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JP-A- 2002 517 157
JP-A- 2002 519 054
US-B2- 8 409 830
US-B2- 9 005 681
GRANDI, GUIDO et al.: "STABILIZATION OF INDUSTRIAL ENZYMES BY PROTEIN ENGINEERING", PROTEIN ENGINEERING IN INDUSTRIAL BIOTECHNOLOGY, 2000, pages 147-157,
TOGNONI, ANGELO et al.: "Cloning and nucleotide sequence of the isoamylase gene from a strain of Pseudomonas sp.", J. Gen. Microbiol., vol. 135, 1989, pages 37-45, XP055553577,
AKIKO TACHIBANA et al.: "Purification, characterization and crystal structure of isoamylase from thermophilic bacteria Rhodothermus marinus", Scientific report of the Faculty of Agriculture, vol. 97, 2008, pages 1-7, XP055583969,

Fortsættes ...

DESCRIPTION

[Field of the Invention]

[0001] The present invention relates to an isoamylase variant improved in heat resistance and a process for producing the isoamylase variant.

[Background of the Invention]

[0002] In the saccharification industry, as an enzyme responsible for hydrolyzing an α -1,6-glucopyranoside bond of starch and amylopectin, pullulanase and isoamylase produced by e.g., *Klebsiella pneumoniae* are known. Among them, isoamylase is an enzyme responsible for hydrolyzing an α -1,6-glucopyranoside bond of starch, amylopectin and glycogen. It is known that since the reaction of isoamylase does not reversibly proceed, high-purity glucose and maltose can be produced if another type of amylase and glucoamylase are used. As a bacterium which produces isoamylase, e.g., *Pseudomonas amyloclavata* (Non Patent Document 1), has been reported. US 9005681 B2 discloses a method of producing starch gel-containing food. US 8409830 B2 discloses isolated polypeptides having isoamylase activity. JP 2002519054 A discloses a genetically engineered variant of parent starch debranching enzyme.

[Prior Art Document]

[Non Patent Document]

[0003] [Non Patent Document 1] Starch (1996), 48: 295-300

[Summary of Invention]

[Problem to be Solved by the Invention]

[0004] However, the optimum temperature of isoamylase produced by e.g., *Pseudomonas amyloclavata* is lower than that of (another type of) amylase. Because of this, it has been difficult to apply such an isoamylase in combination with amylase in the range (level) of a reaction temperature industrially used.

[0005] Thus, an object of the present invention is to provide a novel isoamylase improved in optimum temperature, and more specifically, improved in heat resistance, and a process for producing the isoamylase.

[Means for Solving the Problem]

[0006] The present invention relates to the subject-matter of claims 1 to 9. The present inventors produced a protein partially modified in the amino acid sequence of the isoamylase produced by e.g., *Pseudomonas amyloclavata* and investigated the heat resistance thereof. As a result, the present inventors found that an isoamylase variant improved in heat resistance (increased in heatproof temperature by 5°C to 10°C) can be obtained by changing an amino acid(s) at a predetermined position(s) to another amino acid(s). Based on the finding, the present invention was accomplished.

[0007] More specifically, the present invention provides the following [1] to [9].

[0008]

1. [1] An isoamylase comprising an amino acid mutation selected from the group consisting of A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T in
 1. (i) an isoamylase consisting of an amino acid sequence represented by SEQ ID No: 1 or
 2. (ii) an isoamylase consisting of the amino acid sequence represented by SEQ ID No: 1 and having deletion, substitution or insertion of one to several amino acid residues and having an amino acid sequence identity with the amino acid sequence represented by SEQ ID No: 1 of 80% or more,
 wherein the isoamylase comprising the amino acid mutation selected from the group consisting of A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T expresses improved heat resistance compared to the isoamylase having the amino acid sequence represented by SEQ ID No: 1.
2. [2] The isoamylase according to [1], wherein the amino acid sequence identity of the amino acid sequence having deletion, substitution or insertion with the amino acid sequence represented by SEQ ID No: 1 is 85% or more, preferably 90 % or more.
3. [3] The isoamylase according to [1] or [2], wherein the amino acid mutation includes a mutation selected from the group consisting of A554P/M277I/D268A/A549P and A554P/M277I/D268A/A549P/A580T.

4. [4] A gene encoding the isoamylase according to any one of [1] to [3].
5. [5] A recombinant vector comprising the gene according to [4].
6. [6] A transformant transformed with the recombinant vector according to [5].
7. [7] A process for producing an isoamylase comprising culturing the transformant according to [6] and recovering isoamylase from a culture.
8. [8] An enzyme composition for saccharification of starch comprising the isoamylase according to any one of [1] to [3].
9. [9] The enzyme composition for saccharification of starch according to [8], further comprising an enzyme selected from the group consisting of β -amylase, α -amylase and glucoamylase.

[Effect of Invention]

[0009] The isoamylase of the present invention is improved in heat resistance (increased in heatproof temperature by 5°C or more) and the heatproof temperature overlaps with the optimum temperatures of other amylases. Accordingly, if the isoamylase of the present invention is used in combination with other amylases, to e.g., starch, high purity glucose, maltose and the like can be industrially advantageously produced. In the present invention, "used in combination" refers to the state where at least two enzymes are active in a mixture containing the isoamylase of the present invention and at least one enzyme other than the isoamylase.

[Brief Description of the Drawing]

[0010] FIG. 1 is a graph showing the thermal stability of native enzyme and enzyme variants.

[Mode for Carrying Out the Invention]

[0011] The isoamylase of the present invention is an isoamylase having an amino acid mutation selected from the group consisting of A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T, in an isoamylase consisting of an amino acid sequence represented by SEQ ID No: 1 or an isoamylase consisting of the amino acid sequence represented by SEQ ID No: 1 and having deletion, substitution or insertion of one to several amino acid residues and having an amino acid sequence identity with the amino acid sequence represented by SEQ ID No: 1 of 80% or more,

wherein the isoamylase comprising the amino acid mutation selected from the group consisting of A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T expresses improved heat resistance compared to the isoamylase having the amino acid sequence represented by SEQ ID No: 1.

[0012] Here, the isoamylase consisting of an amino acid sequence represented by SEQ ID No: 1 is the isoamylase produced by *Pseudomonas amyloclavata* described in Non Patent Document 1. Examples of the isoamylase may include an isoamylase not derived from *Pseudomonas amyloclavata* as long as it has the same amino acid sequence, and also include not only a polypeptide but also a glycopeptide as long as it has the same amino acid sequence. Note that, SEQ ID No: 1 represents the amino acid sequence of a matured isoamylase protein.

[0013] In the isoamylase consisting of the amino acid sequence represented by SEQ ID No: 1 and having deletion, substitution or insertion of one to several amino acid residues, the number of amino acid residues constituting deletion, substitution or insertion is preferably 1 to 20, more preferably 1 to 10 and further preferably 1 to 8. The amino acid sequence identity of the isoamylase having deletion, substitution or insertion with the isoamylase of SEQ ID No: 1 is 80% or more, preferably 85% or more, further preferably 90% or more and further more preferably 95% or more. Such a sequence identity expressed by percentages can be computationally obtained by using released or commercially available software having an algorithm for comparing a sequence to a standard/reference sequence. For example, BLAST, FASTA or GENETYX (manufactured by GENETYX Co., Ltd.) can be used.

[0014] The isoamylase of the present invention has at least a double to quintuple amino acid mutation selected from the group consisting of D268A, M277I, A549P, A554P and A580T.

[0015] The amino acid mutations are selected from the group consisting of A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T.

[0016] Examples of preferable amino acid mutations include A554P/M277I/D268A/A549P and A554P/M277I/D268A/A549P/A580T.

[0017] The isoamylase variant of the present invention can be produced by using a gene, which is constructed such that the isoamylase consisting of an amino acid sequence represented by SEQ ID No: 1, or an isoamylase consisting of the amino acid sequence represented by SEQ ID No: 1 and having deletion, substitution or insertion of one to several

amino acid residues has at least two amino acid mutations selected from the group consisting of D268A, M277I, A549P, A554P and A580T as defined above.

[0018] A gene for producing an isoamylase variant of the present invention is a gene having a nucleotide sequence encoding an isoamylase variant as mentioned above and can be constructed, for example, by replacing, in a gene encoding the amino acid sequence represented by SEQ ID No: 1, the nucleotide sequence encoding the amino acid sequence to be replaced by a nucleotide encoding a desired amino acid residue. Various methods for such a site-specific nucleotide sequence substitution are well known in the art, for example, a PCR method using a properly designed primer(s) can be used. Alternatively, a gene encoding a modified amino acid sequence can be fully synthesized.

[0019] The gene thus obtained is inserted in an appropriate expression vector, which is further integrated into an appropriate host (for example, *Escherichia coli*) to transform the host. Many vectors/host systems for expressing an exogenous protein are known in the art. As an expression vector for integrating an isoamylase variant gene, a plasmid vector is mentioned. For example, pET-14b and pBR322 are mentioned for *E. coli*; e.g., pUB110 for *Bacillus subtilis*; e.g., pPTRI for a filamentous fungus; and e.g., pRS403 for a yeast.

[0020] The recombinant plasmid obtained is integrated in a microorganism such as *E. coli*, *Bacillus subtilis*, a mold, a yeast, *Actinomyces*, *Acetobacter* and *Pseudomonas* sp. to transform the microorganism. If the transformant thus obtained is cultured, an isoamylase variant of the present invention can be obtained. In the transformant, the gene for the isoamylase variant may be present in the plasmid or incorporated in the genome of the microorganism.

[0021] The isoamylase of the present invention is improved in heat resistance (increased in heatproof temperature by 5°C to 10°C) compared to the isoamylase produced by e.g., *Pseudomonas amyloclavata* and has the same optimum pH and isoamylase activity as those of the isoamylase produced by e.g., *Pseudomonas amyloclavata*. Accordingly, if an enzyme selected from the group consisting of β -amylase, α -amylase and glucoamylase is applied to starch in combination with the isoamylase of the present invention, high purity glucose and maltose can be easily obtained. As the β -amylase used herein, for example, GODO-GBA2 (GODO SHUSEI CO., LTD.), OPTIMALT BBA (Danisco Japan Ltd.), β -amylase L/R (Nagase ChemteX Corporation) and Hi-Maltosin GL (HBI Enzymes Inc.) can be used. As the α -amylase, for example, KLEISTASE T10 (Daiwa Fine Chemicals Co., Ltd) can be used. As the glucoamylase, for example, Gluczyme (Amano Enzyme Inc.) and GODO-ANGH (GODO SHUSEI CO., LTD.) can be used.

[0022] The isoamylase of the present invention is preferably used as the isoamylase for saccharification and further preferably as the isoamylase for saccharification of starch.

[0023] Preferably, the isoamylase of the present invention is, if necessary, mixed with at least one other enzyme and used as an enzyme composition for saccharification of starch. The at

least one other enzyme can be selected from the group consisting of β -amylase, α -amylase and glucoamylase mentioned above.

[0024] The reaction is carried out, for example, by adding the aforementioned enzyme to starch and an enzyme for starch saccharification such as amylase, and mixing/stirring in the pH and temperature conditions where the enzyme acts. According to the process of the present invention, high purity glucose and maltose can be industrially advantageously produced.

[Examples]

[0025] Now, the present invention will be described in more detail by way of Examples; however, the present invention is not particularly limited to these.

Example 1 (Site-specific mutagenesis of isoamylase)

[0026] Using the genome of *Pseudomonas amyloclavata* as a template and PSTPIA-F (AAACTGCAGATGAAGTGCCCAAAGATTCTC (SEQ ID No: 2)) and HINDPIA-R (CCCAAGCTTCTACTTGGAGATCAACAGCAG (SEQ ID No: 3)) as primers, a fragment (about 2.3 kb) containing an acid-resistant isoamylase gene sequence was obtained. The fragment was digested with restriction enzymes Pst I and Hind III and ligated with a fragment (about 2.2 kb) of pHSG398 (Takara Bio Inc.) digested with restriction enzymes Pst I and Hind III to obtain p-PI. Plasmid p-PI, which is a plasmid expressing native acid-resistant isoamylase, was subjected to site-specific mutagenesis to obtain a single variant (A554P) expression plasmid, p-PIA554P. Similarly, a single variant (M277I) expression plasmid p-PIM277I, a single variant (D268A) expression plasmid, p-PID268A and a single variant (A580T) expression plasmid, p-PIA580T were obtained. In addition, p-PIA554P was subjected to site-specific mutagenesis to obtain a double variant (A554P/M277I) expression plasmid, p-PI2M, a triple variant (A554P/M277I/D268A) expression plasmid, p-PI3M, a quadruple variant (A554P/M277I/D268A/A549P) expression plasmid, p-PI4M and a quintuple variant (A554P/M277I/D268A/A549P/A580T) expression plasmid, p-PI5M.

Example 2 (Production of enzyme)

[0027] *E. coli* DH5 α strains were transformed separately with a native acid-resistant isoamylase expression plasmid, p-PI, a single variant (A554P) expression plasmid, p-PIA554P, a single variant (M277I) expression plasmid, p-PIM277I, a single variant (D268A) expression plasmid, p-PID268A, a single variant (A580T) expression plasmid, p-PIA580T, a double variant (A554P/M277I) expression plasmid, p-PI2M, a triple variant (A554P/M277I/D268A) expression plasmid, p-PI3M, a quadruple variant (A554P/M277I/D268A/A549P) expression plasmid, p-

PI4M and a quintuple variant (A554P/M277I/D268A/A549P/A580T) expression plasmid, p-PI5M, to obtain *E. coli* strains producing individual isoamylases. These *E. coli* strains were each cultured in an LB medium (yeast extract: 0.5%; tryptone: 1.0%; sodium chloride: 0.5%; IPTG: 0.1 mM, pH 7.2) containing a 30 µg/mL chloramphenicol, at 30°C for 3 days to obtain a culture solution (1 L). The bacterium cells were ultrasonically crushed and centrifuged (10,000 g, 10 minutes). The supernatants were subjected to UF concentration (AIP module, manufactured by Asahi Kasei Corporation) so as to obtain a concentration of 1,000 U/mL. These concentrated supernatants were sterilized by a membrane having a pore size of 0.2 µm to separately obtain enzyme solutions of a native acid-resistant isoamylase, a single variant (A554P) isoamylase, a single variant (M277I) isoamylase, a single variant (D268A) isoamylase, a single variant (A580T) isoamylase, a double variant (A554P/M277I) isoamylase, a triple variant (A554P/M277I/D268A) isoamylase, a quadruple variant (A554P/M277I/D268A/A549P) isoamylase and a quintuple variant (A554P/M277I/D268A/A549P/A580T) isoamylase.

Example 3 (Improvement of thermal stability of individual variants)

[0028] These enzyme solutions were kept at 40°C, 50°C, 55°C, 57.5°C, 60°C, 62.5°C, or 65°C for 10 minutes, ice-cooled and the residual activity was measured. Using an approximate expression, which was prepared by plotting residual activity at individual temperatures, the temperature corresponding to a residual activity of 50% was computationally obtained and compared to that of the native acid-resistant isoamylase. A degree of an increase was determined as the degree of heat resistance. The amino acid sequence of the quadruple variant is represented by SEQ ID No: 4. The amino acid sequence of the quintuple variant is represented by SEQ ID No: 5.

<Method for measuring activity>

[0029] A method for measuring the activity of isoamylase is as follows:

With a 0.5% waxy cornstarch solution (0.35 mL), a 0.5 M acetate buffer, pH 4.5 (0.1 mL) was mixed and a diluted enzyme solution (0.1 mL) was added at an appropriate time. The mixture was allowed to react at 45°C for 15 minutes. Thereafter, an iodide solution (0.5 M potassium iodide solution containing 0.05 M iodine) (0.5 mL) diluted 5 fold with 0.1 N HCl was added to terminate the enzyme reaction. After water (10 mL) was added to the reaction solution and the mixture was sufficiently stirred, absorbance was measured at 610 nm by a spectrophotometer. The enzyme activity is represented by the unit, which is the amount of enzyme increasing 0.01 absorbance per minute in the above conditions.

[0030] As a result, as shown in FIG. 1 and Table 1, it was found that the improvement in the degree of heat resistance was verified as follows: that of the single variant (A554P) is about 1.2°C, that of the single variant (M277I) is about 1.1°C, that of the single variant (D268A) is about 1.3°C, that of the single variant (A580T) is about 4.5°C, that of the double variant

(A554P/M277I) is about 3.2°C, that of the triple variant (A554P/M277I/D268A) is about 4.3°C, that of the quadruple variant (A554P/M277I/D268A/A549P) is about 5.1°C, and that of the quintuple variant (A554P/M277I/D268A/A549P/A580T) is about 6°C.

Example 4 (Saccharification of starch)

[0031] As saccharification of starch, a reducing sugar release test was carried out. To a 10 mM acetate buffer (pH 4.5), soluble starch was added so as to obtain a concentration of 30% (weight/weight) and dissolved by heating. (1) Samples each containing 20 mg of a 0.05 mg/mL glucoamylase (manufactured by Wako Pure Chemical Industries, Ltd.) solution per 30% soluble starch (1 g) and 125 U of the native acid-resistant isoamylase per 30% soluble starch (1 g); (2) Samples each containing 20 mg of glucoamylase per 30% soluble starch (1 g) and 125 U of the quadruple variant isoamylase per 30% soluble starch (1 g); and (3) Samples each containing 20 mg of glucoamylase per 30% soluble starch (1 g) and 125 U of the quintuple variant isoamylase per 30% soluble starch (1 g) were prepared. These samples were separately reacted at 55°C, 60°C and 62.5°C for 16 hours.

[0032] These samples were heated at 100°C for 5 minutes to terminate the reaction and the amount of reducing sugar produced was measured by a DNS (3,5-dinitrosalicylic acid) method.

<Measuring method>

[0033] A method for measuring the amount of reducing sugar is as follows:

To a DNS solution (1.5 mL), a diluted sample solution (0.5 mL) was added at an appropriate time, then the mixture was stirred and allowed to react in boiling water for 5 minutes. After the reaction mixture was cooled with water, water (4 mL) was added and the mixture was sufficiently stirred. Absorbance was then measured at 540 nm by a spectrophotometer. The amount of reducing sugar was computationally obtained based on the calibration curve prepared by using a glucose solution. The DNS solution was prepared by dissolving a 4.5% sodium hydroxide solution (1,500 mL) and Rochelle salt (1,275 g) in a 1% DNS solution (4,400 mL), subsequently adding a phenol solution (1% phenol, 2.44% sodium hydroxide) (345 mL) separately prepared and sodium hydrogen carbonate (34.5 g), dissolving them by stirring, storing the solution for 2 days in the dark and filtering the solution through a filter paper, and then put in use.

[0034] The results are shown in Table 2. Assuming that the amount of reducing sugar obtained through the reaction using the native acid-resistant isoamylase at 55°C is 100%, the amount of reducing sugar increased up to 103% when the quadruple variant isoamylase was used and up to 104% when the quintuple variant isoamylase was used. In the reaction at 60°C, the amount of reducing sugar decreased to 98% when the native acid-resistant isoamylase was used; however, the amount was 102% when the quadruple variant isoamylase was used and 104%

when the quintuple variant isoamylase was used. Further in the reaction at 62.5°C, the amount of reducing sugar decreased to 97% when the native acid-resistant isoamylase was used; however, the amount was 102% when the quadruple variant isoamylase was used and 103% when the quintuple variant isoamylase was used. Accordingly, it was found that the amount of reducing sugar to be produced increases when the quadruple variant isoamylase was used compared to the native acid-resistant isoamylase. Furthermore, it was found that the amount of reducing sugar to be produced increases when the quintuple variant isoamylase was used compared to the quadruple variant isoamylase. Moreover, it was found that the debranching effect of the native acid-resistant isoamylase reduces as the temperature increases; however, the debranching effect of the quadruple variant isoamylase or quintuple variant isoamylase does not decrease and the yield increases. From this, the thermal stability effect by mutation was verified.

[Table 1]

Heat resistant variant PIA	Temperature at which residual activity is reduced to half	Degree of heat resistance (°C)
Native	54.1	0.0
Single variant (A554P)	55.3	1.2
Single variant (M277I)	55.2	1.1
Single variant (D268A)	55.4	1.3
Single variant (A580T)	58.6	4.5
Double variant (A554P/M277I)	57.3	3.2
Triple variant (A554P/M277I/D268A)	58.4	4.3
Quadruple variant (A554P/M277I/D268A/A549P)	59.2	5.1
Quintuple variant (A554P/M277I/D268A/A549P/A580T)	60.1	6.0

[0035] The single, double and triple variants are comparative Examples.

[Table 2]

Isoamylase used	Amount of reducing sugar (%)		
	55°C	60°C	62.5°C
Native	100	98	97
Quadruple variant (A554P/M277I/D268A/A549P)	103	102	102
Quintuple variant (A554P/M277I/D268A/A549P/A580T)	104	104	103
*The amount of reducing sugar was indicated by a relative value when measured value of the condition of 55°C (1) using the native acid-resistant isoamylase is taken as 100%.			

[Sequence Listing]

SEQUENCE LISTING

[0036]

<110> GODO SHUSEI CO., LTD.

<120> Heat Resistant Isoamylase

<130> GD0013

<150> JP2016-106198

<151> 2016-5-27

<160> 5

<170> PatentIn version 3.5

<210> 1

<211> 776

<212> PRT

<213> Pseudomonas amyloclavata

<400> 1

Met	Lys	Cys	Pro	Lys	Ile	Leu	Ala	Ala	Leu	Leu	Gly	Cys	Ala	Val	Leu
1				5					10					15	

Ala	Gly	Val	Pro	Ala	Met	Pro	Ala	His	Ala	Ala	Ile	Asn	Ser	Met	Ser
			20					25					30		

Leu	Gly	Ala	Ser	Tyr	Asp	Ala	Gln	Gln	Ala	Asn	Ile	Thr	Phe	Arg	Val
		35					40					45			

Tyr	Ser	Ser	Gln	Ala	Thr	Arg	Ile	Val	Leu	Tyr	Leu	Tyr	Ser	Ala	Gly
	50					55					60				

Tyr	Gly	Val	Gln	Glu	Ser	Ala	Thr	Tyr	Thr	Leu	Ser	Pro	Ala	Gly	Ser
65					70					75					80

Gly	Val	Trp	Ala	Val	Thr	Val	Pro	Val	Ser	Ser	Ile	Lys	Ala	Ala	Gly
			85						90					95	

Ile	Thr	Gly	Ala	Val	Tyr	Tyr	Gly	Tyr	Arg	Ala	Trp	Gly	Pro	Asn	Trp
			100					105					110		

Pro	Tyr	Ala	Ser	Asn	Trp	Gly	Lys	Gly	Ser	Gln	Ala	Gly	Phe	Val	Ser
			115				120					125			

115	120	125
Asp Val Asp Ala Asn Gly Asp Arg Phe Asn Pro Asn Lys Leu Leu Leu		
130	135	140
Asp Pro Tyr Ala Gln Glu Val Ser Gln Asp Pro Leu Asn Pro Ser Asn		
145	150	155 160
Gln Asn Gly Asn Val Phe Ala Ser Gly Ala Ser Tyr Arg Thr Thr Asp		
165	170	175
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Thr Cys Asp Trp Asn Asp Gly Ala Ser Thr Phe Val Ala Pro Gly Ser
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Gly Val Trp Ala Val Thr Val Pro Val Ser Ser Ile Lys Ala Ala Gly
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 35 40 45
 Tyr Ser Ser Gln Ala Thr Arg Ile Val Leu Tyr Leu Tyr Ser Ala Gly
 50 55 60
 Tyr Gly Val Gln Glu Ser Ala Thr Tyr Thr Leu Ser Pro Ala Gly Ser
 65 70 75 80
 Gly Val Trp Ala Val Thr Val Pro Val Ser Ser Ile Lys Ala Ala Gly
 85 90 95
 Ile Thr Gly Ala Val Tyr Tyr Gly Tyr Arg Ala Trp Gly Pro Asn Trp
 100 105 110
 Pro Tyr Ala Ser Asn Trp Gly Lys Gly Ser Gln Ala Gly Phe Val Ser
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Glu Val His Val Arg Gly Phe Thr Glu Gln Asp Thr Ser Ile Pro Ala
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Gln Tyr Arg Gly Thr Tyr Tyr Gly Ala Gly Leu Lys Ala Ser Tyr Leu
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Ala Tyr Asn Lys Ala Ala Gly Gly Pro Thr Ala Glu Phe Gln Ala Met
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 Glu Thr Leu Ile Gly Gly Ala Gly Thr Thr Tyr Gly Gln Cys Gly Gln
 755 760 765

Ser Leu Leu Leu Leu Ile Ser Lys
770 775

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- [US9005681B2 \[0002\]](#)
- [US8409830B2 \[0002\]](#)
- [JP2002519054A \[0002\]](#)
- [JP2016106198A \[0036\]](#)

Non-patent literature cited in the description

- Starch, 1996, vol. 48, 295-300 [\[0003\]](#)

Patentkrav

1. Isoamylase omfattende en aminosyremutation udvalgt fra gruppen bestående af A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T and A554P/M277I/D268A/A549P/A580T i

(i) en isoamylase bestående af en aminosyresekvens repræsenteret ved SEQ ID No: 1 eller

(ii) en isoamylase, der består af aminosyresekvensen repræsenteret ved SEQ ID No: 1 og har deletion, substitution eller insertion af en til flere aminosyrerester og har en aminosyresekvensidentitet med aminosyresekvensen repræsenteret ved SEQ ID No: 1 på 80 % eller mere,

hvor isoamylasen omfatter aminosyremutationen udvalgt fra gruppen bestående af A554P/A580T, M277I/A580T, D268A/A580T, A549P/A580T, A554P/M277I/A580T, A554P/D268A/A580T, A554P/A549P/A580T, M277I/D268A/A580T, D268A/A549P/A580T, A554P/M277I/D268A/A549P, A554P/D268A/A549P/A580T, A554P/M277I/A549P/A580T, A554P/M277I/D268A/A580T, M277I/D268A/A549P/A580T og A554P/M277I/D268A/A549P/A580T ekspri-
merer forbedret varmebestandighed i forhold til den isoamylase, der har aminosyresekvensen repræsenteret ved SEQ ID No: 1.

2. Isoamylase ifølge krav 1, hvor aminosyresekvensidentiteten af aminosyresekvensen, der har deletion, substitution eller insertion, med aminosyresekvensen repræsenteret ved SEQ ID No: 1 er 85 % eller mere, fortrinsvis 90 % eller mere.

3. Isoamylase ifølge krav 1 eller 2, hvor aminosyremutationen indbefatter en mutation udvalgt fra gruppen bestående af A554P/M277I/D268A/A549P og A554P/M277I/D268A/A549P/A580T.

4. Gen, der koder isoamylasen ifølge et af kravene 1 til 3.

5. Rekombinant vektor, der omfatter genet ifølge krav 4.

6. Transformant transformeret med den rekombinante vektor ifølge krav 5.

5 **7.** Fremgangsmåde til fremstilling af en isoamylase omfattende at dyrke transformanten ifølge krav 6 og at udvinde isoamylase fra en kultur.

8. Enzymsammensætning til forsukring af stivelse omfattende isoamylasen ifølge et af kravene 1 til 3.

10

9. Enzymsammensætning til forsukring af stivelse ifølge krav 8, yderligere omfattende et enzym udvalgt fra gruppen bestående af β -amylase, α -amylase og glucoamylase.

DRAWINGS

[Figure 1]

