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(54) **Method for improving the heat stability of a composition containing glucose dehydrogenase (GDH)**

Methode zum Verbessern der Wärmebeständigkeit einer Mischung, die Glukosedehydrogenase enthält.

Méthode pour améliorer la stabilité thermique d'une composition comportant glucose déshydrogénase.

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**Description**Field of the Invention

5 [0001] The present invention relates to a method for improving the heat stability of a composition comprising water-soluble coenzyme-bound glucose dehydrogenase (glucose dehydrogenase sometimes called "GDH" herein), including glucose dehydrogenase having a flavin compound as its coenzyme.

Background of the Invention

10 [0002] Blood glucose self-measurement is important for diabetes patients to constantly assess their own blood glucose levels and used them in treatment. Enzymes having glucose as a substrate are employed in the monitors used for blood glucose self-measurement. One example of such an enzyme is glucose oxidase (EC 1.1.3.4). Glucose oxidase has long been used as the enzyme in blood glucose monitors because of its high specificity for glucose and excellent heat stability, and the first report actually dates back about 40 years. In blood glucose monitors using glucose oxidase, measurement is accomplished by means of electrons generated as glucose is oxidized and converted to D-glucono- $\delta$ -lactone, which are transferred to an electrode via a mediator, but because protons produced during the reaction are likely to be transferred to oxygen, the measurement results are affected by dissolved oxygen.

15 [0003] To avoid this problem NAD(P)-dependent glucose dehydrogenase (EC 1.1.1.47) or pyrroloquinoline quinone (abbreviated herein as PQQ)-dependent glucose dehydrogenase (EC 1.1.5.2 (formerly EC 1.1.99.17) is used as the enzyme in blood glucose monitors. These are superior in that they are not affected by dissolved oxygen, but the former NAD(P)-dependent glucose dehydrogenase (sometimes abbreviated herein as NADGDH) is complicated by poor stability and the need for addition of a coenzyme. On the other hand, PQQ-dependent glucose dehydrogenase (sometimes abbreviated herein as PQQGDH) has poor substrate specificity and acts on sugars other than glucose such as maltose and lactose, detracting from measurement accuracy.

20 [0004] *Aspergillus*-derived flavin-bound glucose dehydrogenase (sometimes abbreviated hereunder as FADGDH) is disclosed in WO 2004-058958. This enzyme is superior in that it has excellent substrate specificity and is not affected by dissolved oxygen. In terms of heat stability, it has an activity survival rate of about 89% following 15 minutes of treatment at 50°C. This stability is still inadequate however considering that heat treatment may be required in some cases in the process of preparing a sensor chip.

25 [0005] EP-A-1146332 and EP-A-0992589 both disclose a method to protect pyrrolo-quinoline quinone dependent glucose dehydrogenase (PQQ-GDH) from the effects of temperature, i.e., to make it more thermostable, by adding to a composition comprising PQQ-GDH either phthalic acid, or maleic acid, or succinic acid, or dimethyl glutaric acid.

30 [0006] EP-A-1584675 discloses a flavin-bound glucose dehydrogenase isolated from the filamentous fungus *Aspergillus oryzae*.

SUMMARY OF THE INVENTION

35 [0007] It is an object of the present invention to overcome the difficulties of heat stability that occur with the aforementioned conventional enzymes for blood glucose monitors, and to provide a composition which can be used in practical situations as a reagent for measuring blood glucose.

40 [0008] From previous studies of PQQGDH the inventors have obtained several multiple variants of this enzyme with improved substrate specificity, but some of these variants have poorer heat stability than the wild-type PQQGDH. After exhaustive research aimed at discovering the cause of this problem, it was found that the three-dimensional structure of PQQGDH could be stabilized and its stability improved by adding a certain kind of compound to the enzyme as described in this patent.

45 [0009] After further study, the inventors perfected the present invention upon discovering that similar effects could be achieved for FADGDH and NADGDH.

50 [0010] Strategies for improving the stability of PQQGDH have already been reported in WO-A-02/072839, which reports on a study using a means of modifying PQQGDH on the genetic level, but the possibility of a means of increasing the stability of an enzyme without modifying it has not heretofore been mentioned.

[0011] The inventors also discovered as a result of exhaustive research that a level of heat stability high enough to allow the composition to be heat dried could be achieved by maintaining the pH of a composition comprising GDH and a flavin compound at a pH in the acid range.

55 [0012] A level of heat stability which allows heat drying is a level at which the active survival rate is 10% or more, or preferably 45% or more, or still more preferably 70% or more after 15 minutes of treatment at 50°C.

[0013] Taking a different perspective from previous studies, the inventors searched for strategies for more easily improving heat stability, and finally perfected the present invention after further exhaustive research when they showed

that the overall stability of a composition comprising GDH could be improved by using a composition with a pH in the acid range and by including one or more dicarboxylic acids or salt compounds.

**[0014]** That is, the present invention consists of the following.

5 [Item 1] A method for improving the heat stability of glucose dehydrogenase (GDH), comprising the step of combining, in a composition comprising soluble flavin-bound glucose dehydrogenase (GDH) derived from *Aspergillus*, the enzyme with a carboxyl group-containing compound, thereby improving the heat stability of GDH over that achieved without the inclusion of the compound, wherein the carboxyl group-containing compound is any one or more selected from the group consisting of calcium glycerate, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3-3'dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.

10 [Item 2] The method for improving heat stability according to Item 1, wherein the final concentration of each included compound is 0.1% or more.

[Item 3] The method according to any one of Items 1 to 2 further comprising the step of maintaining the pH of the composition at pH 3.1 to 7.0, thereby improving the heat stability of the composition over that achieved with the composition at a pH of 7.4.

15 [Item 4] A composition comprising soluble flavin-bound glucose dehydrogenase derived from *Aspergillus*, having improved thermal stability as a result of the methods of Items 1 to 3.

20 [Item 1] A method for improving the heat stability of the flavin-bound glucose dehydrogenase from *Aspergillus* (GDH) in a composition, comprising the step of combining soluble GDH with a carboxyl group-containing compound, thereby improving the heat stability of said GDH over that achieved without the inclusion of said compound, and wherein the carboxyl group-containing compound is any one or more selected from the group consisting of calcium glycerate, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3-3'dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.

25 [Item 2] The method for improving heat stability according to Item 1, wherein the final concentration of each included compound is 0.1% or more.

[Item 3] The method according to any one of Items 1 to 2 further comprising the step of maintaining the pH of the composition at pH 3.1 to 7.0, thereby improving the heat stability of the composition over that achieved with the composition at a pH of 7.4.

30 [Item 4] A composition comprising soluble GDH having improved thermal stability as a result of the methods of Items 1 to 3.

[Item 5] A method for measuring a glucose concentration using the composition according to Item 4.

[Item 6] A glucose sensor comprising the composition according to Item 4.

35 [Item 7] A method for manufacturing a composition comprising GDH having improved heat stability, comprising the step of combining, in a composition soluble GDH with a carboxyl group-containing compound, thereby improving the heat stability of said GDH over that achieved without the inclusion of said compound, and wherein the carboxyl group-containing compound is any one or more selected from the group consisting of calcium glycerate, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3,3-dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.

40 **[0015]** The improvement in heat stability achieved by the present invention makes it possible to reduce thermal deactivation of the enzyme during preparation of the glucose measurement reagent, glucose assay kit and glucose monitor, and to reduce the amount of the enzyme used and improve measurement accuracy. Moreover it also makes it possible to provide a reagent for measuring blood glucose using GDH having excellent storage stability.

45 **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0016]**

50 Figure 1 shows the pH dependence of heat stability of FADGDH derived from an *Aspergillus terreus* subspecies. Figure 2 shows the pH dependence of heat stability of FADGDH derived from *Penicillium lilacinoechinulatum* NBRC6231.

**[0017]** The present invention is explained in more detail below.

55 GDH is an enzyme that catalyzes the following reaction:

D-glucose + electron-transferring substance (oxide form) → D-glucono-5-lactone + electron-transferring substance (reduced form).

It is an enzyme which catalyzes a reaction in which D-glucose is oxidized to produced D-glucono-1,5-lactone, with no

particular limits on its derivation or structure.

**[0018]** There are no particular limits on what GDH can be used in the method of the present invention as long as it is soluble coenzyme-bound glucose dehydrogenase (GDH).

**[0019]** Coenzymes that are used are flavin compounds.

**[0020]** The GDH having FAD as its coenzyme (FAD-bound GDH) that can be used in the present invention include those derived from *Aspergillus*. These microbial strains can be easily obtained by means of a request to a depository authority.

**[0021]** GDH having some of the amino acid residues deleted or replaced or other amino acid residues added in the examples given above can still be used as GDH in the present invention as long as glucose dehydrogenase activity is retained.

**[0022]** Such modification can easily be performed by the skilled artisan according to known techniques in the art. A variety of methods for introducing a site-directed mutagenesis to a protein by substituting or inserting one or more bases to a nucleotide sequence of a gene coding for the protein are disclosed in Sambrook et al, *Molecular Cloning; A Laboratory Manual 2nd Edition*(1989) Cold Spring Harbor Laboratory Press, New York.

For example, naturally-occurring microorganisms producing the GDH, or transformant prepared by inserting a naturally-occurring or modified GDH gene into an expression vector (a variety of vectors including a plasmid are known), followed by transforming a suitable host (a variety of hosts including *E. coli* are known) with the expression vector, are cultured, host cells are collected from a culture medium by centrifugation, cells are broken down mechanically or enzymatically with lysozyme, optionally solubilized by the addition of a chelating agent such as EDTA or a surfactant to obtain a water soluble fraction containing GDH. The expressed GDH can be secreted to a culture medium using a suitable host-vector system.

**[0023]** GDH can be separated and precipitated from the GDH-containing solution by concentration under reduced pressure, membrane concentration, salting out using ammonium sulfate or sodium sulfate, or a fractional precipitation with a hydrophilic solvent such as methanol, ethanol, acetone, etc. Heat treatment and isoelectric treatment are also an effective purification method. Purified GDH can be obtained by gel filtration with adsorbent or gel filtering agent, adsorption chromatography or affinity chromatography. The standard enzyme is preferably purified enough to show a single band in electrophoresis (SDS-PAGE).

**[0024]** The PQQGDH can be heat-treated at 25 to 50°C, preferable 30 to 45°C to increase a proportion of holoenzyme to the total GDH protein before or after the above-mentioned steps.

**[0025]** Concentration of PQQGDH of the invention is not specifically limited.

**[0026]** The appropriate range differs depending on the properties and the like of the enzyme used, but for practical purposes the concentration is one at which a person skilled in the art could measure glucose with adequate reliability using the enzyme.

**[0027]** The concentration of NADGDH in the present invention is also not particularly restricted, but in solution it is preferably 10 to 1000 U/mL or more preferably 20 to 500 U/mL or still more preferably 50 to 150 U/mL. A similar concentration is also desirable in a powder or freeze-dried product, but a concentration of 1000 U/mL or more can also be used when preparing a powder sample.

**[0028]** The concentration of the FADGDH in the present invention is also not particularly restricted, but in solution it is preferably 0.01 to 100 U/mL or more preferably 0.1 to 50 U/mL or still more preferably 0.2 to 10 U/mL. A similar concentration is also desirable in a powder or freeze-dried product, but a concentration of 100 U/mL or more can also be used when preparing a powder sample.

**[0029]** There are no particular limits on the medium for culturing the aforementioned microorganism as long as it is one in which the microorganism can grow and produce the GDH described in the present invention, but preferably it is one containing the necessary carbon sources, inorganic nitrogen sources and/or organic nitrogen sources necessary for the microorganism to grow, and still more preferably it is a liquid medium suited to aeration-agitation. In the case of a liquid medium, examples of carbon sources include glucose, dextran, soluble starch, sucrose and the like while examples of nitrogen sources include ammonium salts, nitrate salts, amino acids, corn steep liquor, peptone, casein, meat extract, defatted soy beans, potato extract and the like. Other nutrient sources (such as calcium chloride, sodium hydrogen phosphate, magnesium chloride and other inorganic salts, vitamins and the like) can also be included as desired.

**[0030]** Culture is by ordinary methods known in the field. For example, spores or growing cells of the microorganism are seeded in liquid medium comprising the aforementioned nutrients, and the bacteria can be made to proliferate by stationary culture or aeration-agitation, but an aeration-agitation culture is preferred. The pH of the culture liquid is preferably 5 to 9 or more preferably 6 to 8. The temperature is normally 14 to 42°C or preferably 20 to 40°C. Culture is normally continued for 14 to 144 hours, and is preferably terminated at the point at which the level of GDH expression peaks under each set of culture conditions. To determine this point, the culture liquid can be sampled and GDH activity in the culture liquid measured to monitor changes, and once GDH activity ceases to rise over time it is considered to have peaked and culture is terminated.

**[0031]** As a method of extracting GDH from the aforementioned culture liquid, to collect GDH which has accumulated

inside the cells the cells alone can be collected by centrifugation, filtration or the like, and re-suspended in a solvent, preferably water or buffer solution. The re-suspended cells can be disrupted by known means and the GDH in the cells collected in the solvent. The cells can be disrupted using a bacteriolytic enzyme or by mechanical means. There are no particular limits on the bacteriolytic enzyme as long as it has the ability to eliminate bacterial cell walls, but one enzyme that can be used is "Lyticase" (Sigma). Means of physical disruption include ultrasound, glass beads, french press and the like. After disruption the solution can be centrifuged or filtered and the residue removed to obtain a raw GDH extract solution.

**[0032]** Culture in the present invention can also be by solid culture. Eukaryotic microorganisms capable of producing the GDH of the present invention are preferably grown on wheat or other bran with the temperature, humidity and the like controlled appropriately. In this case, the culture may be stationary or the culture may be mixed by agitation or the like. GDH is extracted by adding a solvent, preferably water or buffer solution, to the culture to dissolve the GDH, and separating the cells from the bran or other solid matter by centrifugation or filtration.

**[0033]** The GDH can be purified by a combination of various commonly used separation techniques suited to the fraction having GDH activity. A known method such as salting out, solvent precipitation, dialysis, ultrafiltration, gel filtration, unmodified PAGE, SDS-PAGE, ion-exchange chromatography, hydroxyapatite chromatography, affinity chromatography, reverse-phase high-speed liquid chromatography, isoelectric focusing or the like can be selected appropriately for separation from the aforementioned GDH extract.

**[0034]** One mode of the method for improving the heat stability of GDH of the present invention comprises a step of combining, in a composition comprising soluble coenzyme-bound glucose dehydrogenase (GDH), (1) the enzyme and (2) carboxyl group-containing compounds.

**[0035]** Desirable examples of the compound to be added include one or more selected from a group consisting of succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3-3'-dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.

**[0036]** There are no particular limits on the concentration of these compounds to be included, but in solution it is preferably 0.001 to 30% or more preferably 0.01 to 5% or still more preferably 0.01 to 1% by weight. A similar concentration is desirable in the case of a powder or freeze-dried product, but in the case of a powder or freeze-dried product the same effects can be achieved through addition of a lower concentration than in the case of a solution.

**[0037]** The concentrations of the compounds described in the examples are final concentrations of the compounds combined and stored with the GDH enzyme. Examples of desirable combinations a combination of different carboxylic acid-containing compounds, and for example the effects of a salt compound and a carboxylic acid-containing compound reinforce one another when the two are combined.

**[0038]** The pH of the composition can be maintained in the acid range of pH 7 or less in order to further improve heat stability in the aforementioned mode. Alternatively, a dicarboxylic acid such as succinic acid, malonic acid, glutaric acid, phthalic acid or maleic acid or a salt compound such as sodium chloride, sodium sulfate, trisodium citrate or ammonium sulfate can be included.

**[0039]** A different mode of the method for improving the heat stability of GDH of the present invention is a method of improving the heat stability of the composition over that achieved at pH 7.3 or more, comprising a step of maintaining the pH of the composition in the acid range of pH 7 or less in a composition comprising soluble coenzyme-bound glucose dehydrogenase (GDH).

**[0040]** Yet another mode of the method for improving the heat stability of the GDH of the present invention is a method of improving the heat stability of the composition over that achieved at pH 7.4, comprising a step of maintaining pH of the composition at pH 3.1 to 7.0 in a composition comprising soluble coenzyme-bound glucose dehydrogenase (GDH).

**[0041]** The pH is preferably 3.1 to 7.0 or more preferably 4.0 to 6.5 or still more preferably 4.0 to 6.0.

**[0042]** Even after being treated for 15 minutes at 50°C, the aforementioned composition retains GDH activity equal to 10% or more of the GDH activity of the same composition stored at 4°C. In the case of a composition comprising glucose dehydrogenase (GDH) having a flavin compound as the coenzyme, even after 15 minutes of treatment at 50°C the composition retains GDH activity equal to 45% or more of the GDH activity of the same composition stored at 4°C.

**[0043]** In this mode, the composition preferably contains one or more dicarboxylic acids or salt compounds. Examples of dicarboxylic acids include succinic acid, malonic acid, glutaric acid, phthalic acid and maleic acid.

**[0044]** One or more of these compounds can be included in the present invention.

**[0045]** There are no particular limits on the concentrations of these compounds to be included, but in solution it is preferably 1 mM to 10 M or more preferably 5 mM to 5 M or still more preferably 20 mM to 1 M. When preparing a powder or freeze-dried product, a freeze-dried sample with the same effects can be obtained by freeze drying a composition containing the compound in the same concentration as in the solution.

**[0046]** The concentrations of the compounds described in the examples are final concentrations of the compounds combined and stored with the GDH enzyme. Examples of desirable combinations include a combination with a salt compound having similar properties and a combination of different carboxylic acid-containing compounds, and for example it is desirable to combine a salt compound with a carboxylic acid-containing compound because the effects of

the two reinforce one another in combination.

**[0047]** A carboxylic group-containing compound, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3-3'-dimethylglutaric acid, lysine, phthalic acid, maleic acid, glutaric acid, can be included to further improve the heat stability in this mode.

**[0048]** The composition comprising GDH of the present invention can be provided in liquid form or can be made into a powder by freeze drying, vacuum drying, spray drying or the like. In this case, the GDH can be dissolved in a buffer or the like for purposes of use, and sugars, sugar alcohols, amino acids, proteins, peptides and the like other than the aforementioned compounds used in the aforementioned invention can also be added as excipients, stabilizers and the like. The resulting powder can also be granulated. Examples of such substances include trehalose, sucrose, sorbitol, erythritol, glycerol and other sugars and sugar alcohols, glutamic acid, alginic acid and other amino acids and bovine serum albumin, egg white albumin, and various chaperones and other proteins and peptides.

**[0049]** There are no particular limits on the compositions of the buffers used in extracting, refining and powdering the GDH as described above or in stability testing, but preferably they have buffering ability in the pH range of 5 to 8, and examples include buffers such as boric acid, tris-hydrochloric acid and potassium phosphate and Good buffers such as BES, Bicine, Bis-Tris, CHES, EPPS, HEPES, HEPPSO, MES, MOPS, MOPSO, PIPES, POPSO, TAPS, TAPSO, TES and Tricine.

**[0050]** One or two or more of these can be used. A compound of one or more including those other than the above can also be used.

**[0051]** There are no particular limits on the added concentrations of these within the range having buffering ability, but the upper limit is preferably 100 mM or less or more preferably 50 mM or less, and the lower limit is preferably 5 mM or more.

**[0052]** The content of a buffer in a powder or freeze-dried product is not particularly limited but should be in the range of preferably 0.1% or more or more preferably 0.1 to 30% (weight ratio).

**[0053]** A variety of commercial reagents can be used for these.

**[0054]** The various compounds describe above can be added at the time of measurement or can be included in advance when preparing the glucose measurement reagent, glucose assay kit or glucose sensor as described below. They can also be added to the process liquid at any of the various manufacturing steps for extracting, purification, powdering and the like of the GDH. Regardless of the form, whether liquid, dried or the like, they should be able to function during measurement.

**[0055]** Improving heat stability in the present invention means increasing the survival rate (%) of the GDH enzyme maintained after a composition comprising the GDH enzyme has been heat treated for a fixed time at a fixed temperature. In the invention of this application, the survival rate of a sample stored at 4°C, at which almost complete activity is retained, is given as 100%, and the survival rate of the enzyme is calculated by comparing this with the activity rate of a GDH solution which has been heat treated for a fixed time at a fixed temperature. If the survival rate is higher than without addition of the compound, the heat stability of GDH is judged to have been improved.

**[0056]** Specifically, improvements in stability were evaluated as follows.

**[0057]** The GDH activity value (a) of a solution stored at 4°C and the GDH activity value (b) after heat treatment for a fixed time at a fixed temperature were measured by the methods described below for measuring GDH enzyme activity, and the relative value  $((b)/(a) \times 100)$  was calculated given 100 as measurement value (a). This relative value was the survival rate (%). If the survival rate was greater with the compound added than without, heat stability was judged to have been improved.

**[0058]** The effects of the present invention are more conspicuous in a system comprising a mediator. There are no particular limits on the mediator than can be used in the method of the present invention, but examples include a combination of phenazine methosulfate (PMS) with 2,6-dichlorophenol-indophenol (DCPIP), a combination of PMS with nitroblue tetrazolium (NBT), DCPIP alone, ferricyanide ions alone (with potassium ferricyanide for example as the compound) and ferrocene alone. Of these, ferricyanide ions (with potassium ferricyanide or the like as the compound) are preferred.

**[0059]** Because these mediators differ in terms of sensitivity, the added concentration does not need to be determined exactly, but generally it is desirable to add 1 mM or more.

**[0060]** These mediators may be added during measurement or may be included in advance during preparation of the glucose measurement reagent, glucose assay kit or glucose sensor as described below. In this case, regardless of the state, whether liquid, dried or the like, they should be dissociated to produce ions during the measurement reaction.

**[0061]** A variety of components can also be included in the present invention as necessary. For example, surfactants, stabilizers, excipients and the like can be added.

**[0062]** For example, one or two or more amino acids can be selected from the group consisting of glutamic acid, glutamine and lysine. Bovine serum albumin (BSA), egg white albumin (OVA) and the like can also be included.

**[0063]** Glucose can be measured by the following methods in the present invention.

[0064] The glucose measurement reagent, glucose assay kit and glucose sensor of the present invention can be in the form of a liquid (aqueous solution, suspension, etc.), vacuum-dried or spray-dried powder or freeze-dried preparation. There are no particular limits on the method of drying, which may be an ordinary method. The composition comprising an enzyme of the present invention may be a freeze-dried product or may be a solution obtained by re-dissolving a dried product.

[0065] Glucose can be measured by the following methods in the present invention.

#### Glucose measurement reagent

[0066] The glucose measurement reagent of the present invention typically includes GDH, buffers, mediators and other reagents necessary for measurement, a glucose standard solution for preparing the calibration curve, and directions for use. The kit of the present invention can be provided for example as a freeze-dried reagent or as a solution in a suitable storage solution. Preferably the GDH of the present invention is provided in holoenzyme form, but it can also be provided in apoenzyme form and converted to holoenzyme form for use.

#### Glucose assay kit

[0067] The present invention is characterized by the glucose assay kit comprising the GDH according to the present invention. The glucose assay kit of the present invention contains the GDH according to the present invention in the amount enough to assay at least once. Typically, the kit contains the buffer required for the assay, a mediator, glucose standard solutions for making a calibration curve and instructions for the use in addition to the GDH of the present invention. The GDH according to the present invention can be provided in various forms, e.g., as a frozen and dried reagent or a solution in an appropriate storage solution. Preferably, the GDH of the present invention is provided as a holoenzyme, but can be provided as an apoenzyme and converted into the holoenzyme at use.

#### Glucose sensor

[0068] The present invention is characterized by the glucose sensor comprising the GDH according to the present invention. As an electrode, a carbon electrode, a gold electrode or a platinum electrode is used, and the enzyme of the present invention is immobilized on this electrode. As immobilization methods, there are the method of using a crosslinking reagent, the method of including in macromolecular matrix, the method of coating with a dialysis membrane, an optical crosslinking polymer, a conductive polymer, and a redox polymer. Alternatively, the enzyme may be immobilized in the polymer or absorbed/immobilized on the electrode with an electronic mediator typified by ferrocene or derivatives thereof. Or these may be used in combination. Preferably, the GDH of the present invention is immobilized on the electrode as the holoenzyme, but can be immobilized in the apoenzyme form and can be provided as another layer or in another solution. Typically, the GDH of the present invention is immobilized on the carbon electrode using glutaraldehyde, and subsequently glutaraldehyde is blocked by treating with a reagent having an amine group.

[0069] The glucose concentration can also be measured as follows. The buffer is placed in a thermostatic cell, the mediator are added, and the temperature is kept constant. As an action electrode, the electrode on which the PQQGDH has been immobilized is used, and a counter electrode (e.g., platinum electrode) and a reference electrode (e.g., Ag/AgCl electrode) are used. A constant voltage is applied to the carbon electrode, after a current becomes a steady state, a sample containing glucose is added and an increase of the current is measured. The glucose concentration in the sample can be calculated in accordance with the calibration curve made by the glucose solutions with standard concentrations.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0070] The present invention is explained in more detail below using examples.

#### Example 1: Preparation of FAD-dependent GDH sample

[0071] Using an *Aspergillus terreus* subspecies and *Penicillium lilacinoechinulatum* NBRC6231 (purchased from the National Institute of Technology and Evaluation) as the FAD-dependent GDH-producing organisms, the respective L dried samples were seeded on potato dextrose agar medium (Difco) and restored by incubation at 25°C. The hyphae on the restored plates were collected together with the agar and suspended in filter-sterilized water. 6 L of production medium (1% wheat germ extract, 1.5% soy peptides, 0.1% MgSO<sub>4</sub> heptahydrate, 2% glucose, pH 6.5) was prepared in two 10 L jar fermenters, and after 15 minutes of autoclave sterilization at 120°C, the aforementioned respective hyphal suspensions were added to initiate culture. The culture conditions were temperature 30°C, aeration 2 L/minute, agitation

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380 rpm. Culture was stopped after 64 hours, and cells of the respective strains were separately collected on filter paper by suction filtration using a Nutsche filter. 5 L of culture liquid was concentrated to 1/10 volume using a hollow fiber module for ultrafiltration with a molecular weight cut-off of 10,000 and ammonium sulfate was added to a final concentration of 60% saturation (456 g/L) to dissolve each concentrate. This was then centrifuged for 15 minutes at 8000 rpm in a Hitachi high-speed cooled centrifuge to precipitate the residue, the supernatant was adsorbed by an Octyl-Sepharose column and gradient eluted at an ammonium sulfate concentration of 0.6 to 0.0 saturation to collect a fraction having GDH activity. The resulting GDH solution was gel filtered with a G-25 Sepharose column and desalted by collecting the protein fraction, and ammonium sulfate equivalent to 0.6 saturation was added and dissolved in the desalted liquid. This was adsorbed by a Phenyl-Sepharose column, and gradient eluted at an ammonium sulfate concentration of 0.6 to 0.0 saturation to collect a fraction having GDH activity. The resulting GDH solution was further filtered with a G-25 Sepharose column to collect the protein fraction, and the resulting purified enzyme was used as the FAD-dependent GDH evaluation sample.

**[0072]** The mediator used in the composition for glucose measurement, glucose assay kit, glucose sensor or glucose measurement method of the present invention is not particularly limited, but preferably 2,6-dichlorophenol-indophenol (abbrev. DCPIP), ferrocene or derivatives of these (such as potassium ferricyanide, phenazine methosulfate or the like) can be used. These mediators can be obtained commercially.

### Test Example 1: Method for measuring FAD-dependent GDH activity

**[0073]** In the present invention, FAD-dependent GDH activity was measured under the following conditions.

--Reagents--

**[0074]**

50 mM PIPES buffer pH 6.5 (comprising 0.1% Triton X-100)  
14 mM 2,6-dichlorophenol indophenol (DCPIP) solution  
1 M D-glucose solution

**[0075]** 15.8 ml of the aforementioned PIPES buffer, 0.2 ml of DCPIP solution and 4 ml of D-glucose were mixed to make the reaction reagent.

--Measurement conditions--

**[0076]** 2.9 ml of reaction reagent was pre-heated for 5 minutes at 37°C. 0.1 ml of GDH solution was added and slowly mixed, after which changes in absorbancy relative to water were measured for 5 minutes at 600 nm with a spectrometer kept at 37°C, and the change in absorbancy ( $\Delta OD_{\text{TEST}}$ ) per minute beginning with the straight line was measured. In a blind test, the change in absorbancy ( $\Delta OD_{\text{BLANK}}$ ) per minute was measured similarly with the solvent used for dissolving the GDH added to the reagent mixture in place of the GDH solution. GDH activity was calculated from these values by the following formula. One unit (U) of GDH activity here is defined as the amount of enzyme needed to reduce 1 micromole of DCPIP in 1 minute in the presence of D-glucose at a concentration of 200 mM.

$$\text{Activity (U/ml)} = \{-(\Delta OD_{\text{TEST}} - \Delta OD_{\text{BLANK}}) \times 3.0 \times \text{dilution ratio}\} / \{16.3 \times 0.1 \times 1.0\}$$

**[0077]** In the formula, 3.0 is the amount (ml) of reaction reagent + enzyme solution, 16.3 is the millimolar molecular absorption coefficient ( $\text{cm}^2/\text{micromole}$ ) under these activity measurement conditions, 0.1 is the amount of enzyme solution (ml) and 1.0 is the optical light path (cm) of the cell.

**[0078]** Since GDH uses three different coenzymes, improvements in the heat stability of each were investigated under different conditions. For example, in the following Example 2 an enzyme solution adjusted to 5 U/ml with pH 6.5 buffer was first heat treated for 16 hours at 50°C in the case of PQQGDH, and surviving PQQGDH activity was compared to confirm improvement in heat stability. In the same way, in the case of NADGDH, an enzyme liquid adjusted to 85 U/ml with pH 7.2 buffer was heat treated for 1 hour at 50°C, and surviving NADGDH activity was compared to confirm improvement in heat stability. Similarly, in the case of FADGDH, an enzyme solution adjusted to 5 U/ml with pH 7.2 buffer was heated treated for 15 to 30 minutes at 50°C or 55°C, and surviving FADGDH activity was compared to confirm improvement in heat stability.

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**[0079]** In Examples 3 and 4 below, the pH of enzyme solutions prepared with various 50 mM buffers (0.4 to 5.1 U/ml) was observed, the solutions were heat treated for 15 minutes at 50°C or for 30 minutes at 50°C or for 15 minutes at 55°C or for 30 minutes at 55°C, GDH activity was measured, and activity survival (%) was calculated. Activity survival was then compared to confirm improvements in heat stability.

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### Example 2 : Confirming heat stability using glucose measurement system (3)

**[0080]** This was done using the methods for measuring FADGDH activity described in Test Example 1 above.

**[0081]** First, 50 ml of the FADGDH specified in Example 1 dissolved to about 10 U/ml in enzyme diluent (50 mM potassium phosphate buffer (pH 7.2)) was prepared. The respective 2x concentrations of succinic acid, malonic acid, phthalic acid, maleic acid, glutaric acid, sodium chloride, sodium sulfate, trisodium citrate, and ammonium sulfate shown in Tables 1 and 2 were prepared, and 0.5 ml of each was added in the same way to prepare two samples each with a total volume of 1.0 ml. Two control samples were also prepared wherein 0.1 ml of distilled water was added in place of the various compounds.

**[0082]** Of the two samples, one was stored at 4°C, while the other was treated for 30 minutes at 50°C. After treatment, each sample was diluted 21 times with enzyme diluent, and FADGDH activity was measured. In each case, enzyme activity after storage at 4°C was given as 100, and the activity values after 1 hour of treatment at 50°C were compared and given as activity survival values (%).

**[0083]** Improvements in the heat stability of FAD-GDH were also seen from addition of various dicarboxylic acid compounds and salt compounds, and succinic acid and malonic acid of the dicarboxylic acid compounds and sodium sulfate of the salt compounds had the greatest effect (Tables 1 and 2). In the case of succinic acid, malonic acid and sodium sulfate, it is thought that stability could be improved even by addition of a few moles. Since adequate effects were achieved even with a simple salt compound such as sodium chloride, it was discovered for the first time that in the case of FAD-GDH heat stability can be achieved simply by raising the ionic strength.

**[0084]** Table 1 shows survival (%) of FADGDH activity after 30 minutes of treatment at 50°C of FADGDH compositions with dicarboxylic acid compounds included.

**[0085]** Table 2 shows survival (%) of FADGDH activity after 30 minutes of treatment at 50°C of FADGDH compositions with salt compounds included.

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Table 1

	Added compound	Activity survival rate after 30 minutes of treatment at 50°C (%)				
		Dicarboxylic acid concentration				
		0mM	20mM	50mM	100mM	200mM
<i>Aspergillus terreus</i> - derived FAD-GDH	Succinic acid	12.5	19.9	33.9	51.1	64.0
	Malonic acid	12.5	16.0	26.3	41.2	58.7
	Phthalic acid	12.5	11.5	14.6	21.1	35.0
	Maleic acid	12.5	13.0	17.6	25.8	40.4
	Glutaric acid	12.5	13.9	18.9	28.5	45.2
<i>Penicillium</i> NBRC6231 strain FAD-GDH	Succinic acid	11.1	26.7	47.8	69.2	76.3
	Malonic acid	11.1	21.8	38.9	59.1	70.6
	Phthalic acid	11.1	13.3	22.0	28.2	38.7
	Maleic acid.	11.1	14.9	25.1	35.7	50.2
	Glutaric acid	11.1	16.0	28.1	43.7	59.7

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Table 2

	Added compound	GDH activity (U/ml)		Activity survival (%)	
		Stored at 4°C	50°C, 15 min Heat treatment		
5	<i>Aspergillus terreus</i> -derived FAD-GDH	Not added	5.1	0.8	15.8
10	20 mM sodium chloride	4.8	0.8	17.3	
	20 mM sodium sulfate	4.8	1	20.6	
	20 mM trisodium citrate	5.1	0.8	16.6	
	20 mM ammonium sulfate	5.2	0.9	18.1	
15	50 mM sodium chloride	4.8	1	19.8	
	50 mM sodium sulfate	4.9	1.3	26.0	
	50 mM trisodium citrate	4.9	1.1	22.1	
	50 mM ammonium sulfate	5.1	1.1	21.7	
20	100 mM sodium chloride	4.7	1.1	23.0	
	100 mM sodium sulfate	4.7	1.7	35.9	
	100 mM trisodium citrate	4.8	1.5	32.5	
25	100 mM ammonium sulfate	5.2	1.5	28.1	

Example 3 : Study of heat stability improvement effects of GDH composition with pH maintained in the acid range

30 **[0086]** This was done according to the methods for measuring FADGDH activity described in Test Example 1 above.

35 **[0087]** First, 10 ml of the two kinds of FADGDH obtained in Example 5 dissolved to about 10 U/ml in enzyme diluent (50 mM potassium phosphate buffer (pH 6.5)) was prepared. 1.8 ml of 50 mM potassium phosphate buffer (pH 4.3) or 50 mM potassium phosphate buffer (pH 5.6) or 50 mM potassium phosphate buffer (pH 7.0) was added to 0.2 ml of this enzyme solution, to a total volume of 2.0 ml. When the pH values of each of the enzyme solutions were measured, they fluctuated around pH 5.6, 6.0 and 7.2. 1 ml each was dispensed to prepare 2 samples, one of which was stored at 4°C, while the other was heat treated at 50°C. After treatment, each sample was diluted 2x with enzyme diluent, and FADGDH activity was measured. In each case, enzyme activity after storage at 4°C was given as 100%, and the GDH activity values after 15 minutes or 30 minutes of treatment at 50°C were compared and given as GDH activity survival values (%).

40 **[0088]** As a result, it was shown that the heat stability of a composition containing FADGDH is improved by maintaining the pH of a composition containing GDH in the acid range (Table 3).

**[0089]** Moreover, to investigate pH conditions in more detail, the enzyme liquid was diluted 10 times with the various buffers shown in Table 4, and after pH had been confirmed by measurement these were treated for 15 minutes at 50°C, GDH activity was measured, and survival (%) was calculated.

45 **[0090]** As result, it was shown that heat stability was higher at a pH in the range of 3.14 to 6.97 than at 7.4 whether the composition contained FADGDH derived from the *Aspergillus terreus* subspecies or *Penillium lilacinoechinulatum* NBRC6231 (Table 4, Figure 1, Figure 2).

50 **[0091]** The preparation of glucose sensors and sensor chips, which are widespread applications of GDH compositions, includes a step of heat drying, and the fact that the stability of a composition containing FADGDH can be improved by maintaining its pH in the acid range is extremely important from the standpoint of improving the stability and measurement accuracy of sensor chip products which employ FADGDH.

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Table 3

	Actual pH	GDH activity (U/ml)			Activity survival (%)	
		Stored at 4°C	50°C, 15 min heat treatment	50°C, 30 min heat treatment	50°C, 15 min heat treatment	50°C, 30 min heat treatment
<i>Aspergillus terreus</i> -derived FAD-GDH	5.65	0.9318	0.7702	0.661	82.7	70.9
	5.95	0.9302	0.7646	0.637	82.2	68.5
	7.17	0.9206	0.1868	0.0337	20.3	3.7
<i>Penicillium</i> NBRC6231 strain FAD-GDH	5.59	0.8822	0.641	0.5916	72.7	67.1
	5.94	0.8792	0.592	0.5238	67.3	59.6
	7.17	0.885	0.051	0.0173	5.8	2.0
In 50 mM potassium phosphate buffer						

Table 4

	Buffer	Actual pH	GDH activity		Activity survival (%)
			Stored at 4°C	50°C, 15 min heat treatment	
<i>Aspergillus terreus</i> -derived FAD-GDH	Glycine-hydrochloric acid	2.17	0.47	0.00	0.0
		3.14	0.78	0.09	11.0
	Sodium acetate	4.20	0.76	0.53	70.3
		5.11	0.79	0.67	84.7
	Potassium phosphate	5.84	0.84	0.68	80.9
		6.12	0.82	0.62	76.0
6.60		0.81	0.58	70.9	
<i>Penicillium</i> NBRC6231 strain FAD-GDH	Glycine-hydrochloric acid	2.17	0.57	0.00	0.7
		3.14	0.80	0.12	15.3
	Sodium acetate	4.20	0.81	0.72	89.3
		5.11	0.82	0.76	93.0
	Potassium phosphate	5.84	0.81	0.65	80.5
		6.13	0.81	0.61	76.2
6.60		0.81	0.39	48.5	
	6.97	0.83	0.10	12.6	
	7.39	0.79	0.01	1.6	

Example 4 : Study of improvements achieved in heat stability of a GDH composition by including one or more salt compounds or dicarboxylic acids in a GDH composition with the pH maintained in the acid range

**[0092]** This was done according to the methods for measuring FADGDH activity described in Test Example 1 above.

**[0093]** First, compound solutions were prepared consisting of sodium sulfate, trisodium citrate and ammonium sulfate each dissolved to 1 M in 50 mM potassium phosphate buffer (pH 6.5). 20 ml each of the two kinds of FADGDH obtained in Example 1 dissolved to about 10 U/ml in enzyme diluent (50 mM potassium phosphate buffer (pH 6.5)) was also prepared. 8 kinds of enzyme samples (2 ml) were then prepared consisting of each of the two kinds of FADGDH mixed

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1:1 with each of the 4 kinds of compound solutions. Control enzyme liquid samples were also prepared with the pH adjusted to near 6.25 and 6.65 with 50 mM potassium phosphate buffer as controls with no compound solution added. The actual pH values of the various enzyme liquid samples were measured to confirm that the pH remained near that of the control. The enzyme liquid samples were each dispensed in 1 ml amounts to prepare two samples, one of which was stored at 4°C, while the other was heat treated for 30 minutes at 55°C. After treatment, each sample was diluted 10 times with enzyme diluent, and FADGDH activity was measured. In each case, enzyme activity after storage at 4°C was given as 100%, and the GDH activity values after 30 minutes of treatment at 55°C were compared and given as GDH activity survival values (%).

**[0094]** As a result, it was shown that greater heat stability was achieved when the pH of a composition containing GDH was maintained in the acid range and sodium sulfate, trisodium citrate, ammonium sulfate or the like was added thereto than in the case of the control enzyme liquid sample with the pH maintained in the acid range (Table 5).

**[0095]** Next, an equal amount of 2M sodium chloride or 1M sodium sulfate or 1M trisodium citrate or 1M ammonium sulfate was added to enzyme liquid samples (5 U/ml) consisting of the two kinds of FADGDH with the pH adjusted to 5.3 with succinic acid buffer, to prepared 8 kinds of enzyme liquid samples (2 ml). As controls with no compound solution added, control enzyme liquid samples were also prepared by 2x dilution using 50 mM sodium succinate buffer (pH 5.3).. The actual pH values of the various enzyme liquid samples were measured to confirm that the pH remained near that of the control. The enzyme liquid sample were dispensed in 1 ml amounts to prepare two samples, one of which was stored at 4°C, while the other was heat treated for 30 minutes at 55°C. After treatment, each sample was diluted 5 times with enzyme diluent, and FADGDH activity was measured. In each case, enzyme activity after storage at 4°C was given as 100%, and the GDH activity values after 30 minutes of treatment at 55°C were compared and given as activity survival values (%).

**[0096]** As a result, it was confirmed that heat stability can be improved by addition of salt compounds such as sodium chloride, sodium sulfate, trisodium citrate, ammonium sulfate and the like even at low pH values around 5. The effect seen from addition even of such an unremarkable compound as sodium chloride suggests that heat stability of an FADGDH composition can be improved by inclusion of a wide range of salt compounds (Table 6).

**[0097]** Since greater improvement in heat stability was achieved using the succinic acid buffer than using potassium phosphate buffer as the base, it appears that the dicarboxylic acid compound contained in the succinic acid buffer has the effect of improving heat stability in the same way as the salt compounds described above. Therefore, fluctuations in heat stability were investigated when dicarboxylic acid compounds were added with the pH of a GDH-containing composition maintained in the acid range.

**[0098]** First, 100 mM sodium acetate (pH 5.0) and 100 mM potassium phosphate buffer (pH 5.6) were prepared as the base buffers. 20 ml samples of the two kinds of FADGDH obtained in Example 5 dissolved to about 10 U/ml in enzyme diluent (50 mM potassium phosphate buffer (pH 6.5)) were also prepared. Next, 0.4 M succinic acid (adjusted to pH 7.0 with NaOH), 0.4 M malonic acid (adjusted to pH 7.0 with NaOH) and 0.4 M glutaric acid (adjusted to pH 7.0 with NaOH) were prepared.

**[0099]** 0.8 ml of base buffer was mixed with 0.2 ml of enzyme liquid (10 U/ml), 1 ml of each of the various dicarboxylic acid compounds was added, and the pH of each was measured. Distilled water was also added instead of a dicarboxylic acid compound to obtain controls without a compound solution added, and the pH of each sample was adjusted to near the measurement value. The enzyme liquid samples were dispensed in 1 ml amounts to prepare two samples, one of which was stored at 4°C, while the other was heat treated at 55°C. After treatment, each sample was diluted 2 times with enzyme diluent, and FADGDH activity was measured. In each case, enzyme activity after storage at 4°C was given as 100%, and the GDH activity values after 15 minutes or 30 minutes of treatment at 55°C were compared and given as activity survival values (%).

**[0100]** As a result, it was shown that heat stability could be achieved by addition of a dicarboxylic acid such as succinic acid, malonic acid, glutaric acid or the like at a pH of around 5.5 or 6.0. These results suggest that the heat stability of a FADGDH composition can be improved by inclusion of a wide range of dicarboxylic acid compounds (Table 7).

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Table 5

	Added compound	Actual pH	GDH activity (U/ml)		Activity survival (%)	
			Stored at 4°C	55°C, 30 min heat treatment		
5  10	<i>Aspergillus terreus</i> -derived FAD-GDH	None	6.25	5.1	0.4	8.7
		None	6.63	5.1	0.2	3.3
		0.5 M sodium sulfate	6.43	5.1	2.4	47.3
		0.5 M trisodium citrate	6.84	5.0	3.0	60.1
		0.5 M ammonium sulfate	6.31	5.0	1.7	33.6
15  20	<i>Penicillium</i> NBRC6231 strain FAD-GDH	None	6.28	4.8	0.9	17.9
		None	6.64	4.8	0.1	2.0
		0.5 M sodium sulfate	6.46	4.5	2.6	57.0
		0.5 M trisodium citrate	6.87	4.5	2.5	56.4
		0.5 M ammonium sulfate	6.33	4.6	3.0	65.0
In 50 mM potassium phosphate buffer						

Table 6

	Added compound	Actual pH	GDH activity (U/ml)		Activity survival (%)	
			Stored at 4°C	55°C, 30 min heat treatment		
30  35	<i>Aspergillus terreus</i> -derived FAD-GDH	None	5.32	2.8	0.5	19.2
		1 M sodium chloride	4.97	2.9	1.0	35.3
		0.5 M sodium sulfate	5.02	2.9	1.9	63.5
		0.5 M trisodium citrate	6.46	2.6	1.8	68.1
		0.5 M ammonium sulfate	5.06	2.8	1.4	49.5
40  45	<i>Penicillium</i> NBRC6231 strain FAD-GDH	None	5.31	2.3	1.0	42.0
		1 M sodium chloride	4.97	2.4	1.7	72.0
		0.5 M sodium sulfate	5.02	2.4	2.1	87.0
		0.5 M trisodium citrate	6.46	2.3	1.8	76.3
		0.5 M ammonium sulfate	5.05	2.3	2.0	87.3
50 mM succinic acid buffer						

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Table 7

	Added compound	Actual pH	GDH activity (U/ml)			Activity survival (%)		
			4°C	55° 15 min	55°C 30 min	55° 15 min	55°C 30 min	
5	<i>Aspergillus terreus</i> -derived FAD-GDH	None	5.62 <sup>*1</sup>	0.87	0.39	0.13	45.0	14.4
		0.2M succinic acid	5.43 <sup>*1</sup>	0.82	0.44	0.22	52.9	26.5
10		0.2M malonic acid	5.67 <sup>*1</sup>	0.83	0.43	0.22	52.2	26.9
		0.2M glutaric acid	5.52 <sup>*1</sup>	0.81	0.42	0.21	51.8	26.0
		None	6.12 <sup>*2</sup>	0.81	0.27	0.09	33.6	11.3
15		0.2M succinic acid	5.99 <sup>*2</sup>	0.83	0.39	0.18	46.6	21.4
		0.2M malonic acid	6.19 <sup>*2</sup>	0.81	0.30	0.11	37.0	13.7
		0.2M glutaric acid	6.13 <sup>*2</sup>	0.81	0.31	0.12	37.8	14.6
20		<i>Penicillium</i> NBRC6231 strain FAD-GDH	None	5.62 <sup>*1</sup>	0.84	0.47	0.26	55.2
	0.2M succinic acid		5.43 <sup>*1</sup>	0.82	0.57	0.42	69.6	50.7
25	0.2M malonic acid		5.67 <sup>*1</sup>	0.82	0.54	0.37	65.9	44.7
	0.2M glutaric acid		5.52 <sup>*1</sup>	0.82	0.56	0.39	68.0	47.7
	None		6.12 <sup>*2</sup>	0.81	0.18	0.05	22.7	5.9
30	0.2M succinic acid		5.99 <sup>*2</sup>	0.81	0.41	0.23	51.1	28.8
	0.2M malonic acid		6.19 <sup>*2</sup>	0.81	0.27	0.11	33.0	13.4
	0.2M glutaric acid		6.13 <sup>*2</sup>	0.80	0.30	0.13	37.0	16.2
35	* <sup>1</sup> sodium acetate buffer * <sup>2</sup> potassium phosphate buffer							

[0101] With the improved heat stability achieved by the present invention, it is possible to improve the storage stability and measurement accuracy of glucose measurement reagents, glucose assay kits and glucose sensors.

#### SEQUENCE LISTING

#### [0102]

<110> Toyo Boseki Kabushiki Kaisha

<120> Method for improving heat stability of the composition containing water soluble coenzyme-bound glucose dehydrogenase

<130> 200616EP

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<170> PatentIn version 3.1

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40 gagatggcat atatttgctg gccaacggtt gcaccgtcat cagcatatgt atatacggga 1080  
ggcaaaaaag cgattccagg gtgggaaaat acattattgg tcccatcttt aaaacgtggg 1140  
45  
gtgattttcc gtattaaatt ggacccgaca tatagcacga ctttggatga tgctatccca 1200  
50 atgtttaaaa gcaataaccg ttaicgtgat gtcacgcta gtccagaag taatacctta 1260  
tatgtgctga ctgatacagc ggggaatgta caaaaagatg atggttctgt cactcact 1320  
55 ttagagaatc ccggttctct cattaaattt acatataacg gtaagtaa 1368

**Claims**

1. A method for improving the heat stability of the flavin-bound glucose dehydrogenase from *Aspergillus* (GDH) in a composition, comprising the step of combining soluble GDH with a carboxyl group-containing compound, thereby improving the heat stability of said GDH over that achieved without the inclusion of said compound, and wherein the carboxyl group-containing compound is any one or more selected from the group consisting of calcium glycerate, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3,3-dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.
2. The method for improving heat stability according to Claim 1, wherein the final concentration of each included compound is 0.1% or more.
3. The method according to any one of claims 1 to 2 further comprising the step of maintaining the pH of the composition at pH 3.1 to 7.0, thereby improving the heat stability of the composition over that achieved with the composition at a pH of 7.4.
4. A composition comprising soluble GDH having improved thermal stability as a result of the methods of claims 1 to 3.
5. A method for measuring a glucose concentration using the composition according to Claim 4.
6. A glucose sensor comprising the composition according to Claim 4.
7. A method for manufacturing a composition comprising GDH having improved heat stability, comprising the step of combining, in a composition soluble GDH with a carboxyl group-containing compound, thereby improving the heat stability of said GDH over that achieved without the inclusion of said compound, and wherein the carboxyl group-containing compound is any one or more selected from the group consisting of calcium glycerate, succinic acid, diammonium hydrogen citrate, fumaric acid, malonic acid, pimelic acid, 3,3-dimethylglutaric acid, phthalic acid, maleic acid, and glutaric acid.

**Patentansprüche**

1. Verfahren zur Verbesserung der Wärmestabilität der flavingebundenen Glucose-Dehydrogenase aus *Aspergillus* (GDH) in einer Zusammensetzung, umfassend den Schritt des Kombinierens von löslichem GDH mit einer Carboxygruppen enthaltenden Verbindung, wodurch die Wärmestabilität des GDH gegenüber der ohne die Gegenwart dieser Verbindung erreichten verbessert wird, wobei es sich bei der Carboxygruppen enthaltenden Verbindung um eine oder mehrere handelt, die aus der Gruppe ausgewählt sind, die aus Calciumglycerat, Bernsteinsäure, Diammoniumhydrogencitrat, Fumarsäure, Malonsäure, Pimelinsäure, 3,3-Dimethylglutarsäure, Phthalsäure, Maleinsäure und Glutarsäure besteht.
2. Verfahren zur Verbesserung der Wärmestabilität gemäß Anspruch 1, wobei die Endkonzentration jeder vorhandenen Verbindung 0,1% oder mehr beträgt.
3. Verfahren gemäß einem der Ansprüche 1 bis 2, das weiterhin den Schritt des Haltens des pH-Werts der Zusammensetzung auf pH 3,1 bis 7,0 umfasst, wodurch die Wärmestabilität der Zusammensetzung gegenüber der mit der Zusammensetzung bei einem pH-Wert von 7,4 erreichten verbessert wird.
4. Zusammensetzung, die lösliches GDH mit einer aufgrund der Verfahren gemäß Anspruch 1 bis 3 verbesserten Wärmestabilität umfasst.
5. Verfahren zur Messung einer Glucosekonzentration unter Verwendung der Zusammensetzung gemäß Anspruch 4.
6. Glucose-Sensor, der die Zusammensetzung gemäß Anspruch 4 umfasst.
7. Verfahren zur Herstellung einer Zusammensetzung, die GDH mit verbesserter Wärmestabilität umfasst, umfassend den Schritt des Kombinierens von löslichem GDH mit einer Carboxygruppen enthaltenden Verbindung in einer Zusammensetzung, wodurch die Wärmestabilität des GDH gegenüber der ohne die Gegenwart dieser Verbindung erreichten verbessert wird, wobei es sich bei der Carboxygruppen enthaltenden Verbindung um eine oder mehrere

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handelt, die aus der Gruppe ausgewählt sind, die aus Calciumglycerat, Bernsteinsäure, Diammoniumhydrogencitrat, Fumarsäure, Malonsäure, Pimelinsäure, 3,3-Dimethylglutarsäure, Phthalsäure, Maleinsäure und Glutarsäure besteht.

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### Revendications

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1. Procédé pour améliorer la stabilité thermique de la glucose déshydrogénase liée à la flavine provenant d'*Aspergillus* (GDH) dans une composition, comprenant l'étape consistant à combiner de la GDH soluble avec un composé contenant des groupes carboxyles, améliorant ainsi la stabilité thermique de ladite GDH par rapport à celle obtenue en absence dudit composé, dans lequel le composé contenant des groupes carboxyles est l'un quelconque ou plusieurs des composés choisis dans le groupe consistant en le glycérate de calcium, l'acide succinique, le citrate d'ammonium dibasique, l'acide fumarique, l'acide malonique, l'acide pimélique, l'acide 3,3-diméthylglutarique, l'acide phtalique, l'acide maléique et l'acide glutarique.

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2. Procédé pour améliorer la stabilité thermique selon la revendication 1, dans lequel la concentration finale de chaque composé présent est de 0,1 pour cent ou plus.

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3. Procédé selon l'une quelconque des revendications 1 à 2, comprenant en outre l'étape consistant à maintenir le pH de la composition entre 3,1 et 7,0, améliorant ainsi la stabilité thermique de la composition par rapport à celle obtenue avec la composition à un pH de 7,4.

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4. Composition comprenant de la GDH soluble ayant une stabilité thermique améliorée au moyen des procédés selon les revendications 1 à 3.

5. Procédé pour mesurer une concentration de glucose en utilisant la composition selon la revendication 4.

6. Senseur de glucose comprenant la composition selon la revendication 4.

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7. Procédé pour préparer une composition comprenant de la GDH ayant une stabilité thermique améliorée, comprenant l'étape consistant à combiner, dans une composition, de la GDH soluble avec un composé contenant des groupes carboxyles, améliorant ainsi la stabilité thermique de ladite GDH par rapport à celle obtenue en absence dudit composé, dans lequel le composé contenant des groupes carboxyles est l'un quelconque ou plusieurs des composés choisis dans le groupe consistant en le glycérate de calcium, l'acide succinique, le citrate d'ammonium dibasique, l'acide fumarique, l'acide malonique, l'acide pimélique, l'acide 3,3-diméthylglutarique, l'acide phtalique, l'acide maléique et l'acide glutarique.

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Fig. 1

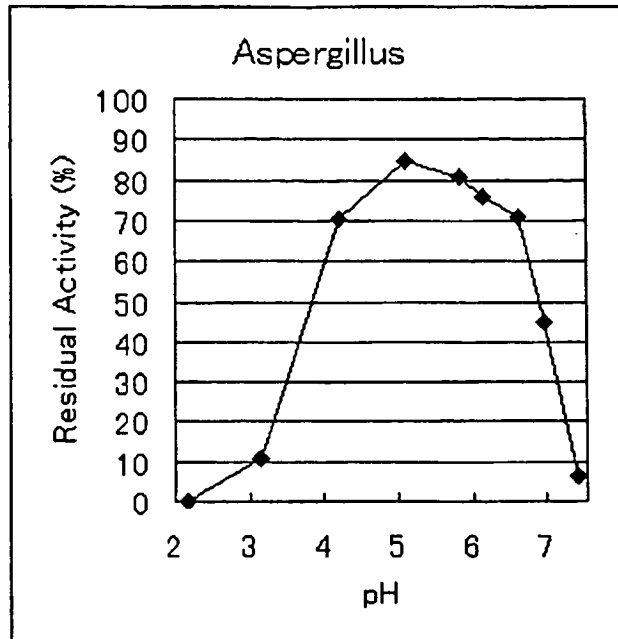
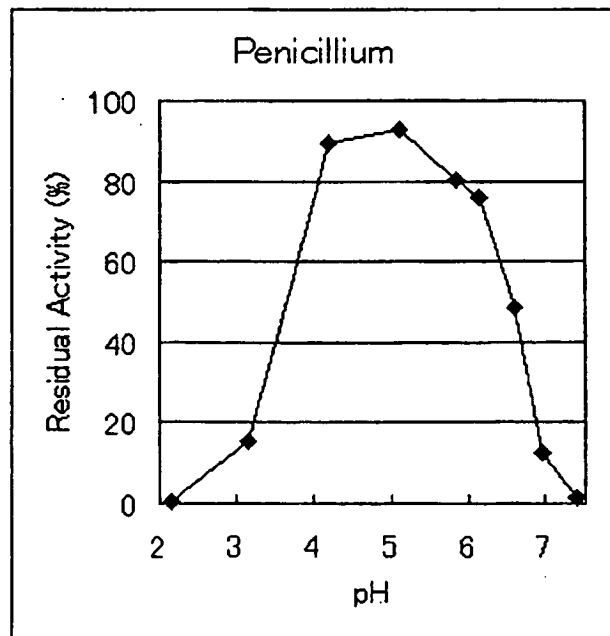


Fig. 2



**REFERENCES CITED IN THE DESCRIPTION**

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专利名称(译)	提高含有葡萄糖脱氢酶 ( GDH ) 的组合物的热稳定性的方法		
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摘要(译)

本发明涉及一种改善葡萄糖脱氢酶 ( GDH ) 的热稳定性的方法，包括在包含可溶性辅酶结合的葡萄糖脱氢酶 ( GDH ) 的组合中将酶与选自下组中的任何一种或多种的组合的步骤。糖醇，含羧基化合物，含碱金属化合物，碱土金属化合物，铵盐，硫酸盐和蛋白质，从而改善GDH的热稳定性，而不是不含化合物。

$$\text{Activity (U/ml)} = \frac{-(A_{405}^{\text{test}} - A_{405}^{\text{blank}})}{\text{ratio}} \times 3.0 \times \text{dilution}$$

$$\text{ratio} = 1/16.3 \times 0.1 \times 1.0$$