Appeal decision

Appeal No. 2017-3283

Osaka, Japan

Appellant TOYOBO CO. LTD.

Osaka, Japan

Patent Attorney SAEGUSA AND PARTNERS

The case of appeal against the examiner's decision of refusal of Japanese Patent Application No. 2011-222278, entitled "Novel Glucose Dehydrogenase" (the application published on May 9, 2013, Japanese Unexamined Patent Application Publication No. 2013-81399) has resulted in the following appeal decision:

Conclusion

The appeal of the case was groundless.

Reason

1 History of the procedures

The application was filed on October 6, 2011, a notice of reasons for refusal was issued on May 30, 2016, and despite submission of a written opinion on August 4, 2016 ,

an examiner's decision of refusal was issued on November 29, 2016 , and an appeal against the examiner's decision of refusal was made on March 6, 2017.

2 The Invention

The inventions according to Claims 1 to 10 of the application are specified by the matters described in Claims 1 to 10 described in the scope of claims originally attached to the application, and the invention according to Claim 1 is as follows.

"[Claim 1] A flavin-bound glucose dehydrogenase having the following characteristics (1) and (2).

(1) Molecular weight: The polypeptide moiety of the enzyme measured by SDS-polyacrylamide gels electrophoresis has a molecular weight of about 88 kDa.

(2) Substrate specificity: Reactivity to D-xylose is 1.3% or less, based on the reactivity to D-glucose at the same concentration taken as 100%." (hereinafter referred to as "the Invention")

3 Reasons for the decision of refusal

Reasons 1 and 2 of notice of reasons for refusal issued on May 30, 2016 (Heisei 28); i.e. reasons for the decision of refusal, are as outlined below.

(1) Reason 1 A flavin-bound glucose dehydrogenase of the Invention includes one derived from any organism; however, only a flavin-bound glucose dehydrogenase derived from Mucor guilliermondii is specifically described in the detailed description of the invention.

In light of common general technical knowledge at the time of filing the application that the difference in origin causes various characteristics even if each polypeptide catalyzes the same reaction, the matters described in the detailed

description of the invention cannot be expanded and generalized to the Invention, and thus it cannot be said that the Invention is described in the detailed description of the invention, and regarding the application according to the Invention, the description of the scope of claims does not meet the requirement stipulated in Article 36(6)(i) of the Patent Act.

(2) Reason 2 Even taking into account the detailed description of the invention and common general technical knowledge at the time of filling the application, obtaining a flavin-bound glucose dehydrogenase of the Invention other than one derived from *Mucor guilliermondii* requires trial and error, and/or complicated and sophisticated experimentation beyond the extent to which a person skilled in the art should be reasonably expected to conduct, and thus regarding the application according to the Invention, the description of the detailed description of the invention does not meet the requirement stipulated in Article 36(4)(i) of the Patent Act.

4 Judgment by the body

(1) The detailed description of the invention

The following descriptions are described in the detailed description of the invention.

A Problem to be solved by the invention

"An object of the present invention is to provide a novel glucose dehydrogenase that has excellent characteristics (for example, reduced reactivity to D-xylose) and a sensor and the like using the same. The present inventors conducted intensive research to provide a more suitable SMBG glucose sensor, and as a result, they found that using an enzyme with higher affinity to glucose (i.e., an enzyme with low Km) enables shortening the measurement time while accurately measuring blood glucose levels with a small amount of enzyme." (paragraph [0008])

B Means for solving the problem

"The present inventors conducted intensive research to achieve the above objects, and they succeeded in purifying GDH, derived from *Mucor guilliermondii*, with excellent substrate specificity and affinity to substrate. The present invention has been accomplished as a result of further research and improvement based on this finding." (paragraph [0009])

C Effect of the Invention

"The flavin-bound glucose dehydrogenase of the present invention (hereinafter referred to as "FGDH," in some cases) has excellent substrate specificity. That is, the FGDH of the present invention has significantly reduced reactivity to D-xylose, D-galactose, and maltose, and thus, even when D-glucose and one or two or more of these saccharides are present in a sample, the amount or concentration of glucose can be accurately measured. In addition, the FGDH of the present invention has high affinity to D-glucose (i.e. the FGDH has significantly small Km to D-glucose), and thus, using the FGDH enables measurement of the concentration of D-glucose in a sample in a shorter time with a smaller amount of enzyme." (paragraph [0011])

D Description of Embodiments

"1-1. Glucose dehydrogenase activity

A flavin-bound glucose dehydrogenase is an enzyme that has a physicochemical property of catalyzing a reaction in which hydroxyl groups of glucose are oxidized to produce glucono-δ-lactone in the presence of an electron acceptor. In this specification, this enzyme activity represents glucose dehydrogenase activity, and unless otherwise noted, the term "enzyme activity" or "activity" represents this enzyme activity. ... More specifically, the activity can be measured using the following reagents and measurement conditions. ..." (paragraphs [0014] to [0015])

"1-2. Molecular weight

The polypeptide moiety constituting the FGDH of the present invention has a

molecular weight of about 88 kDa as measured by SDS-PAGE. 'About 88 kDa' includes a range in which a person skilled in the art would usually determine that the band is present at a position of 88 kDa when a molecular weight is measured by SDS-PAGE. 'Polypeptide moiety' refers to FGDH substantially not having an attached glycan. ...

The molecular weight measurement by SDS-PAGE may be performed using general techniques and devices with the use of commercially available molecular weight markers." (paragraphs [0021] to [0023])

"1-3 Substrate specificity

The FGDH of the present invention has excellent substrate specificity. In particular, the FGDH of the present invention has significantly reduced reactivity at least to D-xylose, D-galactose, and maltose, as compared to the reactivity to D-glucose. ...

The reactivity of FGDH to each saccharide can be measured by replacing D-glucose with another saccharide (e.g., D-xylose, D-galactose, or maltose) in the method for measuring the glucose dehydrogenase activity described in Section 1-1 above, and comparing the results with the results of the D-glucose activity. For comparison, the concentration of each saccharide is 50 mM." (paragraphs [0024] to [0027])

"1-9. Origin

The origin of the FGDH of the present invention is not particularly limited so long as the FGDH has the characteristics described above; examples of the origin of the FGDH of the present invention are microorganisms belonging to the family Mucoraceae, more specifically, microorganisms belonging to the genus Mucor, the genus Absidia, the genus Actinomucor, and the like. More specifically, examples of the origin of the FGDH include microorganisms belonging to *Mucor guilliermondii*, *Mucor prainii*, *Mucor javanicus*, *Mucor circinelloides*, and the like. More specifically, examples of the origin of the FGDH include microorganisms belonging to *Mucor guilliermondii* NBRC9403. Many microorganisms belonging to the genus Mucor including *Mucor*

guilliermondii NBRC9403 are maintained in NBRC (NITE Biological Resource Center) of the National Institute of Technology and Evaluation, and can be obtained after completing predetermined procedures. Examples of other organisms from which the FGDH of the present invention is derived include microorganisms living in soil, water systems such as rivers and lakes, and other; microorganisms living in oceans; microorganisms indigenously present in the surface of or inside various animals or plants, and the like. As an isolation source, it is also possible to use microorganisms that thrive in low-temperature environments; high-temperature environments such as volcanoes; anoxic, high-pressure, and aphotic environments such as deep seas; and special environments such as oil fields." (paragraph [0035])

"2. Method for producing FGDH

A method for producing FGDH of the present invention is not particularly limited so long as the FGDH of the present invention can be obtained; for example, the FGDH can be produced by culturing organisms producing the FGDH of the present invention and purifying the FGDH from the culture supernatant or the cells. As indicated in the following Examples, the FGDH of the present invention was isolated from microorganisms belonging to the genus Mucor. Thus, the FGDH of the present invention can be produced by isolating the FGDH from microorganisms belonging to the family Mucoraceae, more specifically, microorganisms belonging to the genus Mucor, the genus Absidia, the genus Actinomucor, and the like, more specifically, microorganisms belonging to *Mucor guilliermondii*, *Mucor prainii*, *Mucor javanicus*, *Mucor circinelloides*, and the like, more specifically, microorganisms belonging to *Mucor guilliermondii* NBRC9403. ...

Isolation of the FGDH of the present invention from microorganisms can be performed in accordance with conventional methods taking into account the following Examples. For example, the culture medium including microorganisms producing FGDH therein can be directly collected and this medium can be used as FGDH; on the other hand, in a case where FGDH is present in the culture medium, the culture medium is generally is separated into FGDH-containing solution and microorganisms using

centrifugation, and the FGDH-containing solution can be used as FGDH. In a case where FGDH is present in cells, the cells are collected from the culture using filtration or centrifugation, the cells are disrupted by a mechanical technique or techniques using enzymes such as lysozyme, and a surfactant and a chelating agent such as EDTA are optionally added to solubilize FGDH, which is separated and collected as an aqueous solution.

The FGDH-containing solution may be purified by vacuum concentration or membrane concentration, salting out with ammonium sulfate, sodium sulfate, or the like, or fractional precipitation with a hydrophilic organic solvent such as methanol, ethanol, or acetone. Heat treatment or isoelectric focusing is effective purification means. Thereafter, the purified FGDH can be obtained by gel filtration with an adsorbent or a gel filtration agent, adsorption chromatography, ion-exchange chromatography, affinity chromatography, or the like. When column chromatography is used, for example, gel-filtration column chromatography using Sephadex gel (GE Healthcare Bioscience) and column chromatography using DEAE Sepharose CL-6B (GE Healthcare Bioscience) or Octyl Sepharose CL-6B (GE Healthcare Bioscience) may be used to separate and purify the FGDH-containing solution, thereby obtaining a purified enzyme preparation. It is preferable that the purified enzyme preparation is purified to the extent that the enzyme migrates as a single band on electrophoresis (SDS-PAGE).

In recovering (e.g., extracting or purifying) a protein having FGDH activity from a culture medium, any of the following may be used as indices: FDGH activity, reactivity to maltose, thermal stability, and the like, as described in Section 1." (paragraphs [0036] to [0043])

E Examples

"[Example 4] Purification of FGDH derived from *Mucor guilliermondii* NBRC9403

50 mL of DP medium was placed in a 500-mL shake flask and sterilized in an autoclave, thereby preparing a preculture medium. A loopful of *Mucor guilliermondii* NBRC9403 reconstituted in Example 1 was inoculated in the preculture medium and

subjected to shaking culture for 3 days at 25°C and 180 rpm, thereby obtaining a seed culture solution. Next, 6.0 L of a production medium (yeast extract 2.0%, glucose 1%, pH 6.0) was placed in a 10-L jar fermenter and sterilized in an autoclave, thereby obtaining a main culture medium. 50 mL of the seed culture solution was inoculated in the main culture medium and was cultured for 3 days under the following conditions: culture temperature=25°C, stirring speed=600 rpm, air flow rate=2.0 L/min, and tube internal pressure=0.2 MPa. Thereafter, the culture solution was filtrated with a membrane, and the cells were collected. The cells were suspended in 50 mM phosphate buffer solution (pH 6.0).

The resulting suspension was sent to French Press (Niro Soavi) at 160 mL/min, and was disrupted at 1000 to 1300 bar. Subsequently, ammonium sulfate (Sumitomo Chemical Co., Ltd.) was gradually added to the disrupted solution to give 0.4 saturation, the resulting solution was stirred at room temperature for 30 minutes, and the suspended substance was removed using a filter aid (Showa Chemical Industry Co., Ltd.), thereby obtaining a clear filtrate. Next, the filtrate was concentrated using a UF membrane (Millipore Co., Ltd.) having a molecular weight cutoff of 10,000, and the concentrated solution was desalinated using a Sephadex G-25 gel. Next, ammonium sulfate was gradually added to the desalinated solution to give 0.5 saturation, and the resulting solution was subjected to linear gradient elution with 50 mM phosphate buffer solution (pH 6.0) by being passed through a 400-mL SP Sepharose Fast Flow column (GE Healthcare) equilibrated in advance with 50 mM potassium phosphate buffer solution (pH 6.0) containing 0.5-saturated ammonium sulfate. The eluted GDH fraction was concentrated using a hollow fiber membrane (Spectrum Laboratories, Inc.) having a molecular weight cutoff of 10,000, and the concentrated solution was desalinated using a Sephadex G-25 gel. Thereafter, the desalinated solution was subjected to DEAE Sepharose Fast Flow column (GE Healthcare), thereby obtaining a purified enzyme." (paragraphs [0056] to [0057])

"[Example 6] Molecular weight of peptide moiety of enzyme

The purified FGDH purified in Example 4 was denatured by heating at 100°C

for 10 minutes, and then treated with 5 U of N-glycosidase F (Roche Diagnostics K.K.) at 37°C for 6 hours, thereby decomposing glycan added to the protein. Thereafter, the same measurement as in Example 5 was performed using SDS-polyacrylamide gels electrophoresis. The results revealed that the molecular weight of the polypeptide moiety of the purified FDGH was about 88,000 Da." (paragraph [0061])

"[Example 7] Substrate specificity

According to the GDH activity measurement method described in Section 1-1 above, regarding the activity of the FGDH purified in Example 4, the substrate specificity was determined by comparing the activity with D-glucose as a substrate with the apparent activity with maltose, D-galactose, or D-xylose as a substrate. Conditions of the measurement are as follows: the concentration of substrate was 50 mM, pH was 6.5 and temperature was 37°C. Table 2 shows the results.

[Table 2]

	相対活性(%)
D-Glucose	100.0
Maltose	2.9
D-Galactose	1.2
D-Xylose	0.8

相対活性　　relative activity

The results showed that each apparent activity of the substrate specification of the FGDH of the present invention to maltose, D-galactose, and D-xylose was 3% or less, in a case where its activity to D-glucose was 100%." (paragraphs [0062] to [0064])

"[Example 13] Confirmation of flavin-bound enzyme

The enzyme purified in Example 4 was dialyzed with 10 mM acetate buffer solution (pH 5.0), and the absorption spectrum at 250 to 800 nm was measured using a spectrophotometer U-3210 (Hitachi High-Technologies Corporation). The results

revealed that the enzyme had two peaks (maximum absorption wavelength) approximately at a wavelength of 340 to 350 nm and at a wavelength of approximately 420 to 430 nm. The shape of the absorption spectrum strongly suggested that the GDH of the present invention is a flavin-bound protein." (paragraph [0074])

(2) Reason 1 (Violation of Article 36(6)(i) of the Patent Act)

As described in Section 2 above, the Invention includes various flavin-bound glucose dehydrogenases, such as a naturally occurring enzyme derived from any organism, a modified enzyme, or an artificially designed enzyme based thereon, as long as they have a molecular weight and a substrate specificity specified in the invention.

On the other hand, even though the glucose dehydrogenase derived from *Mucor guilliermondii* NBRC9403 is described as only one example in Examples of the detailed description of the invention (the above (1)E), there is only general description that the FGDH derived from microorganisms other than *Mucor guilliermondii* NBRC9403, for example, the FGDH derived from microorganisms belonging to the family Mucoraceae (such as the genus Mucor, the genus Absidia and the genus Actinomucor) can be isolated in accordance with conventional methods taking into account Examples (the above (1)D).

At the time of filing the application, in light of the fact that it was widely known that various microorganisms belonging to the genus Aspergillus, the genus Penicillium, the genus Mucor, and the like have a flavin-bound glucose dehydrogenase (for example, International Publication No. WO2010/140431, International Publication No. WO2011/068050), it is thought that the Invention in which the origin is not specified includes at least a flavin-bound glucose dehydrogenase derived from microorganisms belonging to various genera or a mutant thereof. On the other hand, it was common general technical knowledge at the time of filling the application that enzymes derived from different organisms are different each other in a chemical structure even if each enzyme catalyzes the same reaction, and the enzymes differ from each other in a

molecular weight, substrate specificity, and other characteristics, and it cannot be always said that enzymes derived from closed microorganisms are similar in substrate specificity. For example, it is described in International Publication No. WO2010/140431 (especially, Table 3) that flavin-bound glucose dehydrogenases derived from each of three kinds of the genus Mucor, M. prainii, M. javanicus, and M. circinelloides differ from each other in substrate specificity (such as reactivity to xylose). In addition, it is common general technical knowledge that mutation, such as substitution of an amino acid residue, causes change of characteristics, as described in International Publication No. WO2011/034108 (especially, Tables 5 to 8, 10 to 17) that a flavin-bound glucose dehydrogenase derived from *Aspergillus oryzae* in which one amino acid is substituted has substrate specificity (such as reactivity to xylose) different from that of a naturally occurring flavin-bound glucose dehydrogenase derived from *Aspergillus oryzae*.

Taking into account such common general technical knowledge, if a flavin-bound glucose dehydrogenase is isolated from microorganisms belonging to the genus Mucor closely related to microorganisms described in Examples or a flavin-bound glucose dehydrogenase is obtained by mutating the enzyme in Examples, it cannot be always said that this enzyme does not have a molecular weight and substrate specificity specified in the Invention. Actually, *M. prainii, M. javanicus,* and *M.circinelloides* are illustrated as an isolated source of a flavin-bound glucose dehydrogenase of the Invention ("1-9. Origin" and "2. Method for producing FGDH" in the above (1)D); on the other hand, it is described in International Publication No. WO2010/140431 that each polypeptide moiety of a flavin-bound glucose dehydrogenase derived from three kinds of microorganism has a molecular weight of about 80 kDa measured by SDS-polyacrylamide gels electrophoresis, and the reactivity to D-xylose is 1.53%, 1.43%, and 2.00% respectively, based on the reactivity to D-glucose at the same concentration taken as 100%, each enzyme does not fall within the Invention in terms of molecular weight and substrate specificity, and it can be said that a flavin-bound glucose dehydrogenase derived from the illustrated microorganisms belonging to the genus Mucor may not be the Invention. In addition, a flavin-bound glucose dehydrogenase

derived from microorganisms distantly related to *Mucor guilliermondii* NBRC9403 described in Examples may not be the Invention.

As described above, even in light of common general technical knowledge at the time of filling the application, the matters of glucose dehydrogenase derived from *Mucor guilliermondii* NBRC9403 which are disclosed in the detailed description of the invention cannot be expanded and generalized to the Invention.

(3) Reason 2 (Violation of Article 36(4)(i) of the Patent Act)

Taking into account the judgment in the above (2), it cannot be acknowledged that the FGDH of the Invention other than one derived from *Mucor guilliermondii* NBRC9403 is described in the detailed description of the invention such that a person skilled in the art can make the enzyme.

(4) Appellant's allegation

In the written opinion submitted on August 4, 2016 and the written request for appeal submitted on March 6, 2017, the appellant alleges as follows.

A It is disclosed in the detailed description of the invention that an enzyme of the Invention is obtained from *Mucor guilliermondii* NBRC9403, and this strain has been deposited in the National Institute of Technology and Evaluation, and thus a person skilled in the art can easily obtain a flavin-bound glucose dehydrogenase having a molecular weight and substrate specificity specified in the Invention.

B A person skilled in the art can predict an amino acid sequence required to maintain the physicochemical characteristics by specifying the amino acid sequence of the flavin-bound glucose dehydrogenase obtained in the above allegation A, and comparing the amino acid sequence of the other publicly known flavin-bound glucose dehydrogenase, and a person skilled in the art can obtain a flavin-bound glucose dehydrogenase of the

Invention derived from microorganisms other than *Mucor guilliermondi* NBRC9403 by mutating an amino acid residue other than amino acid residues present in the amino acid sequence required to maintain the physicochemical characteristics.

First, the allegation A is to only describe the reproducibility of Examples, and thus this allegation does not affect the judgments regarding the Reasons 1 and 2.

In addition, regarding the allegation B, the amino acid sequence of the flavin-bound glucose dehydrogenase derived from *Mucor guilliermondi* NBRC9403 described in Examples and the amino acid sequence of the other publicly known flavin-bound glucose dehydrogenase are not described in the detailed description of the invention, and further, there is no technical idea that an enzyme of the Invention can be obtained by mutating an amino acid residue other than amino acid residues present in the amino acid sequence required to maintain the physicochemical characteristics on the basis of information obtained by comparing the amino acid sequences of an enzyme derived from different organisms. Since considerable creativity is required to create a mutant of an enzyme having desired characteristics based on information obtained by comparing the amino acid sequences of an enzyme derived from different organisms, even taking into account common general technical knowledge, the claimed inventions are not within the matters described in the detailed description of the invention, and the allegation B cannot be accepted.

5 Closing

As described above, regarding the application according to the Invention, the description of the detailed description of the invention does not meet the requirement stipulated in Article 36(4)(i) of the Patent Act, and the description of the scope of claims does not meet the requirement stipulated in Article 36(6)(i) of the Patent Act.

Therefore, the appeal decision shall be made as described in the conclusion.

April 24, 2017

Chief administrative judge: OTAKU, Ikuji

Administrative judge: NAGAI, Keiko

Administrative judge: YAMAZAKI, Toshinao