｆｔｗａｒｅ Ｐａｃｋａｇｅ，Ｇｅｎｅｔｉｃｓ Ｃｏｍｐ

ｕｔｅｒ Ｇｒｏｕｐ，５７５ Ｓｃｉｅｎｃｅ ＤＲ．，Ｍａｄｉｓｏｎ，Ｗｌ）、または外観検査により、比較のための最良の配列の並べ方を処理することができる。

【００５１】

類似配列を特定するために適したアルゴリズムの例は、Ａｌｔｓｃｈｕｌらの Ｊ．Ｍｏｌ．Ｂｉｏｌ．２１５：４０３－４１０（１９９０）に記載されている、ＢＬＡＳＴアルゴリズムである。ＢＬＡＳＴ解析を実行するソフトウェアは、全米バイオテクノロジー情報センター（ＮＣＢＩ）（ｈｔｔｐ：／／ｗｗｗ．ｎｃｂｉ．ｎｌｍ．ｎｉｈ．ｇｏｖ／）を通じて公的に入手可能である。このアルゴリズムは、最初にデータベース配列中の同じ長さの単語に並べられると、何らかの陽性の閾値Ｔに一致するか、または満たす問合せ配列の長さＷの短い単語を識別することにより、高スコア配列ペア（ＨＳＰｓ）の識別を伴う。これらの最初にヒットした近傍文字は、これらを含んだより長いＨＳＰｓを発見するための出発点として行動する。ヒットした単語は、累積する整列スコアが増加することができる所まで、比較されている各々２つの配列の両方向に延ばされる。ヒットした単語の延長は以下の場合に停止する：累積整列スコアが最大達成値から数量Ｘだけ低下した時；累積スコアが０またはそれ以下に達した時；もしくは、いずれかの配列の末端に達した時。ＢＬＡＳＴアルゴリズムパラメータＷ、Ｔ及びＸは、配列の感受性と速度とを決定する。ＢＬＡＳＴプログラムは、単語長さ（Ｗ）を１１、ＢＬＯＳＵＭ６２ ｓｃｏｒｉｎｇ ｍａｔｒｉｘ（Ｈｅｎｉｋｏｆｆ＆Ｈｅｎｉｋｏｆｆ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８９：１０９１５（１９８９）を参照）、整列（Ｂ）を５０、期待値（Ｅ）を１０、Ｍ’５、Ｎ’－４及び両ストランドの比較値をデフォルト値として使用する。

【００５２】

それから、ＢＬＡＳＴアルゴリズムは、２つの配列間の類似に関する統計分析を実行する（例えば、Ｋａｒｌｉｎ＆Ａｌｔｓｃｈｕｌ，Ｐｒｏｃ．Ｎａｔ‘ｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ９０：５８７３－５７８７（１９９３）を参照）。ＢＬＡＳＴアルゴリズムによって与えられる類似の指標の１つは、最小の和確立（Ｐ（Ｎ））であり、この最小の和確立（Ｐ（Ｎ））は、２つのヌクレオチド配列間または２つのアミノ酸配列間に偶然に起きるであろう一致の確率の目安を与える。例えば、プロテアーゼアミノ酸配列のようなタンパク質に対する試験アミノ酸配列の比較における最小の和確率が、約０．１より小さい、好ましくは約０．０１より小さい、最も好ましくは約０．００１より小さい場合に、アミノ酸配列はプロテアーゼのようなタンパク質と類似すると考えられる。

【００５３】

また“同一残基”は、その三次構造がＸ線結晶構造解析によって確認されている前駆体タンパク質の三次構造レベルにおける相同性を特定することによって、割り出すことができる。同一残基は、プロテアーゼのような前駆体タンパク質及びバシラス・アミロリケファシエンス・ズブチリシンの特定アミノ酸残基における２つ以上の主鎖原子の原子座標（ＮとＮ、ＣＡとＣＡ、ＣとＣ、ＯとＯ）が、配列後０．１３ｎｍ内であり、好ましくは０．１ｎｍ内であるアミノ酸残基として特定される。配列は、バシラス・アミロリケファシエンス・ズブチリシンに対して、問題のプロテアーゼのようなタンパク質の水素以外のタンパク質原子の原子座標の重なりを最大にするように、そのベストモデルを配向させ、配置させた後に達成される。ベストモデルとは、利用可能な最高の分解能での実験回折データにおいて、最小のＲ因子を与えるような結晶モデルである。

【００５４】

バシラス・アミロリケファシエンス・ズブチリシンの特定残基に対して、機能的に類似する同一残基は、ある意味で、バシラス・アミロリケファシエンス・ズブチリシンの特定残基によって確定され、かつそれに帰属しているタンパク質構造、基質認識または触媒作用を、変化させ、修飾し、またはそれらに寄与する配置を取り得るような、プロテアーゼのような前駆体タンパク質のアミノ酸として特定される。さらに、それらは、所定残基の主鎖原子が相同的位置を占めることに基づく等価の基準を満足するものではないが、その残基の２つ以上の側鎖原子の原子座標が、バシラス・アミロリケファシエンス・ズブチリシ

ンの対応側鎖原子の０．１３ｎｍ内にある程度の類似の位置を占める、プロテアーゼのような前駆体タンパク質（その三次構造はＸ線結晶構造解析によって特定されている）の残基である。バシラス・アミロリケファシエンス・ズブチリシンの三次構造の座標は、欧州特許出願公開第０　２５１　４６６号（米国特許第５，１８２，２０４号に等しく、かかる公報はこの中に引例として組み込まれている）に開示されており、先に概説したように、三次構造のレベルにおいて同一残基を特定するために使用できる。

【００５５】

置換、挿入または欠失させるために特定される残基のいくつかは、保存残基である。それに対し他は保存残基ではない。保存残基でない場合、１つ以上のアミノ酸の置換は、天然に見出されるアミノ酸配列に対応しないアミノ酸配列を有する変異体を作成する置換に限定される。保存残基の場合、このような置換は結果として天然の配列とはならないだろう。本発明の変異体は、このような変異タンパク質のプロ及びプレプロ型と同様に、変異タンパク質の成熟体を含む。プレプロ型は、その変異タンパク質の発現、分泌及び成熟を促進するため、好ましい構造である。

【００５６】

“プロ配列（ｐｒｏｓｅｑｕｅｎｃｅ）”とは、除去された場合に結果としてタンパク質の“成熟”体が生じる、タンパク質成熟体のＮ末端部分に結合しているアミノ酸配列をいう。多くのタンパク質分解酵素は、翻訳上のプロ酵素産物として天然に見出されており、翻訳後のプロセシングがない場合にはこの形態で発現している。変異プロテアーゼのような変異タンパク質を作成するための好ましいプロ配列は、他のプロ配列も使用できるが、バシラス・アミロリケファシエンス・ズブチリシンの推定上のプロ配列である。

【００５７】

“シグナル配列”または“プレ配列（ｐｒｅｓｅｑｕｅｎｃｅ）”とは、タンパク質の成熟体または前駆体の分泌に関与し得るタンパク質のＮ末端部分またはプロタンパク質のＮ末端部分に対して、結合するアミノ酸の任意の配列をいう。このシグナル配列の定義は、機能的な定義であり、天然の状態で、タンパク質分泌の遂行に関与しているタンパク質遺伝子のＮ末端部分によってコードされる全てのアミノ酸配列を含むことを意味する。本発明は、この中で明らかにされているような変異タンパク質の分泌をもたらすための配列を利用する。１つの可能なシグナル配列は、バシラス・レンタス（ＡＴＣＣ　２１５３６）由来ズブチリシンのシグナル配列残基と融合している枯草菌ズブチリシン由来シグナル配列の最初の７個のアミノ酸残基を含む。

【００５８】

変異タンパク質の“プレプロ”型は、そのタンパク質のアミノ末端に動作可能に連結したプロ配列及びそのプロ配列のアミノ末端に動作可能に連結した“プレ”または“シグナル”配列を有するタンパク質の成熟体から成る。

【００５９】

“発現ベクター”とは、適当な宿主において、前記ＤＮＡを発現し得る適当な制御配列に、動作可能に連結しているＤＮＡ配列を包含するＤＮＡ構造をいう。このような制御配列として、転写をもたらすためのプロモーター、このような転写を制御するための任意のオペレーター配列、適当なｍＲＮＡのリボソーム結合部位をコードする配列、並びに転写および翻訳の終了を制御する配列が挙げられる。ベクターは、プラスミド、ファージ粒子、または単に潜在的なゲノム挿入であってもよい。いったん適当な宿主に形質転換されると、ベクターは、宿主ゲノムとは関係なく複製及び機能することができ、または、ある場合には、ゲノム自身に一体化し得る。プラスミドは、現在最も一般的に用いられているベクターの形態であるため、本明細書において、“プラスミド”と“ベクター”は、時に同じ意味で用いる。しかしながら、本発明は、同等の機能を果たし、当業者に公知である、または公知となったような発現ベクターの別の形態を含むことを意図する。

【００６０】

本発明で使用されている“宿主細胞”は、一般的に、酵素的に活性のあるエンドプロテアーゼを分泌できないようにするため、米国特許第４，７６０，０２５号（ＲＥ３４，６０

６）に開示されている方法によって、好ましくは操作されている原核細胞または真核細胞である。タンパク質を発現させるために好ましい宿主細胞は、酵素的に活性な中性タンパク質及びアルカリプロテアーゼ（ズブチリシン）が欠損しているバシラス菌株ＢＧ２０３６である。ＢＧ２０３６株の構造は、米国特許第５，２６４，３６６号に詳細に記載されている。タンパク質を発現させるための他の宿主細胞として、Ｂ．リケニフォルミス、Ｂ．レンタス等のような任意の適当なバシラス菌株と同様に、枯草菌Ｉ１６８（米国特許第４，７６０，０２５号（ＲＥ３４，６０６）及び米国特許第５，２６４，３６６号に記載されており、その公報がこの中に引例によって組み込まれている）が含まれる。

【００６１】

宿主細胞は、組換えＤＮＡ技術を用いて構築したベクターで形質転換させ、または核酸導入する。これらの技術は、いずれの分子生物学の標準手引書からも知ることができる。例えば、Ｓａｍｂｒｏｏｋらの Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ - Ａ Ｌａｂｏｒａｔｏｒｙ Ｍａｎｕａｌ（２ｎｄ ｅｄ．）Ｖｏｌ．１－３，Ｃｏｌｄ Ｓｐｒｉｎｇｓ Ｈａｒｂｏｒ Ｐｕｂｌｉｓｈｉｎｇ（１９８９）（”Ｓａｍｂｒｏｏｋ”）；そして Ｃｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ，Ａｕｓｕｂｅｌらｅｄｓ．），Ｃｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ，ａ ｊｏｉｎｔ ｖｅｎｔｕｒｅ ｂｅｔｗｅｅｎ Ｇｒｅｅｎｅ Ｐｕｂｌｉｓｈｉｎｇ Ａｓｓｏｃｉａｔｅｓ，Ｉｎｃ．ａｎｄ Ｊｏｈｎ Ｗｉｌｅｙ ＆ Ｓｏｎｓ，Ｉｎｃ．，（１９９７ Ｓｕｐｐｌｅｍｅｎｔ）（”Ａｕｓｕｂｅｌ”）である。このような形質転換宿主細胞は、変異タンパク質をコードするベクターを複製できるか、または望ましい変異タンパク質を発現できる。変異タンパク質のプレ、またはプレプロ型をコードするベクターの場合、このような変異体が発現される場合、該変異体は通常、宿主細胞から宿主細胞培養液へ分泌される。

【００６２】

“動作可能に連結する”とは、２つのＤＮＡ領域間の関係を説明する場合には、単にそれらが機能的にお互いに関連しあっていることを意味する。例えば、プレ配列は、もしそれがシグナル配列として機能する場合には、ほとんどの場合シグナル配列の分裂を伴うタンパク質成熟体の分泌に関係するペプチドに動作可能に連結する。プロモーターは、それが配列の転写を制御する場合に、コード配列に対して動作可能に連結しており；リボソーム結合部位は、それが翻訳を可能とするために配置されている場合に、コード配列に対して動作可能に連結している。

【００６３】

天然の前駆体タンパク質をコードする遺伝子は、当業者に公知の一般的な方法に従って得られる。その方法は、一般的には、目的プタンパク質領域をコードする推定上の配列を有する標識されたプローブを合成し、そのタンパク質を発現している生物体からゲノムライブラリーを調製し、該プローブにハイブリダイズさせて目的遺伝子のためにライブラリーをスクリーニングすることを含む。陽性にハイブリダイズしたクローンを、その後マッピングし、そして配列決定する。

【００６４】

“ハイブリッド形成”は、ここで記載されるＤＮＡ配列と所定のＤＮＡ断片または遺伝子が一致するかどうか、すなわち本発明の範囲内に収まるかどうかを分析するために使用する。ハイブリダイズさせるべき試料を、ＤＮＡ断片が目に見えるサイズに分離できるように、アガロースゲル（例えば、０．８％アガロース）を通して電気泳動させる。ＤＮＡ断片は、一般的には、臭化エチジウム染色により目に見えるようになる。前記ゲルは、蒸留水で簡単にすすぐことができ、その次に適当な溶液（例えば、０．２５Ｍ ＨＣｌ）中で穏やかに振とうさせながら脱プリン化し、さらに、（例えば、０．４Ｍ ＮａＯＨ中で）３０分間穏やかに振とうさせながら変性させる。また、前記ゲルを１．５Ｍ ＮａＣｌ、１Ｍトリス、ｐＨ７．０中で３０分間穏やかに振とうさせることで、再生ステップを含むことができる。

【００６５】

前記ＤＮＡは、その後、転写液を用いて（例えば、６ＸＳＳＣ（９００ｍＭ　ＮａＣｌ、９０ｍＭ　クエン酸三ナトリウム）、例えば、Ｍａｘｉｍｕｍ　Ｓｔｒｅｎｇｔｈ　Ｎｙｔｒａｎ　Ｐｌｕｓ　メンブレン（Ｓｃｈｌｅｉｃｈｅｒ　＆　Ｓｃｈｕｅｌｌ，　Ｋｅｅｎｅ，　Ｎ．Ｈ．）のような適当な陽性に荷電した薄膜上に転用するのがよい。いったん、転用が完結すると、一般的には約２時間後、該薄膜を、例えば２Ｘ　ＳＳＣ（２Ｘ　ＳＳＣ＝３００ｍＭ　ＮａＣｌ、３０ｍＭ　クエン酸三ナトリウム）ですすぎ、かつ室温で空気乾燥する。それから、該薄膜は、適当なプレハイブリダイズ溶液中（例えば、１００ｍＬあたり以下のものを含む水溶液：ホルムアミド　２０－５０ｍＬ、２０Ｘ　ＳＳＰＥ（１Ｘ　ＳＳＰＥ＝０．１８Ｍ　ＮａＣｌ、１ｍＭ　ＥＤＴＡ、１０ｍＭ NaH_2PO_4、ｐＨ７．７）２５ｍＬ、２０％ＳＤＳ　２．５ｍＬ及び１０ｍｇ／ｍＬの剪毛したニシンまたはサケの精子のＤＮＡ　１ｍＬ）で、プレハイブリダイズする。当業者に公知の通り、プレハイブリダイズ溶液中のホルムアミドの量は、所定の手順に従って得られる自然反応によって様々である。従って、ホルムアミドの量が少ない場合の方が、同じ手順でより多い量のホルムアミドを使った場合よりも、ハイブリダイズした分子の特定という点に関しては、結果としてより完全なハイブリッド形成となる。一方、より多くのホルムアミドを用いることにより、強力なハイブリッドバンドがより簡単に視覚的に検出できる。

【００６６】

目的ＤＮＡ配列に相補的またはほぼ相補的であり、かつ一般的に長さ１００から１０００塩基であるＤＮＡプローブを、ＤＮＡの３２Ｐと混合するために、標識する（例えば、製品の取扱説明書に従ったラベリングシステム（Ｍｅｇａｐｒｉｍｅ　Ｌａｂｅｌｉｎｇ　Ｓｙｓｔｅｍ）を用いて）。標識されたプローブを、５分間９５℃に加熱することにより変性させ、直ちに薄膜及びプレハイブリッド溶液に加える。ハイブリッド反応は適当な条件下、適当な時間で進行させるべきである。例えば、１８時間、３７℃で穏やかに振とうまたは回転させる。前期薄膜をすすぎ（例えば、２Ｘ　ＳＳＣ／０．３％　ＳＤＳ中で）、その後、緩やかに攪拌しながら適当な洗浄液で洗浄した。望ましいストリンジェンシーは薄膜（フィルター）の洗浄条件に反映する。

【００６７】

具体的に言うと、所定の反応におけるストリンジェンシー（すなわち、ハイブリッド形成の成功に必要な相同性の程度：厳密度）は、ハイブリッド形成の後にフィルターが受ける洗浄条件による。ここで定義される“低ストリンジェンシー”条件は、１５分間、２０℃で、０．２Ｘ　ＳＳＣ／０．１％　ＳＤＳ溶液を用いてフィルターを洗浄することを含む。“高ストリンジェンシー”条件は、さらに、３０分間、３７℃で、０．２Ｘ　ＳＳＣ／０．１％　ＳＤＳ溶液を用いてフィルターを２回洗浄する洗浄ステップを含む。

【００６８】

洗浄後、薄膜を乾燥させ、結合プローブを検出する。もし、標識剤として３２Ｐまたは他の放射性同位元素を使用すると、結合プローブはオートラジオグラフィーによって検出できる。その他のプローブの視覚化のための他の技術は、当業者に公知である。核酸配列を示す結合プローブの検出は、望ましい相同性を有し、また、本発明の範囲内に含まれる。

【００６９】

クローン化タンパク質は、その後タンパク質を発現させる目的で、宿主細胞を形質転換させるために使用する。タンパク質遺伝子は、多コピープラスミドに連結させる。このプラスミドは、以下のプラスミドの複製に必要である周知の要素を含むことで、宿主において複製する：問題の遺伝子に対して動作可能に連結しているプロモーター（もしそれが宿主によって認識、すなわち転写される場合、その遺伝子自身の相同プロモーターとして提供され得る）、外因性、またはタンパク質遺伝子の内因性の終止領域によって提供される転写終了領域とポリアデニル化領域（ある真核宿主細胞において、タンパク質遺伝子から宿主細胞によって転写されたｍＲＮＡの安定性のために必要である）、及び、望ましくは、抗生物質含有培地において培養することによるプラスミド－感染宿主細胞の連続培養維持を可能とする抗生物質耐性遺伝子のような選択遺伝子。また、多コピープラスミドは、宿

主のための複製起点を含み、従って多くのプラスミドは染色体の制限なしに細胞質内で発生することが可能となる。しかしながら、宿主ゲノムにタンパク質遺伝子の多様なコピーを一体化させることは、本発明の範囲内である。これは、特に相同的組換えを受けやすい原核生物及び真核生物によって容易になされる。

【００７０】

１つの実施形態において、その遺伝子はＢ．レンタスまたはＢ．アミロリケファシエンス由来遺伝子のような天然の遺伝子であってよい。或いは、天然の、または変異の前駆体タンパク質をコードする合成遺伝子を作成してもよい。このようなやり方の中で、前駆体タンパク質のＤＮＡ及び／またはアミノ酸配列が特定される。そしてその後、ハイブリッド形成及び連結反応によって、前駆体タンパク質をコードする合成ＤＮＡを作成するような、多様で、重複した合成１本鎖ＤＮＡ断片を合成する。合成遺伝子構造の例が、米国特許第５，２０４，０１５号の実施例３に公開されており、その公報が引例として本発明の中に組み込まれている。

【００７１】

いったん天然または合成の前駆体タンパク質遺伝子がクローン化されると、天然の前駆体タンパク質を合成する域を越えて、その遺伝子の使用を増進するために、多くの修飾が開始される。このような修飾は、米国特許第４，７６０，０２５号（ＲＥ３４，６０６）及び欧州特許出願公開第０　２５１　４４６号に開示されている組換えタンパク質の作成、及びこの中に記載されている変異タンパク質の作成を含む。

【００７２】

他の方法も使用できるが、本発明の変異タンパク質の構築を容易にするため、以下のカセット変異誘発法が使用できる。最初に、該タンパク質をコードする天然の遺伝子を入手し、その全体または一部の配列を決定する。次に、コードされている酵素中における１つ以上のアミノ酸の変異（欠失、挿入または置換）を生じさせることが望ましい地点を示すために、その配列をスキャンする。発現された場合に種々の変異体をコードするオリゴヌクレオチドプールで、その遺伝子の短い断片を置換するための制限酵素認識部位を導入するために、この地点に隣接する配列を評価する。このような制限酵素認識部位は、好ましくは、遺伝子断片の置換を容易にするために、タンパク質遺伝子内の特定の部位に存在する。しかしながら、タンパク質遺伝子内の過度に冗長でない任意の都合のよい制限酵素認識部位を使用でき、制限消化によって作成される遺伝子断片を適当な配列中に再構築できる。もし制限酵素認識部位が、選択された地点から都合のよい距離内（１０から１５ヌクレオチド）の位置に存在しない場合には、最終構造においてリーディング・フレームまたはコードされているアミノ酸のいずれもが変化しないような方法で、その遺伝子中のヌクレオチドを置換することによって、そのような認識部位を作成する。望ましい配列に適合させる目的でその配列を変化させるための遺伝子の変異は、公知の方法に従って、Ｍ１３プライマーの伸長によって達成される。適当な隣接領域の位置の特定、及び２つの都合の良い制限酵素認識部位配列を導入するために必要な変化を評価する作業は、遺伝暗号の冗長性、遺伝子の制限酵素地図、及び多くの異なった制限酵素に基づく、機械的なものとなっている。ここで留意すべきは、もし都合の良い隣接制限酵素認識部位が利用可能であれば、認識部位を含まない隣接領域に関連してのみ上記方法を使用する必要があることである。

【００７３】

いったん天然ＤＮＡまたは合成ＤＮＡがクローン化されると、変異させるべき位置に隣接する制限酵素認識部位を同起源の制限酵素で消化し、多数の最終末端－相補的オリゴヌクレオチドカセット（ｅｎｄ　ｔｅｒｍｉｎｉ－ｃｏｍｐｌｅｍｅｎｔａｒｙ　ｏｌｉｇｏｎｕｃｌｅｏｔｉｄｅ　ｃａｓｓｅｔｔｅｓ）をその遺伝子に連結させる。この方法によると、すべてのオリゴヌクレオチドが同じ制限酵素認識部位を持つように合成でき、制限酵素認識部位を作成するために合成リンカーを必要としないため、突然変異誘発が簡素化される。

【００７４】

本発明の１つの特徴において、その目的は、アレルギー性が低下するとその酵素をより安全に使用できるため、前駆体タンパク質と比較してアレルギー性が変化した変異タンパク質を得ることである。本発明はアレルギー性を低下させるのに有用であるが、この中で明確にされる変異は、熱安定性及び／または基質特異性、修飾活性またはアルカリ安定性を結果として変化させる当業界で公知の変異と組み合わせて利用できる。

【００７５】

従って、本発明は、Ｔ細胞増殖を誘導するバシラス・レンタスにおける１７０－１７３番残基を含むＴ細胞エピトープの性質を変化させることを目的としている。本発明の特に好ましい１つの実施形態は、Ｒ１７０Ｄ、Ｙ１７１Ｑ及び／またはＮ１７３Ｄのうちの１つまたは全てに修飾を加えることを含む。同様に、先に詳細に説明したように、任意のタンパク質における対応残基の修飾は、結果として、該タンパク質における主要Ｔ細胞エピトープを中和すると考えられる。従って、本発明の変異タンパク質のアレルギー性を低下させることに加え、１７０－１７３番アミノ酸残基に対応する領域における現在開示されている変異と組み合わせて、全体的に酵素の安定性及び／またはタンパク質分解活性を調節するために、バシラス・アミロリケファシエンス・ズブチリシンのＶ６８Ａ、Ｔ２１３Ｒ、Ａ２３２Ｖ、Ｑ２３６Ｈ、Ｑ２４５Ｒ及びＴ２６０Ａに対応する位置からなる群より選択される１つ以上の置換と必要に応じて組み合わせて、Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄに対応する位置における置換を用いることができる。同様に、結果として生じる変異酵素の安定性及び／または活性を高めさせるために、Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄの変異と組み合わせ、及び必要に応じてバシラス・アミロリケファシエンス・ズブチリシンのＶ６８Ａ、Ｔ２１３Ｒ、Ａ２３２Ｖ、Ｑ２３６Ｈ、Ｑ２４５Ｒ及びＴ２６０Ａに対応する位置からなる群より選択された１つ以上の置換と組み合わせて、この中で提供される置換と、＋７６の同じ位置おけるバシラス・レンタス・ズブチリシンのアスパラギン（Ｎ）をアスパラギン酸（Ｄ）にする変異と組み合わせることができる。

【００７６】

本発明の最も好ましい実施例は、以下の位置に対応する残基の置換における具体的な組み合わせを含む：バシラス・アミロリケファシエンス・ズブチリシンのＮ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ；

Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｑ２３６Ｈ；

Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｑ２３６Ｈ／Ｑ２４５Ｒ；

Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ａ２３２Ｖ／Ｑ２３６Ｈ／Ｑ２４５Ｒ；及び

Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｔ２１３Ｒ／Ａ２３２Ｖ／Ｑ２３６Ｈ／Ｑ２４５Ｒ／Ｔ２６０Ａ

これらの置換は、任意のバシラス菌タンパク質おいて実施できるが、好ましくはバシラス・レンタス（組換え型又は天然型）において実施する。

【００７７】

変異タンパク質を用いて得られたスクリーニング結果によると、上記のバシラス・アミロリケファシエンス・ズブチリシンにおいて言及した注目の変異が、これら酵素におけるタンパク質分解活性、性能及び／または安定性、並びにこのような変異酵素における浄化または洗浄能力にとって重要である。

【００７８】

本発明の多くの変異タンパク質は、種々の洗剤組成物の形成において有用である。多くの公知の化合物は、本発明の変異タンパク質を含む組成物において有用で適当な界面活性剤となる。米国特許第４，４０４，１２８号（Ｂａｒｒｙ　Ｊ．Ａｎｄｅｒｓｏｎ）及び米国特許第４，２６１，８６８号（Ｊｉｒｉ　Ｆｌｏｒａ　ら）に開示されているように、これらの界面活性剤は、非イオン、陰イオン、陽イオンまたは両性イオン洗剤を含む。適当な洗剤処方は、米国特許第５，２０４，０１５号（すでに引例として記載している）の実

施例７に記載されている。この技術は、洗濯組成物として使用することができるような種々の剤形に通じている。典型的な洗濯組成物に加えて、天然型または野生型タンパク質が使用されるいずれの目的においても、本発明の変異タンパク質を使用できることは当然のことである。従って、これらの変異体は、例えば、固形または液状石鹸の用途、食器用洗剤の形態、コンタクトレンズ洗浄液または手入れ用品、ペプチド加水分解、廃棄物処理、織物製品用途において、またはタンパク質生成における融合－分解酵素等として使用できる。本発明の変異体は、アレルギー性の低下に加えて、洗剤組成物において、強化された性能を持つことができる（前駆体と比較して）。ここで用いられている、高められた洗剤の性能は、標準の洗濯サイクルを行った後に通常の評価をして決定したとき、草または血液のような特定の酵素に敏感な汚れの洗浄力の強化として確認される。

【００７９】

本発明のタンパク質、特にプロテアーゼは、約０．０１～約５重量％（好ましくは０．１重量％～０．５重量％）で、ｐＨが６．５～１２．０の間である公知の粉末及び液体洗剤に処方できる。また、これらの洗剤組成物は、ビルダー及び安定剤と同様に、公知のプロテアーゼ、アミラーゼ、セルラーゼ、リパーゼまたはエンドグリコシダーゼのような他の酵素を含んでいてもよい。

【００８０】

本発明のタンパク質、特にプロテアーゼを従来の洗濯用組成物に添加することは、特別の用途限定とはならない。言い換えると、その洗剤に適した任意の温度及びｐＨは、そのｐＨが上記の範囲にあり、かつその温度が記載されているタンパク質の変性温度より低い限りにおいては、該組成物にも適している。さらに、本発明のタンパク質は、洗浄剤を含まない洗剤組成物において、この場合も同様に、単独またはビルダー及び安定剤と組み合わせて用いることができる。

【００８１】

本発明の変異タンパク質は、例えば、米国特許第５，６１２，０５５号；米国特許第５，３１４，６９２号；及び米国特許第５，１４７，６４２号に記載されているように、動物飼料添加物の一部として、動物飼料に含めることができる。

【００８２】

本発明の特徴の１つは、本発明の変異タンパク質を含む織物加工用の組成物である。該組成物は、ＲＤ２１６，０３４；欧州特許第１３４，２６７号；米国特許第４，５３３，３５９号；及び欧州特許第３４４，２５９号のような公開公報に記載されているように、例えば、絹織物、毛織物を手入れするために使用できる。

【００８３】

前記変異体は、当業者に公知の方法に従って、タンパク質分解活性についてスクリーニングできる。好ましい変異プロテアーゼは、以下に対応する位置に多様な置換を含む：バシラス・アミロリケファシエンス・ズブチリシンのＮ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ；
Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｑ２３６Ｈ；
Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｑ２３６Ｈ／Ｑ２４５Ｒ；
Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ａ２３２Ｖ／Ｑ２３６Ｈ／Ｑ２４５Ｒ；及び
Ｖ６８Ａ／Ｎ７６Ｄ／Ｓ１０３Ａ／Ｖ１０４Ｉ／Ｇ１５９Ｄ／Ｋ１７０Ｄ／Ｙ１７１Ｑ／Ｓ１７３Ｄ／Ｔ２１３Ｒ／Ａ２３２Ｖ／Ｑ２３６Ｈ／Ｑ２４５Ｒ／Ｔ２６０Ａ

【００８４】

本発明のタンパク質は、それらの前駆体タンパク質と比較すると、修正された免疫原性を示す。好ましい実施形態においては、該タンパク質は減少したアレルギー性を示す。他の実施形態においては、該タンパク質は増加した免疫原性を示す。この免疫原性の増加は、Ｂ細胞または体液免疫反応の増加、Ｔ細胞または細胞免疫反応、またはＢ及びＴ細胞免疫

反応の両方における増加によって明示される。本発明のタンパク質の使用は、主に、該タンパク質の免疫特性によって決定されることが、当業者は容易に理解できるであろう。例えば、減少したアレルギー性を示す酵素は洗剤組成物に使用できる。“洗剤組成物”とは、例えば繊物、皿、コンタクトレンズ、その他の固体基質、毛髪（シャンプー）、肌（石鹸及びクリーム）等のような基質から望ましくない化合物を取り除くために用いることができる組成物をいう。アレルギー性の減少したタンパク質、特にセルラーゼ、プロテアーゼ及びアミラーゼもまた、繊物加工に用いることができる。“繊物加工”とは、繊物において、編み込んだり、フェルト加工またはニット加工して布地や衣服にできるような特定の毛糸または繊維を、望ましい特性をもたらすように加工する方法を含む。このような望ましい特性の例としては、“ストーンウオッシュ”、毛羽取り（ｄｅｐｉｌｌｉｎｇ）、脱毛、のり抜き、柔軟加工及び当業者に公知のその他の繊物加工がある。

【００８５】

また、免疫反応を起こす特定のヒトに対する治療用タンパク質も本発明に含まれる。特に、該タンパク質の内生的製造を十分にできない特定のヒトは、中和抗体を形成しやすく、治療しにくい。同様に、タンパク質の修飾は、潜在的免疫原性である新しいエピトープを取り込むことができる。本発明の方法は、例えば、反応の中和を防ぐヒト因子ＶＩＩＩのようなエピトープを同定及び修飾するために用いることができる。

【００８６】

薬剤組成物は、例えば顆粒、タブレット、錠剤、座剤、カプセル、懸濁液、軟膏、ローション、及び類似の形態のような、種々の形状で調製できる。経口及び局所使用に適当な薬剤レベルの有機または無機の運搬剤及び／または希釈剤は、薬剤活性化合物を含む組成物を製造するために使用できる。希釈剤は、公知のものとして、水溶性媒体、植物性及び動物性油並びに油脂が挙げられる。助剤としては、安定化剤、湿潤及び乳化剤、浸透圧を変化させるための塩、または適切なｐＨ値を得るための緩衝液、及び経皮浸透促進剤を用いることができる。また、薬剤組成物は以下の１つ以上を含むことができる：血清アルブミンのような運搬タンパク質；緩衝液；マイクロクリスタリン・セルロース、ラクトース、穀類及びその他でんぷんのような充填剤；結合剤；甘味料及びその他の香料添加剤；着色剤；並びにポリエチレングリコール。添加剤は公知のものを、種々の剤形において使用する。

【００８７】

この中で引用されているすべての出版物及び特許は、そのすべてを参考のためこの明細書に添付する。以下の実施例によって本発明を示しているが、特許請求の範囲を限定するものではない。

【００８８】

実施例

実施例１　ナイーブヒトＴ細胞を用いたペプチドＴ細胞エピトープを同定するための測定法

バシラス・レンタス由来プロテアーゼ及びヒトズブチリシンにおける抗原エピトープを特定するために、“未刺激（ｎａｉｖｅ）”のヒト、すなわちバシラス・レンタスプロテアーゼに対して暴露されまたは感作されたことが知られていないヒトから、新鮮なヒト末梢血細胞を採取する。未刺激のヒトとは、過去にプロテアーゼに対する暴露、または反応を開始していることが知られていないヒトを意味する。末梢血単核細胞（室温で保存し、採取後２４時間以上たっていない）は以下のように調製した：単一の全血由来である軟膜調製物（ｂｕｆｆｙ ｃｏａｔ ｐｒｅｐａｒａｔｉｏｎ）の溶液約３０ｍｌを、ダルベッコのリン酸緩衝液（ＤＰＢＳ）で５０ｍｌとし、さらに２本のチューブに分けた。その試料を室温で１２．５ｍｌリンフォプレップ密度分離媒体（ｌｙｍｐｈｏｐｒｅｐ ｄｅｎｓｉｔｙ ｓｅｐａｒａｔｉｏｎ ｍｅｄｉａ）（ニコメッド（Ｎｙｃｏｍｅｄ）密度 １．０７７ｇ／ｍｌ）下に置いた。そのチューブを６００Ｇで３０分間遠心分離機にかけた。２つの層の境界部分を回収し、溜めてから、さらにＤＰＢＳで洗浄した。その結果として生じた溶液中の細胞密度を血球計算盤で測定した。生存率をトリパンブルー排除によっ

て測定した。
【００８９】
結果として生じた溶液の、７５ｍｌ培養フラスコ当たり10^8の細胞密度を有する末梢血単核細胞試料から、以下のように、分化樹状細胞培地を調製した：
（１）５０ｍｌの無血清ＡＩＭ Ｖ培地（Ｇｉｂｃｏ）に１：１００希釈したβ－メルカプトエタノール（Ｇｉｂｃｏ）を足した。フラスコ壁に単核球を吸着させるために、５％CO_2中、３７℃で２時間、そのフラスコを平面に横にして置いた。
【００９０】
（２）単核細胞の樹状細胞への分化は、以下の通りである：非吸着細胞を除去し、残った吸着細胞（単核球）に３０ｍｌのＡＩＭ Ｖ、８００ｕｎｉｔｓ／ｍｌのＧＭ－ＣＳＦ（内因性（Ｅｎｄｏｇｅｎ））、及び５００ｕｎｉｔｓ／ｍｌのＩＬ－４（Ｅｎｄｏｇｅｎ）を混合した；その混合液を５％CO_2中、３７℃の条件下で５日間培養した。５日後、サイトカインＴＮＦα（Ｅｎｄｏｇｅｎ）を０．２ｕｎｉｔｓ／ｍｌになるまで添加し、さらにサイトカインＩＬ－１α（Ｅｎｄｏｇｅｎ）を最終濃度５０ｕｎｉｔｓ／ｍｌとなるまで添加し、その混合液を５％CO_2中、３７℃でさらに２日間インキュベーションした。
【００９１】
（３）７日目に、分化樹状細胞培地の成長を止めるために、マイトマイシンＣを５０μｇ／ｍｌの濃度になるまで添加した。その溶液を５％CO_2中、３７℃で６０分間インキュベーションした。樹状細胞を、セルスクレーパーを用いてフラスコ底から吸着細胞を優しくこすり取ることによって回収した。吸着細胞及び非吸着細胞は、その後６００Ｇで５分間遠心分離機にかけ、ＤＰＢＳで洗浄し、さらに細胞数を数えた。
【００９２】
（４）調製した樹状細胞を、全容積１００μｌ ＡＩＭ Ｖ培地中、2×10^4／ｗｅｌｌで、９６ウェル丸底プレートに分注した。
【００９３】
ＣＤ４陽性Ｔ細胞は、ヒトＣＤ４陽性セレクトキット（ｈｕｍａｎ ＣＤ４＋ Ｃｅｌｌｅｃｔ Ｋｉｔ）（Ｂｉｏｔｅｘ）を用いて、製品の取扱説明書の方法に以下の変更を加えて、樹状細胞の調製に用いた末梢血細胞試料の凍結分取した部分（ｆｒｏｚｅｎ ａｌｉｑｕｏｔｓ）より調製した：その凍結分取した部分を解凍し、セレクトカラム（Ｃｅｌｌｅｃｔ ｃｏｌｕｍｎ）当たり約10^8細胞が添加されるように洗浄した；その細胞をＤＰＢＳ４ｍｌ及びセレクトキットからの細胞試薬１ｍｌ中に再懸濁させ、その溶液を室温で２０分間保持した。その結果として生じた溶液を室温、６００Ｇで５分間遠心分離し、そのペレットをＤＰＢＳ２ｍｌに再懸濁させ、さらにセレクトカラムに充填した。カラムからの溶出液を、２％ヒト血清含有ＤＰＢＳ中に回収した。生じたＣＤ４陽性細胞溶液を遠心分離し、ＡＩＭＶ培地に再懸濁させ、そして細胞密度を測定した。
【００９４】
前記ＣＤ４陽性Ｔ細胞懸濁液は、９６ウェルプレートの効率的な操作を容易にするため、ＡＩＭ Ｖ培地中において2×10^6／ｍｌとなるように再懸濁させた。
【００９５】
ペプチド抗原は、ＡＩＭ Ｖ培地中に１：１０の比率で希釈することによって、ＤＭＳＯ中の１Ｍの原液から調製した。原液１０μｌを、分化樹状細胞を含む９６ウェルプレートの各ウェルに添加した。上記のように調製した希釈ＣＤ４陽性Ｔ細胞溶液１００μｌを、さらに各ウェルに加えた。有用なコントロールとして、希釈したＤＭＳＯブランク、及び破傷風トキソイドの陽性コントロールが挙げられる。
【００９６】
総容積２１０μｌにおける各ウェルの最終的な濃度は以下の通りである：
2×10^4 ＣＤ４陽性
2×10^5 樹状細胞（Ｒ：Ｓは１０：１）
５ｍＭ ペプチド

【００９７】
実施例２　バシラス・レンタス由来プロテアーゼ及びヒトズブチリシンにおけるＴ細胞エピトープの同定
実施例１に記載の測定法において使用するためのペプチドは、バシラス・レンタスのアミノ酸配列及びヒトズブチリシンのアミノ酸配列に基づき調製した。ペプチド抗原は、以下のように設計した。図１に示されているヒトズブチリシン、またはバシラス・レンタスプロテアーゼの完全長アミノ酸配列から、１５配列（１５ｍｅｒｓ）が合成によって調製され、各１５配列は、３つの残基を除いて、その前の１５配列及びその後の１５配列と重複する。
【００９８】
使用されるペプチドは、図８に示されているバシラス・レンタスのアミノ酸残基列に対応し、そしてペプチドは、図７に示されているヒトズブチリシンのアミノ酸残基に対応する。プロテアーゼに対応して用いられるペプチドは、図６に示す。すべての試験は少なくとも２重に行った。報告されたすべての試験は、破傷風トキソイド抗原に対して強い陽性コントロール反応を示した。反応は、各実験の範囲内において平均化し、基準となる反応に対して標準化した。もしその反応が基準反応の３倍より大きい場合は、陽性の事象が記録される。
【００９９】
ヒトズブチリシン及びバシラス・レンタス由来調製ペプチドに対する免疫反応（すなわちＴ細胞増殖）を記録し、それぞれ図４及び図５に示した。Ｔ細胞増殖は、トリチウム法を組み込んで測定した。その結果が、種々のペプチドに対する１０人の検体（図４）及び１６人の検体（図５）における免疫付加反応の比較として、図４及び図５に示されている。反応は、各試料の基準反応を１．０とした付加反応として示す。従って、図４では、１０．０以下が基準反応であり、図５では１６．０以下が基準反応であると解釈できる。反応が大きいほど、Ｔ細胞エピトープのアレルギー性も高まると考えられる。
【０１００】
図４及び図５に示すように、感作されていないヒト由来未刺激血液試料の免疫反応は、バシラス・アミロリケファシエンスプロテアーゼの１７０－１７３番残基に対応するバシラス・レンタス由来ペプチド断片に対して、顕著なアレルギー反応を示した。予想される通り、ヒトズブチリシンにおける対応断片は、単に基本反応を引き起こしただけである。
【０１０１】
図９は、バシラス・レンタスプロテアーゼに対して過敏症であることが分かっているヒトから採取した試料における、バシラス・レンタスプロテアーゼ由来ペプチドに対するＴ細胞反応を示す。ペプチドＥ０５は、バシラス・アミロリケファシエンス由来プロテアーゼにおける１７０－１７３番残基に対応する領域を表わす。図９に示すように、過敏症のヒトは、ペプチドＥ０５によって表わされているＴ細胞エピトープに対して極めて反応しやすい。この結果は、本発明に基づく測定法を実施することによって、過敏症のヒトのＴ細胞によって認識される主なエピトープの予測が可能であることを確認する。
【０１０２】
図１０は、バシラス・レンタスプロテアーゼに対して過敏症であることが分かっているヒトから採取した試料における、バシラス・レンタスプロテアーゼ由来Ｅ０５ペプチドの種々のアラニン置換体に対する、Ｔ細胞反応を示す。アラニン置換は、エピトープ内における任意の特定残基の役割を特定するための置換として用いた。図１０の凡例（ｌｅｇｅｎｄ）は、アラニンで置換されているペプチドの位置を示しており、すなわち、ペプチドＥ０６（配列　ＧＳＩＳＹＰＡＲＹＡＮＡＭＡＶ）において、以下の置換をした；ＧをＡ＝２、ＳをＡ＝３、ＩをＡ＝４、ＳをＡ＝５、ＹをＡ＝６、ＰをＡ＝７、ＲをＡ＝８、ＹをＡ＝９、ＮをＡ＝１０、ＭをＡ＝１１、ＶをＡ＝１２。図１０に示すように、バシラス・レンタス由来プロテアーゼにおいてＲ１７０Ａ、Ｙ１７１Ａ及び／またはＮ１７３Ａ残基のいずれかを置換すると、結果として、過敏症のヒトの血液試料において反応が劇的に低下する。

【０１０３】
これらの結果から、１７０、１７１及び１７３番残基は、バシラス・レンタス由来プロテアーゼ内において、アレルギー反応の開始のために大きな役割を担っていることが明らかである。
【０１０４】
実施例３　フミコーラ・インソレンス（ケアザイム（登録商標）（Ｃａｒｅｚｙｍｅ（登録商標））由来セルラーゼにおけるＴ細胞エピトープの同定
上記と同様の手順をフミコーラ・インソレンス（ケアザイム（登録商用）、ノボ　ノルディスク（Ｎｏｖｏ　Ｎｏｒｄｉｓｋ）社製）由来セルラーゼから得られるペプチドに行った。図１３からわかるように、２つのＴ細胞エピトープ、すなわちＡ０１及びＦ０６が見つかった。
【０１０５】
実施例４　サーモマイセス・ラヌギノーサ（リポラーゼ（登録商標）（Ｌｉｐｏｌａｓｅ（登録商標））由来リパーゼにおけるＴ細胞エピトープの同定
実施例２に記載と同様の手順をサーモマイセス・ラヌギノーサ（リポラーゼ（登録商標）、ノボ　ノルディスク社製）由来リパーゼから得られるペプチドに行った。図１４からわかるように、２つのＴ細胞エピトープ、すなわちＡ１２及びＣ０６が見つかった。ペプチドＥ０３は、未刺激のドナーにおいてＴ細胞増殖の若干の増加をもたらした。しかしながら、このペプチドはＡ１２まで連続しており、１つのエピトープを示す。この点で、エピトープの長さは様々であり、天然または変異のいずれにおいてもそのエピトープの正確なアレルギー潜在性が、この中に記載されている方法によって特定できることは、当業者にとって容易に理解される。
【０１０６】
実施例５　ストレプトマイセス・プリケイタス（Ｓｔｒｅｐｔｏｍｙｃｅｓ　ｐｌｉｃａｔｕｓ）由来エンドグルカナーゼＨにおけるＴ細胞エピトープの同定
実施例２に記載と同様の手順をストレプトマイセス・プリケイタス由来エンドグルカナーゼＨから得られるペプチドに行った。図１５からわかるように、１つのＴ細胞エピトープ、すなわちＣ０６が見つかった。
【０１０７】
実施例６　混合プロテアーゼ（ＧＧ３６－ＢＰＮ’）におけるＴ細胞エピトープの同定
Ｔ細胞エピトープの位置を特定した後、従来のタンパク質工学技術を用いて混合プロテアーゼを作成した。かかる混合は、タンパク質の高アレルギー性アミノ酸配列を、対応する低アレルギー性相同物由来配列に置換するように作成した。この場合に、プロテアーゼの最初の１２２個のアミノ酸はＧＧ３６から、そして残りのアミノ酸配列はＢＰＮ’から得た。
【０１０８】
前記混合プロテアーゼはまず、１００ｐｐｍの試料から試験を行い、北アメリカの気候条件で、０．５ｐｐｍで２４ウェル測定、ｓｕｐｅｒｆｉｘｅｄ　ｓｗａｔｃｈｅｓ、３Ｋ　ｓｗａｔｃｈｅｓを用いた２４ウェル測定において液体（タイドＫＴ（Ｔｉｄｅ　ＫＴ））０．５を試験し、さらにＮ’Ｎ’－ジメチル　カゼイン測定、ＤＭＣ５ｇ／ｌを含むＮＡ洗剤、ＴＮＢＳ検出法を行った。
【０１０９】
図１６、１７及び１８に結果を示す。
【０１１０】
実施例７　低免疫原性である天然タンパク質の同定
この中に記載されている方法を用いて、他の市販のプロテアーゼよりも低い免疫反応を生じさせるものとして、プロテイナーゼＫを同定した。ここで同定したプロテイナーゼＫはＴｒｉｔｉｒａｃｈｉｕｍ　Ａｌｂｕｍ　ｌｉｍｂｅｒに由来する。プロテアーゼ及び手順の概要は、Ｍａｔｈｅｗ、Ｃ．Ｇ．Ｐ．Ｉｓｏｌａｔｉｏｎ　ｏｆ　ｈｉｇｈ　ｍｏｌｅｃｕｌａｒ　ｗｅｉｇｈｔ　ｅｕｋａｒｙｏｔｉｃ　ＤＮＡ、ｉｎ　Ｍｅｔｈｏｄｓ

ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ、ｖｏｌ２：Ｎｕｃｌｅｉｃ Ａｃｉｄｓ（Ｗａｌｋｅｒ、Ｊ．Ｍ．，ｅｄ．）、Ｈｕｍａｎａ、Ｃｌｉｆｔｏｎ、ＮＪ、（１９８４） ｐ．３１－３４を参照。

【０１１１】

実施例８ 非アレルギー性タンパク質へ導入するＴ細胞エピトープ

バシラス・アミロリケファシエンス・ズブチリシンはハートレイ系モルモットで試験した場合、比較的、免疫原性を示さないことがわかった。バシラス・レンティス由来の関連タンパク質は高い免疫原性を示す。目的の配列が高い相同性を示すことにかかわらず、我々はＢ．アミロリケファシエンス分子中にはないＢ．レンティス分子の機能的Ｔ細胞エピトープをあらかじめ特定した。機能的Ｔ細胞エピトープが存在すると抗体作成の相対レベルを制御できるという原理を試験するために、我々はＢ．アミロリケファシエンス分子中にＢ．レンティス様のＴ細胞エピトープを作成した。この変化は、Ｂ．アミロリケファシエンス配列中のただ１つのアミノ酸の置換により実現できた。Ｂ．アミロリケファシエンス・ズブチリシン及びＢ．アミロリケファシエンス・ズブチリシンの変異体を修飾するＴ細胞エピトープは、免疫原性のモデルとなるモルモットにおいて試験した。

【０１１２】

Ｂ．レンティス及びＢ．アミロリケファシエンス・ズブチリシンＴ細胞エピトープのマッピング：モルモットに、バシラス・レンティス又はバシラス・アミロリケファシエンスいずれかから得られたズブチリシン２０μｇ／回の免疫付与で、免疫性を与えた。補助的に、２週間ごとに１０～１２週間、モルモットの皮下に免疫性を与えた。モルモット脾細胞の単個細胞浮遊液（ｓｉｎｇｌｅ ｃｅｌｌ ｓｕｓｐｅｎｓｉｏｎ）を各モルモットの脾臓から作成する。細胞は、丸底９６ウェルプレートにおいて、ウェル当たり5×10^5脾細胞をプレートした。３アミノ酸を除いた１５マー（ｍｅｒｓ）のペプチドをＭｉｍｏｔｏｐｅｓによって合成した。ペプチドをＤＭＳＯ中１ｍＭになるまで再懸濁した。そして、最終濃度が５μＭになるようにペプチドを前記細胞に添加した。培地を５日間、３７℃、５％CO_2でインキュベーションした。ウェルは０．５μＣｉトリチウムチミジンでパルスして、さらに１８時間インキュベーションさせた。ウェルを収穫し、そしてチミジン結合を評価した。

【０１１３】

２つのＴ細胞エピトープがＢ．レンティス・ズブチリシン中に見つかった、そして、Ｂ．アミロリケファシエンス・ズブチリシン中には見つからなかった（これらのエピトープのために１０匹未満を試験した）。Ｂ．レンティスＴ細胞エピトープは以下の配列を含むことがわかった：ＩＡＡＬＮＮＳＩＧＶＬＧＶＡＰ（ＳＥＱ ＩＤ ＮＯ：２３７）及びＬＥＷＡＧＮＮＧＭＨＶＡＮＬＳＬＧＳ （ＳＥＱ ＩＤ ＮＯ：２３８）。

【０１１４】

ＳＥＱ ＩＤ ＮＯ：２３７に対して、Ｂ．アミロリケファシエンス・ズブチリシンにおける類似配列はＶＡＡＬＮＮＳＩＧＶＬＧＶＡＰ（ＳＥＱ ＩＤ ＮＯ：２３９）である。ＳＥＱ ＩＤ ＮＯ：２３８に対するＢ．アミロリケファシエンス・ズブチリシンの類似領域はほとんど相同性がなかった：ＩＥＷＡＩＡＮＮＭＤＶＩＮＭＳＬＧ （ＳＥＱ ＩＤ ＮＯ：２４０）。

【０１１５】

ＳＥＱ ＩＤ ＮＯ：２３７及びＢ．アミロリケファシエンス・ズブチリシン分子の相同領域（ＳＥＱ ＩＤ ＮＯ：２３９）はアミノ酸が１つ異なる：最初のアミノ酸はバシラス・アミロリケファシエンスではＶだが、Ｂ．レンティス・ズブチリシンにおいてはＩである。従って、もしＢ．アミロリケファシエンス配列においてＶをＩに変えれば、バシラス・アミロリケファシエンスを基幹としてバシラス・レンティスＴ細胞エピトープを作成できると推論した。

【０１１６】

この分子は、標準的な分子生物学技術によって作成し、かかる分子をＢ．アミロリケファシエンスＶ７２１とした。これはＧＰ００２としても知られる。

【０１１７】
モルモット免疫付与：メスの成体ハートレイ系モルモットにＢ．アミロリケファシエンス・ズブチリシン及びＧＰ００２を様々に投与して免疫性を与えた。投与量は１、５、１０及び２０μｇ／投与とした。各投与に対して４匹用意した。モルモットは最初の免疫付与において、完全フロイントアジュバントの酵素を用いて皮下から免疫性を与えた。その後に続くすべての免疫付与は、不完全フロイントアジュバントによって実施した。モルモットに免疫性を与え、そして２週間毎に血清試料を採取した。
【０１１８】
ＥＬＩＳＡ（酵素免疫測定法）：直接吸着ＥＬＩＳＡ法を実施した。Ｃｏｓｔａｒｔ　ＥＩＡプレートをＰＩＢの免疫酵素１０μｇ／ｍｌで、４℃、一晩中コーティングした。プレートを洗浄し、ＰＢＳの１％ＢＳＡで塞いだ。血清試料は１％ＢＳＡ／ＰＢＳで希釈し、１時間、酵素でコーティングされたプレート上でインキュベーションした。血清試料を洗い落とし、ビオチン化抗モルモットＩｇＧを１％ＢＳＡ／ＰＢＳ中１：１０，０００に希釈して加えた。派生的試薬を１時間インキュベーションした。ウェルを洗浄し、アビジンを結合した西洋ワサビ・ペルオキシダーゼを１％ＢＳＡ／ＰＢＳ中１：１０００に希釈して、ウェルに加えた。３０分後、基質（ＡＢＴＳ）を加え、OD_{405}を３０分後に読み取った。
【０１１９】
滴定量の計算：読み取ったＯＤ値からバックグラウンドを差し引き、それぞれのモルモット検体毎にその結果をプロットした。曲線の直線部に線形回帰分析を行った。ＯＤ＝０．５に対する線形回帰方程式から滴定値を計算した。それから、これら個々の滴定量を平均した。
【０１２０】
実験中、１０μｇのＧＰ００１を投与したモルモット２匹が２週間で死亡した。従って、１０μｇ投与量のデータは破棄した。
【０１２１】
２つの結論が直ちに明らかとなった：まず、ＧＰ００２変異体は、すべての時間経過にわたって、より少ない酵素投与に対し、抗原特異的抗体の滴定量を増加させた；さらに、ＧＰ００２変異体は、早い時点でのすべての酵素投与に対し、抗原特異的抗体の滴定量を増加させた。
【０１２２】
長時間が経った時点及びより多く投与した時点においては、Ｂ．アミロリケファシエンス・ズブチリシンとその変異体の間における違いはもはや明白ではない。図１９及び２０を参照。
【０１２３】
図より、Ｂ．アミロリケファシエンス・ズブチリシンのアミノ酸配列のたった１つの変化でその免疫原性が顕著に変化したということが明らかである。
【０１２４】
実施例９　ｉｎ　Ｖｉｖｏによるアレルギー性の減少
ヒトＴ細胞エピトープの同定が可能であるとすると、Ｔ細胞の活性化及びそれに続いて起こるタンパク質に対する免疫反応を減少させるために、それらのアミノ酸配列を修飾することが可能である。しかしながら、これらの変化におけるｉｎ　ｖｉｖｏ効果を評価するために、エピトープを提示するヒトＨＬＡ分子の能力を示す、動物モデルを使用することが必要である。例えば、ヒトＴ細胞エピトープはＢＰＮ’分子の７０－８４及び１０９－１２２番残基領域（２０００年２月８日に出願された米国特許出願第０９／５００，１３５号；図１６を参照）中に同定されている。
【０１２５】
これら主題におけるアミノ酸配列の置換は、広範囲のヒトＨＬＡハプロタイプを示すヒト細胞を用いたｉｎ　ｖｉｔｒｏにおけるＴ細胞増殖を結果として減少させた。ＥＢＶ形質転換Ｂ細胞株を用いたｉｎ　ｖｉｔｒｏ結合測定法は、ＨＬＡ　ＤＱ２分子に結合したペ

プチドの７０－８４番及び１０９－１２３番を明示した。Ｔ細胞増殖を減少させることがわかった置換は、低免疫原性ＦＮＡ変異体を作成するためにＦＮＡ（Ｙ２１７Ｌを置換したＢＰＮ’）をコードする配列に導入した。

【０１２６】

ヒトＨＬＡ遺伝子を発現する遺伝子導入マウスは、ｉｎ　ｖｉｖｏにおいて免疫系を提示するエピトープを調査するために使われてきた。反応する免疫細胞はマウス由来のものだが、ヒト及びマウスに認識されるエピトープの間には強い相関関係がある。しかしながら、ＨＬＡ遺伝子電子導入マウスの新規な使用は、変異タンパク質をテストする際に、どの程度ヒトが反応するかの予測として、アレルギー潜在性を減少させるために用いることができる。

【０１２７】

この効果を実証するために、ＦＮＡ及びエピトープ７０－８４及び１０９－１２３番におけるアミノ酸配列の変化を含むＦＮＡ変異体の両方を、１－Ａｂノックアウトマウス（Ｃ２Ｄとして言及した、内生的Ｉ－ＡクラスＩＩ分子の発現性が欠如しているマウス）に戻し交配させたＨＬＡ　ＤＲ３／ＤＱ２遺伝子導入マウスに、免疫性を与えるために用いた。オスの成体ＨＬＡ‐ＤＲ３／ＤＱ２／Ｃ２Ｄマウスに、完全フロイントアジュバントのＦＮＡまたはＦＮＡ乳化変異体５０μｇを用いて、免疫性を与えた。免疫付与は腹腔内に投与した。２週間後、マウスに、さらに５０μｇの不完全フロイントアジュバントにおけるＦＮＡまたはその乳化変異体の免疫付与を腹腔内に与えた。１週間後、球後ブロック手段（ｒｅｔｒｏ‐ｏｒｂｉｔａｌ　ｒｏｕｔｅ）によってマウスを出血させ、血清を回収した。血清は、直接吸着ＥＬＩＳＡプロトコルにおいて、抗原特異的ＩｇＧ抗体を確認した。簡単に言うと、９６ウェル平底ＥＩＡプレートを１０μｇ／ｍｌの変性ＦＮＡで一晩中コーティングした。プレートを洗浄し、１％のウシ胎仔血清で塞いだ。そして、血清を滴定して１：１０に希釈した。血清をウェルから洗い落とし、抗マウス免疫グロブリンＧと結合した西洋ワサビ・ペルオキシダーゼを用いて抗原特異的ＩｇＧを検出した。結果を平均光学濃度（×１０００）に対する血清希釈率として、表１及び図２１に示す。

【０１２８】

【表１】

表１

希釈率	ＦＮＡ	ＦＮＡ変異体
１０	２９３７．５	８８
１００	２４７６	１２０
１０００	１６９５	１０３
１００００	６４１．５	８０
１０００００	２０７	８５
１０００００００	１２９．５	７６
１００００００００	８８．５	８５

【０１２９】

本結果は、７０－８４及び１０９－１２３番領域に導入した変化が、ＤＱ２遺伝子導入マウスのその変異体に対して体液反応を開始する可能性を著しく減少させることを示し、そして、ヒトの免疫原性の減少を示すために本発明の方法を用いて予測した人工タンパク質のｉｎ　ｖｉｖｏ特定のための方法を提供する。

【図面の簡単な説明】

【図１】

図１Ａ、Ｂ１、Ｂ２及びＢ３は、バシラス・アミロリケファシエンス・ズブチリシン（Ｂａｃｉｌｌｕｓ　ａｍｙｌｏｌｉｑｕｅｆａｃｉｅｎｓ　ｓｕｂｔｉｌｉｓｉｎ）（ＢＰＮ’）のＤＮＡ配列（ＳＥＱ　ＩＤ：ＮＯ１）及びアミノ酸配列（ＳＥＱ　ＩＤ：ＮＯ２）、並びにこの遺伝子の部分的制限酵素地図を示す。

【図２】
図２は、バシラス・アミロリケファシエンス（ＳＥＱ　ＩＤ：ＮＯ３）とバシラス・レンタス（野生型）（ＳＥＱ　ＩＤ：ＮＯ４）由来ズブチリシン間の保存アミノ酸残基を示す。
【図３】
図３Ａ及び３Ｂは、バシラス・アミロリケファシエンス（ＢＰＮ’）、枯草菌、バシラス・リケニフォルミス（ＳＥＱ　ＩＤ：ＮＯ５）及びバシラス・レンタスに由来する、ズブチリシン様プロテアーゼのアミノ酸配列を並べて示す。＊印は、ＢＰＮ’ズブチリシンに照らして、特定のアミノ酸残基の欠失を示す。
【図４】
図４は、バシラス・レンタスプロテアーゼ（ＧＧ３６）に対応するペプチドに対する、１６の末梢血単核細胞試料の付加Ｔ細胞反応を示す。ペプチドＥ０５は、バシラス・アミロリケファシエンス由来プロテアーゼにおける１７０－１７３番残基に対応する残基を含む領域を包含する。
【図５】
図５は、ヒトズブチリシン分子に対応するペプチドに対する、１０の末梢血単核細胞試料の付加Ｔ細胞反応を示す。ペプチドＦ１０、Ｆ９、Ｆ８及びＦ７はいずれも、図３のアミノ酸配列において、バシラス・アミロリケファシエンス由来プロテアーゼにおける１７０－１７３番残基に対応する残基を含む領域に対応するアミノ酸配列ＤＱＭＤを包含する。
【図６】
図６Ａ及び６Ｂ／６Ｃは、それぞれ、バシラス・レンタスプロテアーゼ配列及びヒトズブチリシン配列に由来するペプチドに対応するアミノ酸配列を示す。
【図７】
図７は、ヒトズブチリシンのアミノ酸配列（ＳＥＱ　ＩＤ：ＮＯ６）を示す。
【図８】
図８は、ＢＰＮ’（バシラス・アミロリケファシエンス）プロテアーゼ、サビナーゼ（ＳＡＶＩＮＡＳＥ）（バシラス・レンタス）プロテアーゼ及びヒトズブチリシン（Ｓ２ＨＳＢＴ）のアミノ酸配列を並べて示す。
【図９】
図９は、バシラス・レンタスプロテアーゼに対して過敏症であることが分かっているヒトから採取した試料における、バシラス・レンタスプロテアーゼ由来ペプチドに対するＴ細胞反応を示す。ペプチドＥ０５は、バシラス・アミロリケファシエンス由来プロテアーゼにおける１７０－１７３番残基に対応する領域を示す。
【図１０】
図１０は、バシラス・レンタスプロテアーゼに対して過敏症であることが分かっているヒトから採取した試料において生じる、バシラス・レンタスプロテアーゼペプチドＥ０５の種々のアラニン置換体に対するＴ細胞反応を示す。
【図１１】
図１１は、プロテアーゼに対して過敏症であることが分かっているヒトから採取した試料において生じる、プロテアーゼペプチドＥ０５（未修飾配列によって示されるＴ細胞エピトープの実施形態）の種々のアラニン置換体に対するＴ細胞反応を示す。また、各ペプチドの配列を示す。
【図１２】
図１２は、ヒトズブチリシン分子に対する反応率を示す。
【図１３】
図１３は、フミコーラ・インソレンス・エンドグルカナーゼ（Ｈｕｍｉｃｏｌａ　ｉｎｓｏｌｅｎｓ　ｅｎｄｏｇｌｕｃｏｎａｓｅ）（寄託番号 Ａ２３６３５）由来ペプチドのＴ細胞反応を示す。ペプチドＡ０２及びＦ０６はそれぞれ、フミコーラ・インソレンス・エンドグルカナーゼの、Ｔ細胞エピトープの実施形態における、７０－８４及び３７－５１番残基に対応する領域を示す。ここで、図１３Ｂはその全長配列を示し、下線かつ太字

はＡ０２及びＦ０６を示している。
【図１４】
図１４は、サーモマイセス・ラヌギノーサ・リパーゼ（Ｔｈｅｒｍｏｍｙｃｅｓ ｌａｎｕｇｉｎｏｓａ ｌｉｐａｓｅ）（寄託番号 ＡＡＣ０８５８８及びＰＩＤ番号 ｇ２９９７７３３）由来ペプチドに対するＴ細胞反応を示す。ペプチドＢ０２及びＣ０６はそれぞれ、サーモマイセス・ラヌギノーサ・リパーゼの、Ｔ細胞エピトープの実施形態における、８３－１００及び１０８－１２１番残基に対応する領域を示す。ここで、図１４Ｂはその全長配列を示し、下線かつ太字はＢ０２及びＣ０６を示している。
【図１５】
図１５は、ストレプトマイセス・プリケイタス・エンド－β－Ｎ－アセチルグルコサミニダーゼ（Ｓｔｒｅｐｔｏｍｙｃｅｓ ｐｌｉｃａｔｕｓ ｅｎｄｏ－ｂｅｔａ－Ｎ－ａｃｅｔｙｌｇｌｕｃｏｓａｍｉｎｉｄａｓｅ）（寄託番号 Ｐ０４０６７）由来ペプチドに対するＴ細胞反応を示す。ペプチドＣ０６は、ストレプトマイセス・プリケイタス・エンド－β－Ｎ－アセチルグルコサミニダーゼの、Ｔ細胞エピトープの実施形態における、１２６－１４０番残基に対応する領域を示す。ここで、図１５Ｂはその全長配列を示し、下線かつ太字はＣ０６を示している。
【図１６】
図１６は、２２人の検体における混合ＢＰＮ’由来ペプチドに対するＴ細胞反応を示す。ここで、好ましいＴ細胞エピトープの配列が示されている。
【図１７】
図１７は、２２人の検体における混合ＧＧ３６由来ペプチドに対するＴ細胞反応を示す。ここで、Ｔ細胞エピトープの実施形態における配列が示され、ＧＳＩＳＹＰＡＲＹＡＮＡＭＡＶＧＡ及びＧＡＧＬＤＩＶＡＰＧＶＮＶＱＳが好ましい。
【図１８】
図１８は、Ｎ末端がＮ末端ＧＧ３６配列を含み、Ｃ末端がＣ末端ＢＰＮ’配列を含む、本発明で提供されるハイブリッドタンパク質の実施形態である。また、図８に表したそれらの配列の比較は、ハイブリッド型がそれぞれのタンパク質の好ましいＴ細胞エピトープを除外することを示す。
【図１９】
図１９は、Ｂ．アミロリケファシエンス・ズブチリシンとＢ．レンティス・ズブチリシン由来Ｔ細胞エピトープを含むよう改変された同じズブチリシンとのＥＬＩＳＡ滴定量の比較である。図１９ａは、４週間後、図１９ｂは６週間後、図１９ｃは８週間後、図１９ｄは１０週間後における滴定量を示す。
【図２０】
図２０は、Ｂ．アミロリケファシエンス・ズブチリシンとＢ．レンティス・ズブチリシン由来Ｔ細胞エピトープを含むよう改変された同じズブチリシンとのＥＬＩＳＡ滴定量の時間的経過調査である。図２０ａは酵素１μｇ投与、図２０ｂは５μｇ投与、図２０ｃは２０μｇ投与における滴定量を示す。

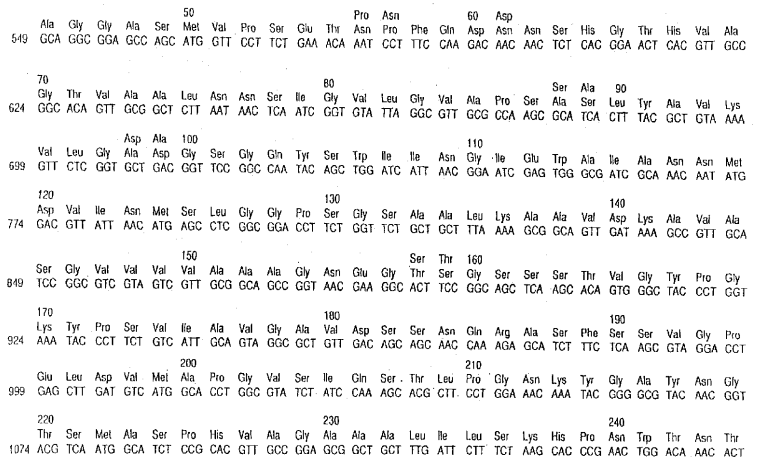


図１Ｂ－２

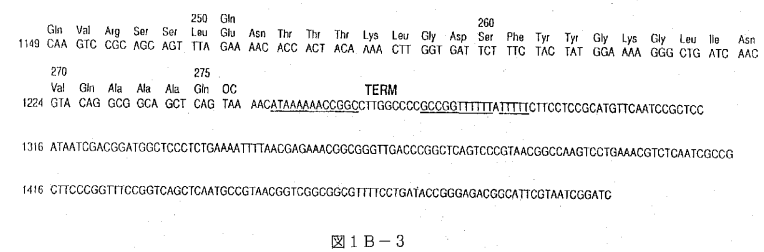
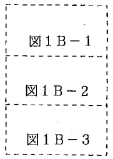


図１Ｂ－３

図１Ｂ



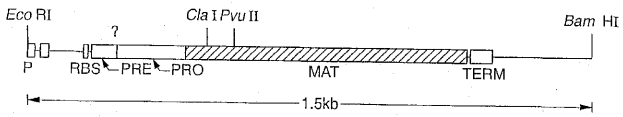


図１Ａ

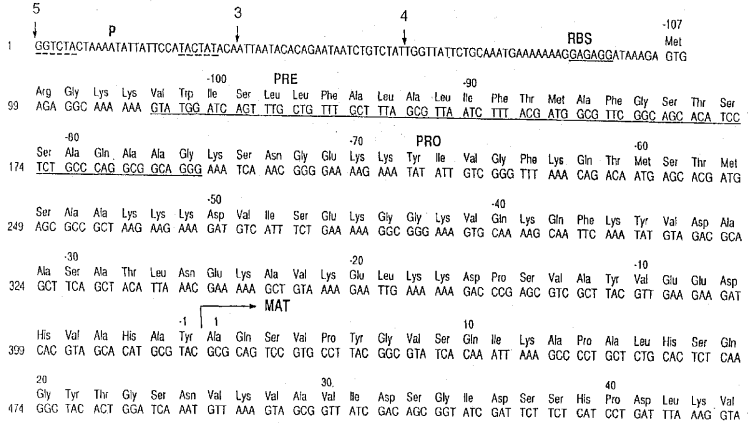


図１Ｂ－１

(29) JP 2004-528013 A 2004.9.16

【図２】

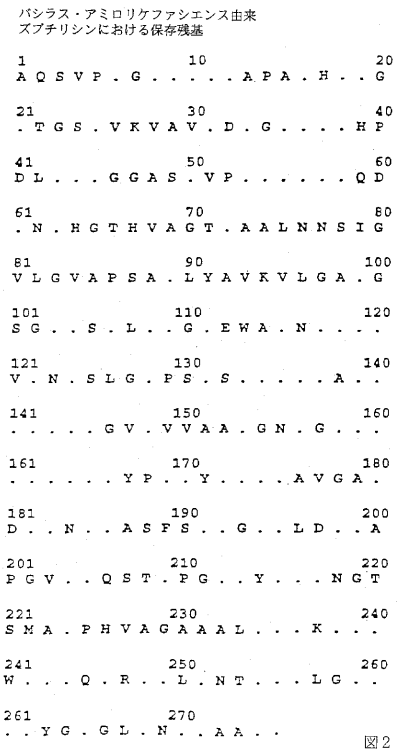


図２

【図３】

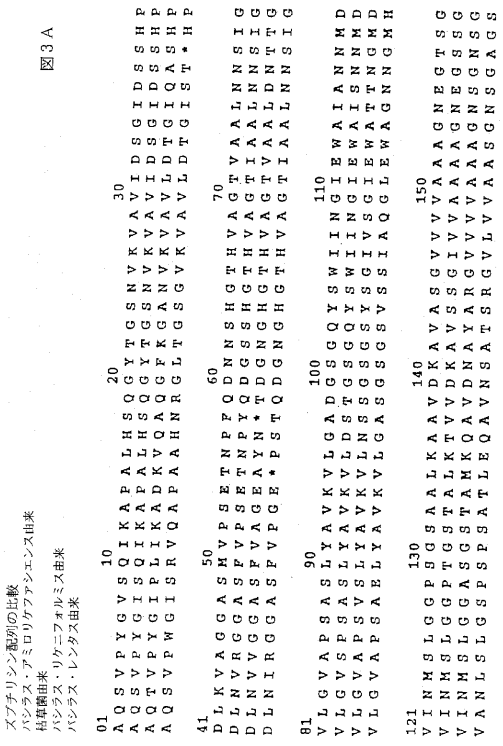


図３Ａ

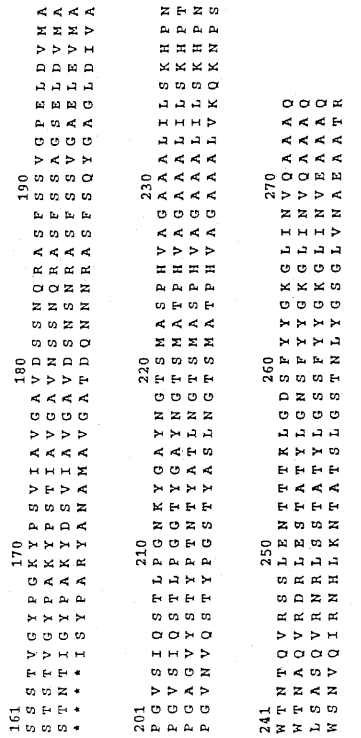


図３Ｂ

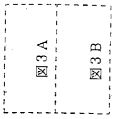


図３

【図４】

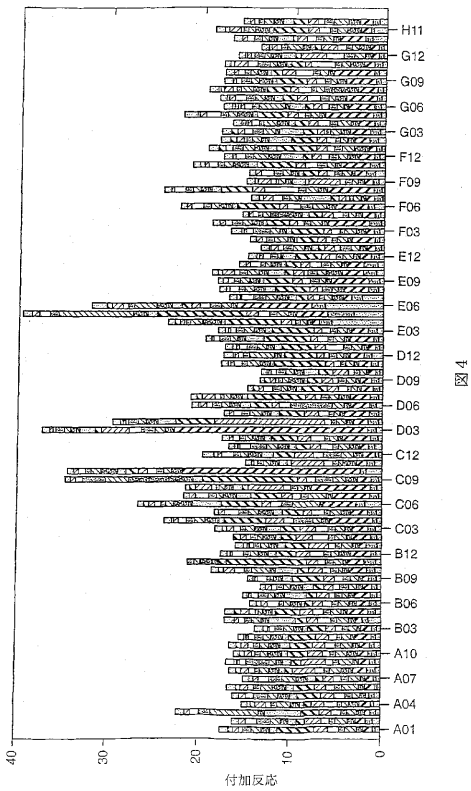


図４

【図５】

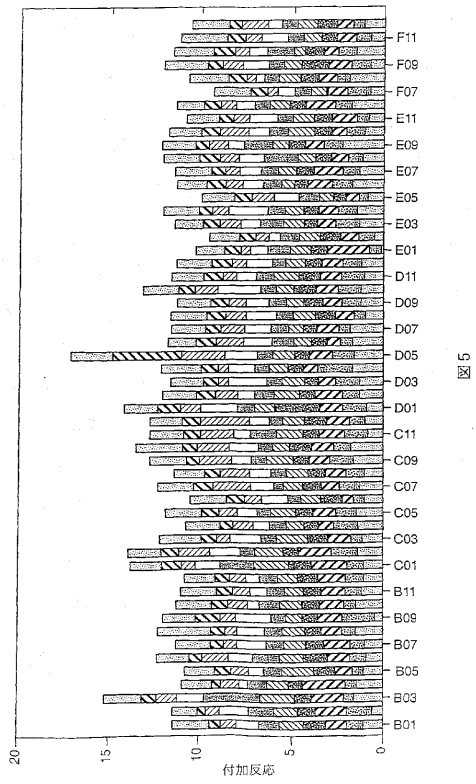


図５

【図６】

1	A12	IKDFHVYFRESRDAG	49	E12	SATSRGVLVVAASGN
2	A11	LEQAVNSATSRGVLV	50	E11	SRGVLVVAASGNSGA
3	A10	AQSVPWGISRVQAPA	51	E10	VLVVAASGNSGAGSI
4	A9	VPWGISRVQAPAAHN	52	E9	VAASGNSGAGSISYP
5	A8	GISRVQAPAAHNRGL	53	E8	SGNSGAGSISYPARY
6	A7	RVQAPAAHNRGLTGS	54	E7	SGAGSISYPARYANA
7	A6	APAAHNRGLTGSGVK	55	E6	GSISYPARYANAMAV
8	A5	AHNRGLTGSGVKVAV	56	E5	SYPARYANAMAVGAT
9	A4	RGLTGSGVKVAVLDT	57	E4	ARYANAMAVGATDQN
10	A3	TGSGVKVAVLDTGIS	58	E3	ANAMAVGATDQNNNR
11	A2	GVKVAVLDTGISTHP	59	E2	MAVGATDQNNNRASF
12	A1	VAVLDTGISTHPDLN	60	E1	GATDQNNNRASFSQY
13	B12	LDTGISTHPDLNIRG	61	F12	DQNNNRASFSQYGAG
14	B11	GISTHPDLNIRGGAS	62	F11	NNRASFSQYGAGLDI
15	B10	THPDLNIRGGASFVP	63	F10	ASFSQYGAGLDIVAP
16	B9	DLNIRGGASFVPGEP	64	F9	SQYGAGLDIVAPGVN
17	B8	IRGGASFVPGEPSTQ	65	F8	GAGLDIVAPGVNVQS
18	B7	GASFVPGEPSTQDGN	66	F7	LDIVAPGVNVQSTYP
19	B6	FVPGEPSTQDGNGHG	67	F6	VAPGVNVQSTYPGST
20	B5	GEPSTQDGNGHGTHV	68	F5	GVNVQSTYPGSTYAS
21	B4	STQDGNGHGTHVAGT	69	F4	VQSTYPGSTYASLNG
22	B3	DGNGHGTHVAGTIAA	70	F3	TYPGSTYASLNGTSM
23	B2	GHGTHVAGTIAALNN	71	F2	GSTYASLNGTSMATP
24	B1	THVAGTIAALNNSIG	72	F1	YASLNGTSMATPHVA
25	C12	AGTIAALNNSIGVLG	73	G12	LNGTSMATPHVAGAA
26	C11	IAALNNSIGVLGVAP	74	G11	TSMATPHVAGAAALV
27	C10	LNNSIGVLGVAPSAE	75	G10	ATPHVAGAAALVKQK
28	C9	SIGVLGVAPSAELYA	76	G9	HVAGAAALVKQKNPS
29	C8	VLGVAPSAELYAVKV	77	G8	GAAALVKQKNPSWSN
30	C7	VAPSAELYAVKVLGA	78	G7	ALVKQKNPSWSNVQI
31	C6	SAELYAVKVLGASGS	79	G6	KQKNPSWSNVQIRNH
32	C5	LYAVKVLGASGSGSV	80	G5	NPSWSNVQIRNHLKN
33	C4	VKVLGASGSGSVSSI	81	G4	WSNVQIRNHLKNTAT
34	C3	LGASGSGSVSSIAQG	82	G3	VQIRNHLKNTATSLG
35	C2	SGSGSVSSIAQGLEW	83	G2	RNHLKNTATSLGSTN
36	C1	GSVSSIAQGLEWAGN	84	G1	LKNTATSLGSTNLYG
37	D12	SSIAQGLEWAGNNGM	85	H12	TATSLGSTNLYGSGL
38	D11	AQGLEWAGNNGMHVA	86	H11	SLGSTNLYGSGLVNA
39	D10	LEWAGNNGMHVANLS	87	H10	STNLYGSGLVNAEAA
40	D9	AGNNGMHVANLSLGS	88	H9	NLYGSGLVNAEAATR
41	D8	NGMHVANLSLGSPSP			
42	D7	HVANLSLGSPSPSAT			
43	D6	NLSLGSPSPSATLEQ			
44	D5	LGSPSPSATLEQAVN			
45	D4	PSPSATLEQAVNSAT			
46	D3	SATLEQAVNSATSRG			
47	D2	LEQAVNSATSRGVLV			
48	D1	AVNSATSRGVLVVAA			

図６Ａ

1	A12	IKDFHVYFRESRDAG	49	E12	KKIDVLNLSIGGPDF
2	A11	DAELHIFRVFTNNQV	50	E11	DVLNLSIGGPDFMDH
3	A10	PLRRASLSLGSGFWH	51	E10	NLSIGGPDFMDHPFV
4	A9	RASLSLGSGFWHATG	52	E9	IGGPDFMDHPFVDKV
5	A8	LSLGSGFWHATGRHS	53	E8	PDFMDHPFVDKVWEL
6	A7	GSGFWHATGRHSSRR	54	E7	MDHPFVDKVWELTAN
7	A6	FWHATGRHSSRRLLR	55	E6	PFVDKVWELTANNVI
8	A5	ATGRHSSRRLLRAIP	56	E5	DKVWELTANNVIMVS
9	A4	RHSSRRLLRAIPRQV	57	E4	WELTANNVIMVSAIG
10	A3	SRRLLRAIPRQVAQT	58	E3	TANNVIMVSAIGNDG
11	A2	LLRAIPRQVAQTLQA	59	E2	NVIMVSAIGNDGPLY
12	A1	AIPRQVAQTLQADVL	60	E1	MVSAIGNDGPLYGTJ
13	B12	RQVAQTLQADVLWQM	61	F12	AIGNDGPLYGTLNNP
14	B11	AQTLQADVLWQMGYT	62	F11	NDGPLYGTLNNPADQ
15	B10	LQADVLWQMGYTGAN	63	F10	PLYGTLNNPADQMDV
16	B9	DVLWQMGYTGANVRV	64	F9	GTLNNPADQMDVIGV
17	B8	WQMGYTGANVRVAVF	65	F8	NNPADQMDVIGVGGI
18	B7	GYTGANVRVAVFDTG	66	F7	ADQMDVIGVGGIDFE
19	B6	GANVRVAVFDTGLSE	67	F6	MDVIGVGGIDFEDNI
20	B5	VRVAVFDTGLSEKHP	68	F5	IGVGGIDFEDNIARF
21	B4	AVFDTGLSEKHPHFK	69	F4	GGIDFEDNIARFSSR
22	B3	DTGLSEKHPHFKNVK	70	F3	DFEDNIARFSSRGMT
23	B2	LSEKHPHFKNVKERT	71	F2	DNIARFSSRGMTTWE
24	B1	KHPHFKNVKERTNWT	72	F1	ARFSSRGMTTWELPG
25	C12	HFKNVKERTNWTNER	73	G12	SSRGMTTWELPGGYG
26	C11	NVKERTNWTNERTLD	74	G11	GMTTWELPGGYGRMK
27	C10	ERTNWTNERTLDDGL	75	G10	TWELPGGYGRMKPDI
28	C9	NWTNERTLDDGLGHG	76	G9	LPGGYGRMKPDIVTY
29	C8	NERTLDDGLGHGTFV	77	G8	GYGRMKPDIVTYGAG
30	C7	TLDDGLGHGTFVAGV	78	G7	RMKPDIVTYGAGVRG
31	C6	DGLGHGTFVAGVIAS	79	G6	PDIVTYGAGVRGSGV
32	C5	GHGTFVAGVIASMRE	80	G5	VTYGAGVRGSGVKGG
33	C4	TFVAGVIASMRECQG	81	G4	GAGVRGSGVKGGCRA
34	C3	AGVIASMRECQGFAP	82	G3	VRGSGVKGGCRALSG
35	C2	IASMRECQGFAPDAE	83	G2	SGVKGGCRALSGTSV
36	C1	MRECQGFAPDAELHI	84	G1	KGGCRALSGTSVASP
37	D12	CQGFAPDAELHIFRV	85	H12	CRALSGTSVASPVVA
38	D11	FAPDAELHIFRVFTN	86	H11	LSGTSVASPVVAGAV
39	D10	DAELHIFRVFTNNQV	87	H10	TSVASPVVAGAVTLL
40	D9	LHIFRVFTNNQVSYT	88	H9	ASPVVAGAVTLLVST
41	D8	FRVFTNNQVSYTSWF	89	H8	VVAGAVTLLVSTVQK
42	D7	FTNNQVSYTSWFLDA	90	H7	GAVTLLVSTVQKREL
43	D6	NQVSYTSWFLDAFNY	91	H6	TLLVSTVQKRELVNP
44	D5	SYTSWFLDAFNYAIL	92	H5	VSTVQKRELVNPASM
45	D4	SWFLDAFNYAILKKI	93	H4	VQKRELVNPASMKQA
46	D3	LDAFNYAILKKIDVL	94	H3	RELVNPASMKQALIA
47	D2	FNYAILKKIDVLNLS	95	H2	VNPASMKQALIASAR
48	D1	AILKKIDVLNLSIGG	96	H1	ASMKQALIASARRLP

図６Ｂ

97	I12	IKDFHVYFRESRDAG
98	I11	DAELHIFRVFTNNQV
99	I10	KQALIASARRLPGVN
100	I9	LIASARRLPGVNMFE
101	I8	SARRLPGVNMFEQGH
102	I7	RLPGVNMFEQGHGKL
103	I6	GVNMFEQGHGKLDLL
104	I5	MFEQGHGKLDLLRAY
105	I4	QGHGKLDLLRAYQIL
106	I3	GKLDLLRAYQILNSY
107	I2	DLLRAYQILNSYKPQ
108	I1	RAYQILNSYKPQASL
109	J12	QILNSYKPQASLSPS
110	J11	NSYKPQASLSPSYID
111	J10	KPQASLSPSYIDLTE
112	J9	ASLSPSYIDLTECPY
113	J8	SPSYIDLTECPYMWP
114	J7	YIDLTECPYMWPYCS
115	J6	LTECPYMWPYCSQPI
116	J5	CPYMWPYCSQPIYYG

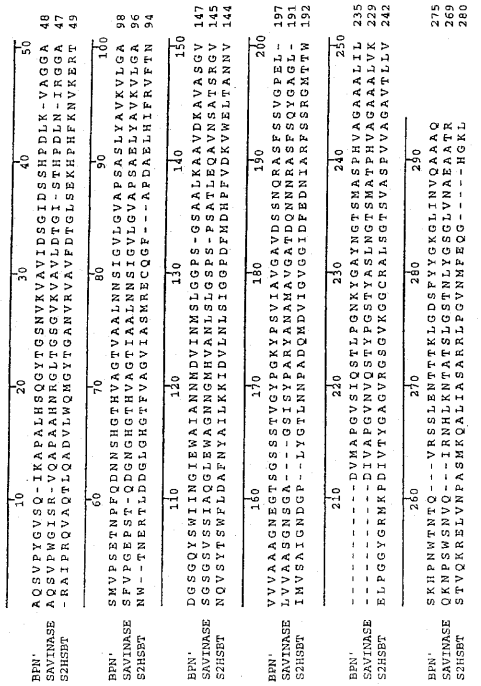
図６Ｃ

【図７】

図７

【図８】

図８



【図９】

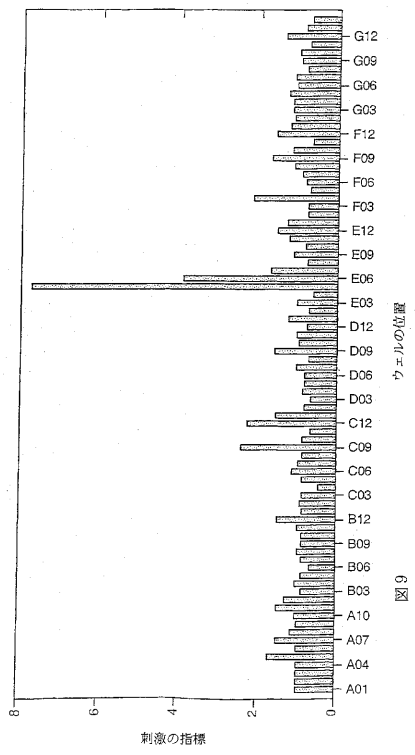


図９

【図１０】

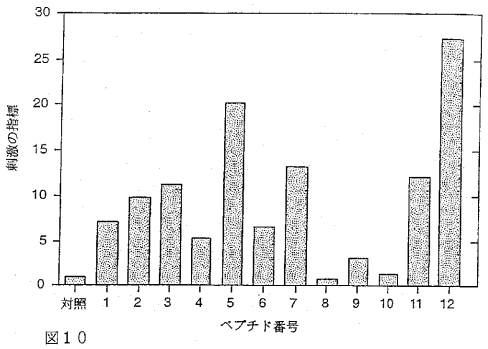


図１０

【図１１】

ペプチド番号		配列
1	(未修飾配列)	GSISYPARYANAMAV
	2	ASISYPARYANAMAV
	3	GAISYPARYANAMAV
	4	GSASYPARYANAMAV
	5	GSIAYPARYANAMAV
	6	GSISAPARYANAMAV
	7	GSISYAARYANAMAV
	8	GSISYPAAYANAMAV
	9	GSISYPARAANAMAV
	10	GSISYPARYAAAMAV
	11	GSISYPARYANAAAV
	12	GSISYPARYANAMAA

図１１

【図１２】

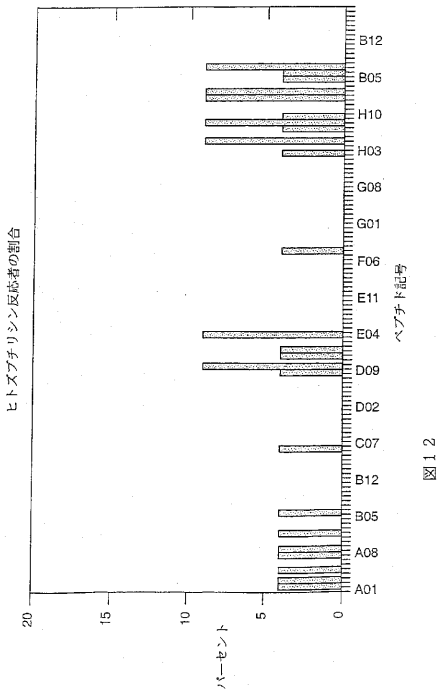


図１２

【図１３】

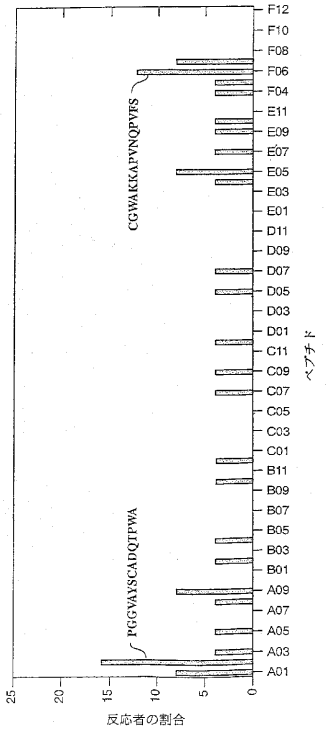


図１３Ａ

```
          1          2          3          4          5
1234567890 1234567890 1234567890 1234567890 1234567890
MRSSPLLPSA VVAALPVLAL AADGRSTRYW DCCKPSCGWA KKAPVNQPVF
SCNANPQRIT DFDAKSGCEP GGVAYSCADQ TPWAVNDDFA LGFAATSIAG
SNEAGWCCAC YELTFTSGPV AGKKMVVQST STGGDLGSNH FDLNIPGGGV
GIFDGCTPQF GGLPGQRYGG ISSRNECDRF PDALKPGCYW RFDWFKNADN
PSFSFRQVQC PAELVARTGC RRNDDGNFPA VQIPSSSTSS PVNQPTSTST
TSTSTTSSPP VQPTTPSGCT AERWAQ
```

図１３Ｂ

【図１４】

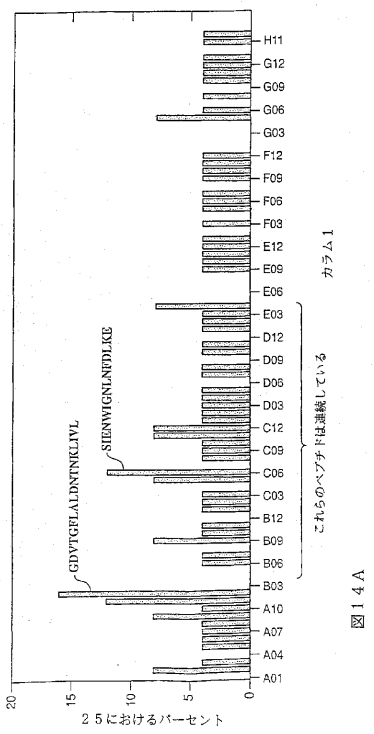


図１４Ａ

```
  1 mrsslvlffv sawtalaspi rrevsqdlfn qfnlfaqysa aaycgknnda
 51 pagtnitctg nacpevekad atflysfeds gvgdvtgfla ldntnklivl
101 sfrgsrsien wiqnlnfdlk eindicsgcr ghdgftsswr svadtlrqkv
151 edavrehpdy rvvftghslg galatvagad lrgngydidv fsygaprvgn
201 rafaefltvq tggtlyrith tndivprlpp refgyshssp eywiksgtlv
251 pvtrndivki egidatggnn qpnipdipah lwyfgligtc l
```

図１４Ｂ

【図１５】

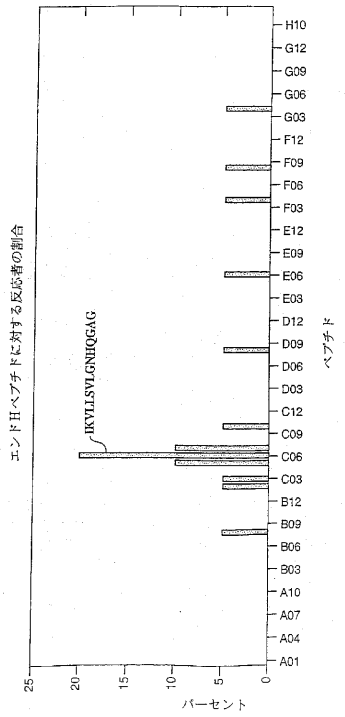


図１５Ａ

```
  1 mftpvrrrvr taalalsaaa alvlgstaas gasatpspap apapapvkqg
 51 ptsvayvevn nnsmlnvgky tladgggnaf dvavifaani nydtgtktay
101 lhfnenvqrv ldnavtqirp lqqqgikvll gvlqnhqqaq fanfpsqqaa
151 safakqlsda vakygldgvd fddeyaeygn ngtaqpndss fvhlvtalra
201 nmpdkiisly nigpaasrls yggvdvsdkf dyawnpyygt wqvpgialpk
251 aqlspaavei grtsrstvad larrtvdegy gvyltynldg gdrtadvsaf
301 trelygseav rtp
```

図１５Ｂ

【図１６】

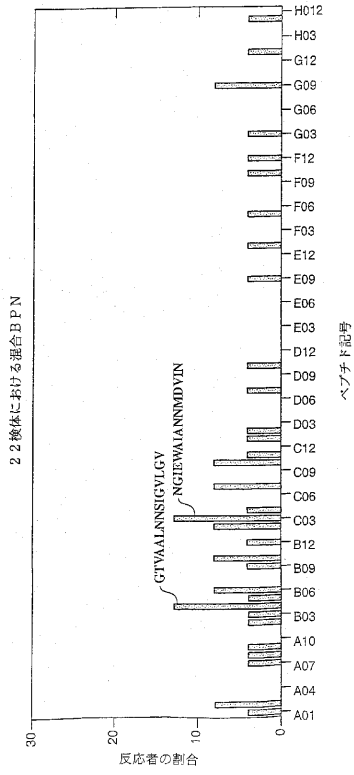


図１６

【図１７】

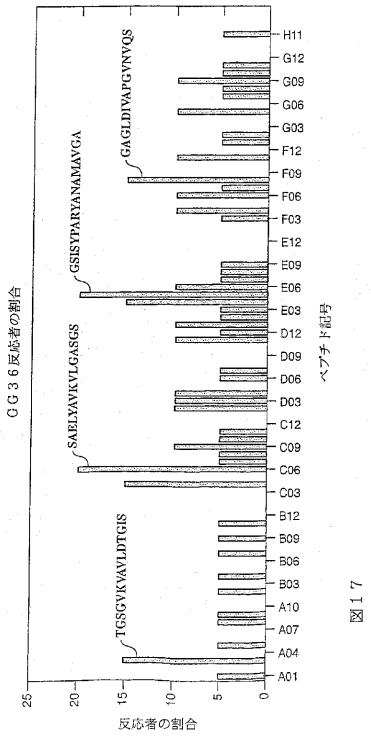
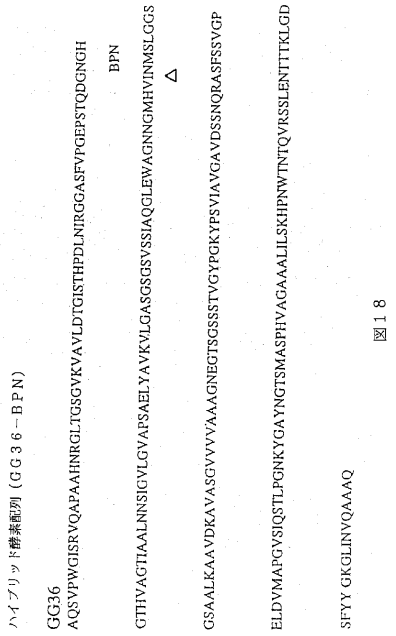


図１７

【図１８】



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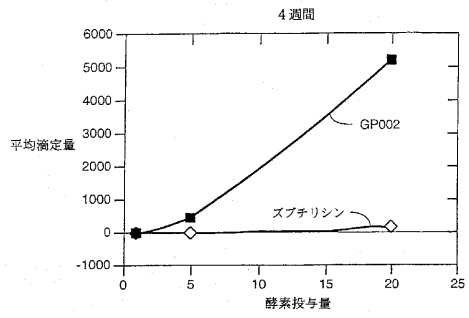

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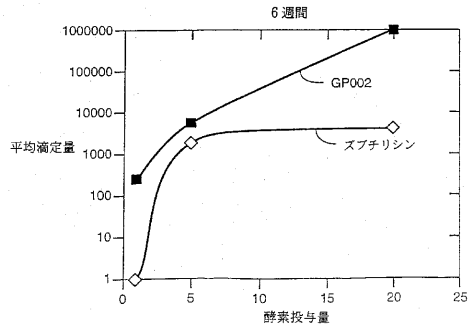

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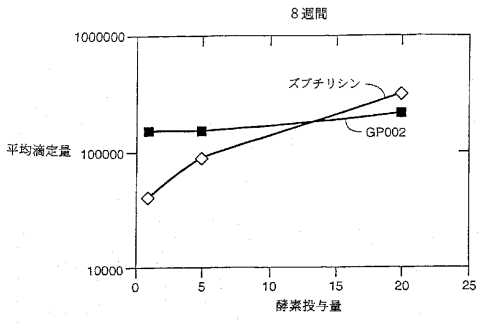

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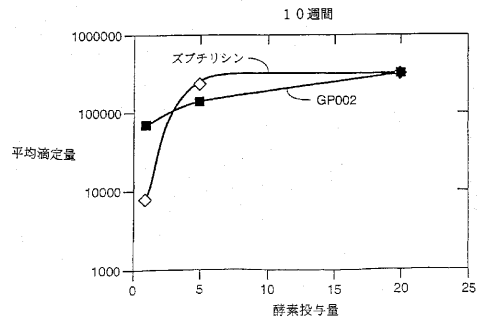

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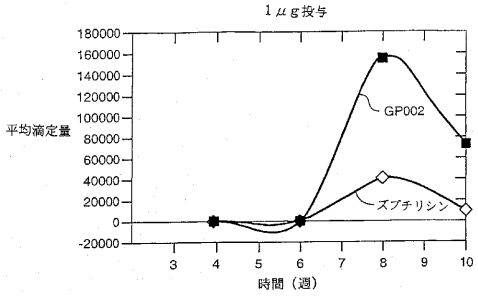

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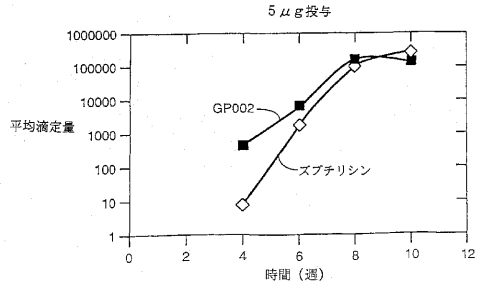

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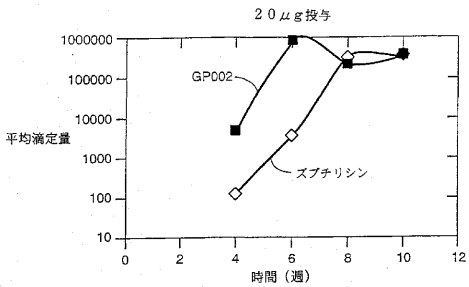

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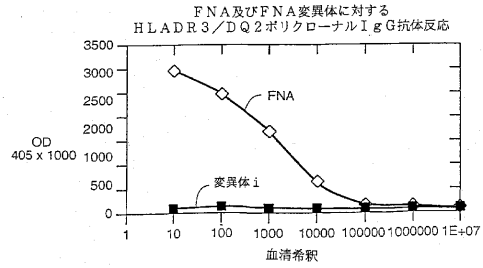

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(71) Applicant *(for all designated States except US)*: **GENENCOR INTERNATIONAL, INC.** [US/US]; 925 Page Mill Road, Palo Alto, CA 94304 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ESTELL, David, A.** [UG/UG]; 248 Woodbridge Circle, San Mateo, CA 94403 (US). **HARDING, Fiona, A.** [US/US]; 772 Lewis Street, Santa Clara, CA 95050 (US).

(74) Agent: **STONE, Christopher, L.**; GENENCOR INTERNATIONAL, INC., 925 Page Mill Road, Palo Alto, CA 94304 (US).

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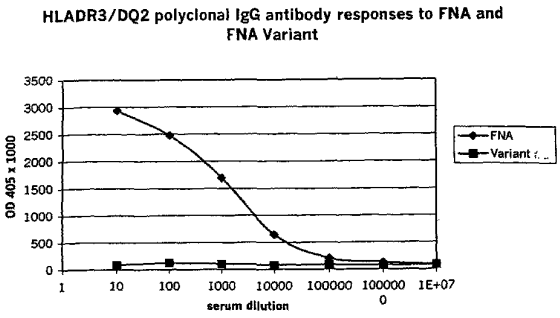


(57) Abstract: The present invention relates to a novel methods and compositions for producing hyper and hypo allergenic compositions. Specifically, the present invention comprises neutralizing or reducing the ability of T-cells to recognize epitopes and thus prevent sensitization of an individual to the protein. Alternatively, T-cell epitopes are mutated to produce increased immunogenic reactions.

WO 02/40997 A2

PROTEINS PRODUCING AN ALTERED IMMUNOGENIC RESPONSE AND METHODS OF MAKING AND USING THE SAME

BACKGROUND OF THE INVENTION

Proteins used in industrial, pharmaceutical and commercial applications are of increasing prevalence. As a result, the increased exposure due to this prevalence has been responsible for some safety hazards caused by the sensitization of certain persons to those peptides, whereupon subsequent exposure causes extreme allergic reactions which can be injurious and even fatal. For example, proteases are known to cause dangerous hypersensitivity in some individuals. As a result, despite the usefulness of proteases in industry, e.g., in laundry detergents, cosmetics, textile treatment etc., and the extensive research performed in the field to provide improved proteases which have, for example, more effective stain removal under detergency conditions; the use of proteases in industry has been problematic due to their ability to produce a hypersensitive allergenic response in some humans.

Much work has been done to alleviate these problems. Among the strategies explored to reduce immunogenic potential of protease use have been improved production processes which reduce potential contact by controlling and minimizing workplace concentrations of dust particles or aerosol carrying airborne protease, improved granulation processes which reduce the amount of dust or aerosol actually produced from the protease product, and improved recovery processes to reduce the level of potentially allergenic contaminants in the final product. However, efforts to reduce the allergenicity of protease, per se, have been relatively unsuccessful. Alternatively, efforts have been made to mask epitopes in protease which are recognized by immunoglobulin E (IgE) in hypersensitive individuals (PCT Publication No. WO 92/10755) or to enlarge or change the nature of the antigenic determinants by attaching polymers or peptides/proteins to the problematic protease.

When an adaptive immune response occurs in an exaggerated or inappropriate form, the individual experiencing the reaction is said to be hypersensitive. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately and sometimes cause inflammatory reactions and tissue damage. They can be provoked by many antigens; and the cause of a hypersensitivity reaction will vary from one individual to the next. Hypersensitivity does not normally manifest itself upon first contact with the antigen, but usually appears upon subsequent contact. One form of hypersensitivity occurs when an IgE response is directed against innocuous environmental antigens, such as pollen, dust-mites or animal dander. The resulting release of pharmacological mediators by IgE-sensitized mast cells produces an acute inflammatory reaction with symptoms such as asthma or rhinitis.

Nonetheless, a strategy comprising modifying the IgE sites will not generally be successful in preventing the cause of the initial sensitization reaction. Accordingly, such strategies, while perhaps neutralizing or reducing the severity of the subsequent hypersensitivity reaction, will not reduce the number or persons actually sensitized. For example, when a person is known to be hypersensitive to a certain antigen, the general, and only safe, manner of dealing with such a

situation is to isolate the hypersensitive person from the antigen as completely as possible. Indeed, any other course of action would be dangerous to the health of the hypersensitive individual. Thus, while reducing the danger of a specific protein for a hypersensitive individual is important, for industrial purposes it would be far more valuable to render a protein incapable of initiating the hypersensitivity reaction in the first place.

T-lymphocytes (T-cells) are key players in the induction and regulation of immune responses and in the execution of immunological effector functions. Specific immunity against infectious agents and tumors is known to be dependent on these cells and they are believed to contribute to the healing of injuries. On the other hand, failure to control these responses can lead to auto aggression. In general, antigen is presented to T-cells in the form of antigen presenting cells which, through a variety of cell surface mechanisms, capture and display antigen or partial antigen in a manner suitable for antigen recognition by the T-cell. Upon recognition of a specific epitope by the receptors on the surface of the T-cells (T-cell receptors), the T-cells begin a series of complex interactions, including proliferation, which result in the production of antibody by B-cells. While T-cells and B-cells are both activated by antigenic epitopes which exist on a given protein or peptide, the actual epitopes recognized by these mononuclear cells are generally not identical. In fact, the epitope which activates a T-cell to initiate the creation of immunologic diversity is quite often not the same epitope which is later recognized by B-cells in the course of the immunologic response. Thus, with respect to hypersensitivity, while the specific antigenic interaction between the T-cell and the antigen is a critical element in the initiation of the immune response to antigenic exposure, the specifics of that interaction, i.e., the epitope recognized, is often not relevant to subsequent development of a full blown allergic reaction.

PCT Publication No. WO 96/40791 discloses a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity using polyalkylene oxide as a starting material.

PCT Publication No. WO 97/30148 discloses a polypeptide conjugate with reduced allergenicity which comprises one polymeric carrier molecule having two or more polypeptide molecules coupled covalently thereto.

PCT Publication No. WO 96/17929 discloses a process for producing polypeptides with reduced allergenicity comprising the step of conjugating from 1 to 30 polymolecules to a parent polypeptide.

PCT Publication No. WO 92/10755 discloses a method of producing protein variants evoking a reduced immunogenic response in animals. In this application, the proteins of interest, a series of proteases and variants thereof, were used to immunize rats. The sera from the rats was then used to measure the reactivity of the polyclonal antibodies already produced and present in the immunized sera to the protein of interest and variants thereof. From these results, it was possible to determine whether the antibodies in the preparation were comparatively more or less reactive with the protein and its variants, thus permitting an analysis of which changes in the protein are likely to neutralize or reduce the ability of the Ig to bind. From these tests on rats, the conclusion was arrived at that changing any of subtilisin 309 residues corresponding to 127, 128, 129, 130, 131,

151, 136, 151, 152, 153, 154, 161, 162, 163, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 186, 193, 194, 195, 196, 197, 247, 251, 261 will result in a change in the immunological potential.

PCT Publication No. WO 94/10191 discloses low allergenic proteins comprising oligomeric forms of the parent monomeric protein, wherein the oligomer has substantially retained its activity.

While some studies have provided methods of reducing the allergenicity of certain proteins and identification of epitopes which cause allergic reactions in some individuals, the assays used to identify these epitopes generally involve measurement of IgE and IgG antibody in blood sera previously exposed to the antigen. However, once an Ig reaction has been initiated, sensitization has already occurred. Accordingly, there is a need for a method of determining epitopes which cause sensitization in the first place, as neutralization of these epitopes will result in significantly less possibility for sensitization to occur, thus reducing the possibility of initial sensitization. There is also a need to produce proteins which produce an enhanced immunogenic response, and a need to identify naturally occurring proteins which produce a low immunogenic response. This invention meets these and other needs.

SUMMARY OF THE INVENTION

The present invention provides proteins which produce immunogenic responses as desired, methods of identifying and making such proteins, and methods of using such proteins. For example, as will be become apparent from the detailed description below, the methods and compositions provided herein are useful in forming hyper- and hypo-allergenic compositions. As used herein, hyper and hypo means the composition produces a greater or lesser immunogenic response, respectively, than the same composition without the proteins of the present invention. Such compositions may include cleaning compositions, textile treatments, contact lens cleaning solutions or products, peptide hydrolysis products, waste treatment products, cosmetic formulations including for skin, hair and oral care, pharmaceuticals such as blood clot removal products, research products such as enzymes and therapeutics including vaccines.

In one aspect of the invention, a polypeptide of interest is selected and provided herein. The polypeptide of interest is preferably one having a T-cell epitope and is then varied as described below. However, polypeptides of interest may also be selected based on naturally occuring properties and not altered. Moreover, polypeptides of interest may be selected which do not have a T-cell epitope, and altered so as to have a T-cell epitope.

In one aspect of the invention provided herein is a variant of a polypeptide of interest comprising a T-cell epitope. The variant differs from the polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual. The variant can be prepared and selected to produce either a greater or lesser immunogenic response than said polypeptide of interest.

The polypeptide of interest can be any polypeptide of interest. In one aspect, the polypeptide is selected from the group consisting of enzymes, hormones, factors, vaccines and cytokines. In one embodiment, the polypeptide of interest is not recognized by said individual as endogenous to said individual, or not recognized as "self". As indicated herein, the polypeptide of

interest may be an enzyme. In one embodiment, the enzyme is selected from the group consisting of lipase, cellulase, endo-glucosidase H, protease, carbohydrase, reductase, oxidase, isomerase, transferase, kinase and phosphatase. In preferred embodiments, the polypeptide of interest and the variant of said polypeptide of interest each comprise at least some of the same activity. For example, if a variant of a protease is provided, said variant will produce an altered immunogenic response, but will retain detectable, and preferably comparable, protease activity.

Wherein a variant of a polypeptide of interest is provided, the T-cell epitope may be altered in a number of ways including by amino acid substitutions, deletions, additions and combinations thereof. Preferably, the T-cell epitope is altered by having amino acid substitutions. In one embodiment herein, the amino acid substitutions are made to corresponding amino acids of a homolog of the polypeptide of interest, wherein the homolog does not comprise the same T-cell epitope in the corresponding position as the polypeptide of interest. In one aspect, the terminal portion of the polypeptide of interest comprising at least one T-cell epitope is replaced with a corresponding terminal portion of the homolog of the polypeptide of interest, wherein the replacement produces said different immunogenic response.

In another embodiment provided herein, the nucleic acids encoding the polypeptides producing the desired immunogenic response are provided herein. Moreover, the invention includes expression vectors and host cells comprising the nucleic acids provided herein. Moreover, once the polypeptides and variants thereof of the present invention are identified, substantially homologous sequences of or those sequences which hybridize to the polypeptides and variants can be identified and are provided herein. Homologous is further defined below, and can refer to similarity or identity, with identity being preferred. Preferably, the homologous sequences are amino acid sequences or nucleic acids encoding peptides having the activity of the polypeptides and variants provided herein.

In yet another aspect of the invention is a method for determining the immunogenic response produced by a protein. In one embodiment, the method comprises (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with said protein; and (d) measuring the proliferation of T-cells in said step (c).

The methods of determining immunogenic responses produced by proteins can also be used to identify comparative immunogenic responses of proteins. Therefore, in one aspect, the method of determining immunogenic responses of proteins further comprises comparing immunogenic responses of one or more proteins. The proteins can be homologs of each other, variants of the same protein, different types of the same protein, for example, different proteases, or different peptides of the same protein.

The invention further provides a method of altering the immunogenicity of a polypeptide of interest comprising determining the immunogenicity of said polypeptide; identifying a T-cell epitope in a said polypeptide; and altering said T-cell epitope so as to alter the immunogencity of said polypeptide. As described herein, said altering can be done by altering a single amino acid or

switching a portion of the polypeptide of interest with a corresponding portion of a homolog, wherein the switch produces an altered immunogenic response.

Other aspects of the invention will be understood by the skilled artisan by the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A, B1, B2 and B3 illustrate the DNA (SEQ ID:NO 1) and amino acid (SEQ ID: NO 2) sequence for *Bacillus amyloliquefaciens* subtilisin (BPN') and a partial restriction map of this gene.

Fig. 2 illustrates the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (SEQ ID:NO 3) and *Bacillus lentus* (wild-type) (SEQ ID:NO 4).

Figs. 3A and 3B illustrate an amino acid sequence alignment of subtilisin type proteases from *Bacillus amyloliquefaciens* (BPN'), *Bacillus subtilis*, *Bacillus licheniformis* (SEQ ID:NO 5) and *Bacillus lentus*. The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 illustrates the additive T-cell response of 16 peripheral mononuclear blood samples to peptides corresponding to the *Bacillus lentus* protease (GG36). Peptide E05 includes the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 5 illustrates the additive T-cell response of 10 peripheral mononuclear blood samples to peptides corresponding to the human subtilisin molecule. Peptides F10, F9, F8 and F7 all contain the amino acid sequence DQMD corresponding to the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens* in the sequence alignment of Fig. 3.

Fig. 6A and 6B/6C illustrate amino acid strings corresponding to peptides derived from the sequence of *Bacillus lentus* protease and a human subtilisin, respectively.

Fig. 7 illustrates the amino acid sequence of human subtilisin (SEQ ID:NO 6).

Fig. 8 illustrates an amino acid sequence alignment of BPN' (*Bacillus amyloliquefaciens*) protease, SAVINASE (*Bacillus lentus*) protease and human subtilisin (S2HSBT).

Fig. 9 illustrates the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 10 illustrates the T-cell response to various alanine substitutions in the E05 *Bacillus lentus* protease peptide set in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease.

Fig. 11 illustrates the T-cell response to various alanine substitutions in the E05 protease peptide (an embodiment of the T-cell epitope designated unmodified sequence) set in a sample taken from an individual known to be hypersensitive to the protease; the sequences for each peptide are also shown.

Fig. 12 illustrates the percent responders to the human subtilisin molecule.

Fig. 13A illustrates the T-cell response of peptides derived from *Humicola insolens* endogluconase (Accession number A23635). Peptides A02 and F06 represent the region corresponding to residues 70-84 and 37-51, respectively, embodiments of the T-cell epitope, of

Humicola insolens endogluconase, wherein the full length sequence is shown in Fig.13B and A02 and F06 are shown underlined and in bold.

Fig. 14A illustrates the T-cell response to peptides derived from *Thermomyces lanuginosa* lipase (Accession number AAC08588 and PID number g2997733). Peptides B02 and C06 represent the regions corresponding to residues 83-100 and 108-121, respectively, embodiments of the T-cell epitope, of *Thermomyces lanuginosa* lipase, wherein the full length sequence is shown in Fig.14B and B02 and C06 are shown underlined and in bold.

Fig. 15A illustrates the T-cell response to peptides derived from *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase. (Accession number P04067). Peptide C06 represents the region corresponding to residues 126-140, an embodiment of the T-cell epitope, of *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase, wherein the full length sequence is shown in Fig.15B and C06 is shown underlined and in bold.

Fig. 16 illustrates the T-cell response to peptides derived from BPN' compiled for 22 individuals, wherein the sequences of preferred T-cell epitopes are indicated.

Fig. 17 illustrates the T-cell response to peptides derived from GG36 compiled for 22 individuals, wherein the sequences of embodiments of T-cell epitopes are indicated, GSISYPARYANAMAVGA and GAGLDIVAPGVNVQS being preferred.

Fig. 18 is an embodiment of a hybrid protein provided herein, where the N-terminus comprises N-terminal GG36 sequence and the C-terminus comprises C-terminal BPN' sequence, and wherein a comparison of the sequences with those shown in Fig. 8 indicates that the hybrid formed omits preferred T-cell epitopes of each protein.

Figure 19 is a comparison of ELISA titers for *B. amyloliquefaciens* subtilisin and the same subtilisin but engineered to contain a T-cell epitope from *B. lentis* subtilisin. Figure 19a represents the titer at 4 weeks; Figure 19b at 6 weeks, Figure 19c at 8 weeks and Figure 19d at 10 weeks.

Figure 20 is a time course study of ELISA titers for *B. amyloliquefaciens* subtilisin and the same subtilisin but engineered to contain a T-cell epitope from *B. lentis* subtilisin. Figure 20a represents the titer for a 1μg dose of enzyme, Figure 20b a 5 μg dose and Figure 20c a 20 μg dose.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method for identifying T-cell epitopes is provided. Moreover, proteins including naturally occurring proteins which have relatively impotent or potent T-cell epitopes or no T-cell epitopes may be identified in accordance with the methods of the present invention. Thus, the present invention allows the identification and production of proteins which produce immunogenic responses as desired, including naturally occurring proteins as well as proteins which have been mutated to produce the appropriate response. It is understood that the terms protein, polypeptide and peptide are sometimes used herein interchangeably. Wherein a peptide is a portion of protein, the skilled artisan can understand this by the context in which the term is used.

In one embodiment, the present invention provides an assay which identifies epitopes and non-epitopes as follows: differentiated dendritic cells are combined with naïve human CD4+ and/or

CD8+ T-cells and with a peptide of interest. More specifically, a method is provided wherein a T-cell epitope is recognized comprising the steps of: (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest; (d) measuring the proliferation of T-cells in said step (c).

In one embodiment, the peptide of interest to be analyzed is derived from a polypeptide of interest. In the practice of the invention, it is possible to identify with precision the location of an epitope which can cause sensitization in an individual or sampling of individuals. In a preferred embodiment of the invention, a series of peptide oligomers which correspond to all or part of the polypeptide of interest are prepared. For example, a peptide library is produced covering the relevant portion or all of the protein. In one embodiment, the manner of producing the peptides is to introduce overlap into the peptide library, for example, producing a first peptide corresponds to amino acid sequence 1-10 of the subject protein, a second peptide corresponds to amino acid sequence 4-14 of the subject protein, a third peptide corresponds to amino acid sequence 7-17 of the subject protein, a fourth peptide corresponds to amino acid sequence 10-20 of the subject protein etc. until representative peptides corresponding to the entire molecule are created. By analyzing each of the peptides individually in the assay provided herein, it is possible to precisely identify the location of epitopes recognized by T-cells. In the example above, the greater reaction of one specific peptide than its neighbors' will facilitate identification of the epitope anchor region to within three amino acids. After determining the location of these epitopes, it is possible to alter the amino acids within each epitope until the peptide produces a different T-cell response from that of the original protein. Alternatively, the epitope may be used in its original form to stimulate an immune response against a target, e.g. infectious agent or tumor cell. Moreover, proteins may be identified herein which have desired high or low T-cell epitope potency which may be used in their naturally occurring forms.

"Antigen presenting cell" as used herein means a cell of the immune system which present antigen on their surface which is recognizable by receptors on the surface of T-cells. Examples of antigen presenting cells are dendritic cells, interdigitating cells, activated B-cells and macrophages.

"T-cell proliferation" as used herein means the number of T-cells produced during the incubation of T-cells with the antigen presenting cells, with or without antigen.

"Baseline T-cell proliferation" as used herein means T-cell proliferation which is normally seen in an individual in response to exposure to antigen presenting cells in the absence of peptide or protein antigen. For the purposes herein, the baseline T-cell proliferation level was determined on a per sample basis for each individual as the proliferation of T-cells in response to antigen presenting cells in the absence of antigen.

"T-cell epitope" means a feature of a peptide or protein which is recognized by a T-cell receptor in the initiation of an immunologic response to the peptide comprising that antigen. Recognition of a T-cell epitope by a T-cell is generally believed to be via a mechanism wherein T-cells recognize peptide fragments of antigens which are bound to class I or class II major

histocompatability (MHC) molecules expressed on antigen-presenting cells (*see e.g.*, Moeller, G. ed., "Antigenic Requirements for Activation of MHC-Restricted Responses," *Immunological Review*, Vol. 98, p. 187 (Copenhagen; Munksgaard) (1987).

"Sample" as used herein comprises mononuclear cells which are naïve, *i.e.*, not sensitized, to the antigen in question.

"Homolog" as used herein means a protein or enzyme which has similar catalytic action, structure and/or use as the protein of interest. For purposes of this invention, a homolog and a protein of interest are not necessarily related evolutionarily, *e.g.*, same functional protein from different species. It is desirable to find a homolog that has a tertiary and/or primary structure similar to the protein of interest as replacement of the epitope in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change. Thus, closely homologous enzymes will provide the most desirable source of epitope substitutions. Alternatively, if possible, it is advantageous to look to human analogs for a given protein. For example, substituting a specific epitope in a bacterial subtilisin with a sequence from a human analog to subtilisin (*i.e.*, human subtilisin) should result in less allergenicity in the bacterial protein.

An "analogous" sequence may be determined by ensuring that the replacement amino acids show a similar function, the tertiary structure and/or conserved residues to the amino acids in the protein of interest at or near the epitope. Thus, where the epitope region contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids should maintain that specific structure.

The epitopes determined according to the assay provided herein are then modified to reduce or augment the immunologic potential of the protein of interest. In a preferred embodiment, the epitope to be modified produces a level of T-cell proliferation of greater than three times the baseline T-cell proliferation in a sample. When modified, the epitope produces less than three times the baseline proliferation, preferably less than two times the baseline proliferation and most preferably less than or substantially equal to the baseline proliferation in a sample.

Preferably, the epitope is modified in one of the following ways: (a) the amino acid sequence of the epitope is substituted with an analogous sequence from a human homolog to the protein of interest; (b) the amino acid sequence of the epitope is substituted with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; (c) the amino acid sequence of the epitope is substituted with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but which produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; or (d) with any sequence which produces lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest.

However, one of skill will readily recognize that epitopes can be modified in other ways depending on the desired outcome. For example, if a T-cell vaccine is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids which increase the immulogic response to the peptide via enhanced MHC binding and/or T-cell recognition. In another

example, if altering an autoimmune response against self -antigens is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids that decrease or cause a shift in an inflammatory or other immune response.

The present invention extends to all proteins against which it is desired to modulate the immunogenic response, for example, peptides to be used as T-cell vaccines, or peptides or proteins to be used as therapeutic agents against, *e.g.,* cancer, infectious diseases and autoimmune diseases. One of skill in the art will readily recognize the proteins and peptides of this invention are not necessarily native proteins and peptides. Indeed, in one embodiment of this invention, the assay described herein is used to determine the immunologic response of proteins from shuffled genes. For descriptions of gene shuffling and expression of such genes see, Stemmer, *Proc. Nat'l Acad. Sci. USA* 91:10747 (1994); Patten, *et al., Current Opinion in Biotechnol.* 8:724 (1997); Kuchner & Arnold, *Trends Biotechnol.* 15:523 (1997); Moore, *et al., J. Mol, Biol.* 272:336 (1997); Zhao, *et al., Nature Biotechnol.* 16:258 (1998); Giver, *et al., Proc. Nat'l Acad. Sci. USA* 95:12809 (1998); Harayama, *Trends Biotechnol.* 16:76 (1998); Lin, *et al., Biotechnol., Prog.* 15:467 (1999); and Sun, *J. Comput. Biol.* 6:77 (1999). The assay is used to predict the immunologic response of proteins encoded by shuffled genes. Once determined, the protein can be altered to modulate the immunolgic response to that protein.

In addition to the above proteins and peptides, the present invention can be used to reduce the allergenicity of proteins. These proteins include, but are not limited to, glucanases, lipases, cellulases, endo-glucosidase Hs (endo-H), proteases, carbohydrases, reductases, oxidases, isomerases, transferases, kinases, phosphatases, amylases, etc. In addition to reducing the allergenicity to an animal, such as a human, of naturally occurring amino acid sequences, this invention encompasses reducing the allergenicity of a mutated human protein, *e.g.,* a protein that has been altered to change the functional activity of the protein. In many instances, the mutation of human proteins to *e.g.,* increase activity, results in the incorporation of new T-cell epitope in the mutated protein. The assay of this invention can be used to determine the presence of the new T-cell epitope and determine substitute amino acids that will reduce the allergenicity of the mutated protein.

Although this invention encompasses the above proteins and many others, for the sake of simplicity, the following will describe a particularly preferred embodiment of the invention, the modification of protease. Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include a-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

In one embodiment herein, hybrid polypeptides are provided. "Hybrid polypeptides" are proteins engineered from at least two different proteins, which are preferably homologs of one another. For example, a preferred hybrid polypeptide might have the N-terminus of a protein and the C-terminus of a homolog of the protein. In a preferred embodiment, the two terminal ends can be

combined to correspond to the full-length active protein. In a preferred embodiment, the homologs share substantial similarity but do not have identical T-cell epitopes. Therefore, in one embodiment, for example, a polypeptide of interest having one or more T-cell epitopes in the C-terminus may have the C-terminus replaced with the C-terminus of a homolog having a less potent T-cell epitope in the C-terminus, less T-cell epitopes, or no T-cell epitope in the C-terminus. Thus, the skilled artisan understands that by being able to identify T-cell epitopes among homologs, a variety of variants producing different immunogenic responses can be formed. Moreover, it is understood that internal portions, and more than one homolog can be used to produce the variants of the present invention.

More generally, the variants provided herein can be derived from the precursor amino acid sequence by the substitution, deletion, insertion, or combination thereof of one or more amino acids of the precursor amino acid sequence. Such modification is preferably of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor enzyme, but can be by the manipulation of the precursor protein. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1.

"Recombinant", "recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram

negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

"Human subtilisin" means proteins of human origin which have subtilisin type catalytic activity, e.g., the kexin family of human derived proteases. An example of such a protein is represented by the sequence in Fig. 7. Additionally, derivatives or homologs of proteins provided herein, including those from non-human sources such as mouse or rabbit, which retain the essential activity of the peptide, such as the ability to hydrolyze peptide bonds, etc., have at least 50%, preferably at least 65% and most preferably at least 80%, more preferably at least 90%, and sometimes as much as 95 or 98% homology to the polypeptide of interest. In one embodiment, the polypeptide of interest is shown in the Figures.

The amino acid position numbers used herein refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically). "Corresponding" as used herein generally refers to an analogous position along the peptide.

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which the sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* can be aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. The conserved residues as between BPN' and *B. lentus* are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +170 is lysine (K) in both *B. amyloliquefaciens* and *B. licheniformis* subtilisins and arginine (R) in Savinase. In one embodiment of the protease variants of the invention, however, the amino acid equivalent to +170 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartic acid (D). The abbreviations and one letter codes for all amino acids in the present invention conform to the PatentIn User Manual (GenBank, Mountain View, CA) 1990, p.101.

Homologous sequences can also be determined by using a "sequence comparison algorithm." Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, an amino acid sequence is considered similar to a protein such as a protease if the smallest sum probability in a comparison of the test amino acid sequence to a protein such as a protease amino acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protein whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protein such as the protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protein such as the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protein such as a protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protein, for example, protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The variants of the present invention include the mature forms of protein variants, as well as the pro- and prepro- forms of such protein variants. The prepro- forms are the preferred construction since this facilitates the expression, secretion and maturation of the protein variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protein which when removed results in the appearance of the "mature" form of the protein. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protein variants such as protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protein or to the N-terminal portion of a proprotein which may participate in the secretion of the mature or pro forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protein gene which participate in the effectuation of the secretion of protein under native conditions. The present invention utilizes such sequences to effect the secretion of the protein variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protein variant consists of the mature form of the protein having a prosequence operably linked to the amino terminus of the protein and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protein is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protein and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protein include *Bacillus subtilis* I168 (also described in US Patent 4,760,025 (RE 34,606) and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. These techniques can be found in any molecular biology practice guide, for example,

Sambrook *et al.* Molecular Cloning - A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Springs Harbor Publishing (1989) ("Sambrook"); and Current Protocols in Molecular Biology, Ausubel *et al.* (eds.), Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1997 Supplement) ("Ausubel"). Such transformed host cells are capable of either replicating vectors encoding the protein variants or expressing the desired protein variant. In the case of vectors which encode the pre- or prepro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked", when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protein may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protein of interest, preparing genomic libraries from organisms expressing the protein, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

"Hybridization" is used to analyze whether a given DNA fragment or gene corresponds to a DNA sequence described herein and thus falls within the scope of the present invention. Samples to be hybridized are electrophoresed through an agarose gel (for example, 0.8% agarose) so that separation of DNA fragments can be visualized by size. DNA fragments are typically visualized by ethidium bromide staining. The gel may be briefly rinsed in distilled H2O and subsequently depurinated in an appropriate solution (such as, for example, 0.25M HCl) with gentle shaking followed by denaturation for 30 minutes (in, for example, 0.4 M NaOH) with gentle shaking. A renaturation step may be included, in which the gel is placed in 1.5 M NaCl, 1MTris, pH 7.0 with gentle shaking for 30 minutes.

The DNA should then be transferred onto an appropriate positively charged membrane, for example, Maximum Strength Nytran Plus membrane (Schleicher & Schuell, Keene, N.H.), using a transfer solution (such as, for example, 6XSSC (900 mM NaCl, 90 mM trisodium citrate). Once the transfer is complete, generally after about 2 hours, the membrane is rinsed in *e.g.*, 2X SSC (2X SSC = 300 mM NaCl, 30 mM trisodium citrate) and air dried at room temperature. The membrane should then be prehybridized (for approximately 2 hours or more) in a suitable prehybridization solution (such as, for example, an aqueous solution containing per 100 mL: 20-50 mL formamide, 25 mL of 20X SSPE (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH2PO4, pH 7.7), 2.5 mL of 20% SDS, and 1 mL of 10 mg/mL sheared herring or salmon sperm DNA). As would be known to one of skill in the art, the amount of formamide in the prehybridization solution may be varied depending on the nature of the reaction obtained according to routine methods. Thus, a lower amount of formamide may result in more complete hybridization in terms of identifying hybridizing molecules than the

same procedure using a larger amount of formamide. On the other hand, a strong hybridization band may be more easily visually identified by using more formamide.

A DNA probe that is complementary or is nearly complementary to the DNA sequence of interest and is generally between 100 and 1000 bases in length is labeled (using, for example, the Megaprime labeling system according to the instructions of the manufacturer) to incorporate 32P in the DNA. The labeled probe is denatured by heating to 95°C for 5 minutes and immediately added to the membrane and prehybridization solution. The hybridization reaction should proceed for an appropriate time and under appropriate conditions, for example, for 18 hours at 37°C with gentle shaking or rotating. The membrane is rinsed (for example, in 2X SSC/0.3% SDS) and then washed in an appropriate wash solution with gentle agitation. The stringency desired will be a reflection of the conditions under which the membrane (filter) is washed.

Specifically, the stringency of a given reaction (*i.e.*, the degree of homology necessary for successful hybridization) will depend on the washing conditions to which the filter is subjected after hybridization. "Low-stringency" conditions as defined herein will comprise washing a filter with a solution of 0.2X SSC/0.1% SDS at 20°C for 15 minutes. "High-stringency" conditions comprise a further washing step comprising washing the filter a second time with a solution of 0.2X SSC/0.1% SDS at 37°C for 30 minutes.

After washing, the membrane is dried and the bound probe detected. If 32P or another radioisotope is used as the labeling agent, the bound probe can be detected by autoradiography. Other techniques for the visualization of other probes are well-known to those of skill. The detection of a bound probe indicates a nucleic acid sequence has the desired homology and is encompassed within this invention.

The cloned protein is then used to transform a host cell in order to express the protein. The protein gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, *i.e.*, transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protein gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protein gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the protein gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

In one embodiment, the gene can be a natural gene such as that from *B lentus* or *B. amyloliquefaciens*. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protein may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor protein is determined. Multiple, overlapping synthetic single-stranded DNA fragments

are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protein. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protein gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protein. Such modifications include the production of recombinant proteins as disclosed in US Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of protein variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protein variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protein is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protein gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protein gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

In one aspect of the invention, the objective is to secure a variant protein having altered allergenic potential as compared to the precursor protein, since decreasing such potential enables safer use of the enzyme. While the instant invention is useful to lower allergenic potential, the mutations specified herein may be utilized in combination with mutations known in the art to result altered thermal stability and/or altered substrate specificity, modified activity or altered alkaline stability as compared to the precursor.

Accordingly, the present invention is directed to altering the capability of the T-cell epitope which includes residue positions 170-173 in *Bacillus lentus* to induce T-cell proliferation. One particularly preferred embodiment of the invention comprises making modification to either one or all of R170D, Y171Q and/or N173D. Similarly, as discussed in detail above, it is believed that the modification of the corresponding residues in any protein will result in a the neutralization of a key T-cell epitope in that protein. Thus, in combination with the presently disclosed mutations in the region corresponding to amino acid residues 170-173, substitutions at positions corresponding to N76D/S103A/V104I/G159D optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin may be used, in addition to decreasing the allergenic potential of the variant protein of the invention, to modulate overall stability and/or proteolytic activity of the enzyme. Similarly, the substitutions provided herein may be combined with mutation at the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 to Aspartate (D) in combination with the mutations S103A/V104I/G159D and optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin, to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred embodiments of the invention include the following specific combinations of substituted residues corresponding to positions:

N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H/Q245R;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and

V68A/N76D//S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protein.

Based on the screening results obtained with the variant proteins, the noted mutations noted above in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protein variants of the invention are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the protein mutants of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protein variants of the present invention may be used for any purpose that native or wild-type proteins are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis,

waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise, in addition to decreased allergenicity, enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteins, particularly proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteins, particularly proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protein's denaturing temperature. In addition, proteins of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The variant proteins of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteins of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The variants can be screened for proteolytic activity according to methods well known in the art. Preferred protease variants include multiple substitutions at positions corresponding to:
N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H/Q245R;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin.

The proteins of this invention exhibit modified immunogenicity when compared to their precursor proteins. In preferred embodiments, the proteins exhibit reduced allergenicity. In other embodiments, the proteins exhibit increased immunogenicity. The increase in immunogenicity is manifested by an increase in B-cell or humoral immunological response, by an increase in T-cell or cellular immunological response, or by an increase in both B and T cell immunological responses. One of skill will readily recognize that the uses of the proteins of this invention will be determined, in large part, on the immunological properties of the proteins. For example, enzymes that exhibit reduced allergenicity can be used in cleaning compositions. "Cleaning compositions" are compositions that can be used to remove undesired compounds from substrates, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin (soaps and creams), etc.

Proteins, in particular, cellulases, proteases, and amylases, with reduced allergenicity can also be used in the treatment of textiles. "Textile treatment" comprises a process wherein textiles, individual yarns or fibers that can be woven, felted or knitted into textiles or garments are treated to effect a desired characteristic. Examples of such desired characteristics are "stone-washing", depilling, dehairing, desizing, softening, and other textile treatments well known to those of skill in the art.

Therapeutic proteins against which individuals mount an immune response are also included in the invention. In particular, individuals who lack endogenous production of the protein are susceptible to forming neutralizing antibodies and become refractile to treatment. Likewise, modifications of a protein may introduce new epitopes that are potentially immunogeneic. Methods of the invention can be used to identify and modify epitopes in , e.g., human Factor VIII, to prevent neutralizing responses.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically_active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

All publications and patents referenced herein are hereby incorporated by reference in their entirety. The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

EXAMPLES

Example 1

Assay for the Identification of Peptide T-Cell Epitopes Using Naïve Human T-Cells

Fresh human peripheral blood cells were collected from "naïve" humans, *i.e.,* persons not known to be exposed to or sensitized to *Bacillus lentus* protease, for determination of antigenic epitopes in protease from *Bacillus lentus* and human subtilisin. Naïve humans is intended to mean that the individual is not known to have been exposed to or developed a reaction to protease in the past. Peripheral mononuclear blood cells (stored at room temperature, no older than 24 hours) were prepared for use as follows: Approximately 30 mls of a solution of buffy coat preparation from one unit of whole blood was brought to 50 ml with Dulbecco's phosphate buffered solution (DPBS) and split into two tubes. The samples were underlaid with 12.5 ml of room temperature lymphoprep density separation media (Nycomed density 1.077 g/ml). The tubes were centrifuged for thirty minutes at 600G. The interface of the two phases was collected, pooled and washed in DPBS. The

cell density of the resultant solution was measured by hemocytometer. Viability was measured by trypan blue exclusion.

From the resulting solution, a differentiated dendritic cell culture was prepared from the peripheral blood mononuclear cell sample having a density of 108 cells per 75 ml culture flask in a solution as follows:

(1) 50 ml of serum free AIM V media (Gibco) was supplemented with a 1:100 dilution beta-mercaptoethanol (Gibco). The flasks were laid flat for two hours at 37°C in 5% CO2 to allow adherence of monocytes to the flask wall.

(2) Differentiation of the monocyte cells to dendritic cells was as follows: nonadherent cells were removed and the resultant adherent cells (monocytes) combined with 30 ml of AIM V, 800 units/ml of GM-CSF (Endogen) and 500 units/ml of IL-4 (Endogen); the resulting mixture was cultured for 5 days under conditions at 37°C in 5% CO2. After five days, the cytokine TNFa (Endogen) was added to 0.2 units/ml, and the cytokine IL-1a (Endogen) was added to a final concentration of 50 units/ml and the mixture incubated at 37°C in 5% CO2 for two more days.

(3) On the seventh day, Mitomycin C was added to a concentration of 50 microgram/ml was added to stop growth of the now differentiated dendritic cell culture. The solution was incubated for 60 minutes at 37°C in 5% CO2. Dendritic cells were collected by gently scraping the adherent cells off the bottom of the flask with a cell scraper. Adherent and non-adherent cells were then centrifuged at 600G for 5 minutes, washed in DPBS and counted.

(4) The prepared dendritic cells were placed into a 96 well round bottom array at 2x104/well in 100 microliter total volume of AIM V media.

CD4+ T cells were prepared from frozen aliquots of the peripheral blood cell samples used to prepare the dendritic cells using the human CD4+ Cellect Kit (Biotex) as per the manufacturers instructions with the following modifications: the aliquots were thawed and washed such that approximately 108 cells will be applied per Cellect column; the cells were resuspended in 4 ml DPBS and 1 ml of the Cell reagent from the Cellect Kit, the solution maintained at room temperature for 20 minutes. The resultant solution was centrifuged for five minutes at 600G at room temperature and the pellet resuspended in 2 ml of DPBS and applied to the Cellect columns. The effluent from the columns was collected in 2% human serum in DPBS. The resultant CD4+ cell solution was centrifuged, resuspended in AIMV media and the density counted.

The CD4+ T-cell suspension was resuspended to a count of 2x106/ml in AIM V media to facilitate efficient manipulation of the 96 well plate.

Peptide antigen is prepared from a 1M stock solution in DMSO by dilution in AIM V media at a 1:10 ratio. 10 microliters of the stock solution is placed in each well of the 96 well plate containing the differentiated dendritic cells. 100 microliter of the diluted CD4+ T-cell solution as prepared

above is further added to each well. Useful controls include diluted DMSO blanks, and tetanus toxoid positive controls.

The final concentrations in each well, at 210 microliter total volume are as follows:

2x104 CD4+

2x105 dendtritic cells (R:S of 10:1)

5 mM peptide

Example 2

Identification of T-Cell Epitopes in Protease from *Bacillus lentus* and Human subtilisin

Peptides for use in the assay described in Example 1 were prepared based on the *Bacillus lentus* and human subtilisin amino acid sequence. Peptide antigens were designed as follows. From the full length amino acid sequence of either human subtilisin or *Bacillus lentus* protease provided in Figure 1, 15mers were synthetically prepared, each 15mer overlapping with the previous and the subsequent 15mer except for three residues.

Peptides used correspond to amino acid residue strings in *Bacillus lentus* as provided in Figure 8, and peptides correspond to amino acid residues in human subtilisin as provided in Figure 7. The peptides used corresponding to the proteases is provided in Fig. 6. All tests were performed at least in duplicate. All tests reported displayed robust positive control responses to the antigen tetanus toxoid. Responses were averaged within each experiment, then normalized to the baseline response. A positive event was recorded if the response was at least 3 times the baseline response.

The immunogenic response (*i.e.*, T-cell proliferation) to the prepared peptides from human subtilisin and *Bacillus lentus* was tallied and is provided in Figures 4 and 5, respectively. T-cell proliferation was measured by the incorporated tritium method. The results shown in Figures 4 and 5 as a comparison of the immunogenic additive response in 10 individuals (Figure 4) and 16 individuals (Figure 5) to the various peptides. Response is indicated as the added response wherein 1.0 equals a baseline response for each sample. Thus, in Figure 4, a reading of 10.0 or less is the baseline response and in Figure 5 a reading of 16.0 or less the baseline response. The greater the response, the more potent the T-cell epitope is considered.

As indicated in Figures 4 and 5, the immunogenic response of the naïve blood samples from unsensitized individuals showed a marked allergenic response at the peptide fragment from *Bacillus lentus* corresponding to residues 170-173 of *Bacillus amyloliquefaciens* protease. As expected, the corresponding fragment in human subtilisin evokes merely baseline response.

Fig. 9 shows the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens.* As shown in Fig. 9, the hypersensitive individual was highly responsive to the T-cell epitope represented by the peptide E05. This result confirms that, by practicing the assay according to the invention, it is possible to predict the major epitopes identified by the T-cells of a hypersensitive individual.

Fig. 10 shows the T-cell response to various alanine substitutions in the E05 peptide derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Alanine substitutions were used as substitutions for the purpose of determining the role of any specific residue within the epitope. The legend of Figure 10 refers to the position of the peptide in which an alanine was substituted, i.e., in peptide E06 (sequence GSISYPARYANAMAV), G to A = 2, S to A = 3, I to A = 4, S to A = 5, Y to A = 6, P to A = 7, R to A = 8, Y to A = 9, N to A = 10, M to A = 11 and V to A = 12. As indicated in Figure 10, substitution of either of the residues R170A, Y171A and/or N173A in protease from *Bacillus lentus* results in dramatically reduced response in the hypersensitive individual's blood sample.

From these results, it is apparent that the residues 170, 171 and 173 are largely responsible for the initiation of allergic reaction within the protease from *Bacillus lentus*.

Example 3

Identification of T-Cell Epitopes in Cellulase from *Humicola insolens* (Carezyme®)

The procedure described above was performed on peptides derived from a cellulase from *Humicola insolens* (Carezyme ® from Novo Nordisk). As can be seen from Figure 13, 2 T-cell epitopes were discovered, A01 and F06.

Example 4

Identification of T-Cell Epitopes in Lipase from *Thermomyces Lanuginosa* (Lipolase®)

The procedure described in Example 2 was performed on peptides derived from a lipase from *Thermomyces lanuginosa* (Lipolase ® from Novo Nordisk). As can be seen from Figure 14, two T-cell epitopes were discovered, A12 and C06. Peptide E03 effected slightly increased T-cell proliferation in the naïve donors, however, this peptide is consecutive to A12 and they represent one epitope. In this regard, the skilled artisan understands that the length of the epitopes can be varied, and the precise potency of the epitope, naturally occuring or mutated can be determined by the methods herein.

Example 5

Identification of T-Cell Epitopes in Endoglucanase H from Streptomyces plicatus

The procedure described in Example 2 was performed on peptides derived from endoglucanase H from *Streptomyces plicatus*. As can be seen from Figure 15, a single T-cell epitope was discovered, C06.

Example 6

Identification of T-Cell Epitopes in a Protease Hybrid (GG36-BPN')

After determining the location of a T-cell epitope, a protease hybrid was constructed using established protein engineering techniques. The hybrid was constructed so that a highly allergenic amino acid sequence of the protein was replaced with a corresponding sequence from a less

allergenic homolog. In this instance, the first 122 amino acids of the protease were derived from GG36, and the remaining amino acid sequence was derived from BPN'.

The hybrid was first tested from a 100 ppm sample in North American condition in 24 well assay at .5 ppm, superfixed swatches, liquid (Tide KT) at .5 in 24 well assay with 3K swatches, and in the N'N'-dimethyl Casein Assay, 5 g/l DMC in NA detergent, TNBS dectection method.

The results are shown in Figures 16, 17 and 18.

Example 7

Identification of a Naturally Occuring Low Immunogenic Protein

Using the methods herein, proteinase K was identified as producing a lower immunogenic response than other commercially available proteases. Proteinase K as identified herein is from Tritirachium Album limber. For a general description of proteases and methodologies, see, Mathew, C.G.P. Isolation of high molecular weight eukaryotic DNA, in Methods in Molecular Biology, vol. 2: Nucleic Acids (Walker, J.M.,ed.), Humana, Clifton, NJ, (1984) pp. 31-34.

Example 8:

T-cell Epitope Introduced into a Non-allergenic Protein

It has been found that *Bacillus amyloliquefaciens* subtilisin is comparatively non-immunogenic when tested in Hartley strain guinea pigs. A related protein from *Bacillus lentis* is highly immunogenic. We had previously defined functional T cell epitopes in the *B. lentis* molecule which were not found in the *B. amyloliquefaciens* molecule, even though the sequences of interest were highly homologous. In order to test the principle that the presence of a functional T cell epitope can control the relative levels of antibody production, we created a *B. lentis*-like T cell epitope in the *B. amyloliquefaciens* molecule. This change was accomplished by the substitution of a single amino acid in the *B. amyloliquefaciens* sequence. *B. amyloliquefaciens* subtilisin and the T cell epitope modified variant of *B. amyloliquefaciens* subtilisin were tested in a guinea pig model of immungenicity.

B. lentis and *B. amyloliquefaciens* subtilisin T cell epitope mapping: Guinea pigs were immunized with 20 µg/immunization of subtilisin from either *B. lentis* or *B. amyloliquefaciens*. Animals were immunized subcutaneously in adjuvant every two weeks for 10 to 12 weeks. A single cell suspension of guinea pig splenocytes was created from each animal's spleen. Cells were plated at 5×10^5 splenocytes per well in round bottom 96 well plates. 15-mer peptides off-set by 3 amino acids were synthesized by Mimotopes. Peptides were resuspended to 1 mM in DMSO. Peptides were added to the cells at a final concentration of 5 µM. Cultures were incubated for 5 days at 37 °, 5% CO_2. Wells were pulsed with 0.5 µCi tritiated thymidine, and allowed to incubate for an additional 18 hours. Wells were harvested, and thymidine incorporation assessed.

Two T cell epitopes were found in *B. lentis* subtilisin, and none were found in *B. amyloliquefaciens* subtilisin (>10 animals tested for these epitopes). The *B. lentis* T cell epitopes were found to comprise the following sequences:

IAALNNSIGVLGVAP (SEQ ID NO:237) and LEWAGNNGMHVANLSLGS (SEQ ID NO:238)

For SEQ ID NO:237, the similar sequence in *B. amyloliquefaciens* subtilisin is VAALNNSIGVLGVAP (SEQ ID NO:239). The similar region in *B. amyloliquefaciens* subtilisin for SEQ ID NO:238 was the much less homologous: IEWAIANNMDVINMSLG (SEQ ID NO:240).

SEQ ID NO:237 and the homologous region in the *B. amyloliquefaciens* subtilisin molecule (SEQ ID NO: 239) differ by one amino acid: In *B. lentis* subtilisin the first amino acid is an I, while it is a V in *B. amyloliquefaciens*. Therefore, we reasoned that if we changed the V in the *B. amyloliquefaciens* sequence to an I, we would create the *B. lentis* T cell epitope in the *B. amyloliquefaciens* backbone.

This molecule was created by standard molecular biological techniques, and was called *B. amyloliquefaciens* V72I. It was also known as GP002.

Guinea pig immunizations: Adult female Hartley guinea pigs were immunized with various doses of *B. amyloliquefaciens* subtilisin and GP002. The doses were 1, 5, 10, and 20 µg/dose. There were four animals for each dose. Animals were immunized subcutaneously with enzyme in Complete Freund's Adjuvant for the first immunization. All subsequent

immunizations were performed in Incomplete Freund's adjuvant. Animals were immunized, and a serum sample taken, every two weeks.

ELISA: A direct ELISA was performed. Costart EIA plates were coated with 10 µg/ml of the immunizing enzyme in PBS overnight at 4 °C. Plates were washed and blocked with 1% BSA in PBS. Serum samples were diluted in 1% BSA/PBS, and incubated on the enzymes coated plates for 1 hour. Serum samples were washed out, and biotinylated anti-guinea pig IgG was added at a 1:10,000 dilution in 1% BSA/PBS. The secondary reagent was incubated for 1 hour. The wells were washed, and avidin conjugated horse radish peroxidase was added to the wells at a 1:1000 dilution in 1% BSA/PBS. After 30 minutes, the substrate (ABTS) was added and the OD_{405} was read after 30 minutes.

Calculation of titers: Background was subtracted from the OD readings, and the results plotted for each individual guinea pig. A linear regression analysis was performed on the linear portion of the curve. The titer value was calculated from the linear regression equation for an OD = 0.5. These individual titers were then averaged.

Two guinea pigs in the 10 µg dose of GP001 died at 2 weeks into the study. The data for the 10 µg dose was therefore thrown out.

Two results are immediately apparent: first, the GP002 variant increased the titers of antigen-specific antibody over the entire time course for the lower doses of enzymes; and the GP002 variant increased titers of antigen-specific antibody for all doses of enzymes in the earliest time points.

At the extended time points and for the higher doses, the difference between *B. amyloliquefaciens* subtilisin and its variant were no longer apparent. See Figures 19 and 20.

From the Figures it is apparent that a single change in the amino acid sequence of *B. amyloliquefaciens* subtilisin significantly altered its immunogenicity.

Example 9

Reduction of Allergenicity *in Vivo*

Given the ability to identity of human T cell epitopes, it is possible to modify their amino acid sequence to reduce activation of T cells and the subsequent immune response to the protein. However, to evaluate the *in vivo* effect of these changes, it is necessary to use an animal model that represents the ability of human HLA molecules to present the epitopes. For example, human T cell epitopes have been identified in the molecule BPN' in the regions 70-84 and 109-122 (see USSN 09/500,135, filed February 8, 2000; Figure 16).

Substitutions in the amino acid sequence of these motifs led to reduced T cell proliferation *in vitro* using human cells representing a broad range of human HLA haplotypes. *In vitro* binding assays using EBV-transformed B cell lines demonstrated the peptides 70-84 and 109-123 bound to HLA DQ2 molecules. The substitutions that were found to reduce T-cell proliferation were introduced into the coding sequence for FNA (BPN' with a Y217L substitution) for production of reduced immunogenic FNA variants.

Transgenic mice expressing human HLA genes have been used to study epitopes presented to the immune system *in vivo*. Although the responding immune cells are of mouse origin, there is a strong correlation between the epitopes recognized in humans and mice. However, a novel use of HLA transgenic mice is in the testing of variant proteins for reduced allergenic potential as a prediction of how human individuals will respond.

To demonstrate this effect, both FNA and the FNA variant containing amino acid changes in the epitopes 70-84 and 109-123 were used to immunize HLA DR3/DQ2 transgenic mice that had been backcrossed onto I-Ab knockout mice (lacking the expression of endogenous I-A class II molecules, referred to as C2D). Adult male HLA-DR3/DQ2/C2D mice were immunized with 50 µg of FNA or FNA Variant emulsified in Complete Freund's Adjuvant. The immunization was administered intraperitoneally. Two weeks later, the mice received another intraperitoneal immunization of 50 µg FNA or the Variant emulsified in Incomplete Freund's Adjuvant. One week later, the mice were bled via the retro-orbital route, and the serum collected. Serum was assessed for antigen-specific IgG antibodies in a direct ELISA protocol. Briefly, 96 well flat-bottomed EIA plates were coated overnight with10 µg/ml of denatured FNA. Plates were washed, blocked with 1% Fetal Calf Serum, and serum was titered out at 1:10 dilutions. The serum was washed out of the wells, and antigen-specific IgG was detected with horse radish peroxidase conjugated anti-mouse IgG. Results are presented as serum dilution versus average optical density (x 1000) in Table 1 and Figure 21.

Table 1

Dilution	FNA	FNA Variant
10	2937.5	88
100	2476	120
1000	1695	103
10000	641.5	80
100000	207	85
1000000	129.5	76
10000000	88.5	85

The results indicated the changes introduced into regions 70-84 and 109-123 significantly reduced the ability of DQ2 transgenic mice to mount a humoral response to the variant and provide a method for *in vivo* characterization of engineered proteins predicted with the methods of this invention to show reduced immunogenicity in humans.

CLAIMS

1. A method to determine the allergenic potential of an engineered protein comprising the steps of,

a) immunizing a first transgenic mouse with a protein of interest and immunizing a second transgenic mouse with an engineered protein wherein said engineered protein is a variant of said protein of interest and said protein of interest includes a T-cell epitope wherein the variant differs from the protein of interest by having an altered T cell epitope;

b) collecting serum of said first and said second immunized transgenic mice;

c) measuring the serum for antigen specific immunoglobulins; and

d) comparing the immunogenic response of said variant and said protein of interest wherein the variant and the protein of interest produce a different immunogenic response in said transgenic mice.

2. The method according to claim 1, wherein said protein of interest is an enzyme.

3. The method according to claim 2, wherein said enzyme is a protease.

4. The method according to claim 1, wherein the antigen specific immunoglobulin is IgG.

5. The method according to claim 1, wherein the first transgenic mouse and second transgenic mouse are HLA DR3/DQ2.

6. The method according to claim 5, wherein the HLA DR3/DQ2 transgenic mice have been backcrossed with mice lacking the expression of endogenous I-A class II molecules.

7. The method according to claim 1, wherein said T-cell epitope is altered with amino acid substitutions.

8. The method according to claim 1, wherein said T-cell epitope is altered by having a terminal portion of said protein of interest which includes said T-cell epitope replaced with a corresponding terminal portion of a homolog of said protein of interest wherein said homolog does not comprise a T-cell epitope identical to said replaced T-cell epitope.

9. The method according to claim 1, wherein said immunogenic response produced by the variant is less than the immunogenic response produced by the protein of interest.

10. The method according to claim 1, wherein said immunogenic response produced by the variant is more than the immunogenic response produced by the protein of interest.

11. A method of using transgenic mice to predict the allergenic response of a human to an engineered protein comprising the steps of,

a) immunizing a first transgenic mouse with a protein of interest and immunizing a second transgenic mouse with an engineered protein, wherein said engineered protein is a variant of said protein of interest and the protein of interest includes a T-cell epitope, wherein the variant differs from the protein of interest by having an altered T cell epitope;

b) collecting serum of the first and the second immunized transgenic mice;

c) measuring the serum for antigen specific immunglobulins; and

d) comparing the immunogenic response of the variant and the protein of interest, wherein the variant and the protein of interest produce a different immunogenic response in said transgenic mice, and wherein said immunogenic response is predictive of the allergenic response in humans.

12. The method according to claim 11, wherein said protein of interest is a protease.

13. A variant of a polypeptide of interest comprising a T-cell epitope, wherein said variant differs from said polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual.

14. The variant of claim 13, wherein said immunogenic response produced by said variant is greater than said immunogenic response produced by said protein of interest.

15. A method for determining the immunogenic response produced by a protein, comprising;

a) obtaining from a single blood source a solution of dendritic cells and a solution of naive CD4+ and/or CD8+ T-cells;

b) promoting differentiation in said dendritic cells;

c) combining said solution of differentiated dendritic cells and said naive CD4+ and/or CD8+ T cells with said protein; and

d) measuring the proliferation of said T-cells in step c).

16. The method according to claim 15 further comprising comparing the proliferation of said T-cells to the proliferation of a second protein.

17. The method according to claim 16, wherein the protein of interest and the second protein are homologs of one another.

18. The method of claim 17, wherein the protein of interest and the second protein are proteases.

19. The method of claim 18, wherein the protein of interest and the second protein are each different peptides of the same protein.

20. A method of altering the immunogenicity of a polypeptide of interest comprising, a) determining the immunogenicity of said polypeptide; b) identifying a T-cell epitope in said polypeptide; and c)altering said T-cell epitope so as to alter the immunogenicity of said polypeptide.

21. The method according to claim 20, wherein said T-cell epitope is altered by having at least one amino acid substitution.

22. The method according to claim 20, wherein said T-cell epitope is altered by replacing a portion of said polypeptide of interest which includes said T-cell epitope with a corresponding portion of a homolog of said polypeptide of interest, where the corresponding portion does not contain said T-cell epitope.

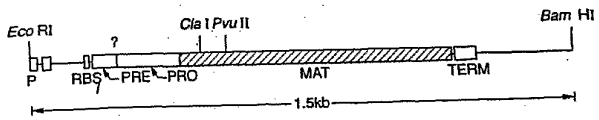


FIG._1A

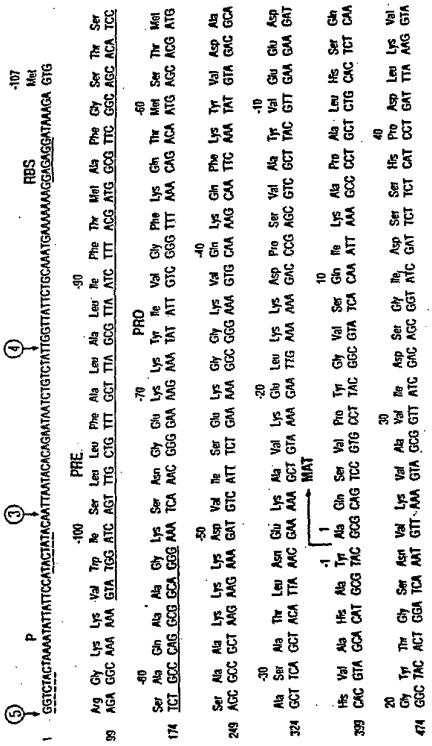


FIG._1B - 1

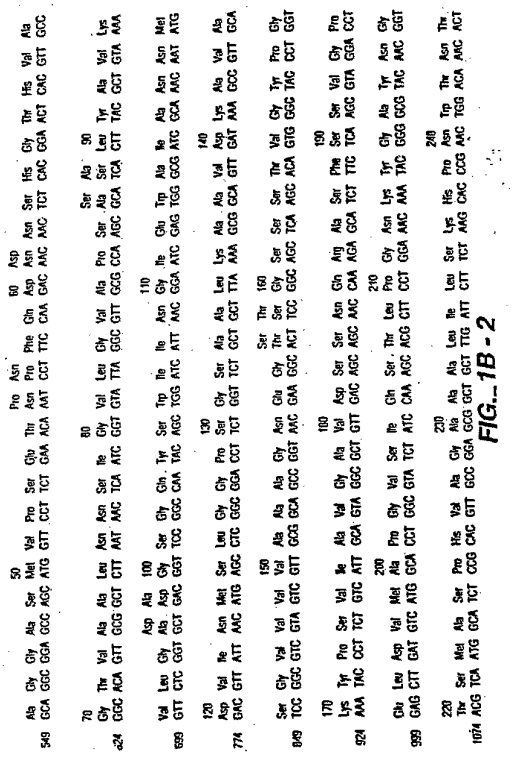


FIG._1B - 2

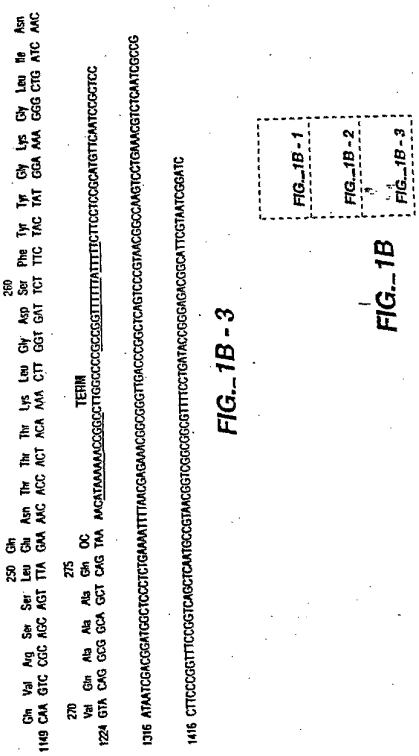


FIG._1B - 3

FIG._1B

FIG._1B - 1
FIG._1B - 2
FIG._1B - 3

CONSERVED RESIDUES IN SUBTILISINS FROM *BACILLUS AMYLOLIQUEFACIENS*

```
1                   10                  20
A Q S V P . G . . . . . A P A . H . . G

21                  30                  40
. T G S . V K V A V . D . G . . . . H P

41                  50                  60
D L . . . G G A S . V P . . . . . Q D

61                  70                  80
. N . H G T H V A G T . A A L N N S I G

81                  90                  100
V L G V A P S A . L Y A V K V L G A . G

101                 110                 120
S G . . S . L . . G . E W A . N . . . .

121                 130                 140
V . N . S L G . P S . S . . . . . A . .

141                 150                 160
. . . . . G V . V V A A . G N . G . . .

161                 170                 180
. . . . . Y P . . Y . . . . A V G A .

181                 190                 200
D . . N . . A S F S . . G . . L D . . A

201                 210                 220
P G V . . Q S T . P G . . Y . . . N G T

221                 230                 240
S M A . P H V A G A A A L . . . K . . .

241                 250                 260
W . . . Q . R . . L . N T . . . L G . .

261                 270
. . Y G . G L . N . . A A . .
```

FIG._2

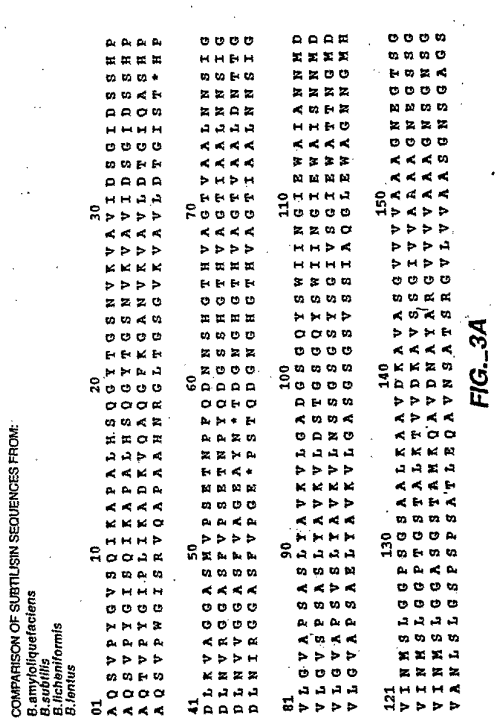

COMPARISON OF SUBTILISIN SEQUENCES FROM:
B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

FIG._3A

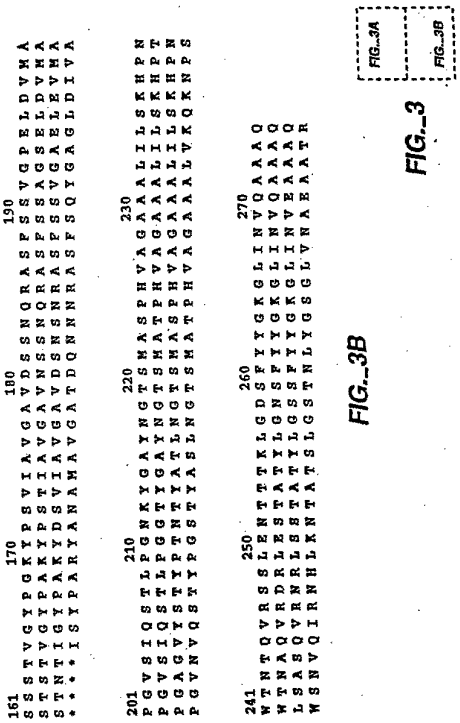


FIG_3B

FIG_3

FIG_3A

FIG_3B

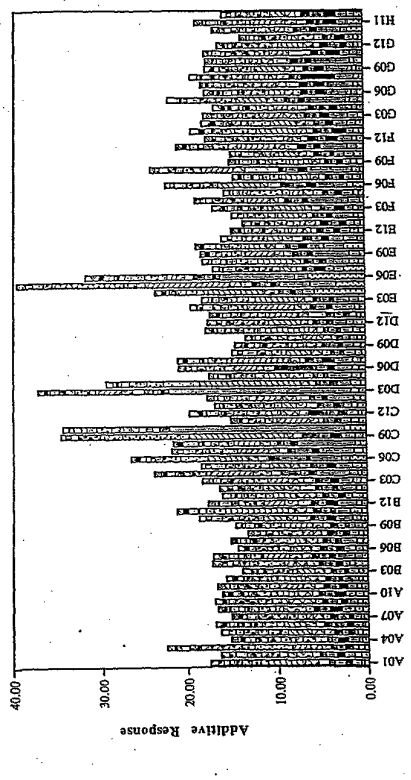

Additive Response
40.00
30.00
20.00
10.00
0.00
A01
A04
A07
A10
B03
B06
B09
B12
C03
C06
C09
C12
D03
D06
D09
D12
E03
E06
E09
E12
F03
F06
F09
F12
G03
G06
G09
G12
H11

FIG. 4

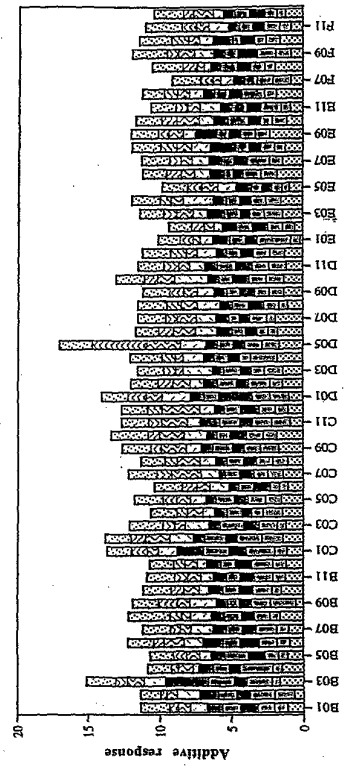

Additive response
0
5
10
15
20
B01
B03
B05
B07
B09
B11
C01
C03
C05
C07
C09
C11
D01
D03
D05
D07
D09
D11
E01
E03
E05
E07
E09
E11
F07
F09
F11

FIG. 5

1	A12	IKDFHVYFRESRDAG	49	E12	SATSRGVLVVAASGN
2	A11	LEQAVNSATSRGVLV	50	E11	SRGVLVVAASGNSGA
3	A10	AQSVPWGISRVQAPA	51	E10	VLVVAASGNSGAGSI
4	A9	VPWGISRVQAPAAHN	52	E9	VAASGNSGAGSISYP
5	A8	GISRVQAPAAHNRGL	53	E8	SGNSGAGSISYPARY
6	A7	RVQAPAAHNRGLTGS	54	E7	SGAGSISYPARYANA
7	A6	APAAHNRGLTGSGVK	55	E6	GSISYPARYANAMAV
8	A5	AHNRGLTGSGVKVAV	56	E5	SYPARYANAMAVGAT
9	A4	RGLTGSGVKVAVLDT	57	E4	ARYANAMAVGATDQN
10	A3	TGSGVKVAVLDTGIS	58	E3	ANAMAVGATDQNNNR
11	A2	GVKVAVLDTGISTHP	59	E2	MAVGATDQNNNRASF
12	A1	VAVLDTGISTHPDLN	60	E1	GATDQNNNRASFSQY
13	B12	LDTGISTHPDLNIRG	61	F12	DQNNNRASFSQYGAG
14	B11	GISTHPDLNIRGGAS	62	F11	NNRASFSQYGAGLDI
15	B10	THPDLNIRGGASFVP	63	F10	ASFSQYGAGLDIVAP
16	B9	DLNIRGGASFVPGEP	64	F9	SQYGAGLDIVAPGVN
17	B8	IRGGASFVPGEPSTQ	65	F8	GAGLDIVAPGVNVQS
18	B7	GASFVPGEPSTQDGN	66	F7	LDIVAPGVNVQSTYP
19	B6	FVPGEPSTQDGNGHG	67	F6	VAPGVNVQSTYPGST
20	B5	GEPSTQDGNGHGTHV	68	F5	GVNVQSTYPGSTYAS
21	B4	STQDGNGHGTHVAGT	69	F4	VQSTYPGSTYASLNG
22	B3	DGNGHGTHVAGTIAA	70	F3	TYPGSTYASLNGTSM
23	B2	GHGTHVAGTIAALNN	71	F2	GSTYASLNGTSMATP
24	B1	THVAGTIAALNNSIG	72	F1	YASLNGTSMATPHVA
25	C12	AGTIAALNNSIGVLG	73	G12	LNGTSMATPHVAGAA
26	C11	IAALNNSIGVLGVAP	74	G11	TSMATPHVAGAAALV
27	C10	LNNSIGVLGVAPSAE	75	G10	ATPHVAGAAALVKQK
28	C9	SIGVLGVAPSAELYA	76	G9	HVAGAAALVKQKNPS
29	C8	VLGVAPSAELYAVKV	77	G8	GAAALVKQKNPSWSN
30	C7	VAPSAELYAVKVLGA	78	G7	ALVKQKNPSWSNVQI
31	C6	SAELYAVKVLGASGS	79	G6	KQKNPSWSNVQIRNH
32	C5	LYAVKVLGASGSGSV	80	G5	NPSWSNVQIRNHLKN
33	C4	VKVLGASGSGSVSSI	81	G4	WSNVQIRNHLKNTAT
34	C3	LGASGSGSVSSIAQG	82	G3	VQIRNHLKNTATSLG
35	C2	SGSGSVSSIAQGLEW	83	G2	RNHLKNTATSLGSTN
36	C1	GSVSSIAQGLEWAGN	84	G1	LKNTATSLGSTNLYG
37	D12	SSIAQGLEWAGNNGM	85	H12	TATSLGSTNLYGSGL
38	D11	AQGLEWAGNNGMHVA	86	H11	SLGSTNLYGSGLVNA
39	D10	LEWAGNNGMHVANLS	87	H10	STNLYGSGLVNAEAA
40	D9	AGNNGMHVANLSLGS	88	H9	NLYGSGLVNAEAATR
41	D8	NGMHVANLSLGSPSP			
42	D7	HVANLSLGSPSPSAT			
43	D6	NLSLGSPSPSATLEQ			
44	D5	LGSPSPSATLEQAVN			
45	D4	PSPSATLEQAVNSAT			
46	D3	SATLEQAVNSATSRG			
47	D2	LEQAVNSATSRGVLV			
48	D1	AVNSATSRGVLVVAA			

FIG. 6A

1	A12	IKDFHVYFRESRDAG
2	A11	DAELHIFRVFTNNQV
3	A10	PLRRASLSLGSGFWH
4	A9	RASLSLGSGFWHATG
5	A8	LSLGSGFWHATGRHS
6	A7	GSGFWHATGRHSSRR
7	A6	FWHATGRHSSRRLLR
8	A5	ATGRHSSRRLLRAIP
9	A4	RHSSRRLLRAIPRQV
10	A3	SRRLLRAIPRQVAQT
11	A2	LLRAIPRQVAQTLQA
12	A1	AIPRQVAQTLQADVL
13	B12	RQVAQTLQADVLWQM
14	B11	AQTLQADVLWQMGYT
15	B10	LQADVLWQMGYTGAN
16	B9	DVLWQMGYTGANVRV
17	B8	WQMGYTGANVRVAVF
18	B7	GYTGANVRVAVFDTG
19	B6	GANVRVAVFDTGLSE
20	B5	VRVAVFDTGLSEKHP
21	B4	AVFDTGLSEKHPHFK
22	B3	DTGLSEKHPHFKNVK
23	B2	LSEKHPHFKNVKERT
24	B1	KHPHFKNVKERTNWT
25	C12	HFKNVKERTNWTNER
26	C11	NVKERTNWTNERTLD
27	C10	ERTNWTNERTLDDGL
28	C9	NWTNERTLDDGLGHG
29	C8	NERTLDDGLGHGTFV
30	C7	TLDDGLGHGTFVAGV
31	C6	DGLGHGTFVAGVIAS
32	C5	GHGTFVAGVIASMRE
33	C4	TFVAGVIASMRECQG
34	C3	AGVIASMRECQGFAP
35	C2	IASMRECQGFAPDAE
36	C1	MRECQGFAPDAELHI
37	D12	CQGFAPDAELHIFRV
38	D11	FAPDAELHIFRVFTN
39	D10	DAELHIFRVFTNNQV
40	D9	LHIFRVFTNNQVSYT
41	D8	FRVFTNNQVSYTSWF
42	D7	FTNNQVSYTSWFLDA
43	D6	NQVSYTSWFLDAFNY
44	D5	SYTSWFLDAFNYAIL
45	D4	SWFLDAFNYAILKKI
46	D3	LDAFNYAILKKIDVL
47	D2	FNYAILKKIDVLNLS
48	D1	AILKKIDVLNLSIGG
49	E12	KKIDVLNLSIGGPDF
50	E11	DVLNLSIGGPDFMDH
51	E10	NLSIGGPDFMDHPFV
52	E9	IGGPDFMDHPFVDKV
53	E8	PDFMDHPFVDKVWEL
54	E7	MDHPFVDKVWELTAN
55	E6	PFVDKVWELTANNVI
56	E5	DKVWELTANNVIMVS
57	E4	WELTANNVIMVSAIG
58	E3	TANNVIMVSAIGNDG
59	E2	NVIMVSAIGNDGPLY
60	E1	MVSAIGNDGPLYGTI
61	F12	AIGNDGPLYGTLNNP
62	F11	NDGPLYGTLNNPADQ
63	F10	PLYGTLNNPADQMDV
64	F9	GTLNNPADQMDVIGV
65	F8	NNPADQMDVIGVGGI
66	F7	ADQMDVIGVGGIDFE
67	F6	MDVIGVGGIDFEDNI
68	F5	IGVGGIDFEDNIARF
69	F4	GGIDFEDNIARFSSR
70	F3	DFEDNIARFSSRGMT
71	F2	DNIARFSSRGMTTWE
72	F1	ARFSSRGMTTWELPG
73	G12	SSRGMTTWELPGGYG
74	G11	GMTTWELPGGYGRMK
75	G10	TWELPGGYGRMKPDI
76	G9	LPGGYGRMKPDIVTY
77	G8	GYGRMKPDIVTYGAG
78	G7	RMKPDIVTYGAGVRG
79	G6	PDIVTYGAGVRGSGV
80	G5	VTYGAGVRGSGVKGG
81	G4	GAGVRGSGVKGGCRA
82	G3	VRGSGVKGGCRALSG
83	G2	SGVKGGCRALSGTSV
84	G1	KGGCRALSGTSVASP
85	H12	CRALSGTSVASPVVA
86	H11	LSGTSVASPVVAGAV
87	H10	TSVASPVVAGAVTLL
88	H9	ASPVVAGAVTLLVST
89	H8	VVAGAVTLLVSTVQK
90	H7	GAVTLLVSTVQKREL
91	H6	TLLVSTVQKRELVNP
92	H5	VSTVQKRELVNPASM
93	H4	VQKRELVNPASMKQA
94	H3	RELVNPASMKQALIA
95	H2	VNPASMKQALIASAR
96	H1	ASMKQALIASARRLP

FIG. 6B

97	I12	IKDFHVYFRESRDAG
98	I11	DAELHIFRVFTNNQV
99	I10	KQALIASARRLPGVN
100	I9	LIASARRLPGVNMFE
101	I8	SARRLPGVNMFEQGH
102	I7	RLPGVNMFEQGHGKL
103	I6	GVNMFEQGHGKLDLL
104	I5	MFEQGHGKLDLLRAY
105	I4	QGHGKLDLLRAYQIL
106	I3	GKLDLLRAYQILNSY
107	I2	DLLRAYQILNSYKPQ
108	I1	RAYQILNSYKPQASL
109	J12	QILNSYKPQASLSPS
110	J11	NSYKPQASLSPSYID
111	J10	KPQASLSPSYIDLTE
112	J9	ASLSPSYIDLTECPY
113	J8	SPSYIDLTECPYMWP
114	J7	YIDLTECPYMWPYCS
115	J6	LTECPYMWPYCSQPI
116	J5	CPYMWPYCSQPIYYG

FIG. 6C

MKLVNIWLLLLVVLLCGKKHLGDRLEKKSFEKAPCPGCSHLTLKVEFSSTVVEYEYIVAFNGYFT
AKARNSFISSALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQR
KVFRSLKYAESDPTVPCNETRWSQKWQSSRPLRRASLSLGSGFWHATGRHSSRRLLRAIPRQVAQ
TLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERTNWTNERTLDDGLGHGTFVAGVIASM
RECQGFAPDAELHIFRVFTNNQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWEL
TANNVIMVSAIGNDGPLYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTWELPGGYGRMKPD
IVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLVSTVQKRELVNPASMKQALIASARRLPG
VNMFEQGHGKLDLLRAYQILNSYKPQASLSPSYIDLTECPYMWPYCSQPIYYGGMPTVVNVTILN
GMGVTGRIVDKPDWQPYLPQNGDNIEVAFSYSSVLWPWSGYLAISISVTKKAASWEGIAQGHVMI
TVASPAETESKNGAEQTSTVKLPIKVKIIPTPPRSKRVLWDQYHNLRYPPGYFPRDNLRMKNDPL
DWNGDHIHTNFRDMYQHLRSNGYFVEVLGAPFTCFDASQYGTLLMVDSEEEYFPEEIAKLRRDVD
NGLSLVIFSDWYNTSVMRKVKFYDENTRQWWMPDTGGANIPALNELLSVWNMGFSDGLYEGEFTL
ANHDMYYASGCSIAKFPEDGVVITQTFKDQGLEVLKQETAVVENVPILGLYQIPAEGGGRIVLYG
DSNCLDDSHRQKDCFWLLDALLQYTSYGVTPPSLSHSGNRQRPPSGAGSVTPERMEGNHLHRYSK
VLEAHLGDPKPRPLPACPRLSWAKPQPLNETAPSNLWKHQKLLSIDLDKVVLPNFRSNRPQVRPL
SPGESGAWDIPGGIMPGRYNQEVGQTIPVFAFLGAMVVLAFFVVQINKAKSRPKRRKPRVKRPQL
MQQVHPPKTPSV

FIG. 7

```
                  10        20        30        40        50
BPN'      AQSVPYGVSQ-IKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLK-VAGGA  48
SAVINASE  AQSVPWGISR-VQAPAAHNRGLTGSGVKVAVLDTGI-STHPDLN-IRGGA  47
S2HSET    -RAIPRQVAQTLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERT  49

                  60        70        80        90       100
BPN'      SMVPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGA  98
SAVINASE  SFVPGEPST-QDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGA  96
S2HSET    NW--TNERTLDDGLGHGTFVAGVIASMRECQGF---APDAELHIFRVFTN  94

                 110       120       130       140       150
BPN'      DGSGQYSWIINGIEWAIANNMDVINMSLGGPS-GSAALKAAVDKAVASGV  147
SAVINASE  SGSGSVSSIAQGLEWAGNNGMHVANLSLGSPS-PSATLEQAVNSATSRGV  145
S2HSET    NQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWELTANNV  144

                 160       170       180       190       200
BPN'      VVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPEL-  197
SAVINASE  LVVAASGNSGA----GSISYPARYANAMAVGATDQNNNRASFSQYGAGL-  191
S2HSET    IMVSAIGNDGP--LYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTW  192

                 210       220       230       240       250
BPN'      -----------DVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALIL  235
SAVINASE  -----------DIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVK  229
S2HSET    ELPGGYGRMKPDIVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLV  242

                 260       270       280       290
BPN'      SKHPNWTNTQ---VRSSLENTTTKLGDSFYYGKGLINVQAAAQ          275
SAVINASE  QKNPSWSNVQ---IRNHLKNTATSLGSTNLYGSGLVNAEAATR         269
S2HSET    STVQKRELVNPASMKQALIASARRLPGVNMFEQG-----HGKL          280
```

FIG. 8

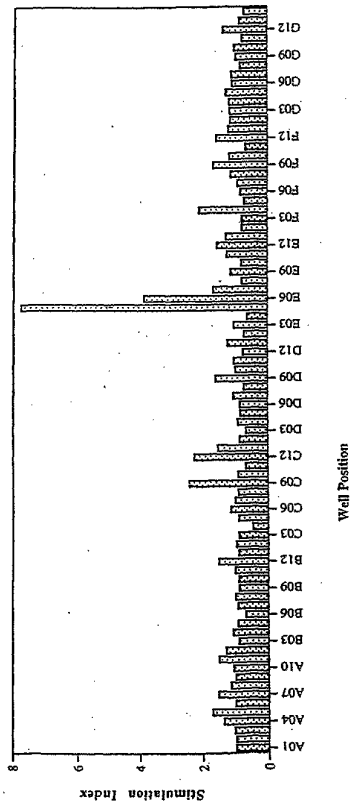

Stimulation Index
0
2
4
6
8
Well Position
A01
A04
A07
A10
B03
B06
B09
B12
C03
C06
C09
C12
D03
D06
D09
D12
E03
E06
E09
E12
F03
F06
F09
F12
G03
G06
G09
G12

FIG. 9

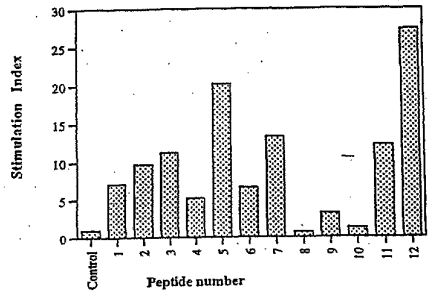
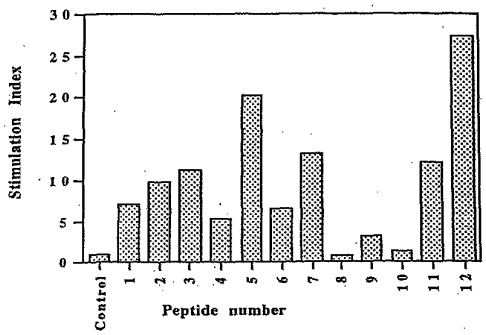

Stimulation Index
30
25
20
15
10
5
0
Control
1
2
3
4
5
6
7
8
9
10
11
12
Peptide number

FIG. 10



Peptide number	Sequence
1 (unmodified sequence)	GSISYPARYANAMAV
2	ASISYPARYANAMAV
3	GAISYPARYANAMAV
4	GSASYPARYANAMAV
5	GSIAYPARYANAMAV
6	GSISAPARYANAMAV
7	GSISYAARYANAMAV
8	GSISYPAAYANAMAV
9	GSISYPARAANAMAV
10	GSISYPARYAAAMAV
11	GSISYPARYANAAAV
12	GSISYPARYANAMAA

FIG. 11

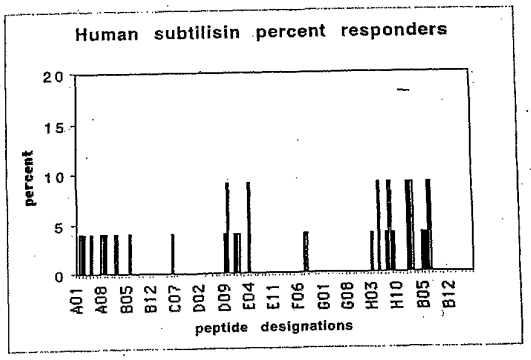

Human subtilisin percent responders
20
15
10
5
0
percent
A01
A08
B05
B12
C07
D02
D09
E04
E11
F06
G01
G08
H03
H10
B05
B12
peptide designations

FIG. 12

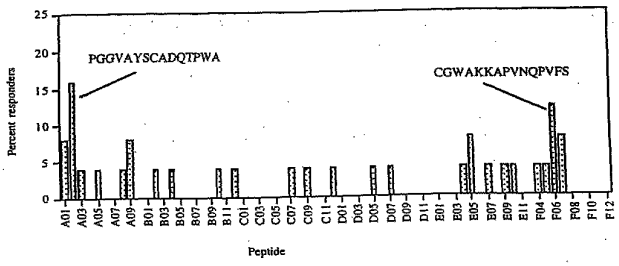

PGGVAYSCADQTPWA
CGWAKKAPVNQPVFS
Percent responders
Peptide

FIG. 13A

```
          1          2          3          4          5
1234567890 1234567890 1234567890 1234567890 1234567890
MRSSPLLPSA VVAALPVLAL AADGRSTRYW DCCKPSCGWA KKAPVNQPVE
SCNANFQRIT DFDAKSGCEP GGVAYSCADQ TPWAVNDDFA LGFAATSIAG
SNEAGWCCAC YELTFTSGPV AGKKMVVQST STGGDLGSNH FDLNIPGGGV
GIFDGCTPQF GGLPGQRYGG ISSRNECDRF PDALKPGCYW RFDWFKNADN
PSFSFRQVQC PAELVARTGC RRNDDGNFPA VQIPSSSTSS PVNQPTSTST
TSTSTTSSPP VQPTTPSGCT AERWAQ
```

FIG. 13B

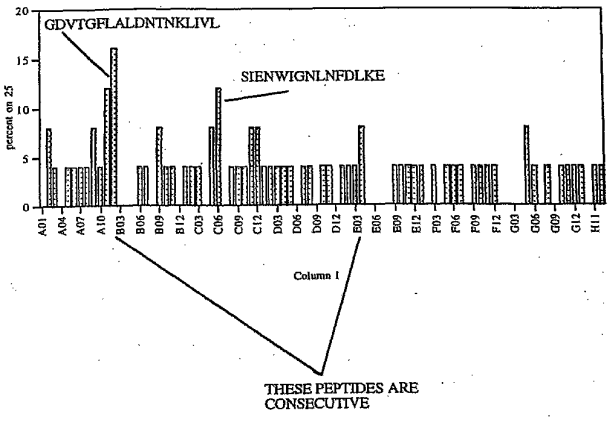

GDVTGFLALDNTNKLIVL
SIENWIGNLNFDLKE
percent on 25
20
15
10
5
0
A01
A04
A07
A10
B03
B05
B09
B12
C03
C06
C09
C12
D03
D06
D09
D12
E03
E06
E09
E12
F03
F06
F09
F12
G03
G06
G09
G12
H11
Column 1
THESE PEPTIDES ARE CONSECUTIVE

FIG. 14A

```
  1 mrsslvlffv sawtalaspi rrevsqdlfn qfnlfaqysa aaycgknnda
 51 pagtnitctg nacpevekad atflysfeds gvgdvtgfla ldntnklivl
101 sfrgsrsien wignlnfdlk eindicsgcr ghdgftsswr svadtlrqkv
151 edavrehpdy rvvftghslg galatvagad lrgngydidv fsygaprvgn
201 rafaefltvq tggtlyrith tndivprlpp refgyshssp eywiksgtlv
251 pvtrndivki egidatggnn qpnipdipah lwyfgligtc l
```

FIG. 14B

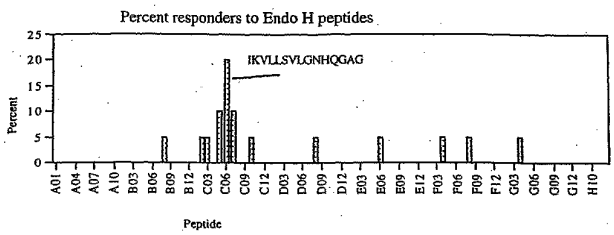

Percent responders to Endo H peptides
IKVLLSVLGNHQGAG
Percent
25
20
15
10
5
0
A01 A04 A07 A10 B03 B06 B09 B12 C03 C06 C09 C12 D03 D06 D09 D12 E03 E06 E09 E12 F03 F06 F09 F12 G03 G06 G09 G12 H10
Peptide

FIG. 15A

```
  1 mftpvrrrvr taalalsaaa alvlgstaas gasatpspap apapapvkqg
 51 ptsvayvevn nnsmlnvgky tladgggnaf dvavifaani nydtgtktay
101 lhfnenvqrv ldnavtqirp lqqqgikvll svlqnhqqaq fanfpsqqaa
151 safakqlsda vakygldgvd fddeyaeygn ngtaqpndss fvhlvtalra
201 nmpdkiisly nigpaasrls yggvdvsdkf dyawnpyygt wqvpgialpk
251 aqlspaavei grtsrstvad larrtvdegy gvyltynldg gdrtadvsaf
301 trelygseav rtp
```

FIG. 15B

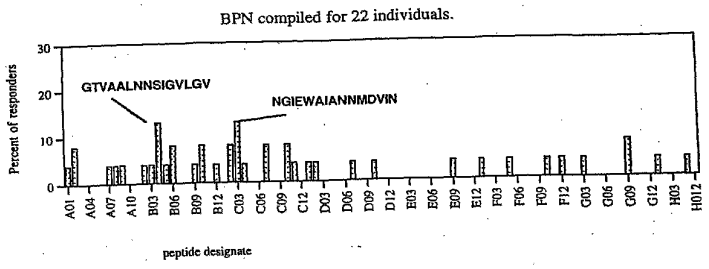

BPN compiled for 22 individuals.
Percent of responders
30
20
10
0
GTVAALNNSIGVLGV
NGIEWAIANNMDVIN
A01
A04
A07
A10
B03
B05
B09
B12
C03
C06
C09
C12
D03
D06
D09
D12
E03
E06
E09
E12
F03
F06
F09
F12
G03
G06
G09
G12
H03
H012
peptide designate

FIG. 16

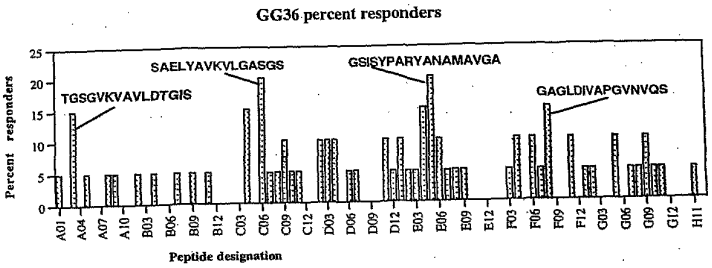

GG36 percent responders
Percent responders
25
20
15
10
5
0
TGSGVKVAVLDTGIS
SAELYAVKVLGASGS
GSISYPARYANAMAVGA
GAGLDIVAPGVNVQS
A01 A04 A07 A10 B03 B06 B09 B12 C03 C06 C09 C12 D03 D06 D09 D12 E03 E06 E09 E12 F03 F06 F09 F12 G03 G06 G09 G12 H11
Peptide designation

FIG. 17

Hybrid enzyme sequence (GG36-BPN)

GG36
AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGH

BPN

GTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVINMSLGGS

Δ

GSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGP

ELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGD

SFYY GKGLINVQAAAQ

FIG. 18

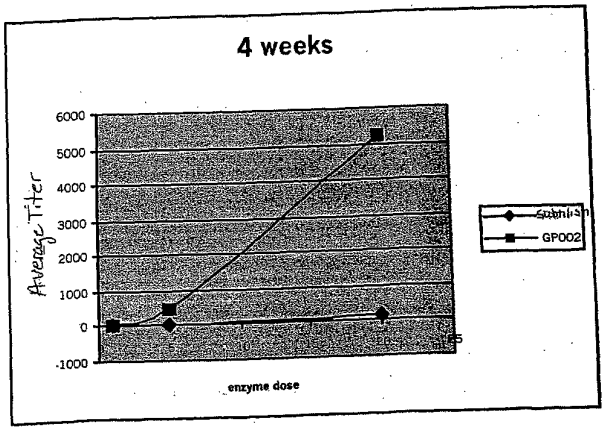

4 weeks
6000
5000
4000
3000
2000
1000
0
-1000
Average Titer
enzyme dose
GP002

FIGURE 19A

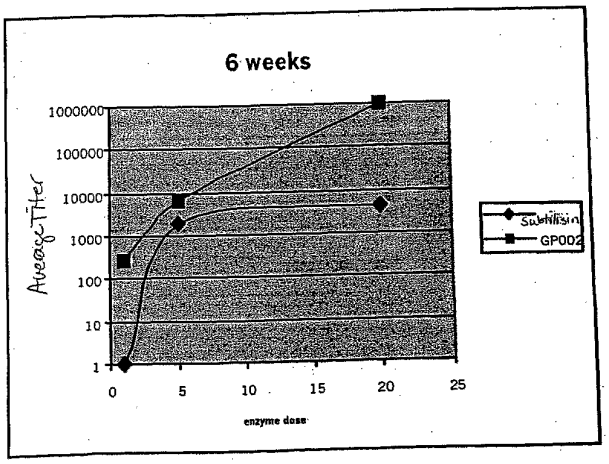

6 weeks
1000000
100000
10000
1000
100
10
1
Average Titer
0
5
10
15
20
25
enzyme dose
GP002

FIGURE 19B

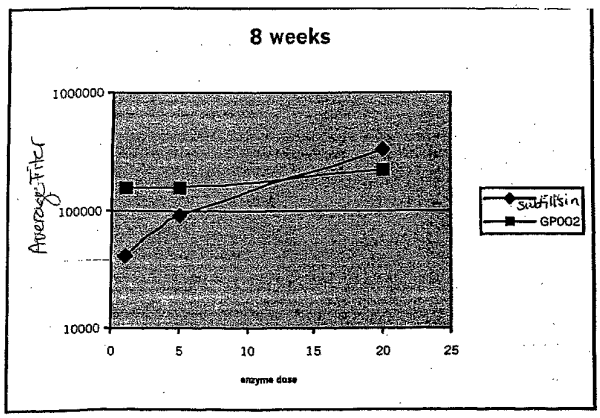

8 weeks
1000000
100000
10000
Average Titer
0
5
10
15
20
25
enzyme dose
GP002

FIGURE 19C

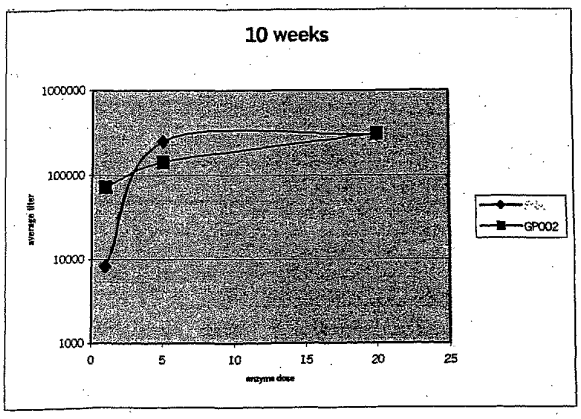

10 weeks
1000000
100000
10000
1000
average titer
0
5
10
15
20
25
enzyme dose
GP002

FIGURE 19D

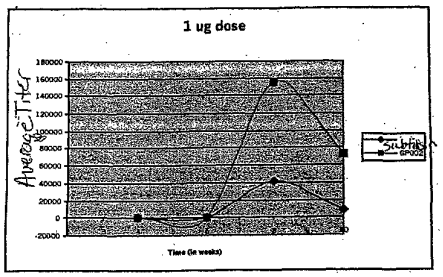

1 ug dose
Average Titer
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
Time (in weeks)
SUBTIL
GP002

FIGURE 20A

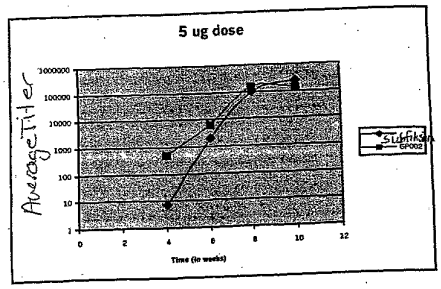

5 ug dose
Average Titer
1000000
100000
10000
1000
100
10
1
0
2
4
6
8
10
12
Time (in weeks)

FIGURE 20B

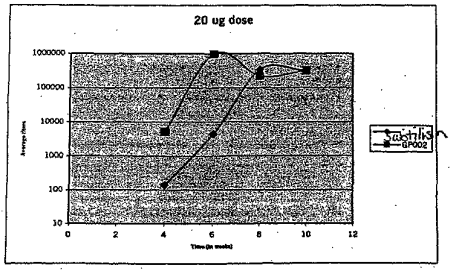


FIGURE 20C

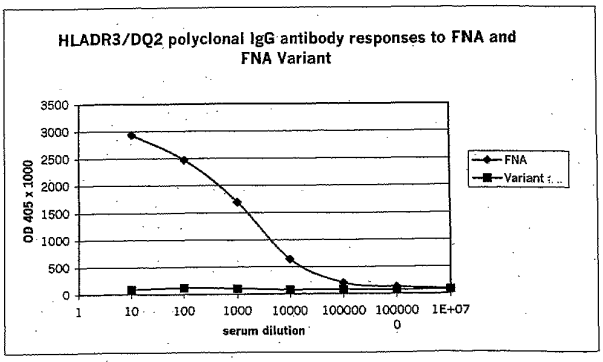

HLADR3/DQ2 polyclonal IgG antibody responses to FNA and FNA Variant
OD 405 x 1000
3500
3000
2500
2000
1500
1000
500
0
1
10
100
1000
10000
100000
1000000
1E+07
serum dilution
FNA
Variant

FIGURE 21

【国際公開パンフレット（コレクトバージョン）】

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(71) Applicant *(for all designated States except US)*: **GENENCOR INTERNATIONAL, INC.** [US/US]; 925 Page Mill Road, Palo Alto, CA 94304 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ESTELL, David, A.** [UG/UG]; 248 Woodbridge Circle, San Mateo, CA 94403 (US). **HARDING, Fiona, A.** [US/US]; 772 Lewis Street, Santa Clara, CA 95050 (US).

(74) Agent: **STONE, Christopher, L.**; GENENCOR INTERNATIONAL, INC., 925 Page Mill Road, Palo Alto, CA 94304 (US).

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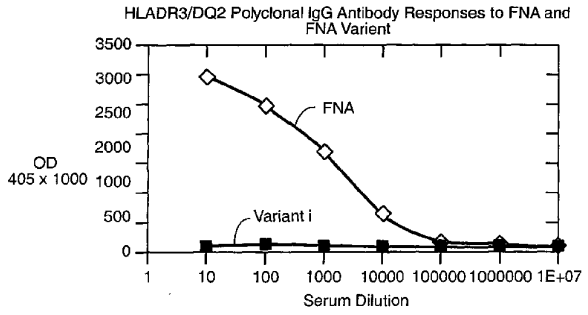
[Continued on next page]

(54) Title: PROTEINS PRODUCING AN ALTERED IMMUNOGENIC RESPONSE AND METHODS OF MAKING AND USING THE SAME



(57) Abstract: The present invention relates to a novel methods and compositions for producing hyper and hypo allergenic compositions. Specifically, the present invention comprises neutralizing or reducing the ability of T-cells to recognize epitopes and thus prevent sensitization of an individual to the protein. Alternatively, T-cell epitopes are mutated to produce increased immunogenic reactions.

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PROTEINS PRODUCING AN ALTERED IMMUNOGENIC RESPONSE AND METHODS OF MAKING AND USING THE SAME

BACKGROUND OF THE INVENTION

Proteins used in industrial, pharmaceutical and commercial applications are of increasing prevalence. As a result, the increased exposure due to this prevalence has been responsible for some safety hazards caused by the sensitization of certain persons to those peptides, whereupon subsequent exposure causes extreme allergic reactions which can be injurious and even fatal. For example, proteases are known to cause dangerous hypersensitivity in some individuals. As a result, despite the usefulness of proteases in industry, e.g., in laundry detergents, cosmetics, textile treatment etc., and the extensive research performed in the field to provide improved proteases which have, for example, more effective stain removal under detergency conditions; the use of proteases in industry has been problematic due to their ability to produce a hypersensitive allergenic response in some humans.

Much work has been done to alleviate these problems. Among the strategies explored to reduce immunogenic potential of protease use have been improved production processes which reduce potential contact by controlling and minimizing workplace concentrations of dust particles or aerosol carrying airborne protease, improved granulation processes which reduce the amount of dust or aerosol actually produced from the protease product, and improved recovery processes to reduce the level of potentially allergenic contaminants in the final product. However, efforts to reduce the allergenicity of protease, per se, have been relatively unsuccessful. Alternatively, efforts have been made to mask epitopes in protease which are recognized by immunoglobulin E (IgE) in hypersensitive individuals (PCT Publication No. WO 92/10755) or to enlarge or change the nature of the antigenic determinants by attaching polymers or peptides/proteins to the problematic protease.

When an adaptive immune response occurs in an exaggerated or inappropriate form, the individual experiencing the reaction is said to be hypersensitive. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately and sometimes cause inflammatory reactions and tissue damage. They can be provoked by many antigens; and the cause of a hypersensitivity reaction will vary from one individual to the next. Hypersensitivity does not normally manifest itself upon first contact with the antigen, but usually appears upon subsequent contact. One form of hypersensitivity occurs when an IgE response is directed against innocuous environmental antigens, such as pollen, dust-mites or animal dander. The resulting release of pharmacological mediators by IgE-sensitized mast cells produces an acute inflammatory reaction with symptoms such as asthma or rhinitis.

Nonetheless, a strategy comprising modifying the IgE sites will not generally be successful in preventing the cause of the initial sensitization reaction. Accordingly, such strategies, while perhaps neutralizing or reducing the severity of the subsequent hypersensitivity reaction, will not reduce the number or persons actually sensitized. For example, when a person is known to be hypersensitive to a certain antigen, the general, and only safe, manner of dealing with such a

situation is to isolate the hypersensitive person from the antigen as completely as possible. Indeed, any other course of action would be dangerous to the health of the hypersensitive individual. Thus, while reducing the danger of a specific protein for a hypersensitive individual is important, for industrial purposes it would be far more valuable to render a protein incapable of initiating the hypersensitivity reaction in the first place.

T-lymphocytes (T-cells) are key players in the induction and regulation of immune responses and in the execution of immunological effector functions. Specific immunity against infectious agents and tumors is known to be dependent on these cells and they are believed to contribute to the healing of injuries. On the other hand, failure to control these responses can lead to auto aggression. In general, antigen is presented to T-cells in the form of antigen presenting cells which, through a variety of cell surface mechanisms, capture and display antigen or partial antigen in a manner suitable for antigen recognition by the T-cell. Upon recognition of a specific epitope by the receptors on the surface of the T-cells (T-cell receptors), the T-cells begin a series of complex interactions, including proliferation, which result in the production of antibody by B-cells. While T-cells and B-cells are both activated by antigenic epitopes which exist on a given protein or peptide, the actual epitopes recognized by these mononuclear cells are generally not identical. In fact, the epitope which activates a T-cell to initiate the creation of immunologic diversity is quite often not the same epitope which is later recognized by B-cells in the course of the immunologic response. Thus, with respect to hypersensitivity, while the specific antigenic interaction between the T-cell and the antigen is a critical element in the initiation of the immune response to antigenic exposure, the specifics of that interaction, i.e., the epitope recognized, is often not relevant to subsequent development of a full blown allergic reaction.

PCT Publication No. WO 96/40791 discloses a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity using polyalkylene oxide as a starting material.

PCT Publication No. WO 97/30148 discloses a polypeptide conjugate with reduced allergenicity which comprises one polymeric carrier molecule having two or more polypeptide molecules coupled covalently thereto.

PCT Publication No. WO 96/17929 discloses a process for producing polypeptides with reduced allergenicity comprising the step of conjugating from 1 to 30 polymolecules to a parent polypeptide.

PCT Publication No. WO 92/10755 discloses a method of producing protein variants evoking a reduced immunogenic response in animals. In this application, the proteins of interest, a series of proteases and variants thereof, were used to immunize rats. The sera from the rats was then used to measure the reactivity of the polyclonal antibodies already produced and present in the immunized sera to the protein of interest and variants thereof. From these results, it was possible to determine whether the antibodies in the preparation were comparatively more or less reactive with the protein and its variants, thus permitting an analysis of which changes in the protein are likely to neutralize or reduce the ability of the Ig to bind. From these tests on rats, the conclusion was arrived at that changing any of subtilisin 309 residues corresponding to 127, 128, 129, 130, 131,

151, 136, 151, 152, 153, 154, 161, 162, 163, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 186, 193, 194, 195, 196, 197, 247, 251, 261 will result in a change in the immunological potential.

PCT Publication No. WO 94/10191 discloses low allergenic proteins comprising oligomeric forms of the parent monomeric protein, wherein the oligomer has substantially retained its activity.

While some studies have provided methods of reducing the allergenicity of certain proteins and identification of epitopes which cause allergic reactions in some individuals, the assays used to identify these epitopes generally involve measurement of IgE and IgG antibody in blood sera previously exposed to the antigen. However, once an Ig reaction has been initiated, sensitization has already occurred. Accordingly, there is a need for a method of determining epitopes which cause sensitization in the first place, as neutralization of these epitopes will result in significantly less possibility for sensitization to occur, thus reducing the possibility of initial sensitization. There is also a need to produce proteins which produce an enhanced immunogenic response, and a need to identify naturally occurring proteins which produce a low immunogenic response. This invention meets these and other needs.

SUMMARY OF THE INVENTION

The present invention provides proteins which produce immunogenic responses as desired, methods of identifying and making such proteins, and methods of using such proteins. For example, as will be become apparent from the detailed description below, the methods and compositions provided herein are useful in forming hyper- and hypo-allergenic compositions. As used herein, hyper and hypo means the composition produces a greater or lesser immunogenic response, respectively, than the same composition without the proteins of the present invention. Such compositions may include cleaning compositions, textile treatments, contact lens cleaning solutions or products, peptide hydrolysis products, waste treatment products, cosmetic formulations including for skin, hair and oral care, pharmaceuticals such as blood clot removal products, research products such as enzymes and therapeutics including vaccines.

In one aspect of the invention, a polypeptide of interest is selected and provided herein. The polypeptide of interest is preferably one having a T-cell epitope and is then varied as described below. However, polypeptides of interest may also be selected based on naturally occuring properties and not altered. Moreover, polypeptides of interest may be selected which do not have a T-cell epitope, and altered so as to have a T-cell epitope.

In one aspect of the invention provided herein is a variant of a polypeptide of interest comprising a T-cell epitope. The variant differs from the polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual. The variant can be prepared and selected to produce either a greater or lesser immunogenic response than said polypeptide of interest.

The polypeptide of interest can be any polypeptide of interest. In one aspect, the polypeptide is selected from the group consisting of enzymes, hormones, factors, vaccines and cytokines. In one embodiment, the polypeptide of interest is not recognized by said individual as endogenous to said individual, or not recognized as "self". As indicated herein, the polypeptide of

interest may be an enzyme. In one embodiment, the enzyme is selected from the group consisting of lipase, cellulase, endo-glucosidase H, protease, carbohydrase, reductase, oxidase, isomerase, transferase, kinase and phosphatase. In preferred embodiments, the polypeptide of interest and the variant of said polypeptide of interest each comprise at least some of the same activity. For example, if a variant of a protease is provided, said variant will produce an altered immunogenic response, but will retain detectable, and preferably comparable, protease activity.

Wherein a variant of a polypeptide of interest is provided, the T-cell epitope may be altered in a number of ways including by amino acid substitutions, deletions, additions and combinations thereof. Preferably, the T-cell epitope is altered by having amino acid substitutions. In one embodiment herein, the amino acid substitutions are made to corresponding amino acids of a homolog of the polypeptide of interest, wherein the homolog does not comprise the same T-cell epitope in the corresponding position as the polypeptide of interest. In one aspect, the terminal portion of the polypeptide of interest comprising at least one T-cell epitope is replaced with a corresponding terminal portion of the homolog of the polypeptide of interest, wherein the replacement produces said different immunogenic response.

In another embodiment provided herein, the nucleic acids encoding the polypeptides producing the desired immunogenic response are provided herein. Moreover, the invention includes expression vectors and host cells comprising the nucleic acids provided herein. Moreover, once the polypeptides and variants thereof of the present invention are identified, substantially homologous sequences of or those sequences which hybridize to the polypeptides and variants can be identified and are provided herein. Homologous is further defined below, and can refer to similarity or identity, with identity being preferred. Preferably, the homologous sequences are amino acid sequences or nucleic acids encoding peptides having the activity of the polypeptides and variants provided herein.

In yet another aspect of the invention is a method for determining the immunogenic response produced by a protein. In one embodiment, the method comprises (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with said protein; and (d) measuring the proliferation of T-cells in said step (c).

The methods of determining immunogenic responses produced by proteins can also be used to identify comparative immunogenic responses of proteins. Therefore, in one aspect, the method of determining immunogenic responses of proteins further comprises comparing immunogenic responses of one or more proteins. The proteins can be homologs of each other, variants of the same protein, different types of the same protein, for example, different proteases, or different peptides of the same protein.

The invention further provides a method of altering the immunogenicity of a polypeptide of interest comprising determining the immunogenicity of said polypeptide; identifying a T-cell epitope in a said polypeptide; and altering said T-cell epitope so as to alter the immunogencity of said polypeptide. As described herein, said altering can be done by altering a single amino acid or

switching a portion of the polypeptide of interest with a corresponding portion of a homolog, wherein the switch produces an altered immunogenic response.

Other aspects of the invention will be understood by the skilled artisan by the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A, B1, B2 and B3 illustrate the DNA (SEQ ID:NO 1) and amino acid (SEQ ID: NO 2) sequence for *Bacillus amyloliquefaciens* subtilisin (BPN') and a partial restriction map of this gene.

Fig. 2 illustrates the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (SEQ ID:NO 3) and *Bacillus lentus* (wild-type) (SEQ ID:NO 4).

Figs. 3A and 3B illustrate an amino acid sequence alignment of subtilisin type proteases from *Bacillus amyloliquefaciens* (BPN'), *Bacillus subtilis*, *Bacillus licheniformis* (SEQ ID:NO 5) and *Bacillus lentus*. The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 illustrates the additive T-cell response of 16 peripheral mononuclear blood samples to peptides corresponding to the *Bacillus lentus* protease (GG36). Peptide E05 includes the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 5 illustrates the additive T-cell response of 10 peripheral mononuclear blood samples to peptides corresponding to the human subtilisin molecule. Peptides F10, F9, F8 and F7 all contain the amino acid sequence DQMD corresponding to the region comprising residues corresponding to 170-173 in protease from *Bacillus amyloliquefaciens* in the sequence alignment of Fig. 3.

Fig. 6A and 6B/6C illustrate amino acid strings corresponding to peptides derived from the sequence of *Bacillus lentus* protease and a human subtilisin, respectively.

Fig. 7 illustrates the amino acid sequence of human subtilisin (SEQ ID:NO 6).

Fig. 8 illustrates an amino acid sequence alignment of BPN' (*Bacillus amyloliquefaciens*) protease, SAVINASE (*Bacillus lentus*) protease and human subtilisin (S2HSBT).

Fig. 9 illustrates the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*.

Fig. 10 illustrates the T-cell response to various alanine substitutions in the E05 *Bacillus lentus* protease peptide set in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease.

Fig. 11 illustrates the T-cell response to various alanine substitutions in the E05 protease peptide (an embodiment of the T-cell epitope designated unmodified sequence) set in a sample taken from an individual known to be hypersensitive to the protease; the sequences for each peptide are also shown.

Fig. 12 illustrates the percent responders to the human subtilisin molecule.

Fig. 13A illustrates the T-cell response of peptides derived from *Humicola insolens* endogluconase (Accession number A23635). Peptides A02 and F06 represent the region corresponding to residues 70-84 and 37-51, respectively, embodiments of the T-cell epitope, of

Humicola insolens endogluconase, wherein the full length sequence is shown in Fig.13B and A02 and F06 are shown underlined and in bold.

Fig. 14A illustrates the T-cell response to peptides derived from *Thermomyces lanuginosa* lipase (Accession number AAC08588 and PID number g2997733). Peptides B02 and C06 represent the regions corresponding to residues 83-100 and 108-121, respectively, embodiments of the T-cell epitope, of *Thermomyces lanuginosa* lipase, wherein the full length sequence is shown in Fig.14B and B02 and C06 are shown underlined and in bold.

Fig. 15A illustrates the T-cell response to peptides derived from *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase. (Accession number P04067). Peptide C06 represents the region corresponding to residues 126-140, an embodiment of the T-cell epitope, of *Streptomyces plicatus* endo-beta-N-acetylglucosaminidase, wherein the full length sequence is shown in Fig.15B and C06 is shown underlined and in bold.

Fig. 16 illustrates the T-cell response to peptides derived from BPN' compiled for 22 individuals, wherein the sequences of preferred T-cell epitopes are indicated.

Fig. 17 illustrates the T-cell response to peptides derived from GG36 compiled for 22 individuals, wherein the sequences of embodiments of T-cell epitopes are indicated, GSISYPARYANAMAVGA and GAGLDIVAPGVNVQS being preferred.

Fig. 18 is an embodiment of a hybrid protein provided herein, where the N-terminus comprises N-terminal GG36 sequence and the C-terminus comprises C-terminal BPN' sequence, and wherein a comparison of the sequences with those shown in Fig. 8 indicates that the hybrid formed omits preferred T-cell epitopes of each protein.

Figure 19 is a comparison of ELISA titers for *B. amyloliquefaciens* subtilisin and the same subtilisin but engineered to contain a T-cell epitope from *B. lentis* subtilisin. Figure 19a represents the titer at 4 weeks; Figure 19b at 6 weeks, Figure 19c at 8 weeks and Figure 19d at 10 weeks.

Figure 20 is a time course study of ELISA titers for *B. amyloliquefaciens* subtilisin and the same subtilisin but engineered to contain a T-cell epitope from *B. lentis* subtilisin. Figure 20a represents the titer for a 1µg dose of enzyme, Figure 20b a 5 µg dose and Figure 20c a 20 µg dose.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method for identifying T-cell epitopes is provided. Moreover, proteins including naturally occurring proteins which have relatively impotent or potent T-cell epitopes or no T-cell epitopes may be identified in accordance with the methods of the present invention. Thus, the present invention allows the identification and production of proteins which produce immunogenic responses as desired, including naturally occurring proteins as well as proteins which have been mutated to produce the appropriate response. It is understood that the terms protein, polypeptide and peptide are sometimes used herein interchangeably. Wherein a peptide is a portion of protein, the skilled artisan can understand this by the context in which the term is used.

In one embodiment, the present invention provides an assay which identifies epitopes and non-epitopes as follows: differentiated dendritic cells are combined with naïve human CD4+ and/or

CD8+ T-cells and with a peptide of interest. More specifically, a method is provided wherein a T-cell epitope is recognized comprising the steps of: (a) obtaining from a single blood source a solution of dendritic cells and a solution of naïve CD4+ and/or CD8+ T-cells; (b) promoting differentiation in said solution of dendritic cells; (c) combining said solution of differentiated dendritic cells and said naïve CD4+ and/or CD8+ T-cells with a peptide of interest; (d) measuring the proliferation of T-cells in said step (c).

In one embodiment, the peptide of interest to be analyzed is derived from a polypeptide of interest. In the practice of the invention, it is possible to identify with precision the location of an epitope which can cause sensitization in an individual or sampling of individuals. In a preferred embodiment of the invention, a series of peptide oligomers which correspond to all or part of the polypeptide of interest are prepared. For example, a peptide library is produced covering the relevant portion or all of the protein. In one embodiment, the manner of producing the peptides is to introduce overlap into the peptide library, for example, producing a first peptide corresponds to amino acid sequence 1-10 of the subject protein, a second peptide corresponds to amino acid sequence 4-14 of the subject protein, a third peptide corresponds to amino acid sequence 7-17 of the subject protein, a fourth peptide corresponds to amino acid sequence 10-20 of the subject protein etc. until representative peptides corresponding to the entire molecule are created. By analyzing each of the peptides individually in the assay provided herein, it is possible to precisely identify the location of epitopes recognized by T-cells. In the example above, the greater reaction of one specific peptide than its neighbors' will facilitate identification of the epitope anchor region to within three amino acids. After determining the location of these epitopes, it is possible to alter the amino acids within each epitope until the peptide produces a different T-cell response from that of the original protein. Alternatively, the epitope may be used in its original form to stimulate an immune response against a target, e.g. infectious agent or tumor cell. Moreover, proteins may be identified herein which have desired high or low T-cell epitope potency which may be used in their naturally occurring forms.

"Antigen presenting cell" as used herein means a cell of the immune system which present antigen on their surface which is recognizable by receptors on the surface of T-cells. Examples of antigen presenting cells are dendritic cells, interdigitating cells, activated B-cells and macrophages.

"T-cell proliferation" as used herein means the number of T-cells produced during the incubation of T-cells with the antigen presenting cells, with or without antigen.

"Baseline T-cell proliferation" as used herein means T-cell proliferation which is normally seen in an individual in response to exposure to antigen presenting cells in the absence of peptide or protein antigen. For the purposes herein, the baseline T-cell proliferation level was determined on a per sample basis for each individual as the proliferation of T-cells in response to antigen presenting cells in the absence of antigen.

"T-cell epitope" means a feature of a peptide or protein which is recognized by a T-cell receptor in the initiation of an immunologic response to the peptide comprising that antigen. Recognition of a T-cell epitope by a T-cell is generally believed to be via a mechanism wherein T-cells recognize peptide fragments of antigens which are bound to class I or class II major

histocompatability (MHC) molecules expressed on antigen-presenting cells (*see e.g.*, Moeller, G. ed., "Antigenic Requirements for Activation of MHC-Restricted Responses," *Immunological Review*, Vol. 98, p. 187 (Copenhagen; Munksgaard) (1987).

"Sample" as used herein comprises mononuclear cells which are naïve, *i.e.*, not sensitized, to the antigen in question.

"Homolog" as used herein means a protein or enzyme which has similar catalytic action, structure and/or use as the protein of interest. For purposes of this invention, a homolog and a protein of interest are not necessarily related evolutionarily, *e.g.*, same functional protein from different species. It is desirable to find a homolog that has a tertiary and/or primary structure similar to the protein of interest as replacement of the epitope in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change. Thus, closely homologous enzymes will provide the most desirable source of epitope substitutions. Alternatively, if possible, it is advantageous to look to human analogs for a given protein. For example, substituting a specific epitope in a bacterial subtilisin with a sequence from a human analog to subtilisin (*i.e.*, human subtilisin) should result in less allergenicity in the bacterial protein.

An "analogous" sequence may be determined by ensuring that the replacement amino acids show a similar function, the tertiary structure and/or conserved residues to the amino acids in the protein of interest at or near the epitope. Thus, where the epitope region contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids should maintain that specific structure.

The epitopes determined according to the assay provided herein are then modified to reduce or augment the immunologic potential of the protein of interest. In a preferred embodiment, the epitope to be modified produces a level of T-cell proliferation of greater than three times the baseline T-cell proliferation in a sample. When modified, the epitope produces less than three times the baseline proliferation, preferably less than two times the baseline proliferation and most preferably less than or substantially equal to the baseline proliferation in a sample.

Preferably, the epitope is modified in one of the following ways: (a) the amino acid sequence of the epitope is substituted with an analogous sequence from a human homolog to the protein of interest; (b) the amino acid sequence of the epitope is substituted with an analogous sequence from a non-human homolog to the protein of interest, which analogous sequence produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; (c) the amino acid sequence of the epitope is substituted with a sequence which substantially mimics the major tertiary structure attributes of the epitope, but which produces a lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest; or (d) with any sequence which produces lesser immunogenic, e.g., allergenic, response due to T-cell epitope recognition than that of the protein of interest.

However, one of skill will readily recognize that epitopes can be modified in other ways depending on the desired outcome. For example, if a T-cell vaccine is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids which increase the immulogic response to the peptide via enhanced MHC binding and/or T-cell recognition. In another

example, if altering an autoimmune response against self -antigens is desired, it is contemplated the amino acid sequence of an epitope will be substituted with amino acids that decrease or cause a shift in an inflammatory or other immune response.

The present invention extends to all proteins against which it is desired to modulate the immunogenic response, for example, peptides to be used as T-cell vaccines, or peptides or proteins to be used as therapeutic agents against, *e.g.*, cancer, infectious diseases and autoimmune diseases. One of skill in the art will readily recognize the proteins and peptides of this invention are not necessarily native proteins and peptides. Indeed, in one embodiment of this invention, the assay described herein is used to determine the immunologic response of proteins from shuffled genes. For descriptions of gene shuffling and expression of such genes see, Stemmer, *Proc. Nat'l Acad. Sci. USA* 91:10747 (1994); Patten, *et al., Current Opinion in Biotechnol.* 8:724 (1997); Kuchner & Arnold, *Trends Biotechnol.* 15:523 (1997); Moore, *et al., J. Mol, Biol.* 272:336 (1997); Zhao, *et al., Nature Biotechnol.* 16:258 (1998); Giver, *et al., Proc. Nat'l Acad. Sci. USA* 95:12809 (1998); Harayama, *Trends Biotechnol.* 16:76 (1998); Lin, *et al., Biotechnol., Prog.* 15:467 (1999); and Sun, *J. Comput. Biol.* 6:77 (1999). The assay is used to predict the immunologic response of proteins encoded by shuffled genes. Once determined, the protein can be altered to modulate the immunolgic response to that protein.

In addition to the above proteins and peptides, the present invention can be used to reduce the allergenicity of proteins. These proteins include, but are not limited to, glucanases, lipases, cellulases, endo-glucosidase Hs (endo-H), proteases, carbohydrases, reductases, oxidases, isomerases, transferases, kinases, phosphatases, amylases, etc. In addition to reducing the allergenicity to an animal, such as a human, of naturally occurring amino acid sequences, this invention encompasses reducing the allergenicity of a mutated human protein, *e.g.*, a protein that has been altered to change the functional activity of the protein. In many instances, the mutation of human proteins to *e.g.*, increase activity, results in the incorporation of new T-cell epitope in the mutated protein. The assay of this invention can be used to determine the presence of the new T-cell epitope and determine substitute amino acids that will reduce the allergenicity of the mutated protein.

Although this invention encompasses the above proteins and many others, for the sake of simplicity, the following will describe a particularly preferred embodiment of the invention, the modification of protease. Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include a-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

In one embodiment herein, hybrid polypeptides are provided. "Hybrid polypeptides" are proteins engineered from at least two different proteins, which are preferably homologs of one another. For example, a preferred hybrid polypeptide might have the N-terminus of a protein and the C-terminus of a homolog of the protein. In a preferred embodiment, the two terminal ends can be

combined to correspond to the full-length active protein. In a preferred embodiment, the homologs share substantial similarity but do not have identical T-cell epitopes. Therefore, in one embodiment, for example, a polypeptide of interest having one or more T-cell epitopes in the C-terminus may have the C-terminus replaced with the C-terminus of a homolog having a less potent T-cell epitope in the C-terminus, less T-cell epitopes, or no T-cell epitope in the C-terminus. Thus, the skilled artisan understands that by being able to identify T-cell epitopes among homologs, a variety of variants producing different immunogenic responses can be formed. Moreover, it is understood that internal portions, and more than one homolog can be used to produce the variants of the present invention.

More generally, the variants provided herein can be derived from the precursor amino acid sequence by the substitution, deletion, insertion, or combination thereof of one or more amino acids of the precursor amino acid sequence. Such modification is preferably of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor enzyme, but can be by the manipulation of the precursor protein. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1.

"Recombinant", "recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram

negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

"Human subtilisin" means proteins of human origin which have subtilisin type catalytic activity, e.g., the kexin family of human derived proteases. An example of such a protein is represented by the sequence in Fig. 7. Additionally, derivatives or homologs of proteins provided herein, including those from non-human sources such as mouse or rabbit, which retain the essential activity of the peptide, such as the ability to hydrolyze peptide bonds, etc., have at least 50%, preferably at least 65% and most preferably at least 80%, more preferably at least 90%, and sometimes as much as 95 or 98% homology to the polypeptide of interest. In one embodiment, the polypeptide of interest is shown in the Figures.

The amino acid position numbers used herein refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically). "Corresponding" as used herein generally refers to an analogous position along the peptide.

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which the sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* can be aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. The conserved residues as between BPN' and *B. lentus* are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +170 is lysine (K) in both *B. amyloliquefaciens* and *B. licheniformis* subtilisins and arginine (R) in Savinase. In one embodiment of the protease variants of the invention, however, the amino acid equivalent to +170 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartic acid (D). The abbreviations and one letter codes for all amino acids in the present invention conform to the PatentIn User Manual (GenBank, Mountain View, CA) 1990, p.101.

Homologous sequences can also be determined by using a "sequence comparison algorithm." Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, an amino acid sequence is considered similar to a protein such as a protease if the smallest sum probability in a comparison of the test amino acid sequence to a protein such as a protease amino acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protein whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protein such as the protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protein such as the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protein such as a protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protein, for example, protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The variants of the present invention include the mature forms of protein variants, as well as the pro- and prepro- forms of such protein variants. The prepro- forms are the preferred construction since this facilitates the expression, secretion and maturation of the protein variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protein which when removed results in the appearance of the "mature" form of the protein. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protein variants such as protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protein or to the N-terminal portion of a proprotein which may participate in the secretion of the mature or pro forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protein gene which participate in the effectuation of the secretion of protein under native conditions. The present invention utilizes such sequences to effect the secretion of the protein variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protein variant consists of the mature form of the protein having a prosequence operably linked to the amino terminus of the protein and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protein is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protein and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protein include *Bacillus subtilis* I168 (also described in US Patent 4,760,025 (RE 34,606) and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis, B. lentus*, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. These techniques can be found in any molecular biology practice guide, for example,

Sambrook *et al.* Molecular Cloning - A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Springs Harbor Publishing (1989) ("Sambrook"); and Current Protocols in Molecular Biology, Ausubel *et al.*(eds.), Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1997 Supplement) ("Ausubel"). Such transformed host cells are capable of either replicating vectors encoding the protein variants or expressing the desired protein variant. In the case of vectors which encode the pre- or prepro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked", when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protein may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protein of interest, preparing genomic libraries from organisms expressing the protein, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

"Hybridization" is used to analyze whether a given DNA fragment or gene corresponds to a DNA sequence described herein and thus falls within the scope of the present invention. Samples to be hybridized are electrophoresed through an agarose gel (for example, 0.8% agarose) so that separation of DNA fragments can be visualized by size. DNA fragments are typically visualized by ethidium bromide staining. The gel may be briefly rinsed in distilled H2O and subsequently depurinated in an appropriate solution (such as, for example, 0.25M HCl) with gentle shaking followed by denaturation for 30 minutes (in, for example, 0.4 M NaOH) with gentle shaking. A renaturation step may be included, in which the gel is placed in 1.5 M NaCl, 1MTris, pH 7.0 with gentle shaking for 30 minutes.

The DNA should then be transferred onto an appropriate positively charged membrane, for example, Maximum Strength Nytran Plus membrane (Schleicher & Schuell, Keene, N.H.), using a transfer solution (such as, for example, 6XSSC (900 mM NaCl, 90 mM trisodium citrate). Once the transfer is complete, generally after about 2 hours, the membrane is rinsed in *e.g.*, 2X SSC (2X SSC = 300 mM NaCl, 30 mM trisodium citrate) and air dried at room temperature. The membrane should then be prehybridized (for approximately 2 hours or more) in a suitable prehybridization solution (such as, for example, an aqueous solution containing per 100 mL: 20-50 mL formamide, 25 mL of 20X SSPE (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH2PO4, pH 7.7), 2.5 mL of 20% SDS, and 1 mL of 10 mg/mL sheared herring or salmon sperm DNA). As would be known to one of skill in the art, the amount of formamide in the prehybridization solution may be varied depending on the nature of the reaction obtained according to routine methods. Thus, a lower amount of formamide may result in more complete hybridization in terms of identifying hybridizing molecules than the

same procedure using a larger amount of formamide. On the other hand, a strong hybridization band may be more easily visually identified by using more formamide.

A DNA probe that is complementary or is nearly complementary to the DNA sequence of interest and is generally between 100 and 1000 bases in length is labeled (using, for example, the Megaprime labeling system according to the instructions of the manufacturer) to incorporate 32P in the DNA. The labeled probe is denatured by heating to 95°C for 5 minutes and immediately added to the membrane and prehybridization solution. The hybridization reaction should proceed for an appropriate time and under appropriate conditions, for example, for 18 hours at 37°C with gentle shaking or rotating. The membrane is rinsed (for example, in 2X SSC/0.3% SDS) and then washed in an appropriate wash solution with gentle agitation. The stringency desired will be a reflection of the conditions under which the membrane (filter) is washed.

Specifically, the stringency of a given reaction (*i.e.*, the degree of homology necessary for successful hybridization) will depend on the washing conditions to which the filter is subjected after hybridization. "Low-stringency" conditions as defined herein will comprise washing a filter with a solution of 0.2X SSC/0.1% SDS at 20°C for 15 minutes. "High-stringency" conditions comprise a further washing step comprising washing the filter a second time with a solution of 0.2X SSC/0.1% SDS at 37°C for 30 minutes.

After washing, the membrane is dried and the bound probe detected. If 32P or another radioisotope is used as the labeling agent, the bound probe can be detected by autoradiography. Other techniques for the visualization of other probes are well-known to those of skill. The detection of a bound probe indicates a nucleic acid sequence has the desired homology and is encompassed within this invention.

The cloned protein is then used to transform a host cell in order to express the protein. The protein gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, *i.e.*, transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protein gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protein gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the protein gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

In one embodiment, the gene can be a natural gene such as that from *B lentus* or *B. amyloliquefaciens*. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protein may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor protein is determined. Multiple, overlapping synthetic single-stranded DNA fragments

are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protein. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protein gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protein. Such modifications include the production of recombinant proteins as disclosed in US Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of protein variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protein variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protein is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protein gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protein gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

In one aspect of the invention, the objective is to secure a variant protein having altered allergenic potential as compared to the precursor protein, since decreasing such potential enables safer use of the enzyme. While the instant invention is useful to lower allergenic potential, the mutations specified herein may be utilized in combination with mutations known in the art to result altered thermal stability and/or altered substrate specificity, modified activity or altered alkaline stability as compared to the precursor.

Accordingly, the present invention is directed to altering the capability of the T-cell epitope which includes residue positions 170-173 in *Bacillus lentus* to induce T-cell proliferation. One particularly preferred embodiment of the invention comprises making modification to either one or all of R170D, Y171Q and/or N173D. Similarly, as discussed in detail above, it is believed that the modification of the corresponding residues in any protein will result in a the neutralization of a key T-cell epitope in that protein. Thus, in combination with the presently disclosed mutations in the region corresponding to amino acid residues 170-173, substitutions at positions corresponding to N76D/S103A/V104I/G159D optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin may be used, in addition to decreasing the allergenic potential of the variant protein of the invention, to modulate overall stability and/or proteolytic activity of the enzyme. Similarly, the substitutions provided herein may be combined with mutation at the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 to Aspartate (D) in combination with the mutations S103A/V104I/G159D and optionally in combination with one or more substitutions selected from the group consisting of positions corresponding to V68A, T213R, A232V, Q236H, Q245R, and T260A of *Bacillus amyloliquefaciens* subtilisin, to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred embodiments of the invention include the following specific combinations of substituted residues corresponding to positions:

N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D /Q236H/Q245R;

V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and

V68A/N76D//S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protein.

Based on the screening results obtained with the variant proteins, the noted mutations noted above in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protein variants of the invention are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the protein mutants of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protein variants of the present invention may be used for any purpose that native or wild-type proteins are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis,

waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise, in addition to decreased allergenicity, enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteins, particularly proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteins, particularly proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protein's denaturing temperature. In addition, proteins of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The variant proteins of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteins of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The variants can be screened for proteolytic activity according to methods well known in the art. Preferred protease variants include multiple substitutions at positions corresponding to:
N76D/S103A/V104I/G159D/K170D/Y171Q/S173D;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/Q236H/Q245R;
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/A232V/Q236H/Q245R; and
V68A/N76D/S103A/V104I/G159D/K170D/Y171Q/S173D/T213R/A232V/Q236H/Q245R/T260A of *Bacillus amyloliquefaciens* subtilisin.

The proteins of this invention exhibit modified immunogenicity when compared to their precursor proteins. In preferred embodiments, the proteins exhibit reduced allergenicity. In other embodiments, the proteins exhibit increased immunogenicity. The increase in immunogenicity is manifested by an increase in B-cell or humoral immunological response, by an increase in T-cell or cellular immunological response, or by an increase in both B and T cell immunological responses. One of skill will readily recognize that the uses of the proteins of this invention will be determined, in large part, on the immunological properties of the proteins. For example, enzymes that exhibit reduced allergenicity can be used in cleaning compositions. "Cleaning compositions" are compositions that can be used to remove undesired compounds from substrates, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin (soaps and creams), etc.

Proteins, in particular, cellulases, proteases, and amylases, with reduced allergenicity can also be used in the treatment of textiles. "Textile treatment" comprises a process wherein textiles, individual yarns or fibers that can be woven, felted or knitted into textiles or garments are treated to effect a desired characteristic. Examples of such desired characteristics are "stone-washing", depilling, dehairing, desizing, softening, and other textile treatments well known to those of skill in the art.

Therapeutic proteins against which individuals mount an immune response are also included in the invention. In particular, individuals who lack endogenous production of the protein are susceptible to forming neutralizing antibodies and become refractile to treatment. Likewise, modifications of a protein may introduce new epitopes that are potentially immunogeneic. Methods of the invention can be used to identify and modify epitopes in , e.g., human Factor VIII, to prevent neutralizing responses.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically_active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

All publications and patents referenced herein are hereby incorporated by reference in their entirety. The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

EXAMPLES

Example 1

Assay for the Identification of Peptide T-Cell Epitopes Using Naïve Human T-Cells

Fresh human peripheral blood cells were collected from "naïve" humans, *i.e.*, persons not known to be exposed to or sensitized to *Bacillus lentus* protease, for determination of antigenic epitopes in protease from *Bacillus lentus* and human subtilisin. Naïve humans is intended to mean that the individual is not known to have been exposed to or developed a reaction to protease in the past. Peripheral mononuclear blood cells (stored at room temperature, no older than 24 hours) were prepared for use as follows: Approximately 30 mls of a solution of buffy coat preparation from one unit of whole blood was brought to 50 ml with Dulbecco's phosphate buffered solution (DPBS) and split into two tubes. The samples were underlaid with 12.5 ml of room temperature lymphoprep density separation media (Nycomed density 1.077 g/ml). The tubes were centrifuged for thirty minutes at 600G. The interface of the two phases was collected, pooled and washed in DPBS. The

cell density of the resultant solution was measured by hemocytometer. Viability was measured by trypan blue exclusion.

From the resulting solution, a differentiated dendritic cell culture was prepared from the peripheral blood mononuclear cell sample having a density of 108 cells per 75 ml culture flask in a solution as follows:

(1) 50 ml of serum free AIM V media (Gibco) was supplemented with a 1:100 dilution beta-mercaptoethanol (Gibco). The flasks were laid flat for two hours at 37°C in 5% CO2 to allow adherence of monocytes to the flask wall.

(2) Differentiation of the monocyte cells to dendritic cells was as follows: nonadherent cells were removed and the resultant adherent cells (monocytes) combined with 30 ml of AIM V, 800 units/ml of GM-CSF (Endogen) and 500 units/ml of IL-4 (Endogen); the resulting mixture was cultured for 5 days under conditions at 37°C in 5% CO2. After five days, the cytokine TNFa (Endogen) was added to 0.2 units/ml, and the cytokine IL-1a (Endogen) was added to a final concentration of 50 units/ml and the mixture incubated at 37°C in 5% CO2 for two more days.

(3) On the seventh day, Mitomycin C was added to a concentration of 50 microgram/ml was added to stop growth of the now differentiated dendritic cell culture. The solution was incubated for 60 minutes at 37°C in 5% CO2. Dendritic cells were collected by gently scraping the adherent cells off the bottom of the flask with a cell scraper. Adherent and non-adherent cells were then centrifuged at 600G for 5 minutes, washed in DPBS and counted.

(4) The prepared dendritic cells were placed into a 96 well round bottom array at 2x104/well in 100 microliter total volume of AIM V media.

CD4+ T cells were prepared from frozen aliquots of the peripheral blood cell samples used to prepare the dendritic cells using the human CD4+ Cellect Kit (Biotex) as per the manufacturers instructions with the following modifications: the aliquots were thawed and washed such that approximately 108 cells will be applied per Cellect column; the cells were resuspended in 4 ml DPBS and 1 ml of the Cell reagent from the Cellect Kit, the solution maintained at room temperature for 20 minutes. The resultant solution was centrifuged for five minutes at 600G at room temperature and the pellet resuspended in 2 ml of DPBS and applied to the Cellect columns. The effluent from the columns was collected in 2% human serum in DPBS. The resultant CD4+ cell solution was centrifuged, resuspended in AIMV media and the density counted.

The CD4+ T-cell suspension was resuspended to a count of 2x106/ml in AIM V media to facilitate efficient manipulation of the 96 well plate.

Peptide antigen is prepared from a 1M stock solution in DMSO by dilution in AIM V media at a 1:10 ratio. 10 microliters of the stock solution is placed in each well of the 96 well plate containing the differentiated dendritic cells. 100 microliter of the diluted CD4+ T-cell solution as prepared

above is further added to each well. Useful controls include diluted DMSO blanks, and tetanus toxoid positive controls.

The final concentrations in each well, at 210 microliter total volume are as follows:

2x104 CD4+

2x105 dendritic cells (R:S of 10:1)

5 mM peptide

Example 2

Identification of T-Cell Epitopes in Protease from *Bacillus lentus* and Human subtilisin

Peptides for use in the assay described in Example 1 were prepared based on the *Bacillus lentus* and human subtilisin amino acid sequence. Peptide antigens were designed as follows. From the full length amino acid sequence of either human subtilisin or *Bacillus lentus* protease provided in Figure 1, 15mers were synthetically prepared, each 15mer overlapping with the previous and the subsequent 15mer except for three residues.

Peptides used correspond to amino acid residue strings in *Bacillus lentus* as provided in Figure 8, and peptides correspond to amino acid residues in human subtilisin as provided in Figure 7. The peptides used corresponding to the proteases is provided in Fig. 6. All tests were performed at least in duplicate. All tests reported displayed robust positive control responses to the antigen tetanus toxoid. Responses were averaged within each experiment, then normalized to the baseline response. A positive event was recorded if the response was at least 3 times the baseline response.

The immunogenic response (*i.e.*, T-cell proliferation) to the prepared peptides from human subtilisin and *Bacillus lentus* was tallied and is provided in Figures 4 and 5, respectively. T-cell proliferation was measured by the incorporated tritium method. The results shown in Figures 4 and 5 as a comparison of the immunogenic additive response in 10 individuals (Figure 4) and 16 individuals (Figure 5) to the various peptides. Response is indicated as the added response wherein 1.0 equals a baseline response for each sample. Thus, in Figure 4, a reading of 10.0 or less is the baseline response and in Figure 5 a reading of 16.0 or less the baseline response. The greater the response, the more potent the T-cell epitope is considered.

As indicated in Figures 4 and 5, the immunogenic response of the naïve blood samples from unsensitized individuals showed a marked allergenic response at the peptide fragment from *Bacillus lentus* corresponding to residues 170-173 of *Bacillus amyloliquefaciens* protease. As expected, the corresponding fragment in human subtilisin evokes merely baseline response.

Fig. 9 shows the T-cell response to peptides derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Peptide E05 represents the region corresponding to 170-173 in protease from *Bacillus amyloliquefaciens*. As shown in Fig. 9, the hypersensitive individual was highly responsive to the T-cell epitope represented by the peptide E05. This result confirms that, by practicing the assay according to the invention, it is possible to predict the major epitopes identified by the T-cells of a hypersensitive individual.

Fig. 10 shows the T-cell response to various alanine substitutions in the E05 peptide derived from *Bacillus lentus* protease in a sample taken from an individual known to be hypersensitive to *Bacillus lentus* protease. Alanine substitutions were used as substitutions for the purpose of determining the role of any specific residue within the epitope. The legend of Figure 10 refers to the position of the peptide in which an alanine was substituted, i.e., in peptide E06 (sequence GSISYPARYANAMAV), G to A = 2, S to A = 3, I to A = 4, S to A = 5, Y to A = 6, P to A = 7, R to A = 8, Y to A = 9, N to A = 10, M to A = 11 and V to A = 12. As indicated in Figure 10, substitution of either of the residues R170A, Y171A and/or N173A in protease from *Bacillus lentus* results in dramatically reduced response in the hypersensitive individual's blood sample.

From these results, it is apparent that the residues 170, 171 and 173 are largely responsible for the initiation of allergic reaction within the protease from *Bacillus lentus*.

Example 3

Identification of T-Cell Epitopes in Cellulase from *Humicola insolens* (Carezyme®)

The procedure described above was performed on peptides derived from a cellulase from *Humicola insolens* (Carezyme ® from Novo Nordisk). As can be seen from Figure 13, 2 T-cell epitopes were discovered, A01 and F06.

Example 4

Identification of T-Cell Epitopes in Lipase from *Thermomyces Lanuginosa* (Lipolase®)

The procedure described in Example 2 was performed on peptides derived from a lipase from *Thermomyces lanuginosa* (Lipolase ® from Novo Nordisk). As can be seen from Figure 14, two T-cell epitopes were discovered, A12 and C06. Peptide E03 effected slightly increased T-cell proliferation in the naïve donors, however, this peptide is consecutive to A12 and they represent one epitope. In this regard, the skilled artisan understands that the length of the epitopes can be varied, and the precise potency of the epitope, naturally occuring or mutated can be determined by the methods herein.

Example 5

Identification of T-Cell Epitopes in Endoglucanase H from Streptomyces plicatus

The procedure described in Example 2 was performed on peptides derived from endoglucanase H from *Streptomyces plicatus*. As can be seen from Figure 15, a single T-cell epitope was discovered, C06.

Example 6

Identification of T-Cell Epitopes in a Protease Hybrid (GG36-BPN')

After determining the location of a T-cell epitope, a protease hybrid was constructed using established protein engineering techniques. The hybrid was constructed so that a highly allergenic amino acid sequence of the protein was replaced with a corresponding sequence from a less

allergenic homolog. In this instance, the first 122 amino acids of the protease were derived from GG36, and the remaining amino acid sequence was derived from BPN'.

The hybrid was first tested from a 100 ppm sample in North American condition in 24 well assay at .5 ppm, superfixed swatches, liquid (Tide KT) at .5 in 24 well assay with 3K swatches, and in the N'N'-dimethyl Casein Assay, 5 g/l DMC in NA detergent, TNBS dectection method.

The results are shown in Figures 16, 17 and 18.

Example 7

Identification of a Naturally Occuring Low Immunogenic Protein

Using the methods herein, proteinase K was identified as producing a lower immunogenic response than other commercially available proteases. Proteinase K as identified herein is from Tritirachium Album limber. For a general description of proteases and methodologies, see, Mathew, C.G.P. Isolation of high molecular weight eukaryotic DNA, in Methods in Molecular Biology, vol. 2: Nucleic Acids (Walker, J.M.,ed.), Humana, Clifton, NJ, (1984) pp. 31-34.

Example 8:

T-cell Epitope Introduced into a Non-allergenic Protein

It has been found that *Bacillus amyloliquefaciens* subtilisin is comparatively non-immunogenic when tested in Hartley strain guinea pigs. A related protein from *Bacillus lentis* is highly immunogenic. We had previously defined functional T cell epitopes in the *B. lentis* molecule which were not found in the *B. amyloliquefaciens* molecule, even though the sequences of interest were highly homologous. In order to test the principle that the presence of a functional T cell epitope can control the relative levels of antibody production, we created a *B. lentis*-like T cell epitope in the *B. amyloliquefaciens* molecule. This change was accomplished by the substitution of a single amino acid in the *B. amyloliquefaciens* sequence. *B. amyloliquefaciens* subtilisin and the T cell epitope modified variant of *B. amyloliquefaciens* subtilisin were tested in a guinea pig model of immungenicity.

B. lentis and *B. amyloliquefaciens* subtilisin T cell epitope mapping: Guinea pigs were immunized with 20 µg/immunization of subtilisin from either *B. lentis* or *B. amyloliquefaciens*. Animals were immunized subcutaneously in adjuvant every two weeks for 10 to 12 weeks. A single cell suspension of guinea pig splenocytes was created from each animal's spleen. Cells were plated at 5×10^5 splenocytes per well in round bottom 96 well plates. 15-mer peptides off-set by 3 amino acids were synthesized by Mimotopes. Peptides were resuspended to 1 mM in DMSO. Peptides were added to the cells at a final concentration of 5 µM. Cultures were incubated for 5 days at 37 °, 5% CO_2. Wells were pulsed with 0.5 µCi tritiated thymidine, and allowed to incubate for an additional 18 hours. Wells were harvested, and thymidine incorporation assessed.

Two T cell epitopes were found in *B. lentis* subtilisin, and none were found in *B. amyloliquefaciens* subtilisin (>10 animals tested for these epitopes). The *B. lentis* T cell epitopes were found to comprise the following sequences:

IAALNNSIGVLGVAP (SEQ ID NO:237) and LEWAGNNGMHVANLSLGS (SEQ ID NO:238)

For SEQ ID NO:237, the similar sequence in *B. amyloliquefaciens* subtilisin is VAALNNSIGVLGVAP (SEQ ID NO:239). The similar region in *B. amyloliquefaciens* subtilisin for SEQ ID NO:238 was the much less homologous: IEWAIANNMDVINMSLG (SEQ ID NO:240).

SEQ ID NO:237 and the homologous region in the *B. amyloliquefaciens* subtilisin molecule (SEQ ID NO: 239) differ by one amino acid: In *B. lentis* subtilisin the first amino acid is an I, while it is a V in *B. amyloliquefaciens*. Therefore, we reasoned that if we changed the V in the *B. amyloliquefaciens* sequence to an I, we would create the *B. lentis* T cell epitope in the *B. amyloliquefaciens* backbone.

This molecule was created by standard molecular biological techniques, and was called *B. amyloliquefaciens* V72I. It was also known as GP002.

Guinea pig immunizations: Adult female Hartley guinea pigs were immunized with various doses of *B. amyloliquefaciens* subtilisin and GP002. The doses were 1, 5, 10, and 20 µg/dose. There were four animals for each dose. Animals were immunized subcutaneously with enzyme in Complete Freund's Adjuvant for the first immunization. All subsequent

immunizations were performed in Incomplete Freund's adjuvant. Animals were immunized, and a serum sample taken, every two weeks.

ELISA: A direct ELISA was performed. Costart EIA plates were coated with 10 µg/ml of the immunizing enzyme in PBS overnight at 4 °C. Plates were washed and blocked with 1% BSA in PBS. Serum samples were diluted in 1% BSA/PBS, and incubated on the enzymes coated plates for 1 hour. Serum samples were washed out, and biotinylated anti-guinea pig IgG was added at a 1:10,000 dilution in 1% BSA/PBS. The secondary reagent was incubated for 1 hour. The wells were washed, and avidin conjugated horse radish peroxidase was added to the wells at a 1:1000 dilution in 1% BSA/PBS. After 30 minutes, the substrate (ABTS) was added and the OD_{405} was read after 30 minutes.

Calculation of titers: Background was subtracted from the OD readings, and the results plotted for each individual guinea pig. A linear regression analysis was performed on the linear portion of the curve. The titer value was calculated from the linear regression equation for an OD = 0.5. These individual titers were then averaged.

Two guinea pigs in the 10 µg dose of GP001 died at 2 weeks into the study. The data for the 10 µg dose was therefore thrown out.

Two results are immediately apparent: first, the GP002 variant increased the titers of antigen-specific antibody over the entire time course for the lower doses of enzymes; and the GP002 variant increased titers of antigen-specific antibody for all doses of enzymes in the earliest time points.

At the extended time points and for the higher doses, the difference between *B. amyloliquefaciens* subtilisin and its variant were no longer apparent. See Figures 19 and 20.

From the Figures it is apparent that a single change in the amino acid sequence of *B. amyloliquefaciens* subtilisin significantly altered its immunogenicity.

Example 9

Reduction of Allergenicity *in Vivo*

Given the ability to identity of human T cell epitopes, it is possible to modify their amino acid sequence to reduce activation of T cells and the subsequent immune response to the protein. However, to evaluate the *in vivo* effect of these changes, it is necessary to use an animal model that represents the ability of human HLA molecules to present the epitopes. For example, human T cell epitopes have been identified in the molecule BPN' in the regions 70-84 and 109-122 (see USSN 09/500,135, filed February 8, 2000; Figure 16).

Substitutions in the amino acid sequence of these motifs led to reduced T cell proliferation *in vitro* using human cells representing a broad range of human HLA haplotypes. *In vitro* binding assays using EBV-transformed B cell lines demonstrated the peptides 70-84 and 109-123 bound to HLA DQ2 molecules. The substitutions that were found to reduce T-cell proliferation were introduced into the coding sequence for FNA (BPN' with a Y217L substitution) for production of reduced immunogenic FNA variants.

Transgenic mice expressing human HLA genes have been used to study epitopes presented to the immune system *in vivo*. Although the responding immune cells are of mouse origin, there is a strong correlation between the epitopes recognized in humans and mice. However, a novel use of HLA transgenic mice is in the testing of variant proteins for reduced allergenic potential as a prediction of how human individuals will respond.

To demonstrate this effect, both FNA and the FNA variant containing amino acid changes in the epitopes 70-84 and 109-123 were used to immunize HLA DR3/DQ2 transgenic mice that had been backcrossed onto I-Ab knockout mice (lacking the expression of endogenous I-A class II molecules, referred to as C2D). Adult male HLA-DR3/DQ2/C2D mice were immunized with 50 µg of FNA or FNA Variant emulsified in Complete Freund's Adjuvant. The immunization was administered intraperitoneally. Two weeks later, the mice received another intraperitoneal immunization of 50 µg FNA or the Variant emulsified in Incomplete Freund's Adjuvant. One week later, the mice were bled via the retro-orbital route, and the serum collected. Serum was assessed for antigen-specific IgG antibodies in a direct ELISA protocol. Briefly, 96 well flat-bottomed EIA plates were coated overnight with10 µg/ml of denatured FNA. Plates were washed, blocked with 1% Fetal Calf Serum, and serum was titered out at 1:10 dilutions. The serum was washed out of the wells, and antigen-specific IgG was detected with horse radish peroxidase conjugated anti-mouse IgG. Results are presented as serum dilution versus average optical density (x 1000) in Table 1 and Figure 21.

Table 1

Dilution	FNA	FNA Variant
10	2937.5	88
100	2476	120
1000	1695	103
10000	641.5	80
100000	207	85
1000000	129.5	76
10000000	88.5	85

The results indicated the changes introduced into regions 70-84 and 109-123 significantly reduced the ability of DQ2 transgenic mice to mount a humoral response to the variant and provide a method for *in vivo* characterization of engineered proteins predicted with the methods of this invention to show reduced immunogenicity in humans.

CLAIMS

1. A method to determine the allergenic potential of an engineered protein comprising the steps of,

a) immunizing a first transgenic mouse with a protein of interest and immunizing a second transgenic mouse with an engineered protein wherein said engineered protein is a variant of said protein of interest and said protein of interest includes a T-cell epitope wherein the variant differs from the protein of interest by having an altered T cell epitope;

b) collecting serum of said first and said second immunized transgenic mice;

c) measuring the serum for antigen specific immunoglobulins; and

d) comparing the immunogenic response of said variant and said protein of interest wherein the variant and the protein of interest produce a different immunogenic response in said transgenic mice.

2. The method according to claim 1, wherein said protein of interest is an enzyme.

3. The method according to claim 2, wherein said enzyme is a protease.

4. The method according to claim 1, wherein the antigen specific immunoglobulin is IgG.

5. The method according to claim 1, wherein the first transgenic mouse and second transgenic mouse are HLA DR3/DQ2.

6. The method according to claim 5, wherein the HLA DR3/DQ2 transgenic mice have been backcrossed with mice lacking the expression of endogenous I-A class II molecules.

7. The method according to claim 1, wherein said T-cell epitope is altered with amino acid substitutions.

8. The method according to claim 1, wherein said T-cell epitope is altered by having a terminal portion of said protein of interest which includes said T-cell epitope replaced with a corresponding terminal portion of a homolog of said protein of interest wherein said homolog does not comprise a T-cell epitope identical to said replaced T-cell epitope.

9. The method according to claim 1, wherein said immunogenic response produced by the variant is less than the immunogenic response produced by the protein of interest.

10. The method according to claim 1, wherein said immunogenic response produced by the variant is more than the immunogenic response produced by the protein of interest.

11. A method of using transgenic mice to predict the allergenic response of a human to an engineered protein comprising the steps of,

a) immunizing a first transgenic mouse with a protein of interest and immunizing a second transgenic mouse with an engineered protein, wherein said engineered protein is a variant of said protein of interest and the protein of interest includes a T-cell epitope, wherein the variant differs from the protein of interest by having an altered T cell epitope;

b) collecting serum of the first and the second immunized transgenic mice;

c) measuring the serum for antigen specific immunglobulins; and

d) comparing the immunogenic response of the variant and the protein of interest, wherein the variant and the protein of interest produce a different immunogenic response in said transgenic mice, and wherein said immunogenic response is predictive of the allergenic response in humans.

12. The method according to claim 11, wherein said protein of interest is a protease.

13. A variant of a polypeptide of interest comprising a T-cell epitope, wherein said variant differs from said polypeptide of interest by having an altered T-cell epitope such that said variant and said polypeptide produce different immunogenic responses in an individual.

14. The variant of claim 13, wherein said immunogenic response produced by said variant is greater than said immunogenic response produced by said protein of interest.

15. A method for determining the immunogenic response produced by a protein, comprising;

a) obtaining from a single blood source a solution of dendritic cells and a solution of naive CD4+ and/or CD8+ T-cells;

b) promoting differentiation in said dendritic cells;

c) combining said solution of differentiated dendritic cells and said naive CD4+ and/or CD8+ T cells with said protein; and

d) measuring the proliferation of said T-cells in step c).

16. The method according to claim 15 further comprising comparing the proliferation of said T-cells to the proliferation of a second protein.

17. The method according to claim 16, wherein the protein of interest and the second protein are homologs of one another.

18. The method of claim 17, wherein the protein of interest and the second protein are proteases.

19. The method of claim 18, wherein the protein of interest and the second protein are each different peptides of the same protein.

20. A method of altering the immunogenicity of a polypeptide of interest comprising, a) determining the immunogenicity of said polypeptide; b) identifying a T-cell epitope in said polypeptide; and c)altering said T-cell epitope so as to alter the immunogenicity of said polypeptide.

21. The method according to claim 20, wherein said T-cell epitope is altered by having at least one amino acid substitution.

22. The method according to claim 20, wherein said T-cell epitope is altered by replacing a portion of said polypeptide of interest which includes said T-cell epitope with a corresponding portion of a homolog of said polypeptide of interest, where the corresponding portion does not contain said T-cell epitope.

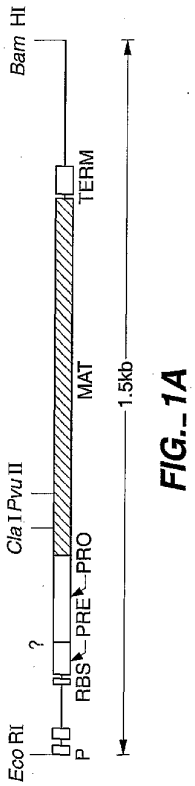

FIG._1A

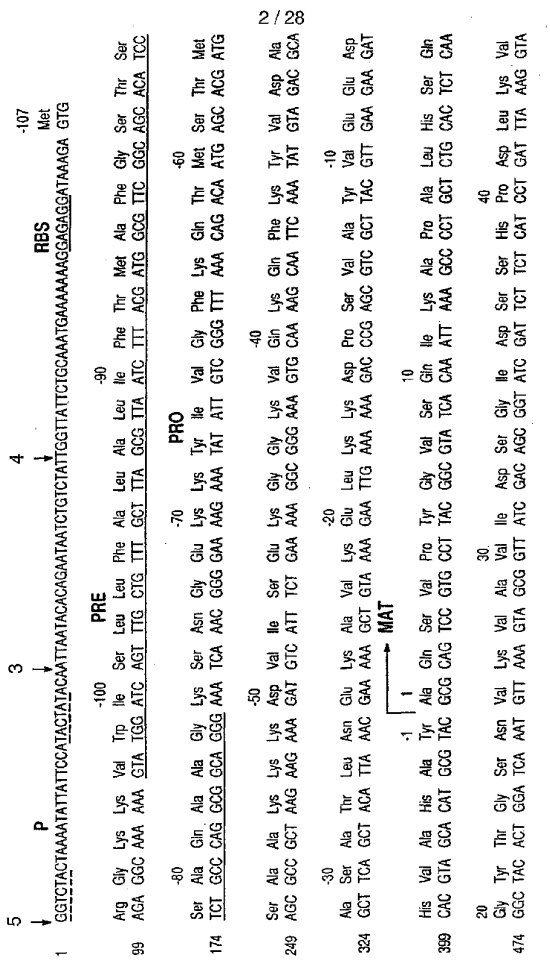
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FIG._1B - 1

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                         50                      Pro Asn         60  Asp
     Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His Gly Thr His Val Ala
549  GCA GGC GGA GCC AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC GGA ACT CAC GTT GCC

     70                                      80                                  Ser Ala 90
     Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys
624  GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA

                 Asp Ala 100                                     110
     Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met
699  GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG

     120                                     130                                     140
     Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
774  GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA

                         150                             Ser Thr 160
     Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly
849  TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT

     170                                     180                                     190
     Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro
924  AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

                         200                                     210
     Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly
999  GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

     220                                     230                                     240
     Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT
```

FIG._1B - 2

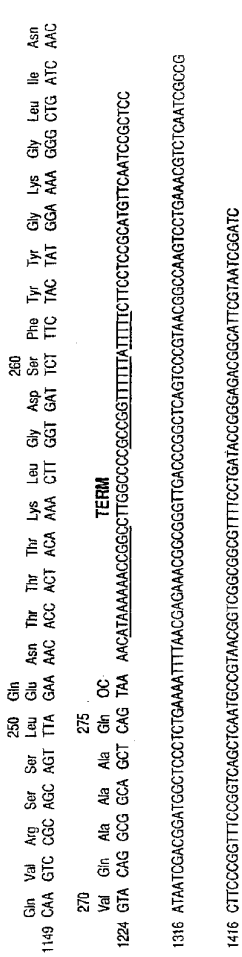


FIG._1B - 3

FIG._1B

FIG._1B - 1

FIG._1B - 2

FIG._1B - 3

CONSERVED RESIDUES IN SUBTILISINS FROM *BACILLUS AMYLOLIQUEFACIENS*

```
1                 10                  20
A Q S V P . G . . . . . A P A . H . . G

21                30                  40
. T G S . V K V A V . D . G . . . . H P

41                50                  60
D L . . . G G A S . V P . . . . . Q D

61                70                  80
. N . H G T H V A G T . A A L N N S I G

81                90                  100
V L G V A P S A . L Y A V K V L G A . G

101               110                 120
S G . . S . L . . G . E W A . N . . . .

121               130                 140
V . N . S L G . P S . S . . . . . A . .

141               150                 160
. . . . . G V . V V A A . G N . G . . .

161               170                 180
. . . . . Y P . . Y . . . . A V G A .

181               190                 200
D . . N . . A S F S . . G . . L D . . A

201               210                 220
P G V . . Q S T . P G . . Y . . . N G T

221               230                 240
S M A . P H V A G A A A L . . . K . . .

241               250                 260
W . . . Q . R . . L . N T . . . L G . .

261               270
. . Y G . G L . N . . A A . .
```

FIG._2

FIG._3A

COMPARISION OF SUBTILISIN SEQUENCES FROM:
B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

```
                                                  10                  20                  30
01  AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHP
    AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHP
    AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHP
    AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHP

41  DLKVAGGASMVPSETNPFQDNNSHGTHVAGTVAALNNSIG
    DLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAALNNSIG
    DLNVVGGASFVAGEAYN*TDGNGHGTHVAGTVAALDNTTG
    DLNIRGGASPVPGE*PSTQDGNGHGTHVAGTIAALNNSIG

81  VLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMD
    VLGVSPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMD
    VLGVAPSVSLYAVKVLNSSGSGSYSGIVSGIEWATTNGMD
    VLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMH

121 VINMSLGGPSGSAALKAAVDKAVASGVVVVAAAGNEGTSG
    VINMSLGGPTGSTALKTVVDKAVSSGIVVVAAAGNEGSSG
    VINMSLGGASGSTAMKQAVDNAYARGVVVVAAAGNSGNSG
    VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGS
```

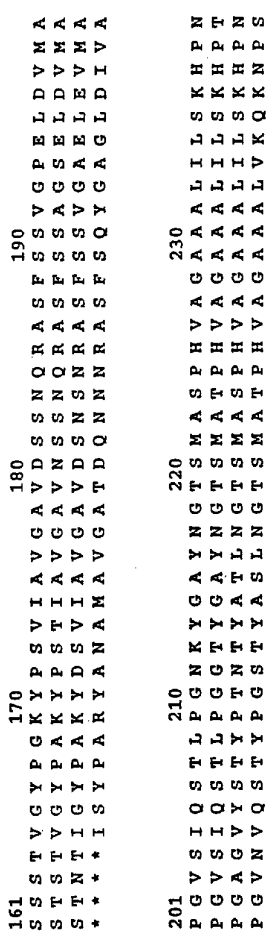


FIG._3B

FIG._3

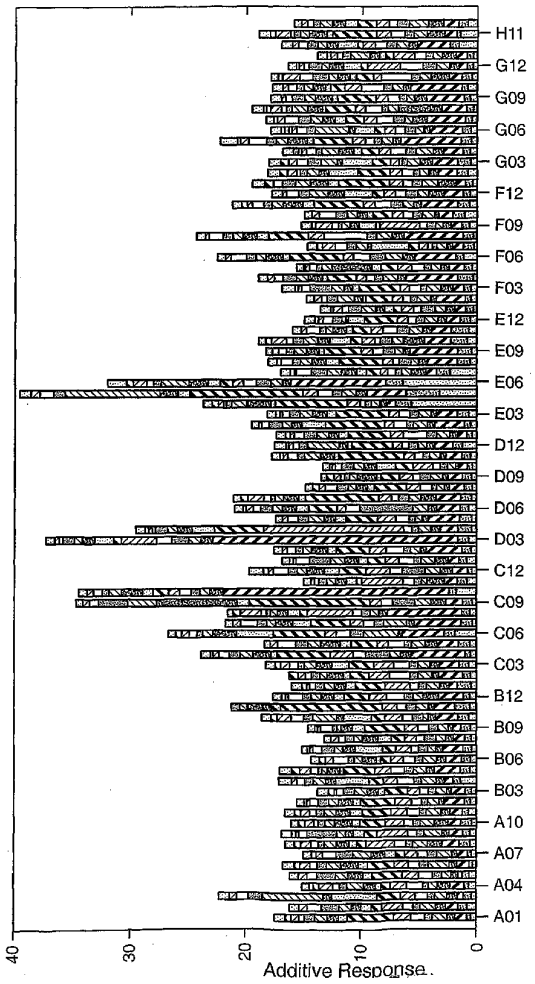

H11
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
40
30
20
10
0
Additive Response

FIG._4

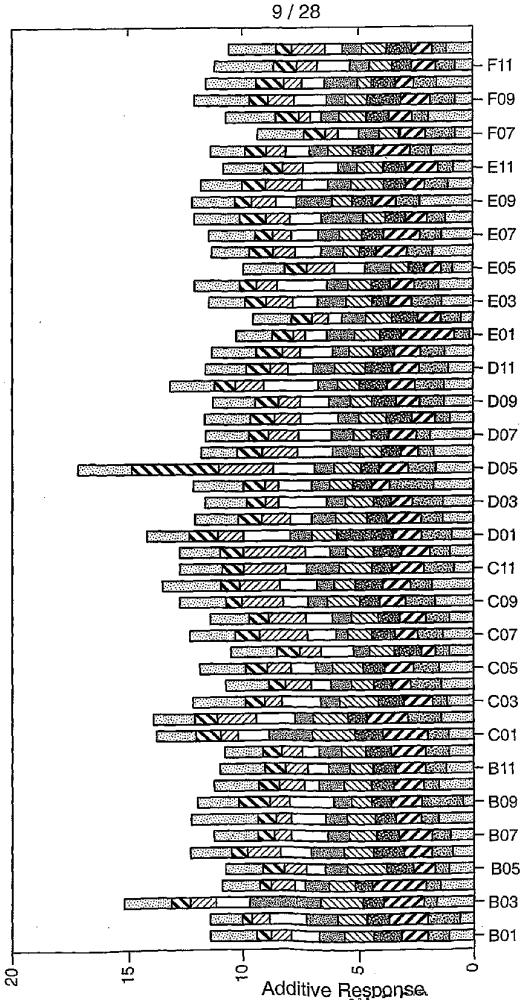

F11
F09
F07
E11
E09
E07
E05
E03
E01
D11
D09
D07
D05
D03
D01
C11
C09
C07
C05
C03
C01
B11
B09
B07
B05
B03
B01
20
15
10
5
0
Additive Response

FIG._5

1	A12	IKDFHVYFRESRDAG	49	E12	SATSRGVLVVAASGN
2	A11	LEQAVNSATSRGVLV	50	E11	SRGVLVVAASGNSGA
3	A10	AQSVPWGISRVQAPA	51	E10	VLVVAASGNSGAGSI
4	A9	VPWGISRVQAPAAHN	52	E9	VAASGNSGAGSISYP
5	A8	GISRVQAPAAHNRGL	53	E8	SGNSGAGSISYPARY
6	A7	RVQAPAAHNRGLTGS	54	E7	SGAGSISYPARYANA
7	A6	APAAHNRGLTGSGVK	55	E6	GSISYPARYANAMAV
8	A5	AHNRGLTGSGVKVAV	56	E5	SYPARYANAMAVGAT
9	A4	RGLTGSGVKVAVLDT	57	E4	ARYANAMAVGATDQN
10	A3	TGSGVKVAVLDTGIS	58	E3	ANAMAVGATDQNNNR
11	A2	GVKVAVLDTGISTHP	59	E2	MAVGATDQNNNRASF
12	A1	VAVLDTGISTHPDLN	60	E1	GATDQNNNRASFSQY
13	B12	LDTGISTHPDLNIRG	61	F12	DQNNNRASFSQYGAG
14	B11	GISTHPDLNIRGGAS	62	F11	NNRASFSQYGAGLDI
15	B10	THPDLNIRGGASFVP	63	F10	ASFSQYGAGLDIVAP
16	B9	DLNIRGGASFVPGEP	64	F9	SQYGAGLDIVAPGVN
17	B8	IRGGASFVPGEPSTQ	65	F8	GAGLDIVAPGVNVQS
18	B7	GASFVPGEPSTQDGN	66	F7	LDIVAPGVNVQSTYP
19	B6	FVPGEPSTQDGNGHG	67	F6	VAPGVNVQSTYPGST
20	B5	GEPSTQDGNGHGTHV	68	F5	GVNVQSTYPGSTYAS
21	B4	STQDGNGHGTHVAGT	69	F4	VQSTYPGSTYASLNG
22	B3	DGNGHGTHVAGTIAA	70	F3	TYPGSTYASLNGTSM
23	B2	GHGTHVAGTIAALNN	71	F2	GSTYASLNGTSMATP
24	B1	THVAGTIAALNNSIG	72	F1	YASLNGTSMATPHVA
25	C12	AGTIAALNNSIGVLG	73	G12	LNGTSMATPHVAGAA
26	C11	IAALNNSIGVLGVAP	74	G11	TSMATPHVAGAAALV
27	C10	LNNSIGVLGVAPSAE	75	G10	ATPHVAGAAALVKQK
28	C9	SIGVLGVAPSAELYA	76	G9	HVAGAAALVKQKNPS
29	C8	VLGVAPSAELYAVKV	77	G8	GAAALVKQKNPSWSN
30	C7	VAPSAELYAVKVLGA	78	G7	ALVKQKNPSWSNVQI
31	C6	SAELYAVKVLGASGS	79	G6	KQKNPSWSNVQIRNH
32	C5	LYAVKVLGASGSGSV	80	G5	NPSWSNVQIRNHLKN
33	C4	VKVLGASGSGSVSSI	81	G4	WSNVQIRNHLKNTAT
34	C3	LGASGSGSVSSIAQG	82	G3	VQIRNHLKNTATSLG
35	C2	SGSGSVSSIAQGLEW	83	G2	RNHLKNTATSLGSTN
36	C1	GSVSSIAQGLEWAGN	84	G1	LKNTATSLGSTNLYG
37	D12	SSIAQGLEWAGNNGM	85	H12	TATSLGSTNLYGSGL
38	D11	AQGLEWAGNNGMHVA	86	H11	SLGSTNLYGSGLVNA
39	D10	LEWAGNNGMHVANLS	87	H10	STNLYGSGLVNAEAA
40	D9	AGNNGMHVANLSLGS	88	H9	NLYGSGLVNAEAATR
41	D8	NGMHVANLSLGSPSP			
42	D7	HVANLSLGSPSPSAT			
43	D6	NLSLGSPSPSATLEQ			
44	D5	LGSPSPSATLEQAVN			
45	D4	PSPSATLEQAVNSAT			
46	D3	SATLEQAVNSATSRG			
47	D2	LEQAVNSATSRGVLV			
48	D1	AVNSATSRGVLVVAA			

FIG._6A

1	A12	IKDFHVYFRESRDAG	49	E12	KKIDVLNLSIGGPDF
2	A11	DAELHIFRVFTNNQV	50	E11	DVLNLSIGGPDFMDH
3	A10	PLRRASLSLGSGFWH	51	E10	NLSIGGPDFMDHPFV
4	A9	RASLSLGSGFWHATG	52	E9	IGGPDFMDHPFVDKV
5	A8	LSLGSGFWHATGRHS	53	E8	PDFMDHPFVDKVWEL
6	A7	GSGFWHATGRHSSRR	54	E7	MDHPFVDKVWELTAN
7	A6	FWHATGRHSSRRLLR	55	E6	PFVDKVWELTANNVI
8	A5	ATGRHSSRRLLRAIP	56	E5	DKVWELTANNVIMVS
9	A4	RHSSRRLLRAIPRQV	57	E4	WELTANNVIMVSAIG
10	A3	SRRLLRAIPRQVAQT	58	E3	TANNVIMVSAIGNDG
11	A2	LLRAIPRQVAQTLQA	59	E2	NVIMVSAIGNDGPLY
12	A1	AIPRQVAQTLQADVL	60	E1	MVSAIGNDGPLYGTI
13	B12	RQVAQTLQADVLWQM	61	F12	AIGNDGPLYGTLNNP
14	B11	AQTLQADVLWQMGYT	62	F11	NDGPLYGTLNNPADQ
15	B10	LQADVLWQMGYTGAN	63	F10	PLYGTLNNPADQMDV
16	B9	DVLWQMGYTGANVRV	64	F9	GTLNNPADQMDVIGV
17	B8	WQMGYTGANVRVAVF	65	F8	NNPADQMDVIGVGGI
18	B7	GYTGANVRVAVFDTG	66	F7	ADQMDVIGVGGIDFE
19	B6	GANVRVAVFDTGLSE	67	F6	MDVIGVGGIDFEDNI
20	B5	VRVAVFDTGLSEKHP	68	F5	IGVGGIDFEDNIARF
21	B4	AVFDTGLSEKHPHFK	69	F4	GGIDFEDNIARFSSR
22	B3	DTGLSEKHPHFKNVK	70	F3	DFEDNIARFSSRGMT
23	B2	LSEKHPHFKNVKERT	71	F2	DNIARFSSRGMTTWE
24	B1	KHPHFKNVKERTNWT	72	F1	ARFSSRGMTTWELPG
25	C12	HFKNVKERTNWTNER	73	G12	SSRGMTTWELPGGYG
26	C11	NVKERTNWTNERTLD	74	G11	GMTTWELPGGYGRMK
27	C10	ERTNWTNERTLDDGL	75	G10	TWELPGGYGRMKPDI
28	C9	NWTNERTLDDGLGHG	76	G9	LPGGYGRMKPDIVTY
29	C8	NERTLDDGLGHGTFV	77	G8	GYGRMKPDIVTYGAG
30	C7	TLDDGLGHGTFVAGV	78	G7	RMKPDIVTYGAGVRG
31	C6	DGLGHGTFVAGVIAS	79	G6	PDIVTYGAGVRGSGV
32	C5	GHGTFVAGVIASMRE	80	G5	VTYGAGVRGSGVKGG
33	C4	TFVAGVIASMRECQG	81	G4	GAGVRGSGVKGGCRA
34	C3	AGVIASMRECQGFAP	82	G3	VRGSGVKGGCRALSG
35	C2	IASMRECQGFAPDAE	83	G2	SGVKGGCRALSGTSV
36	C1	MRECQGFAPDAELHI	84	G1	KGGCRALSGTSVASP
37	D12	CQGFAPDAELHIFRV	85	H12	CRALSGTSVASPVVA
38	D11	FAPDAELHIFRVFTN	86	H11	LSGTSVASPVVAGAV
39	D10	DAELHIFRVFTNNQV	87	H10	TSVASPVVAGAVTLL
40	D9	LHIFRVFTNNQVSYT	88	H9	ASPVVAGAVTLLVST
41	D8	FRVFTNNQVSYTSWF	89	H8	VVAGAVTLLVSTVQK
42	D7	FTNNQVSYTSWFLDA	90	H7	GAVTLLVSTVQKREL
43	D6	NQVSYTSWFLDAFNY	91	H6	TLLVSTVQKRELVNP
44	D5	SYTSWFLDAFNYAIL	92	H5	VSTVQKRELVNPASM
45	D4	SWFLDAFNYAILKKI	93	H4	VQKRELVNPASMKQA
46	D3	LDAFNYAILKKIDVL	94	H3	RELVNPASMKQALIA
47	D2	FNYAILKKIDVLNLS	95	H2	VNPASMKQALIASAR
48	D1	AILKKIDVLNLSIGG	96	H1	ASMKQALIASARRLP

FIG._6B

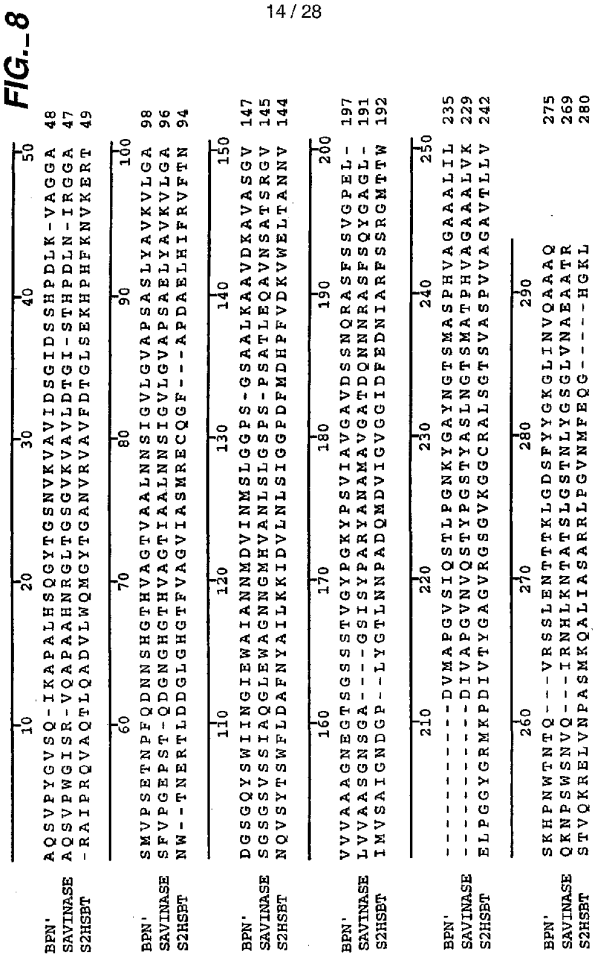
97	I12	IKDFHVYFRESRDAG
98	I11	DAELHIFRVFTNNQV
99	I10	KQALIASARRLPGVN
100	I9	LIASARRLPGVNMFE
101	I8	SARRLPGVNMFEQGH
102	I7	RLPGVNMFEQGHGKL
103	I6	GVNMFEQGHGKLDLL
104	I5	MFEQGHGKLDLLRAY
105	I4	QGHGKLDLLRAYQIL
106	I3	GKLDLLRAYQILNSY
107	I2	DLLRAYQILNSYKPQ
108	I1	RAYQILNSYKPQASL
109	J12	QILNSYKPQASLSPS
110	J11	NSYKPQASLSPSYID
111	J10	KPQASLSPSYIDLTE
112	J9	ASLSPSYIDLTECPY
113	J8	SPSYIDLTECPYMWP
114	J7	YIDLTECPYMWPYCS
115	J6	LTECPYMWPYCSQPI
116	J5	CPYMWPYCSQPIYYG

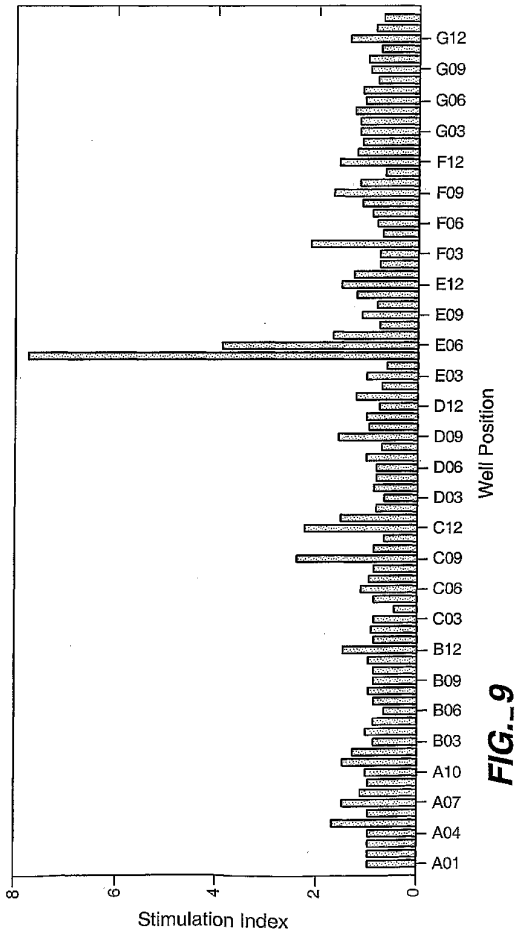
FIG._6C

MKLVNIWLLLLVVLLCGKKHLGDRLEKKSFEKAPCPGCSHLTLKVEFSSTVVEYEYIVAFNGYFT
AKARNSFISSALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQR
KVFRSLKYAESDPTVPCNETRWSQKWQSSRPLRRASLSLGSGFWHATGRHSSSRRLLRAIPRQVAQ
TLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERTNWTNERTLDDGLGHGTFVAGVIASM
RECQGFAPDAELHIFRVFTNNQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWEL
TANNVIMVSAIGNDGPLYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTWELPGGYGRMKPD
IVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLVSTVQKRELVNPASMKQALIASARRLPG
VNMFEQGHGKLDLLRAYQILNSYKPQASLSPSYIDLTECPYMWPYCSQPITYGGMPTVVNVTILN
GMGVTGRIVDKPDWQPYLPQNGDNIEVAFSYSSVLWPWSGYLAISISVTKKAASWEGIAQGHVMI
TVASPAETESKNGAEQTSTVKLPIKVKIIPTPRSKRVLWDQYHNLRYPPGVFPRDNLRMKNDPL
DWNGDHIHTNFRDMYQHLRSMGYFVEVLGAPFTCFDASQYGTLLMVDSEEEYFPEEIAKLRRDVD
NGLSLVIFSDWYNTSVMRKVKFYIDENTRQWWMPDTGGANIPALNELLSVWNMGFSDGLYEGEFTL
ANHDMYYASGCSIAKFPEDGVVITQTFKDQGLEVLKQETAVVENVPILGLYQIPAEGGGRIVLYG
DSNCLDDSHRQKDCFWLLDALLQYTSYGVTPPSLSHSGNRQRPPSGAGSVTPERMEGNHLHRYSK
VLEAHLGDPKFRPLPACPRLSWAKPQPLNETAPSNLWKHQKLLSIDLDKVVLPNFRSNRPQVRPL
SPGESGAWDIPGGIMPGRYNQEVGQTIPVFAFLGAMVVLAFFVVQINKAKSRPKRRKPRVKRPQL
MQQVHPPKTPSV

FIG._7

FIG._8


BPN' AQSVPYGVSQ-IKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLK-VAGGA 48
SAVINASE AQSVPWGISR-VQAPAAHNRGLTGSGVKVAVLDTGI-STHPDLN-IRGGA 47
S2HSBT -RAIPRQVAQTLQADVLWQMGYTGANVRVAVFDTGLSEKHPHFKNVKERT 49
BPN' SMVPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGA 98
SAVINASE SFVPGEPST-QDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGA 96
S2HSBT NW--TNERTLDDGLGHGTFVAGVIASMRECQGF---APDAELHIFRVFTN 94
BPN' DGSGQYSWIINGIEWAIANNMDVINMSLGGPS-GSAALKAAVDKAVASGV 147
SAVINASE SGSGSVSSIAQGLEWAGNNGMHVANLSLGSPS-PSATLEQAVNSATSRGV 145
S2HSBT NQVSYTSWFLDAFNYAILKKIDVLNLSIGGPDFMDHPFVDKVWELTANNV 144
BPN' VVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPEL- 197
SAVINASE LVVAASGNSGA----GSISYPARYANAMAVGATDQNNNRASFSQYGAGL- 191
S2HSBT IMVSAIGNDGP--LYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGMTTW 192
BPN' ----------DVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALIL 235
SAVINASE ----------DIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVK 229
S2HSBT ELPGGYGRMKPDIVTYGAGVRGSGVKGGCRALSGTSVASPVVAGAVTLLV 242
BPN' SKHPNWTNTQ---VRSSLENTTTKLGDSFYYGKGLINVQAAAQ 275
SAVINASE QKNPSWSNVQ---IRNHLKNTATSLGSTNLYGSGLVNAEAATR 269
S2HSBT STVQKRELVNPASMKQALIASARRLPGVNMFEQG----HGKL 280


G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
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D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
Well Position
8
6
4
2
0
Stimulation Index

FIG._9

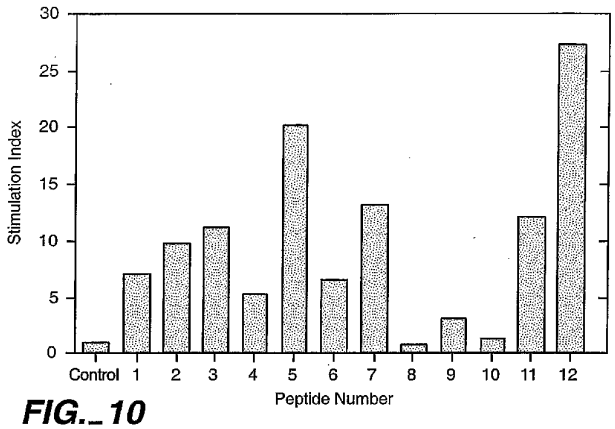


FIG._10

FIG._11

PEPTIDE NUMBER	SEQUENCE
1 (Unmodified Sequence)	GSISYPARYANAMAV
2	**A**SISYPARYANAMAV
3	G**A**ISYPARYANAMAV
4	GS**A**SYPARYANAMAV
5	GSI**A**YPARYANAMAV
6	GSIS**A**PARYANAMAV
7	GSISY**A**ARYANAMAV
8	GSISYPA**A**YANAMAV
9	GSISYPAR**A**ANAMAV
10	GSISYPARYA**A**AMAV
11	GSISYPARYANA**A**AV
12	GSISYPARYANAMA**A**

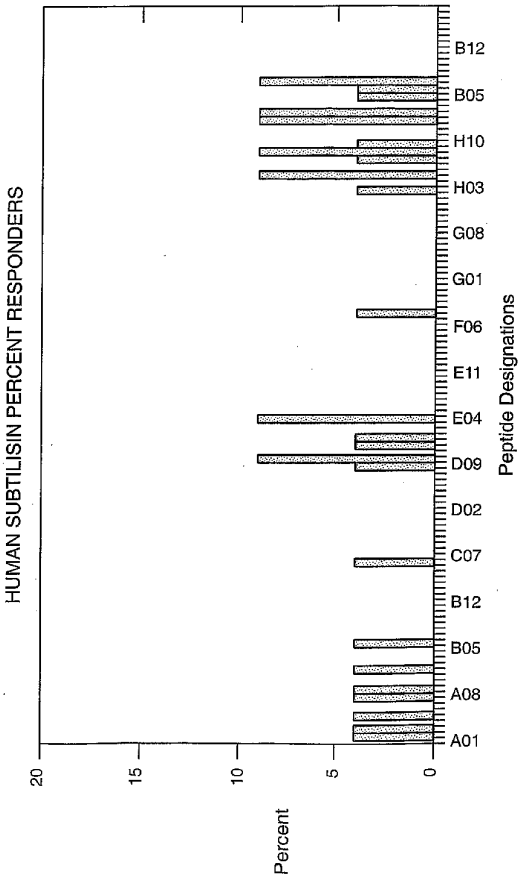
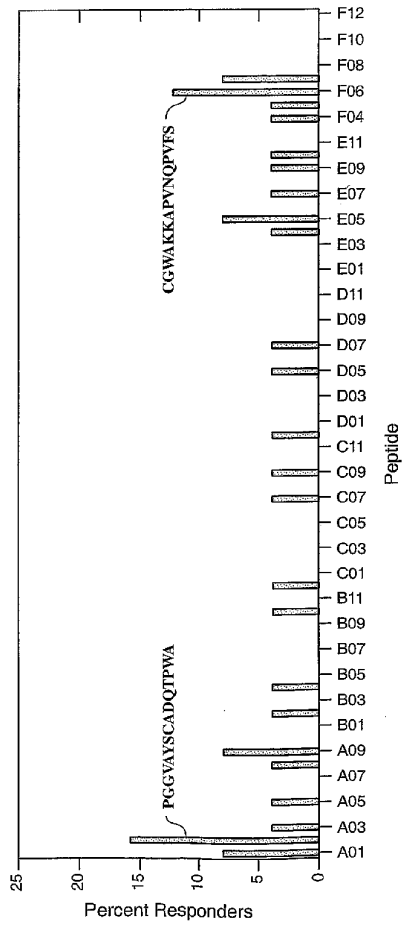

HUMAN SUBTILISIN PERCENT RESPONDERS
Peptide Designations
B12
B05
H10
H03
G08
G01
F06
E11
E04
D09
D02
C07
B12
B05
A08
A01
20
15
10
5
0
Percent

FIG._12


F12
F10
F08
F06
F04
E11
E09
E07
E05
E03
E01
D11
D09
D07
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D03
D01
C11
C09
C07
C05
C03
C01
B11
B09
B07
B05
B03
B01
A09
A07
A05
A03
A01
CGWAKKAPVNQPVFS
PGGVAYSCADQTPWA
Peptide
25
20
15
10
5
0
Percent Responders

FIG._13A

```
         1          2          3          4          5
1234567890 1234567890 1234567890 1234567890 1234567890
MRSSPLLPSA VVAALPVLAL AADGRSTRYW DCCKPSCGWA KKAPVNQPVF
SCNANFQRIT DFDAKSGCEP GGVAYSCADQ TPWAVNDDFA LGFAATSIAG
SNEAGWCCAC YELTFTSGPV AGKKMVVQST STGGDLGSNH FDLNIPGGGV
GIFDGCTPQF GGLPGQRYGG ISSRNECDRF PDALKPGCYW RFDWFKNADN
PSFSFRQVQC PAELVARTGC RRNDDGNFPA VQIPSSSTSS PVNQPTSTST
TSTSTTSSPP VQPTTPSGCT AERWAQ
```

FIG._13B

```
  1 mrsslvlffv sawtalaspi rrevsqdlfn qfnlfaqysa aaycgknnda
 51 pagtnitctg nacpevekad atflysfeds gvqdvtqfla ldntnklivl
101 sfrgsrsien wiqnlnfdlk eindicsgcr ghdgftsswr svadtlrqkv
151 edavrehpdy rvvftghslg galatvagad lrgngydidv fsygaprvgn
201 rafaefltvq tggtlyrith tndivprlpp refgyshssp eywiksgtlv
251 pvtrndivki egidatggnn qpnipdipah lwyfgligtc l
```

FIG._14B

```
  1 mftpvrrrvr taalalsaaa alvlgstaas gasatpspap apapapvkqg
 51 ptsvayvevn nnsmlnvgky tladgggnaf dvavifaani nydtgtktay
101 lhfnenvqrv ldnavtqirp lqqqgikvll svlqnhqqag fanfpsqqaa
151 safakqlsda vakygldgvd fddeyaeygn ngtaqpndss fvhlvtalra
201 nmpdkiisly nigpaasrls yggvdvsdkf dyawnpyygt wqvpgialpk
251 aqlspaavei grtsrstvad larrtvdegy gvyltynldg gdrtadvsaf
301 trelygseav rtp
```

FIG._15B

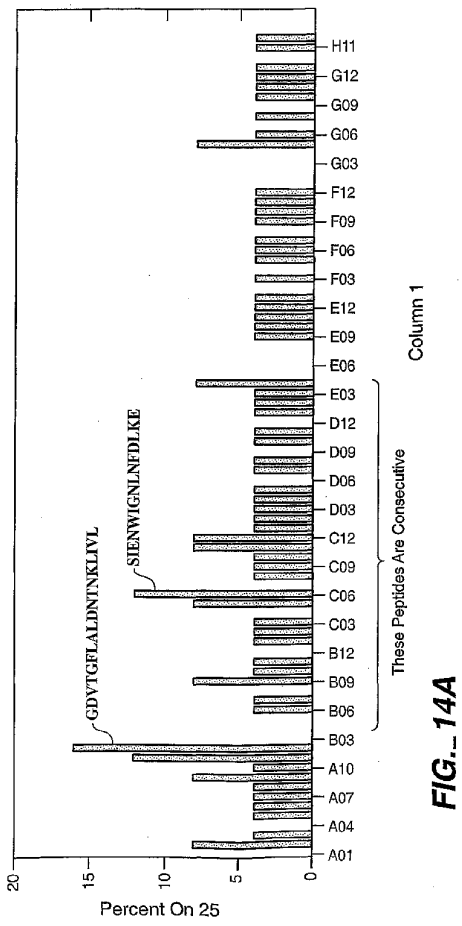
H11
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
GDVTGFLALDNTNKLIVL
SIENWIGNLNFDLKE
These Peptides Are Consecutive
Column 1
20
15
10
5
0
Percent On 25

FIG._14A

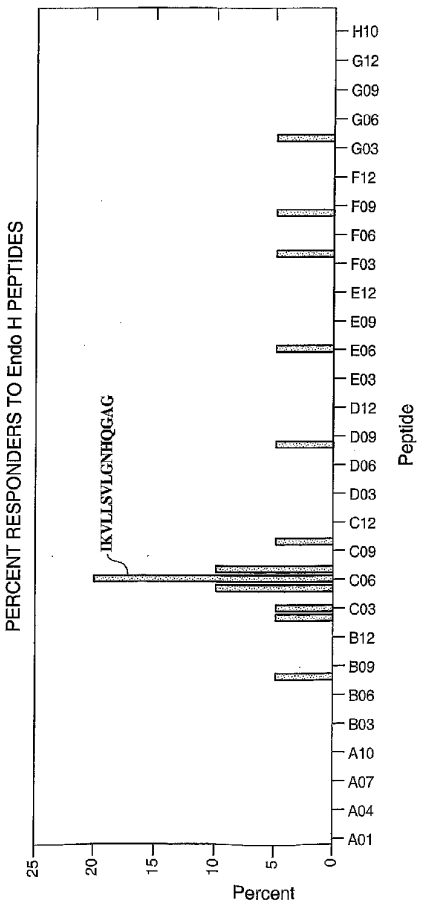
PERCENT RESPONDERS TO Endo H PEPTIDES
IKVLLSVLGNHQGAG
H10
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
Peptide
25
20
15
10
5
0
Percent

FIG._15A

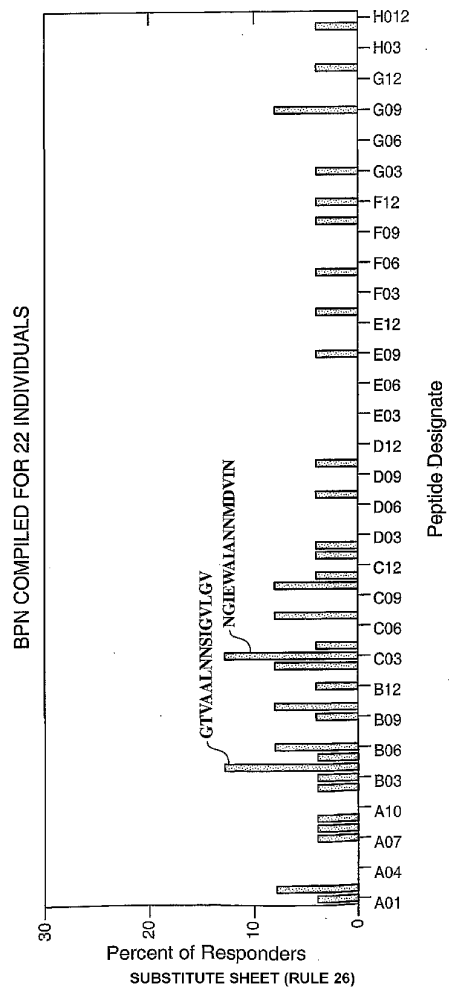
BPN COMPILED FOR 22 INDIVIDUALS
Peptide Designate
H012
H03
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
NGIEWAIANNMDVIN
GTVAALNNSIGVLGV
30
20
10
0
Percent of Responders

FIG._16

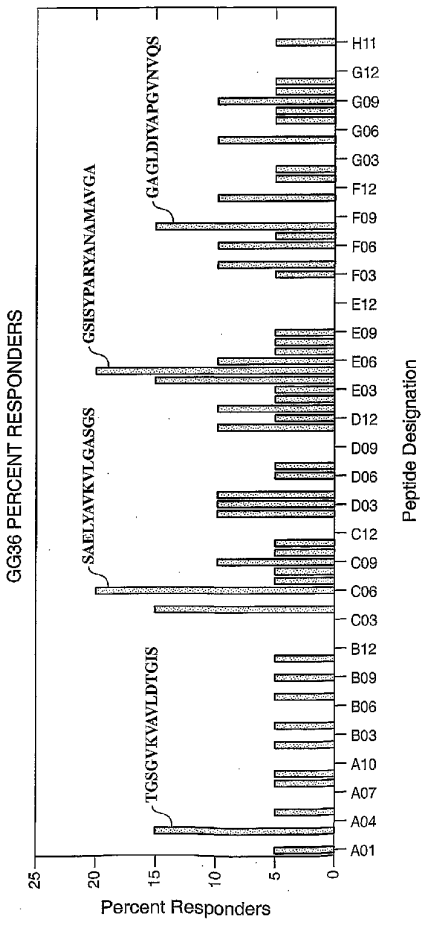
GG36 PERCENT RESPONDERS
Percent Responders
25
20
15
10
5
0
Peptide Designation
H11
G12
G09
G06
G03
F12
F09
F06
F03
E12
E09
E06
E03
D12
D09
D06
D03
C12
C09
C06
C03
B12
B09
B06
B03
A10
A07
A04
A01
GAGLDIYAPGVNVQS
GSISYPARYANAMAVGA
SAELYAVKVLGASGS
TGSGVKVAVLDTGIS

FIG._17

Hybrid enzyme sequence (GG36-BPN)

GG36
AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGH

BPN
GTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVINMSLGGS

Δ

GSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGP

ELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGD

SFYY GKGLINVQAAAQ

FIG._18

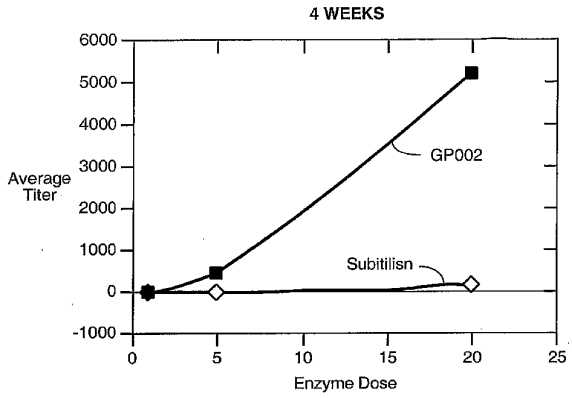

FIG._19A

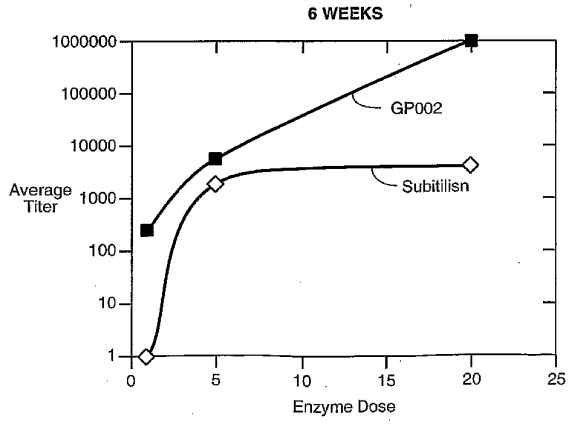

FIG._19B

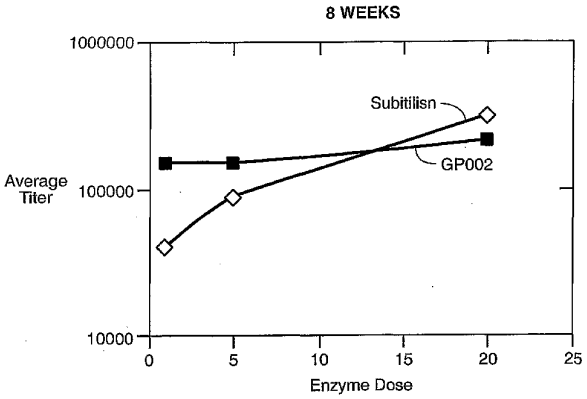

FIG._19C

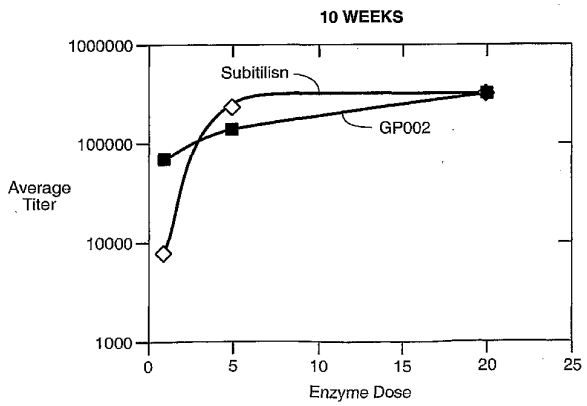

FIG._19D

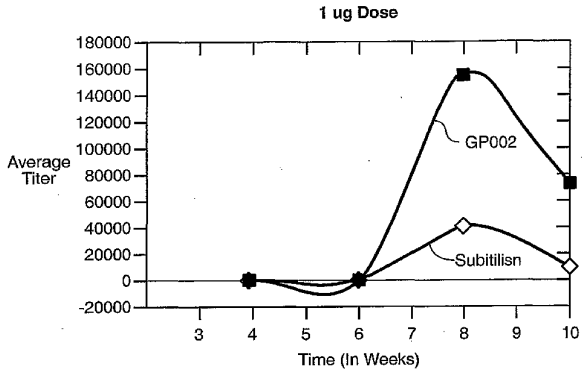

FIG._20A

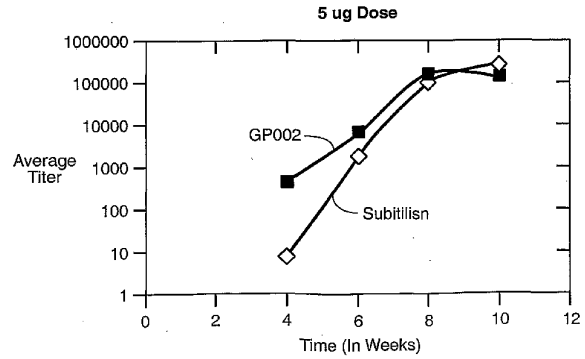

FIG._20B

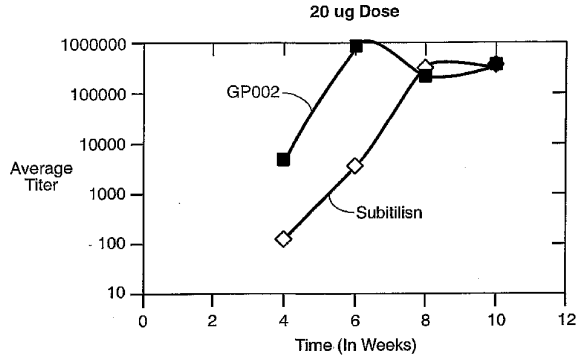

FIG._20C

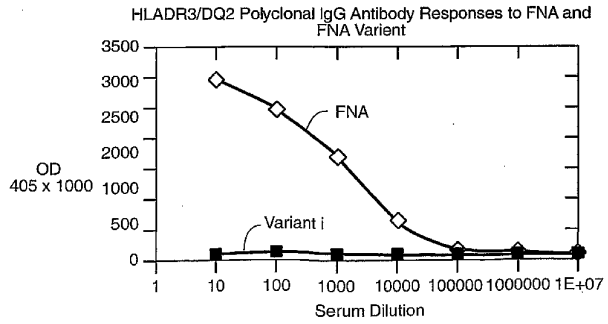

FIG._21

フロントページの続き

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(72)発明者 エステル、デイビッド・エイ
アメリカ合衆国、カリフォルニア州 ９４４０３、サン・マテオ、ウッドブリッジ・サークル ２４８
(72)発明者 ハーディング、フィオーナ・エイ
アメリカ合衆国、カリフォルニア州 ９５０５０、サンタ・クララ、ルイス・ストリート ７７２

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[标]发明人	エステルデイビッドエイ ハーディングフィオーナエイ		
发明人	エステル、デイビッド·エイ ハーディング、フィオーナ·エイ		
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摘要(译)

本发明涉及用于产生过敏和低过敏组合物的新方法和组合物。具体地，本发明包括中和或降低T细胞识别表位的能力，从而防止个体对蛋白质的敏化。或者，突变T细胞表位以产生增加的免疫原性反应。