

SPECIFICATION

Polypeptide possessing cyclomaltodextrin glucanotransferase activity

- 5 The present invention relates to a polypeptide, and more particularly to a polypeptide possessing cyclomaltodextrin glucanotransferase activity. 5
- Throughout the present specification, amino acids, peptides, etc. are designated with abbreviations which are commonly used in the art. Examples of such abbreviations are as follows:
- DNA is the abbreviation for deoxyribonucleic acid; RNA, ribonucleic acid; A, adenine; T, thymine; G, guanine; C, cytosine; dNTP, deoxynucleotide triphosphate; ddNTP, dideoxynucleotide triphosphate; dCTP, deoxycytidine triphosphate; SDS, sodium dodecyl sulfate; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; and 10
- 15 CGTase, cyclomaltodextrin glucanotransferase. 15
- Wherever optical isomers are possible, the abbreviation used to designate an amino acid refers to the L-isomer, unless specified otherwise.
- The term "polypeptide" is used throughout this specification to mean "polypeptide possessing CGTase activity".
- 20 CGTase, or *macerans* amylase, has been known for sometime as any enzyme produced by *Bacillus macerans*. 20
- Recently, it has been found that CGTase is produced by other microorganisms such as those of species *Bacillus stearothermophilus* and *Bacillus circulans*. The saccharide transfer activity of CGTase now has many industrial applications.
- 25 For example, cyclodextrins are produced by subjecting gelatinized starch to the action of CGTase, while glycosylsucrose production utilizes the saccharide transfer reaction from starch to sucrose which is effected by subjecting a solution of liquefied starch and sucrose to the action of CGTase. 25
- There is an expanding demand for cyclodextrins for use as a host to form a stable inclusion complex with an organic compound which is volatile or susceptible to oxidation. There is also an increasing demand for glycosylsucrose as a mildly-sweet low-cariogenic sweetener. This sweetener is sold by Hayashibara Co., Ltd., Okayama, Japan, under the Registered Trademark of "Coupling Sugar".
- 30 In order to meet these demands, there is an urgent need to provide a stationary CGTase supply. This requires determination of the amino acid sequence of the polypeptide that possesses CGTase activity. 35
- The amino acid sequence of CGTase has not been known hitherto.
- The Applicants have performed investigations to determine the amino acid sequence of the polypeptide possessing CGTase activity and have found that the polypeptide comprises one or 40 more partial amino acid sequences selected from 40
- (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
 (b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
 (c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
 (d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
 45 (e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly, 45
- and more particularly, that these partial amino acid sequences (a), (b), (c), (d) and (e) are located in sequence of nearness to the N-terminal end of polypeptide.
- The polypeptide is characterized by the fact that it forms cyclodextrin from soluble starch; that it exhibits a molecular weight of $70,000 \pm 10,000$ daltons on SDS-polyacrylamide electrophoresis; 50 and that it has a specific activity of 200 ± 30 units/mg protein. 50
- We have also found that polypeptides having CGTase activity derived from *Bacillus stearothermophilus* and *Bacillus macerans* have the amino acid sequences as shown in Tables 2-1 and 5-1 respectively. Both amino acid sequences will be detailed more fully below.
- In addition we have determined the amino acid sequences of the signal peptides which 55 regulate secretion of the polypeptide from producer microorganisms. 55
- In the present invention, the amino acid sequence of the polypeptide is determined by cloning the polypeptide gene from a CGTase producer microorganism, and sequencing the polypeptide gene.
- By amino acid sequence containing N-terminal end is determined by analyzing a highly-purified 60 polypeptide with a gas phase protein sequencer. 60

Cloning of polypeptide gene

- In the invention, a DNA fragment (obtained by separating DNA from a donor microorganism capable of producing the polypeptide, and digesting the DNA, for example, with ultrasonic 65 means or restriction enzymes), and a vector fragment (obtained by cleaving a vector in the same 65

way) are ligated, for example, with DNA ligase to obtain a recombinant DNA carrying polypeptide gene.

The donor microorganism is chosen from bacteria which produce the polypeptide. Examples of such bacteria are those of genus *Bacillus* such as *Bacillus macerans*, *Bacillus megaterium*, *Bacillus* 5 *circulans*, *Bacillus polymyxa*, and *Bacillus stearothermophilus*, and those of genus *Klebsiella* such as *Klebsiella pneumoniae*, as described, for example, in Japan Patent Kokai No. 20,373/72, Japan Patent Kokai No. 63,189/75, Japan Patent Kokai No. 88,290/75, and Hans Bender, *Archives of Microbiology*, Vol. 111, pp. 271-282 (1977).

Recombinant microorganisms in which polypeptide producibility has been introduced by genetic 10 engineering techniques can be used as the donor microorganism.

The DNA of the donor microorganism can be prepared by culturing the donor microorganism, for example, with a liquid culture medium for about 1-3 days under aeration-agitation conditions, centrifugally collecting the microorganism from the culture, and lysing the microorganism.

Examples of bacteriolytic procedures are cytohydrolist treatment using lysozyme or β -glucanase, 15 and ultrasonic treatment.

Other enzymes, such as protease, and/or surface active agents, such as sodium lauryl sulfate, can be used in combination, if necessary. Freezing-thawing treatments may also be carried out, if necessary.

In order to isolate DNA from the resultant lysate, two or more conventional procedures such 20 as phenol extraction, protein removal, protease treatment, ribonuclease treatment, alcohol sedimentation, and centrifugation are combined.

Although DNA ligation can be effected by treating DNA- and vector-fragments, for example, with ultrasonic means or restriction enzymes, it is desirable to use restriction enzymes, in particular, those acting specifically on a prescribed nucleotide sequence, for smooth ligation.

Specifically suited are Type II restriction enzymes, for example, *EcoRI*, *HindIII*, *BamHI*, *Sall*, *SalI*, 25 *XmaI*, *MboI*, *XbaI*, *MboI*, *XbaI*, *SacI*, *PstI*, etc.

Bacteriophages and plasmids which autonomically proliferate in the host microorganism are suitable as vectors.

When a microorganism of species *Escherichia coli* is used as the host, bacteriophages such as 30 λ gt.10C and λ gt.10B may be used, while ϕ 11, ϕ 1 and ϕ 105 are suitable when a microorganism of species *Bacillus subtilis* is used as the host.

As regards plasmids, when a microorganism of species *Escherichia coli* is used as the host, plasmids such as pBR322 and pBR325 are suitable, while pUB110, pTZ4 (pTP4) and pC194 35 may be used for a host microorganism of the species *Bacillus subtilis*. Plasmids which autonomically proliferate in two or more different host microorganisms, for example, pHV14, TRp7, YEp7 and pBS7, can be used as the vector. These vectors are cleaved with the same types of restriction enzymes as used in DNA digestion to obtain a vector fragment.

DNA- and vector-fragments are ligated by conventional procedures using DNA ligase. For 40 example, DNA- and vector-fragments are first annealed, then subjected *in vitro* to the action of a suitable DNA ligase to obtain a recombinant DNA. If necessary such recombinant DNA can be prepared by introducing the annealed fragments into the host microorganism to subject them to *in vivo* DNA ligase.

The host microorganisms usable in the invention are those in which recombinant DNA automatically and consistently proliferates to express its characteristics. Specifically, microorganisms 45 which are not capable of producing α -amylase (EC 3.2.1.1.) can be advantageously used because the use of such microorganisms facilitates isolation and purification of the secreted polypeptide.

The recombinant DNA can be introduced into the host microorganism by conventional procedures. For example, when the host microorganism belongs to the species *Escherichia coli*, introduction of recombinant DNA is effected in the presence of calcium ions, while the competent cell- and protoplast-methods are employed when host microorganisms of the genus *Bacillus* 50 are used.

The recombinant microorganism in which recombinant DNA has been introduced is selected by collecting clones which grow on a plate culture containing starch to convert the starch into cyclodextrin.

We have found that the recombinant DNA carrying the polypeptide gene cloned in this way 55 can be easily introduced, after isolation from the recombinant microorganism, into a different host microorganism. We have also found that a DNA fragment carrying the polypeptide gene, obtained by digesting with restriction enzymes a recombinant DNA carrying the gene, can be easily ligated with a vector fragment which has been obtained in the same manner.

Furthermore, we have found that the polypeptide gene in the recombinant DNA obtained 60 according to the invention is cleaved by the restriction enzyme *PvuII*, purchased from Toyobo Co., Ltd., Osaka, Japan, to lose its ability of expressing the polypeptide gene because the recombinant DNA has a *PvuII* restriction cleavage site.

65 Sequence of the polypeptide gene

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The polypeptide gene was sequenced by the chain-terminator method as described in *Gene*, Vol. 9, pp. 259-268 (1982).

This method contains the step of using restriction enzymes to insert a cloned DNA fragment carrying the polypeptide gene into the insertion site of a suitable plasmid such as pUC18. The obtained recombinant plasmid is introduced by transformation into a suitable *Escherichia coli* strain such as *Escherichia coli* JM83, followed by selection of the recombinant microorganism that contains the plasmid.

The recombinant plasmid is prepared from the proliferated recombinant microorganism.

The obtained recombinant plasmid is annealed together with a synthetic primer, and the Klenow fragment is then allowed to act on the mixture to extend the primer, as well as to form the complementary DNA.

Thereafter, the mixture is subjected sequentially to polyacrylamide-electrophoresis and autoradiography from which the polypeptide gene sequence can be determined.

The signal peptide which regulates secretion of the polypeptide from the cell can be sequenced in the same manner.

Amino acid sequence of the polypeptide

The amino acid sequence of the polypeptide is determined from the DNA sequence of the polypeptide gene.

The amino acid sequence of the signal peptide is determined in the same manner.

Partial amino acid sequence of polypeptide containing N-terminal end

A polypeptide producer microorganism of genus *Bacillus* is cultured with a nutrient culture medium to produce the polypeptide. The supernatant, centrifugally obtained from the culture, is purified by ammonium sulfate fractionation, ion exchange chromatography and high-performance liquid chromatography to obtain a high-purity polypeptide specimen. The specimen is then degraded using a gas phase protein sequencer in accordance with the method described in *Journal of Biological Chemistry*, Vol. 256, pp. 7990-7997 (1981), and fixed with high-performance liquid chromatography, followed by determination of the partial amino acid sequence containing N-terminal end.

Preparation of the polypeptide with recombination microorganism

We have found that a large amount of the polypeptide can be consistently produced by culturing a recombinant microorganism with a nutrient culture medium.

To the nutrient culture medium is incorporated, for example, a carbon source, a nitrogen source, minerals, and, if necessary, small amounts of organic nutrients such as amino acids and vitamins.

Starch, partial starch hydrolysate, and saccharides such as glucose, fructose and sucrose are suitable as the carbon source. Examples of suitable sources of nitrogen are inorganic nitrogen sources such as ammonia gas, ammonia water, ammonium salts and nitrates; and organic nitrogen sources such as peptone, yeast extract, and defatted soy-bean, corn steep liquor and meat extracts.

The recombinant microorganism is cultured with a nutrient culture medium for about 1-4 days under aeration-agitation conditions to accumulate the polypeptide while keeping the culture medium, for example, at pH 4-10 and 25-65°C.

Although the polypeptide in the culture may be used intact, generally, the culture is separated into a polypeptide solution and cells by conventional procedures such as filtration and centrifugation, prior to its use. Thus, for example, the cells are first treated with ultrasonic waves, surface active agents and/or cytohydrolyst, filtered and centrifuged to separate a solution containing the polypeptide.

The solution containing the polypeptide thus obtained is purified, for example, by a combination of concentration *in vacuo*, concentration using a membrane filter, salting-out using ammonium sulfate or sodium sulfate, fractional sedimentation using methanol, ethanol or acetone, to obtain a highly-purified polypeptide specimen which is advantageously usable as industrial polypeptide material.

To further improve the quality of the polypeptide, prior to its use, the amino acid sequence of the polypeptide may be partially substituted, removed, added, or modified in such a manner that the polypeptide does not lose its CGTase activity.

One unit of CGTase activity is defined as the amount of polypeptide that diminishes completely the iodine-coloration of 15 mg soluble starch at 40°C over a period of 10 minutes under the following reaction conditions: To 5 ml of 0.3 w/w % soluble starch solution containing 0.02 M acetate buffer (pH 5.5) and 2×10^{-3} M calcium chloride is added 0.2 ml of a diluted enzyme solution, and the mixture is incubated at 40°C for 10 minutes. Thereafter, 0.5 ml of the reaction mixture is sampled and added with 15 ml of 0.02 N aqueous sulfuric acid solution to suspend the enzymatic reaction. The reaction mixture is then added with 0.2 ml of 0.1 N I_2 -KI solution to

effect coloration, and determined for the absorbance at a wavelength of 660 nm.

Deposition of recombinant microorganisms

Recombinant microorganisms *Escherichia coli* TCH201, *Escherichia coli* MAH2, *Bacillus subtilis* MAU210, and *Bacillus subtilis* TCU211 referred to in this specification were deposited as from November 2, 1984 under the accession numbers of FERM P-7924, 7925, 7926 and 7927 respectively as the Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Yatabemachi, Tsukuba-gun, Ibaraki-ken, Japan.

In the accompanying drawings:—

10 Figure 1 shows the restriction map of recombinant DNA pTCH201, in particular, that of the DNA fragment which carries the polypeptide gene derived from *Bacillus stearothermophilus*. 10

Figure 2 shows the restriction map of recombinant DNA pTCU211, in particular, that of the DNA fragment which carries the polypeptide gene derived from *Bacillus stearothermophilus*.

Figure 3 shows the restriction map of recombinant DNA pMAH2, in particular, that of the DNA 15 fragment which carries the polypeptide gene derived from *Bacillus macerans*. 15

Figure 4 shows the restriction map of recombinant DNA pMAU210, in particular, that of the DNA fragment which carries the polypeptide gene derived from *Bacillus macerans*.

The present invention is illustrated further by the following Examples.

20 Example 1 20

Cloning of Bacillus stearothermophilus polypeptide gene into Escherichia coli

Example 1-(1)

Preparation of chromosome DNA carrying heat-resistant-polypeptide gene of *Bacillus stearothermophilus* 25

The chromosome DNA carrying heat-resistant-polypeptide gene of *Bacillus stearothermophilus* was prepared in accordance with the method described by Saito and Miura, *Biochimica et Biophysica Acta*, Vol. 72, pp. 619-629 (1963). A seed culture of *Bacillus stearothermophilus* FERM-P No. 2225 was cultured with brain heart infusion medium at 50°C overnight under 30 vigorous shaking conditions. The cell, centrifugally collected from the culture, was suspended with TES buffer (pH 8.0) containing Tris-aminomethane, hydrochloric acid, EDTA and sodium chloride, added with 2 mg/ml of lysozyme, and incubated at 37°C for 30 minutes. The incubated mixture was frozen, allowed to stand at -20°C overnight, added with TSS buffer (pH 9.0) containing Trisaminomethane, hydrochloric acid, sodium lauryl sulfate and sodium chloride, 35 heated to 60°C, added with a mixture of TES buffer (pH 7.5) and phenol (1:4 by volume), cooled in ice-chilled water, and centrifuged to obtain a supernatant. To the supernatant was added two volumes of cold ethanol to recover a crude chromosome DNA which was then dissolved in SSC buffer (pH 7.1) containing sodium chloride and trisodium citrate, thereafter, the mixture was subjected to both "RNase A", a ribonuclease commercialized by Sigma Chemical 40 Co., MO, USA, and "Pronase E", a protease commercialized by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan, added with a fresh preparation of TES buffer and phenol mixture, cooled, centrifuged, and added with two volumes of cold ethanol to recover a purified chromosome DNA. The chromosome DNA was dissolved in a buffer (pH 7.5) containing Tris-aminomethane, hydrochloric acid and EDTA, and stored at -20°C. 45

Example 1-(2)

Preparation of plasmid pBR322

Plasmid pBR322 (ATCC 37013) was isolated from *Escherichia coli* in accordance with the method described by J. Meyer et al. in *Journal of Bacteriology*, Vol. 127, pp. 1524-1537 50 (1976). 50

Example 1-(3)

Preparation of recombinant DNA carrying polypeptide gene

The purified chromosome DNA carrying heat-resistant-polypeptide gene, prepared in Example 55 1-(1), was partially digested with restriction enzyme *MboI*, purchased from Nippon Gene Co., Ltd., Toyaria, Japan, to give a DNA fragment of 1-20 kbp. Separately, the pBR322 specimen, prepared in Example 1-(2), was completely cleaved with restriction enzyme *BamHI*, purchased from Nippon Gene Co., Ltd., and the cleaved product was subjected to *Escherichia coli* alkali phosphatase, purchased from Takara Shuzo Co., Ltd., Kyoto, Japan, to prevent self-ligation of 60 the plasmid fragment as well as to dephosphorize the 5'-terminal end of the fragment. 60

Both fragments were then ligated by subjecting them to T₄ DNA ligase, purchased from Nippon Gene Co., Ltd., at 4°C overnight to obtain a recombinant DNA.

Example 1-(4)

65 Introduction of recombinant DNA into *Escherichia coli* 65

Escherichia coli HB101 (ATCC 33694), a strain incapable of producing amylase, was used as the host.

The microorganism was cultured with L-broth at 37°C for 4 hours, and the cell, centrifugally collected from the culture, was suspended with 10 mM acetate buffer (pH 5.6) containing 50 mM manganese chloride, centrifugally collected again, resuspended with 10 mM acetate buffer (pH 5.6) containing 125 mM manganese chloride, added with the recombinant DNA prepared in Example 1-(3), and allowed to stand in an ice-chilled water bath for 30 minutes. The mixture was then warmed to 37°C, added with L-broth, spread on L-broth agar plate medium containing 50 µg/ml of ampicillin and 2 mg/ml starch, and incubated at 37°C for 24 hours to form colonies.

The colony which had degraded the starch into cyclodextrin was selected by the iodine-coloration method. Thus, the microorganism in which the recombinant DNA carrying polypeptide gene had been introduced was selected. The recombinant microorganism was then proliferated, and the recombinant DNA was extracted from the proliferated microorganism by the plasmid preparation method in Example 1-(2), subjected to restriction enzymes to determine the restriction cleavage sites, and completely digested with restriction enzyme *EcoRI* purchased from Nippon Gene Co., Ltd. The digested product was subjected to T₄ DNA ligase similarly as in Example 1-(3) to obtain a recombinant DNA, followed by selection of recombinant microorganism in accordance with the method in Example 1-(4). The recombinant microorganism contained a recombinant DNA of a relatively small-size that carries polypeptide gene.

The recombinant DNA and plasmid pBR322 were then completely digested with restriction enzyme *Sall*, purchased from Nippon Gene Co., Ltd., and treated similarly as in the case of *EcoRI* to select recombinant microorganisms containing a recombinant DNA of a much smaller-size that carries polypeptide gene.

One of these microorganisms and its recombinant DNA were named as "*Escherichia coli* TCH201 (FERM P-7924) and "pTCH201".

The restriction map of recombinant DNA pTCH201, in particular, that of the DNA fragment derived from *Bacillus stearothermophilus* microorganism was as shown in Fig. 1.

Fig. 1 clearly shows that this recombinant DNA is cleaved by either restriction enzyme *PvuII* purchased from Toyobo Co., Ltd., *KpnI*, *HindIII* purchased from Nippon Gene Co., Ltd., or *XbaI* purchased from Takara Shuzo Co., Ltd, but not by *EcoRI*, *BamHI*, *PstI*, *XhoI*, *BglII* or *AclI*, all purchased from Nippon Gene Co., Ltd.

Example 2

Cloning of polypeptide gene of *Bacillus stearothermophilus* into *Bacillus subtilis*

Example 2-(1)

Preparation of recombinant DNA pTCH201

Recombinant DNA pTCH201 was isolated from *Escherichia coli* TCH201 (FERM P-7924) in accordance with the method in Example 1-(2).

Example 2-(2)

Preparation of plasmid pUB110

Plasmid pUB110 (ATCC 37015) was isolated from *Bacillus subtilis* in accordance with the method described by Gryczan et al. in *Journal of Bacteriology*, Vol. 134, pp. 318-329 (1978).

Example 2-(3)

Preparation of recombinant DNA carrying polypeptide gene

The recombinant DNA pTCH201 carrying heat-resistant-polypeptide gene, prepared in Example 2-(1), was completely digested by subjecting it simultaneously to restriction enzymes *EcoRI* and *XbaI*.

Separately, the plasmid pUB110 specimen, prepared in Example 2-(2), was completely cleaved by subjecting it to restriction enzymes *EcoRI* and *XbaI* in the same manner.

The resultant fragments were subjected to T₄ DNA ligase similarly as in Example 1-(3) to obtain a recombinant DNA.

Example 2-(4)

Introduction of recombinant DNA into *Bacillus subtilis*

In this Example, *Bacillus subtilis* 715A, a strain incapable of producing amylase, was used as the host. The microorganism was cultured with brain heart infusion medium at 28°C for 5 hours, and the cell, centrifugally collected from the culture, was then prepared into protoplast suspension in accordance with the method described by Schaeffer et al. in *Proceeding of the National Academy of Sciences of the USA*, Vol. 73, pp. 2151-2155 (1976).

To the suspension was added the recombinant DNA, prepared in Example 2-(3), and the mixture was then treated in accordance with the method described by Sekiguchi et al. in *Agricultural and Biological Chemistry*, Vol. 46, pp. 1617-1621 (1982) to effect transformation,

spread on HCP medium containing 250 µg/ml of kanamycin and 10 mg/ml of starch, and incubated at 28°C for 72 hours to form colonies.

From these colonies, recombinant microorganism in which the recombinant DNA carrying heat-resistant-polypeptide gene had been introduced were selected by the method in Example 1-(4).

- 5 One of these microorganisms and its recombinant DNA were named as "*Bacillus subtilis* TCU 211 (FERM P-7927)" and "pTCU211" respectively. 5

The restriction map of recombinant DNA pTCU211, in particular, that of the DNA fragment derived from *Bacillus stearothermophilus* microorganism, was as shown in Fig. 2. Fig. 2 clearly shows that this recombinant DNA is cleaved by either restriction enzyme *PvuII*, *KpnI* or *HindIII*, 10 but not by *EcoRI*, *BamHI*, *PstI*, *XhoI*, *BglII*, *AclI* or *XbaI*. 10

Example 3

Partial amino acid sequence of *Bacillus stearothermophilus* polypeptide containing N-terminal end

15 Example 3-(1) 15

Preparation of polypeptide

Bacillus stearothermophilus FERM-P No. 2225 was cultured with a liquid culture medium by the method in Example 5 to produce polypeptide. The supernatant, centrifugally obtained from the culture, was salted out with ammonium sulphate to obtain a polypeptide fraction which was then 20 purified by column chromatography using "DEAE, Toyopearl 650", an anion exchanger commercialized by Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan, and chromatofocusing using "Mono P", a product of Pharmacia Fine Chemicals AB, Uppsala, Sweden, to obtain a highly-purified polypeptide specimen. 20

On SDS-polyacrylamide electrophoresis in accordance with the method described by K. Weber 25 and M. Osborn in *Journal of Biological Chemistry*, Vol. 244, page 4406 (1969), the polypeptide specimen showed a molecular weight of 70,000 ± 10,00 daltons. 25

The specific activity of the polypeptide specimen was 200 ± 30 units/mg protein.

Example 3-(2)

30 Partial amino acid sequence of polypeptide containing N-terminal end 30

A polypeptide specimen, prepared by the method in Example 3-(1), was fed to "Model 470A", a gasphase protein sequencer, a product of Applied Biosystems Inc., CA, USA, and then analyzed with high-performance liquid chromatography to determine the partial amino acid sequence containing N-terminal end.

35 The partial amino acid sequence was Ala-Gly-Asn-Leu-Asn-Lys-Val-Asn-Phe-Thr. 35

Example 4

Sequence of polypeptide gene derived from *Bacillus stearothermophilus* and amino acid sequence of polypeptide

40 Example 4-(1) 40

Preparation of plasmid pUC18

Plasmid pUC18 was prepared in accordance with the method of Example 1-(2) from *Escherichia coli* JM83 (ATCC 35607) in which the plasmid has been introduced.

45 Example 4-(2) 45

Preparation of recombinant DNA carrying polypeptide gene

The recombinant DNA was prepared by the method in Example 1-(3).

A fragment, obtained by digesting a fragment carrying polypeptide gene, prepared by the method in Example 2-(3), with restriction enzymes, and a plasmid fragment, obtained by cleav- 50 ing a pUC18 specimen, prepared by the method in Example 4-(1), in the same manner, were subjected to T₄ DNA ligase to obtain a recombinant DNA. 50

Example 4-(3)

Introduction of recombinant DNA into *Escherichia coli*

55 In this example, *Escherichia coli* JM83 was used as the host. 55

The recombinant DNA was introduced into the microorganism in accordance with the method in Example 1-(4) to transform the microorganism.

The recombinant microorganisms were inoculated to a culture medium containing 5-bromo-4-chloro-3-indoyl-β-galactoside (Xgal), and the microorganism forming colorless plaque was se- 60 lected. 60

Example 4-(4)

Preparation of recombinant DNA from recombinant microorganism

The recombinant microorganism was cultured on L-broth containing 50 µg/ml of ampicillin, and 65 the obtained cell was then treated with the alkaline mini-preparation method to obtain a recombi- 65

nant DNA.

Example 4-(5)

Sequence of recombinant DNA

- 5 The recombinant DNA was sequenced by the dideoxy chain terminator method. 5
- The recombinant DNA, prepared in Example 4-(4), and a synthetic primer composed of 17 bases were mixed, annealed at 60°C for 20 minutes, added with dNTP, ddNTP, (α -³²P) dCTP and Klenow fragment, and reacted at 37°C for 30 minutes to extend the primer towards the 3' site from the 5' site. Thus, the complementary DNA was obtained. To the complementary DNA was added an excessive amount of dNTP, and the mixture was reacted at 37°C for 30 minutes, followed by addition of a formamide solution of dye mixture to suspend the reaction. The reaction mixture was boiled for 3 minutes, and electrophoresed on 6% polyacrylamide gel at about 25 mA (about 2,000 volts) to separate the extended complementary DNA. After completion of the electrophoresis, the gel was fixed and dehydrated. 10
- 15 The dehydrated gel was then autographed, and the polypeptide gene was determined by analyzing the base bands on the radioautogram. 15
- The results were as shown in Table 1-1.
- The signal peptide gene located upstream at the 5'-terminal end of the polypeptide gene was sequenced in the same manner.
- 20 The results were as shown in Table 1-2. 20

Example 4-(6)

Amino acid sequence of polypeptide

- 25 The amino acid sequence of polypeptide was determined with reference to the sequence as shown in Table 1-1, and the results were as shown in Table 2-1. 25
- The amino acid sequence of the signal peptide was determined in the same manner, and the results were as shown in Table 2-2.
- These evidences confirmed that the polypeptide derived from *Bacillus stearothermophilus* has the amino acid sequence as shown in Table 2-1.
- 30 30

Example 5

Preparation of polypeptide with recombinant microorganism

- Polypeptides were prepared with recombinant microorganisms *Escherichia coli* TCH201 (FERM P-7924) and *Bacillus subtilis* TCU211 (FERM P-7927) both in which recombinant DNA carrying heat-resistant-polypeptide gene derived from *Bacillus stearothermophilus* had been introduced. 35
- The polypeptide productivities of these recombinant microorganisms were compared with those of host microorganism and donor *Bacillus stearothermophilus* microorganism in relation to their CGTase activity.

Table 1-1

10	20	30	40	50	60
GCTGGAAATC	TTAATAAGGT	AAACTTTACA	TCAGATGTTG	TCTATCAAAAT	TGTAGTGGAT
70	80	90	100	110	120
CGATTGTGG	ATGGAATAC	ATCCAATAAT	CCGAGTGGAG	CATTATTTAG	CTCAGGATGT
130	140	150	160	170	180
ACGAATTTAC	GCAAGTATTG	CGGTGGAGAT	TGGCAAGGCA	TCATCAATAA	AATTAACGAT
190	200	210	220	230	240
GGGTATTTAA	CAGATATGGG	TGTGACAGCG	ATATGGATTT	CTCAGCCTGT	AGAAAATGTA
250	260	270	280	290	300
TTTTCTGTGA	TGAATGATGC	AAGCGGTTCC	GCAICCTATC	ATGGTTATTG	GCGCGCGGAT
310	320	330	340	350	360
TTCAAAAAGC	CAACCCGTT	TTTTGGTACC	CTCAGTGATT	TCCAACGTTT	AGTTGATGEC
370	380	390	400	410	420
GCACATGCAA	AAGGAATAAA	GGTAATTATT	GACTTTGCCC	CCAACCATAC	TTCTCCTGCT
430	440	450	460	470	480
TCAGAAACGA	ATCCTTCTTA	TATGGAAAAC	GGACGACTGT	ACGATAATGG	GACATTECTT
490	500	510	520	530	540
GGCGGTTACA	CAATGATGC	CAACATGTAT	TTTCACCATA	ACGGTGGAAC	AACGTTTTC

Table 1-1 continued (1)

550	500	570	500	500	500
AGCTTAGAGG	ATGGGATTTA	TCGAAATCTG	TTTGACTTGG	CGGACCTTAA	CCATCAGAAC
010	020	030	040	050	060
CCTGTTATTG	ATAGGTATTT	AAAAGATGCA	GTAAAAATGT	GGATAGATAT	GGGGATTGAT
070	080	090	700	710	720
GGTATCCGTA	TGGATGCGGT	GAAGCACATG	CCGTTTGGAT	GGCAAAAAATC	TCTGATGGAT
730	740	750	760	770	780
GAGATTGATA	ACTATCGTCC	TGTCTTTACG	TTTGGGGAGT	GGTTTTTGTC	AGAAAAATGAA
790	800	810	820	830	840
GTGGACGCGA	ACAAATCATT	CTTTGCCAAT	GAAAGTGGAA	TGAGTTTGCT	CGATTTTCGT
850	860	870	880	890	900
TTCGGACAAA	AGCTTCGTCA	AGTATTGCGC	AATAACAGCG	ATAATTGGTA	TGGCTTTAAT
910	920	930	940	950	960
CAAAATGATTC	AAGATACGGC	ATCAGCATAT	GACGAGGTTT	TCGATCAAGT	AACATTCTATA
970	980	990	1000	1010	1020
GACAACCATG	ATATGGATCG	GTTTATGATT	GACGGAGGAG	ATCCGCGCAA	GGTGGATATG
1030	1040	1050	1060	1070	1080
GCACCTTGCTG	TATTATTGAC	ATCCCGTGCC	GTACCGAATA	TTTACTATGG	TACAGAGCAA

Table 1-1 continued (2)

1090	1100	1110	1120	1130	1140
TACATGACCG	GTACGGCGA	TCCAAACAAT	CGTAAGATGA	TGAGTTTCATT	CAATAAAAT
1150	1160	1170	1180	1190	1200
ACTCGGCGGT	ATCAAGTGAT	TCAAAAACATA	TCTTCTCTCC	GACGAAACAA	TCCGGCGTTA
1210	1220	1230	1240	1250	1260
GCTTATGGTG	ATACGGAACA	GCGTTGGATC	AATGGCGATG	TGTATGTGTA	TGAGCGACAG
1270	1280	1290	1300	1310	1320
TTTGGCAAAG	ATGTTGTGTT	AGTTCGGGTT	AATCGTAGTT	CAAGCAGTAA	ITACTCGATT
1330	1340	1350	1360	1370	1380
ACTGGCTTAT	TTACAGCTTT	ACCAGCAGGA	ACATATACGG	ATCAGCTTGG	CGGTCTTTTA
1390	1400	1410	1420	1430	1440
GACGGAAATA	CAATTCAAGT	CGGTTCAAAT	GGATCAGTTA	ATGCATTTGA	CTTAGGACCB
1450	1460	1470	1480	1490	1500
GGGGAAGTCG	GTGTATGGGC	ATACAGTGCA	ACAGAAAGCA	CGCCAATTAT	TGTCATGTT
1510	1520	1530	1540	1550	1560
GGACCGATGA	TGGGGCAAGT	CGGTCAATCA	GTAACCATTTG	ATGGCGAAGG	ATTGCGAACA
1570	1580	1590	1600	1610	1620
AATACGGGCA	CTGTGAAGTT	CGGAACGACA	GCTGCCAATG	TTGTGTCTTG	GTCTAACAAAT

Table 1-1 continued (3)

1630	1640	1650	1660	1670	1680
CAAATCGTTG	TGECTGTACC	AAATGTGTCA	CCAGGAAAAAT	ATAATATTAC	CGTCCAAATCA
1690	1700	1710	1720	1730	1740
TCAAGCGGTC	AAACGAGTGC	GGCTTATGAT	AACTTTGAAG	TACTAACAAA	TGATCAAGTG
1750	1760	1770	1780	1790	1800
TCAGTGCGGT	TTGTTGTTAA	TAAAGCGGACT	ACCAATCTAG	GGCAAAATAT	ATACATTGTT
1810	1820	1830	1840	1850	1860
GGCAACGTAT	ATGAGCTCGG	CAACTGGGAC	ACTAGTAAGG	CAATCGGTCC	AATGTTCAAT
1870	1880	1890	1900	1910	1920
CAAGTGTTTT	ACTCCTATCC	TACATGGTAT	ATAGATGTCA	GTGTCCGAGA	AGGAAAGACA
1930	1940	1950	1960	1970	1980
ATTGAGTTTA	AGTTTATTAA	AAAAGACAGC	CAAGGTAATG	TCACCTGGGA	AAGTGGTTCA
1990	2000	2010	2020	2030	2040
AATCATGTTT	ATACGACACC	AACGAATACA	ACCGGAAAAA	TTATAGTGGA	TTGGCAGAAC

Table 2-2

	70	80	90	100
	GTATCTGATA	CGCAGAAAGT	CACCGTTGAA	GCA

1	Met	1	Met	0	Leu	7	Leu	6	Val	0	Leu	10	Met	11	Met	12	Ser	13	Phe	14	Val	15	Phe
1	Arg	2	Arg	3	Arg	4	Trp	5	Leu	6	Ser	7	Ser	8	Val	9	Val	10	Gln	11	Lys	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp	8	Thr	9	Gln	10	Lys	11	Thr	12	Val
1	Ala	2	Ala	3	Ile	4	Phe	5	Ile	6	Val	7	Asp										

A liquid culture medium consisting of 1.0 w/v % corn steep liquor, 0.1 w/v% ammonium sulfate, 1.0 w/v % calcium carbonate, 1 w/v % starch and water was adjusted to pH 7.2, sterilized by heating at 120°C for 20 minutes, and cooled. In the case of *Escherichia coli* TCH201, the liquid culture medium was added with 50 µg/ml of ampicillin, and the microorganism was inoculated to the liquid culture medium. *Escherichia coli* HB101 was inoculated to the liquid culture medium without addition of antibiotic. In the case, microorganism was cultured at 37°C for 48 hours under vigorous shaking conditions.

Separately, *Bacillus subtilis* TCU211 was inoculated to the liquid culture medium additionally containing 5 µg/ml of kanamycin, while *Bacillus subtilis* 715A was inoculated to the liquid culture medium without addition of antibiotic. In each case, microorganism was cultured at 28°C for 72 hours.

Bacillus stearothermophilus FERM-P No. 2225 was cultured with the liquid culture medium at 50°C for 48 hours without addition of antibiotic. After separation of each culture into supernatant and cell by centrifugation, the supernatant was assayed intact for CGTase activity, while the cell was ultrasonically broken, prior to determination of its CGTase activity per culture. The results were as shown in Table 3.

These evidences clearly show that the recombinant microorganism are advantageously usable in industrial-scale production of polypeptide because these microorganism possess an improved polypeptide productivity.

The supernatants were salted out with ammonium sulfate at a saturation degree of 0.6 to obtain crude polypeptide specimens. After studying these polypeptide specimens on their enzymatic properties such as saccharide transfer from starch to sucrose, cyclodextrin production from starch, ratio of α -, β - and γ -cyclodextrins, optimum temperature, optimum pH, stable temperature range and stable pH range, the properties of the polypeptide produced by the recombinant microorganism were in good accordance with those of the polypeptide produced by the donor *Bacillus stearothermophilus* microorganism.

Table 3

Microorganism	CGTase activity (units/ml)		
	Supernatant	Cell	Total
<u>Escherichia coli</u> TCH201 (FERM P-7924)	0.8	13.5	14.3
			Present invention
<u>Bacillus subtilis</u> TCU211 (FERM P-7927)	46.7	20.5	67.2
			Present invention
<u>Escherichia coli</u> HB101	0	0	0
			Control
<u>Bacillus subtilis</u> 715A	0	0	0
			Control
<u>Bacillus stearothermophilus</u> FERM-P No.2225	8.5	0.3	8.8
			Control

Example 6**Cloning of *Bacillus macerans* polypeptide gene into *Escherichia coli* Example 6-(1)****Preparation of chromosome DNA carrying *Bacillus macerans* polypeptide gene**

- 5 The polypeptide gene was prepared in accordance with the method in Example 1-(1), except that *Bacillus macerans* 17A was cultured at 28°C. 5

Example 6-(2)**Preparation of recombinant DNA carrying polypeptide gene**

- 10 The chromosome DNA carrying polypeptide gene derived from *Bacillus macerans*, prepared in Example 6-(1), was partially digested similarly as in Example 1-(3) with restriction enzyme *HindIII*, purchased from Nippon Gene Co., Ltd. 10

- Separately, a plasmid pBR322 specimen, prepared by the method in Example 1-(2), was completely cleaved with restriction enzyme *HindIII*, and the 5'-terminal end of the cleaved product was dephosphorylated by the method in Example 1-(3). The fragments thus obtained were 15 ligated in accordance with the method in Example 1-(3) to obtain a recombinant DNA. 15

Example 6-(3)**Introduction of recombinant DNA into *Escherichia coli***

- 20 The recombinant microorganism in which recombinant DNA had been introduced was cloned in accordance with the method in Example 1-(4) using *Escherichia coli* HB101 (ATCC 33694), a strain incapable of producing amylase, as the host. Thereafter, the recombinant DNA was isolated from the microorganism, subjected to restriction enzymes to determine the restriction cleavage sites, and partially digested with restriction enzyme *Sau3AI* commercialized by Nippon Gene Co., Ltd. 20 25

- Separately, a plasmid pBR322 specimen, obtained by the method in Example 1-(2), was completely cleaved with restriction enzyme *BamHI*, and the 5'-terminal end of the resultant product was dephosphorylated similarly as in Example 1-(3). The obtained fragments were ligated with T_4 DNA ligase to obtain a recombinant DNA, followed by selecting recombinant microorganisms in accordance with the method in Example 1-(4). The recombinant microorganisms contained a recombinant DNA of a relatively small-size that carries polypeptide gene. 30

One of these recombinant microorganisms and its recombinant DNA were named as "*Escherichia coli* MAH2 (FERM P-7925)" and "pMAH2" respectively.

- The restriction map of recombinant DNA pMAH2, in particular, that of the DNA fragment that carries the polypeptide gene derived from *Bacillus macerans*, was as shown in Fig. 3. 35

Fig. 3 shows that this recombinant DNA is cleaved by either restriction enzyme *PvuII*, *Sall*, *AvaI* commercialized by Nippon Gene Co., Ltd., or *PstI* commercialized by Nippon Gene Co., Ltd., but not by *EcoRI*, *HindIII*, *KpnI*, *BamHI*, *XbaI*, *XhoI*, or *SmaI*.

Example 7**Cloning of *Bacillus macerans* polypeptide gene into *Bacillus subtilis*****Example 7-(1)****Preparation of recombinant DNA pMAH2**

- The recombinant DNA pMAH2 was isolated from *Escherichia coli* MAH2 (FERM P-7925) in accordance with the method in Example 1-(2). 40 45

Example 7-(2)**Preparation of recombinant DNA carrying polypeptide gene**

- The recombinant DNA pMAH2 specimen carrying polypeptide gene, prepared in Example 7-(1), was completely digested by subjecting it simultaneously to restriction enzymes *EcoRI* and *BamHI*. 50

Separately, a plasmid pUB110 specimen, prepared by the method in Example 2-(2), was completely cleaved by subjecting it simultaneously to restriction enzymes *EcoRI* and *BamHI*.

- The fragments thus obtained were subjected to T_4 DNA ligase similarly as in Example 1-(3) to obtain a recombinant DNA. 55

Example 7-(3)**Introduction of recombinant DNA into *Bacillus subtilis***

- Recombinant microorganisms in which recombinant DNA carrying the polypeptide gene derived from *Bacillus macerans* had been introduced were cloned in accordance with the method in Example 2-(4) using *Bacillus subtilis* 715A, a strain incapable of producing amylase. 60

- One of the recombinant microorganisms and its recombinant DNA were named as "*Bacillus subtilis* MAU210 (FERM P-7926)" and "pMAU210" respectively. The restriction map of recombinant DNA pMAU210, in particular, that of the DNA fragment that carries the polypeptide gene derived from *Bacillus macerans*, was as shown in Fig. 4. Fig. 4 shows that the recombinant 65

DNA is cleaved by either restriction enzyme *PvuII*, *Sall*, *AvaI* or *PstI*, but not by *EcoRI*, *HindIII*, *KpnI*, *BamHI*, *XbaI*, *XhoI* or *SmaI*.

Example 8

5 Amino acid sequence of polypeptide derived from *Bacillus macerans* containing N-terminal end 5

Example 8-(1)

Preparation of polypeptide

The polypeptide was produced by culturing *Bacillus subtilis* MAU210 (FERM P-7926) with a
10 liquid culture medium similarly as in Example 10, and then purified in accordance with the 10
method in Example 4-(1) to obtain a high-purity polypeptide specimen.

On SDS-polyacrylamide electrophoresis, the polypeptide specimen showed a molecular weight of $70,000 \pm 10,000$ daltons and a specific activity of 200 ± 30 units/mg protein.

15 Example 8-(2) 15

Partial amino acid sequence containing N-terminal end

The partial amino acid sequence containing N-terminal end was determined with the polypeptide specimen, prepared in Example 8-(1), in accordance with the method in Example 3-(2).

20 The partial amino acid sequence was Ser-Pro-Asp-Thr-Ser-Val-Asn-Asn-Lys-Leu. 20

Example 9

Sequence of polypeptide gene derived from *Bacillus macerans* and amino acid sequence of polypeptide

Example 9-(1)

25 Preparation of recombinant DNA carrying polypeptide gene 25

The recombinant DNA was prepared in accordance with the method in Example 4-(3).

More particularly, a DNA fragment, obtained by digesting a DNA fragment carrying polypeptide gene, prepared by the method in Example 7-(2), with restriction enzymes, and a plasmid fragment, obtained by cleaving a plasmid pUC18 specimen, prepared by the method in Example
30 4-(2), in the same manner, were ligated with T_4 DNA ligase to obtain a recombinant DNA. 30

Example 9-(2)

Introduction of recombinant DNA into *Escherichia coli*

The recombinant DNA was introduced in accordance with the method in Example 4-(3) into
35 *Escherichia coli* JM83 as the host microorganism to obtain a recombinant microorganism. 35

Example 9-(3)

Preparation of recombinant DNA from recombinant microorganism

The recombinant DNA was prepared in accordance with the method in Example 4-(4).
40 40

Example 9-(4)

Sequence of recombinant DNA

The polypeptide gene was sequenced in accordance with the method in Example 4-(5).

The results were as shown in Table 4-1.

45 The signal peptide located upstream at the 5'-site of the polypeptide gene was sequenced in 45
the same manner.

The results were as shown in Table 4-2.

Example 9-(5)

50 Amino acid sequence of polypeptide 50

The amino acid sequence of polypeptide was determined with reference to the sequence of polypeptide gene. The results were as shown in Table 5-1.

The amino acid sequence of the signal peptide was determined in the same manner. The results were as shown in Table 5-2.

55 These evidences confirmed that the polypeptide derived from *Bacillus macerans* has the amino 55
acid sequence as shown in Table 5-1.

The evidence as shown in Tables 2-1 and 5-2 show that each polypeptide commonly has the amino acid sequence of

- 60 (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
(b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
(c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
(d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
(e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly,

as well as that these partial amino acid sequences (a), (b), (c), (d) and (e) are located in
65 sequence of nearness to the N-terminal end of polypeptide. 65

Table 4-1

10	20	30	40	50	60
TCCCCGGATA	CGAGCGTGAA	CAACAAGCTC	AATTTTAGCA	CGGATACGGT	TTACCAGATT
70	80	90	100	110	120
GTAACCGACC	GGTTTGTTGA	CGGCAATTCC	GCCAACAACC	CGACCGGAGC	AGCCTTCAGC
130	140	150	160	170	180
AGCGATCATT	CCAACCTGAA	GCTGTATTTC	GGGGGCGACT	GECAGGGGAT	CACGAACAAA
190	200	210	220	230	240
ATCAACGACG	GCTATCTGAC	CGGAATGGGC	ATCACCGCCC	TCTGGATCTC	GCAGCCCGTT
250	260	270	280	290	300
GAGAACATCA	CCGCCGTCAT	CAATTATTCC	GGCGTCAACA	ATACAGCTTA	CCACGGTTAC
310	320	330	340	350	360
TGGCCTCGCG	ACTTCAAGAA	GACCAATGCC	GCCTTCGGCA	GCTTCACCGA	CTTCTCCAAT
370	380	390	400	410	420
TTGATCGCCG	CAGCGCATT	ACACAATATC	AAGGTAGTTA	TGGACTTTGC	ACCTAATCAC
430	440	450	460	470	480
ACCAACCCGG	CTTCGAGTAC	GGACCCCTCG	TTCGCCGAGA	ACGGCGCGCT	CTACAACAAC
490	500	510	520	530	540
GGAACGCTGC	TCGGCAAGTA	TAGCAACGAT	ACCGCCGGCC	TGTTCCACCA	CAATGGCGGC

Table 4-1 continued (1)

550	560	570	580	590	600
ACCGATTCT	CGACGACTGA	AAGCGGTATC	TACAAGAAC	TGTACGATCT	CGCGGATATC
610	620	630	640	650	660
AATCAGAAC	ACAACACCAT	CGACTCGTAT	CTCAAGGAAT	CGATCCAGCT	GTGGCTGAAT
670	680	690	700	710	720
CTCGGAGTCG	ACGGCATCCG	CTTCGACGCC	GTGAAGCATA	TCCCTCAGGG	CTGGCAGAAG
730	740	750	760	770	780
AGCTAGGTCT	CGTCGATCTA	CAGCAGCGCC	AATCCGGTGT	TCACCTTCGG	TGAATGGTTC
790	800	810	820	830	840
CTCGGCCCCG	ACGAAATGAC	CCAGGACAAC	ATCAACTTGG	CGAATCAGAG	CGGCATGCAC
850	860	870	880	890	900
CTGCTGGACT	TTGCGTTTGC	GCAGGAATC	CGTGAAGTGT	TCCGCGACAA	GTGCGAGACG
910	920	930	940	950	960
ATGACCGACC	TGAACTCGGT	GATCTCCAGC	ACCGGCTCCA	GCTATAATTA	CATCAACAAC
970	980	990	1000	1010	1020
ATGTTTACGT	TCATCGACAA	CCATGACATG	GACCGCTTCC	AGCAAGCCGG	AGCGAGCACT
1030	1040	1050	1060	1070	1080
CGCCCGACCG	AGCAGGCTCT	TGCGGTAACG	CTGACTTCCC	GCGGCGTTCC	GGCAATCTAC

Table 4-1 continued (2)

1090	1100	1110	1120	1130	1140
TACGGTACAG	AGCAATATAT	GACCGGCAAC	GGCGACCCGA	ACAAACGGCG	CATGATGACC
1150	1160	1170	1180	1190	1200
GGCTTCGATA	CGAACAAAGAC	AGCGTACAAA	GTGATCAAGG	CGCTGGCTCC	GCTTCGCAAG
1210	1220	1230	1240	1250	1260
TCCAACCCGG	CTCTCGCCTA	CGGCTCGACG	ACCCAGCGTT	GGGTGAACAG	CGACGTCTAC
1270	1280	1290	1300	1310	1320
GTATATGAAC	GCAAGTTCGG	AAGCAACGTA	GCTCTCGTTG	CCGTCAACCG	CAGCTCGACG
1330	1340	1350	1360	1370	1380
ACTGCCTATC	CGATATCGGG	AGCGTTTACT	GCTCTGCCAA	ACGGAACGTA	TACCGACGTT
1390	1400	1410	1420	1430	1440
CTCGGGGGCC	TGCTTAATGG	CAATTCAATT	ACCGTTAACG	GCGGCACGGT	CAGCAACTTT
1450	1460	1470	1480	1490	1500
ACACTTGCAG	CGGGCGGTAC	GGCAGTCTGG	CAGTACACGA	CGACGGAATC	CTCGCCGATT
1510	1520	1530	1540	1550	1560
ATCGGCAACG	TCGGCCCCBAC	TATGGGCAAG	CCCGGCAACA	CCATCAGCAT	CGACGGACGC
1570	1580	1590	1600	1610	1620
GGCTTCGGTA	CTACGAAGAA	CAAAGTTACT	TTCGGTACGA	CAGCCGTTAC	CGGGCGCGAAC

Table 4-1 continued (3)

1630	1640	1650	1660	1670	1680
ATCGTGAGCT	GGGAAGATAC	CGAATCAAG	GTCAAAGTTC	CGAACGTGGC	CGCEGGCAAC
1690	1700	1710	1720	1730	1740
ACGGCCGTTA	CGGTAAACGA	CGCCGCCGGC	ACTACCAGCG	CAGCGTTCAA	CAACTTTAAC
1750	1760	1770	1780	1790	1800
GTACTGACTG	CCGATCAGGT	CACTGTCCGC	TTCAAAGTCA	ACAATGCCAC	CACGGCCCTG
1810	1820	1830	1840	1850	1860
GGACAAACG	TCTACCTGAC	CGGTAACGTC	GCCGAGCTTG	GCAACTGGAC	AGCCGCCAAC
1870	1880	1890	1900	1910	1920
GCAATCGGTC	CBATGTACAA	CCAGGTAGAA	GCCAAGCTATC	CGACTTGGTA	CTTCGACGTC
1930	1940	1950	1960	1970	1980
AGCGTTCGGG	CCAACACGGC	GCTECAATTC	AAGTTCATCA	AAGTGAACGG	CTCGACAGTG
1990	2000	2010	2020	2030	2040
ACTTGGGAAG	GCGGCAACAA	CCACACCTTC	ACCTCGCCTT	CGAGCGGGCT	TGCGACCCGT
2050	2060				
ACGGTCGATT	GCCAGAAC				

Table 4-2

10	20	30	40	50	60
ATGAAAAGC	AAGTCAATG	GTTGACGTCG	GTGTGGATGT	CCGTAGGAT	CGCACTCGGC
70	80	90			
GCGGGCTGC	CTGTATGGC	A			

Table 5-2

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Met	Lys	Lys	Gln	Val	Lys	Trp	Leu	Thr	Ser	Val	Ser	Met	Ser	Val
Gly	Tle	Ala	Leu	Gly	Ala	Ala	Leu	Pro	Val	Trp	Ala			

Example 10**Preparation of polypeptide with recombinant microorganism**

- Polypeptides were prepared with *Escherichia coli* MAH2 (FERM P-7925) and *Bacillus subtilis* MAU210 (FERM P-7926) both in which recombinant DNA carrying the polypeptide gene derived from *Bacillus macerans* had been introduced. The polypeptide productivities of these recombinant microorganisms, host microorganism, and donor *Bacillus macerans* microorganism were compared in relation to their CGTase activity. The used liquid culture medium was prepared by the method in Example 5.
- 10 *Escherichia coli* MAH2 was inoculated to the liquid culture medium additionally containing 50 $\mu\text{g/ml}$ of ampicillin, while *Escherichia coli* HB101 was inoculated in the liquid medium culture without addition of antibiotic. In each case, microorganism was cultured at 35°C for 24 hours under vigorous shaking conditions. 10
- 15 *Bacillus subtilis* MAU210 was inoculated to the liquid culture medium additionally containing 5 $\mu\text{g/ml}$ of kanamycin, while *Bacillus subtilis* 715A was inoculated to the liquid culture medium without addition of antibiotic. In each case, microorganism was cultured at 28°C for 72 hours. 15
- Bacillus macerans* 17A was cultured with the liquid culture medium at 28°C for 72 hours without addition of antibiotic.
- 20 Each culture was treated similarly as in Example 5, and its CGTase activities was then determined. The results were as shown in Table 6. 20
- These evidences clearly show that the recombinant microorganisms are advantageously usable in industrial-scale production of polypeptide because they have improved polypeptide productivity.

Table 6

Microorganism	CGTase activity (units/ml)		
	Supernatant	Cell	Total
<u>Escherichia coli</u> MAH2 (FERM P-7925)	0.6	11.8	12.4
			Present invention
<u>Bacillus subtilis</u> MAU210 (FERM P-7926)	54.6	0.3	54.9
			Present invention
<u>Escherichia coli</u> HB101	0	0	0
			Control
<u>Bacillus subtilis</u> 715A	0	0	0
			Control
<u>Bacillus macerans</u> 17A	7.5	0.4	7.9
			Control

The supernatants were salted out with ammonium sulfate at a saturation degree of 0.6 to obtain crude polypeptide specimens.

On studying these crude polypeptide specimens on their enzymatic properties similarly as in Example 5, the enzymatic properties of the polypeptide produced by the recombinant microorganisms were in good accordance with those of the polypeptide produced by the donor *Bacillus macerans* microorganism.

The principal uses of the polypeptide of the invention are more fully discussed below.

The polypeptide effects intra- or intermolecular saccharide transfer between saccharide donors and saccharide acceptors.

According to one aspect of the present invention, various saccharide-transferred products can be produced by taking advantage of these saccharide transfer reactions.

For example, a partial starch hydrolysate containing α -, β - and γ -cyclodextrins is prepared by subjecting an amylaceous substance as the substrate, such as starch, liquefied starch with a Dextrose Equivalent (DE) of below 10, or amylase, to the action of the polypeptide to induce an intramolecular saccharide transfer. Each cyclodextrin can be isolated from the partial starch hydrolysate, if necessary.

α -Glycosylated saccharide sweeteners, for example, α -glucosyl-, α -maltosyl- and α -maltotriosyl-saccharides, can be prepared by subjecting a mixture of a saccharide donor (for example, an amylaceous substance such as starch, liquefied starch, dextrin, cyclodextrin or amylose) and a saccharide acceptor (for example, a monosaccharide such as xylose, sorbose or fructose, or a disaccharide such as sucrose, maltulose or isomaltulose) to the action of the polypeptide to induce an intermolecular saccharide transfer. The α -glycosylated saccharide sweetener can be advantageously used in foods and beverages because the α -glycosylated saccharide sweetener is much milder in taste, more soluble in water, but less crystallizable in comparison with the unmodified saccharide sweetener. These would expand extremely the use of saccharide sweeteners.

In the intermolecular saccharide transfer reaction, the use of a glycoside, for example, steviol glycoside such as stevioside or rebaudioside, glycyrrhizin, soyasaponin, teasaponin, rutin or esculin, as the saccharide acceptor leads to the formation of α -glycosylated glycosides such as α -glucosyl-, α -maltosyl- and α -maltotriosyl-glycosides. The α -glycosylated glycoside is free of the unpleasant tastes such as bitter- and astringent-tastes which are inherent in the unmodified glycoside, and more readily soluble in water than the unmodified glycoside. These would expand extremely the use of glycosides. Specifically, α -glycosylated steviol glycoside and α -glycosylated glycyrrhizin can be advantageously used in foods, beverages, and pharmaceuticals for peroral administration because the taste improvement in these α -glycosylated glycosides is remarkably high, and because their sweetness is comparable to that of sucrose.

The following Examples further illustrate the preparation of such modified saccharide sweeteners.

Example 11

Corn syrup containing cyclodextrin

A 10 w/w % suspension of potato starch was added with 2 units/g starch of a polypeptide specimen prepared with *Bacillus subtilis* TCU211 in accordance with the method in Example 5, liquefied by heating to 85°C at pH 6.5, cooled to 70°C, further added with the same amount of the polypeptide specimen, and reacted for 40 hours. The reaction mixture was purified by decoloration using activated carbon and deionization using an ion exchange resin, and then concentrated to obtain a corn syrup containing cyclodextrin in a yield of 92% based on the dry solid. The corn syrup can be advantageously incorporated into flavorings and cosmetics wherein fragrance or aroma is one of the important factors because the corn syrup has excellent flavor-locking properties.

The α -, β - and γ -cyclodextrins in the corn syrup can be separated by treating the corn syrup by means of a procedure using an organic precipitant, such as toluene or trichloromethane, or conventional column chromatography.

Example 12

α -Glycosylsucrose

A 35 w/w % suspension of cornstarch was added with 0.2 w/w % oxalic acid, autoclaved to 120°C to give a DE of 20, neutralized with calcium carbonate, and filtered to obtain a dextrin solution. The dextrin solution was then added with a half amount of sucrose based on the dry solid, and the resultant mixture was added with 15 units/g starch of a polypeptide specimen prepared with *Bacillus subtilis* MAU210 in accordance with the method in Example 10, and reacted at pH 6.0 and 55°C for 40 hours. The reaction mixture was purified by decoloration using activated carbon and deionization using ion exchange resin, and then concentrated to obtain a colorless, transparent corn syrup in a yield of 94% based on the dry solid. The corn syrup containing a large amount of α -glycosylsucrose can be advantageously used in confection-

ery because it is mildly sweet and amorphous.

Example 13

α -Glycosyl stevioside

Two-hundred g of stevioside and 600 g of dextrin (DE 8) were dissolved in 3 liters of water by heating, and the resultant solution was cooled to 70°C, added with 5 units/g dextrin of a polypeptide specimen prepared with *Bacillus subtilis* TCU211 in accordance with the method in Example 5, and reacted at pH 6.0 and 65°C for 35 hours. The reaction mixture was then heated at 95°C for 15 minutes, purified by filtration, concentrated, and pulverized to obtain a pulverulent sweetener containing α -glycosyl stevioside in a yield of about 92% based on the dry solid.

The sweetener free of the unpleasant taste which is inherent in the unmodified parent stevioside was comparable to sucrose in taste quality, and the sweetening power of the sweetener was about 100-times higher than that of scurose. The sweetener can be advantageously used as a diet sweetener or for seasoning foods and beverages because of its low-calorigenic and low-calorific properties.

Example 14

α -Glycosyl ginsenoside

Sixty g of a ginseng extract and 180 g of β -cyclodextrin were dissolved in 500 ml of water by heating, and the resultant mixture was cooled to 70°C, adjusted to pH 6.0, added with 3 units/g β -cyclodextrin of a polypeptide specimen prepared with *Escherichia coli* TCH201 in accordance with the method in Example 5, cooled to 65°C, and reacted at pH 6.0 for 40 hours. The reaction mixture was heated for 15 minutes to inactivate the polypeptide, followed by filtration. The filtrate was admitted to a column packed with 3 liters of "Amberlite XAD-7", a synthetic adsorbant commercialized by Rohm & Haas Co., Philadelphia, PA, USA. Thereafter, the column was sufficiently washed with water to remove free saccharides. The column was then admitted with 10 liters of 50 v/v % ethanol, and the eluate was concentrated and dehydrated to obtain about 21 g of a pulverulent product that contains α -glycosyl ginsenoside. Since the product is free of the unpleasant tastes such as bitter-, astringent- and harsh-tastes which are manifest in the unmodified parent ginsenoside, the product can be perorally administered intact, or, if necessary, seasoned with any sweetener or sour, prior to its use. In addition, the product can be advantageously used in health foods and medicines for internal administration because the product possesses invigorating, peptic, intestine-regulating, haematic, anti-inflammatory and expectorant effects similar to the parent ginsenoside.

As described above, we have determined the amino acid sequences of the polypeptide gene and its signal peptide, as well as preparing recombinant DNA having a *PvuII* restriction site from a donor microorganism by *in vitro* genetic engineering technique. Furthermore, we have prepared recombinant microorganisms in which the recombinant DNA is introduced, as well as confirming that the recombinant microorganisms autonomically and consistently proliferate in a nutrient culture medium.

CLAIMS

1. A polypeptide possessing cyclomaltodextrin glucanotransferase (CGTase) activity, comprising one or more partial amino acid sequences selected from the group consisting of

- (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
- (b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
- (c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
- (d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
- (e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly.

2. The polypeptide in accordance with claim 1, wherein partial amino acid sequences of

- (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
- (b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
- (c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
- (d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
- (e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly

are located in sequence of nearness to the N-terminal end of said polypeptide.

3. The polypeptide in accordance with claim 1, which shows a molecular weight of 70,000 $\pm$ 10,000 daltons on SDS-polyacrylamide electrophoresis.

4. The polypeptide in accordance with claim 1, whose partial amino acid sequence containing N-terminal end is Ala-Gly-Asn-Leu-Asn-Lys-Val-Asn-Phe-Thr.

5. The polypeptide in accordance with claim 4, which has the following amino acid sequence:



ery because it is mildly sweet and amorphous.

Example 13

α -Glycosyl stevioside

- 5 Two-hundred g of stevioside and 800 g of dextrin (DE 8) were dissolved in 3 liters of water by heating, and the resultant solution was cooled to 70°C, added with 5 units/g dextrin of a polypeptide specimen prepared with *Bacillus subtilis* TCU211 in accordance with the method in Example 5, and reacted at pH 6.0 and 65°C for 35 hours. The reaction mixture was then heated at 95°C for 15 minutes, purified by filtration, concentrated, and pulverized to obtain a pulverulent
10 sweetener containing α -glycosyl stevioside in a yield of about 92% based on the dry solid.

- The sweetener free of the unpleasant taste which is inherent in the unmodified parent stevioside was comparable to sucrose in taste quality, and the sweetening power of the sweetener was about 100-times higher than that of sucrose. The sweetener can be advantageously used as a diet sweetener or for seasoning foods and beverages because of its low-cariogenic and
15 low-calorific properties.

Example 14

α -Glycosyl ginsenoside

- 20 Sixty g of a ginseng extract and 180 g of β -cyclodextrin were dissolved in 500 ml of water by heating, and the resultant mixture was cooled to 70°C, adjusted to pH 6.0, added with 3 units/g β -cyclodextrin of a polypeptide specimen prepared with *Escherichia coli* TCH201 in accordance with the method in Example 5, cooled to 65°C, and reacted at pH 6.0 for 40 hours. The reaction mixture was heated for 15 minutes to inactivate the polypeptide, followed by filtration. The filtrate was admitted to a column packed with 3 liters of "Amberlite XAD-7", a
25 synthetic adsorbant commercialized by Rohm & Haas Co., Philadelphia, PA, USA. Thereafter, the column was sufficiently washed with water to remove free saccharides. The column was then admitted with 10 liters of 50 v/v % ethanol, and the eluate was concentrated and dehydrated to obtain about 21 g of a pulverulent product that contains α -glycosyl ginsenoside. Since the product is free of the unpleasant tastes such as bitter-, astringent- and harsh-tastes which are
30 manifest in the unmodified parent ginsenoside, the product can be perorally administered intact, or, if necessary, seasoned with any sweetener or sour, prior to its use. In addition, the product can be advantageously used in health foods and medicines for internal administration because the product possesses invigorating, peptic, intestine-regulating, haematic, anti-inflammatory and expectorant effects similar to the parent ginsenoside.

- 35 As described above, we have determined the amino acid sequences of the polypeptide gene and its signal peptide, as well as preparing recombinant DNA having a *PvuII* restriction site from a donor microorganism by *in vitro* genetic engineering technique. Furthermore, we have prepared recombinant microorganisms in which the recombinant DNA is introduced, as well as confirming that the recombinant microorganisms autonomically and consistently proliferate in a nutrient
40 culture medium.

CLAIMS

1. A polypeptide possessing cyclomaltodextrin glucanotransferase (CGTase) activity, comprising one or more partial amino acid sequences selected from the group consisting of

- 45 (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
(b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
(c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
(d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
(e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly.

- 50 2. The polypeptide in accordance with claim 1, wherein partial amino acid sequences of

- (a) Asn-Lys-Ile-Asn-Asp-Gly-Tyr-Leu-Thr,
(b) Pro-Val-Phe-Thr-Phe-Gly-Glu-Trp-Phe-Leu,
(c) Val-Thr-Phe-Ile-Asp-Asn-His-Asp-Met-Asp-Arg-Phe,
(d) Ile-Tyr-Tyr-Gly-Thr-Glu-Gln-Tyr-Met-Thr-Gly-Asn-Gly-Asp-Pro-Asn-Asn-Arg, and
55 (e) Asn-Pro-Ala-Leu-Ala-Tyr-Gly

are located in sequence of nearness to the N-terminal end of said polypeptide.

3. The polypeptide in accordance with claim 1, which shows a molecular weight of 70,000 $\pm$ 10,000 daltons on SDS-polyacrylamide electrophoresis.

4. The polypeptide in accordance with claim 1, whose partial amino acid sequence containing
60 N-terminal end is Ala-Gly-Asn-Leu-Asn-Lys-Val-Asn-Phe-Thr.

5. The polypeptide in accordance with claim 4, which has the following amino acid sequence:



15 Tyr Asn Lys Asp Gln Ser Asn Ala Asn Asn Ser Asp Ala Asp Phe Asn Leu Asn Asp Ile Leu Ser Leu
14 Val Asn Arg Asn Ser Gly Pro Asp pro Glu Thr Phe Ala Asp Met Trp Ala Lys Phe Leu Met Val Glu Met Lys
13 Val Ser Leu Ile Ile Ser Lys Val Ala Met Tyr Thr Leu Lys Arg Leu Glu Phe Gln Gly Val Phe Ala Thr Met Gln
12 Asp Thr Asn Lys Trp Ala Lys Leu Phe Tyr Gly Thr Asp Leu Ile Ser Gly Tyr Gly Tyr Glu Arg Leu Gly Lys Ile
11 Ser Asn Thr Asn Ile Asp Phe Arg Asp Ser Gly Phe Tyr Gly Lys Phe His Phe Trp Asp Asp Ala Tyr Arg Val
10 Thr Gly Cys Ile Ala Asn Asp Gln Ile Pro Leu Gly Leu Arg Asp Gln Thr Asn Arg Asn Tyr Met Met Tyr Asn Gln
9 Phe Asp Gly Ile Thr Met Arg Phe Ile Asn Leu Asn Asp Ile Trp Phe Asn Phe Asp Ala Asp Ile Asn Tyr
8 Asn Val Ser Gly Val Val Ala Asp Val Thr His Arg Ile Gly Gly Val Ala Asp Ser Ser His Val Asn Pro Ala
7 Val Phe Ser Gln Gly Ser Trp Ser Lys Glu His Tyr Val Met Phe Pro Asp Leu Asn Ala Asn Lys Pro Asp Arg
6 Lys Arg Phe Trp Met Phe Tyr Leu Ile Ser Asn Phe Ile Pro Asp Pro Arg Val Leu Asn Thr Asp Arg Val Gly Thr
5 Asn Asp Leu Asp Asp Val Gly Thr Gly Ala Asp Tyr Gly Asn Ile Met Tyr Glu Ser Arg Asp Ile Pro Gly Asn Asn
4 Leu Val Ala Gly Thr Asn His Gly Lys Pro Tyr Met Asp Gln Trp His Asn Asn Met Leu Gln Phe Asp Arg Gly Lys
3 Asn Val Gly Gly Leu Glu Tyr Phe Ala Ser Leu Asn Glu His Met Lys Asp Glu Gly Val Ile Thr Gly Ser Thr Asn
2 Gly Ile Ser Cys Tyr Val Ser Phe His Thr Arg Ala Leu Asn Lys Val Ile Ser Ser Gln Met Val Gly Thr Met Phe
1 Ala Gln Pro Tyr Gly Pro Ala Pro Ala His Gly Asp Ser Leu Val Ala Glu Leu Glu Arg Gln Gln Asp Leu Tyr Ser
11> 10> 31> 46> 61> 76> 81> 106> 121> 130> 151> 168> 181> 196> 211> 226> 241> 256> 271> 286> 301> 316> 331> 346> 361> 376>



* C L M S *

Thr Gln Ser Gly Ile Pro Pro Gln Val Asn Asn Asp Val Val Ile Tyr Phe Ser Ile
Asp Arg Ser Ala Thr Gly Thr His Thr Asn Tyr Tyr Phe Ile Ala Trp Lys Gly Ile
Gly Glu Ser Pro Asn Leu Ser Gly Gly Ser Lys Ala Arg Tyr Lys Thr Phe Ser Lys
Tyr Tyr Arg Leu Gly Asp Glu Val Thr Trp Gly Ala Val Ile Ser Pro Glu Gly
Ala Val Asn Ala Asp Phe Thr Gln Asn Ser Pro Ser Ser Asn Thr Tyr Ile Trp Thr
Leu Tyr Val Thr Leu Ala Ala Gly Thr Val Ser Thr Val Gln Asp Ser Thr Thr Thr
Ala Val Arg Phe Leu Asn Ser Met Gly Val Val Gln Gln Gly Trp Tyr Lys Val Asn
Pro Asp Val Leu Gly Val Tyr Met Phe Asn Asn Gly Asp Leu Asn Val Gly Asn Thr
Asn Gly Leu Gly Gly Ser Ala Pro Gly Ala Pro Ser Asn Asn Gly Val Glu Gly Pro
Asn Asn Val Thr Leu Gly Trp Gly Glu Ala Val Ser Thr Thr Leu Pro Gln Thr
Arg Ile Val Ile Gln Asn Val Val Gly Thr Ala Ser Leu Thr Glu Asn Val Ser Thr Asn
Arg Trp Asp Ser Asp Ser Gly His Asp Thr Val Gln Val Ala Tyr Phe Ser Asp Tyr Gln
Leu Arg Lys Tyr Thr Gly Val Gly Ile Gly Val Val Glu Asn Val Met Val Lys Val Trp
Ser Gln Gly Asn Tyr Val Glu Ile Thr Phe Ile Thr Phe Asn Asn Pro Asp Lys His Asp
Ser Glu Phe Ser Thr Gln Gly Ile Val Lys Gln Ile Asn Val Gly Gly Ile Ile Asn Val
391>> 406>> 421>> 436>> 451>> 466>> 481>> 496>> 511>> 526>> 541>> 556>> 571>> 586>> 601>> 616>> 631>> 646>> 661>> 676>>



6. The polypeptide in accordance with claim 4, wherein a signal peptide having an amino acid sequence of Met-Arg-Arg-Trp-Leu-Ser-Leu-Val-Leu-Ser-Met-Ser-Phe-Val-Phe-Ser-Ala-Ile-Phe-Ile-Val-Ser-Asp-Thr-Gln-Lys-Val-Thr-Val-Glu-Ala is located upstream at the upstream at the N-terminal side of said polypeptide.
- 5 7. The polypeptide in accordance with claim 4, whose partial amino acid sequence containing 5 N-terminal end is Ser-Pro-Asp-Thr-Ser-Val-Asn-Asn-Lys-Leu.
8. The polypeptide in accordance with claim 1, which has the following amino acid sequence:



1> Ser Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 16> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 31> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 46> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 61> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 76> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 91> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 106> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 121> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 136> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 151> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 166> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 181> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 196> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 211> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 226> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 241> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 256> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 271> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 286> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 301> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 316> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 331> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 346> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 361> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 376> Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 1 Ser Thr Ala Leu Ile Gly Lys Phe Leu Phe Lys Thr Asp
 2 Pro Val Asn Lys Asn Ser Val Lys Ile Ala Tyr Asp Leu Lys Arg Tyr Gly Asn Ala Thr Tyr Arg Leu Gly Gly
 3 Asp Tyr Asn Leu Asp Gln Asn Thr Ala Pro Glu Ser Phe Ala Glu Phe Val Glu Phe Gln Asp Ile Phe Ala Thr Met
 4 Thr Gln Pro Tyr Gly Pro Asn Asn Ala Asn Asn Ser Asp Ser Asp Ser Trp Ala Glu Leu Asn Gln Val Glu Met
 5 Ser Ile Thr Phe Tyr Val Thr Ala Ala His Gly Asp Thr Ile Ala Ser Phe Asn Ile Asn Asn Gln Thr Gln Thr
 6 Val Val Gly Gly Leu Glu Ala Ala His Thr Thr Asn Gln Val Ile Leu Gln Arg Ser Met Ala Leu Tyr Gly
 7 Asn Thr Ala Gly Thr Asn Tyr Phe Ser Asn Leu Ala Glu Gln Leu Lys Tyr Gly Ser Glu Val Val Gly Thr Met Phe
 8 Asn Asp Ala Asp Gly Ile His Gly His Pro Gly Ser Asn Trp His Ser Pro Gly Val Ile Thr Ala Ser Thr Asp
 9 Lys Arg Phe Trp Met Thr Gly Ser Asn Ala Asn Leu Gly Asn Leu Met Ser Asp Met Phe Ser Phe Ser Arg Gly Thr
 10 Leu Phe Ser Gln Gly Ala Tyr Phe Ile Ser Asn Phe Ile Asn Asn Pro Ala Glu His Arg Ser Ile Thr Gly Asn Asn
 11 Asn Val Ser Gly Ile Val Trp Thr Lys Ser Gly His Tyr Thr Leu Gln Asn Met Leu Asp Thr Asp Arg Val Gly Lys
 12 Phe Asp Asp Ile Thr Ile Pro Asp Val Thr Thr His Lys Ile Gly Gly Pro Thr Leu Lys Gly Asn Pro Pro Asp Thr
 13 Ser Gly His Thr Ala Asn Arg Phe Val Asp Leu Asn Asn Val Trp Val Gln Asp Ser Ser His Thr Ala Pro Ala
 14 Thr Asn Ser Asn Leu Tyr Asp Ser Met Pro Leu Gly Leu Ser Asp Gln Phe Asp Phe Glu Ser Asp Glu Ile Asn Tyr
 15 Asp Ser Asn Lys Trp Ser Phe Asn Asp Ser Ser Gly Tyr Tyr Gly Lys Thr Asn Ala Thr Tyr Met Gln Tyr Asn Lys



9. The polypeptide in accordance with claim 8, wherein a signal peptide having an amino acid sequence of Met-Lys-Lys-Gln-Val-Lys-Trp-Leu-Thr-Ser-Val-Ser-Met-Ser-Val-Gly-Ile-Ala-Leu-Gly-Ala-Ala-Leu-Pro-Val-Trp-Ala is located upstream at the N-terminal side of said polypeptide.
10. The polypeptide in accordance with claim 1, which originates from a microorganism
5 capable of producing CGTase. 5
11. The polypeptide in accordance with claim 1, which originates from a microorganism of species *Bacillus stearothermophilus*.
12. The polypeptide in accordance with claim 1, which originates from a microorganism of species *Bacillus macerans*.
- 10 13. The polypeptide in accordance with claim 1, which originates from a recombinant micro- 10
organism in which a recombinant DNA carrying CGTase gene has been introduced.
14. A recombinant DNA having a *PvuII* restriction cleavage site and carrying a CGTase gene, said recombinant DNA comprising:
a DNA fragment, obtained by digesting the DNA of a donor microorganism capable of produc-
15 ing CGTase with a restriction enzyme *in vitro*; and 15
a vector fragment, obtained by cleaving a vector with the restriction enzyme, these fragments being ligated.
15. The recombinant DNA in accordance with claim 14, wherein said donor microorganism is of genus *Bacillus*.
- 20 16. The recombinant DNA in accordance with claim 14, wherein said donor microorganism is 20
a member selected from the group consisting of *Bacillus stearothermophilus* and *Bacillus macerans*.
17. The recombinant DNA in accordance with claim 14, which has restriction cleavage sites as shown in Fig. 1, 2, 3 or 4.
- 25 18. A biologically-pure culture of a recombinant microorganism in which a recombinant DNA 25
having a *PvuII* restriction cleavage site and carrying CGTase gene, prepared by ligation of a DNA fragment, obtained by digesting the DNA of a donor microorganism capable of producing CGTase with a restriction enzyme *in vitro*, and a vector fragment, obtained by cleaving a vector with the restriction enzyme, has been introduced.
- 30 19. The culture in accordance with claim 18, wherein said recombinant DNA has restriction 30
cleavage sites as shown in Fig. 1, 2, 3 or 4.
20. The culture in accordance with claim 19, wherein said recombinant microorganism is of *Escherichia* or *Bacillus*.
21. The culture in accordance with claim 19, wherein said recombinant microorganism is a
35 member selected from the group consisting of *Escherichia coli* TCH201 (FERM P-7924) or 35
Escherichia coli MAH2 (FERM P-7925).
22. The culture in accordance with claim 18, wherein said recombinant microorganism is a member selected from the group consisting of *Bacillus subtilis* MAU210 (FERM P-7926) and *Bacillus subtilis* TCU211 (FERM P-7927).
- 40 23. A process for producing CGTase, comprising: 40
culturing with a nutrient culture medium a recombinant microorganism, in which a recombinant DNA having a *PvuII* restriction cleavage site and carrying CGTase gene, prepared by ligation of a DNA fragment, obtained by digesting the DNA of a donor microorganism capable of producing a CGTase with a restriction enzyme *in vitro*, and a vector fragment, obtained by cleaving a vector
45 with the restriction enzyme, has been introduced; and 45
recovering the accumulated CGTase.
24. The process in accordance with claim 23, wherein said recombinant DNA has restriction cleavage sites as shown in Fig. 1, 2, 3 or 4.
25. The process in accordance with claim 23, wherein said recombinant microorganism is of
50 genus *Escherichia* or *Bacillus*. 50
26. The process in accordance with claim 23, wherein said recombinant microorganism is a member selected from the group consisting of *Escherichia coli* TCH201 (FERM P-7924) or *Escherichia coli* MAH2 (FERM P-7925).
27. The process in accordance with claim 23, wherein said recombinant microorganism is a
55 member selected from the group consisting of *Bacillus subtilis* MAU210 (FERM P-7926) and 55
Bacillus subtilis TCU211 (FERM P-7927).
28. The process for producing a saccharide-transferred product, comprising subjecting an amylaceous substance to the action of a polypeptide possessing CGTase activity; said polypeptide being a polypeptide as claimed in any one of claims 1 to 13 and 35.
- 60 29. The process in accordance with claim 28, wherein said saccharide-transferred product is 60
cyclodextrin.
30. The process in accordance with claim 28, wherein said amylaceous substance is subjected to the action of said polypeptide in the presence of a saccharide acceptor.
31. The process in accordance with claim 28, wherein said amylaceous substance is selected
65 from the group consisting of starch, amylose, cyclodextrin, dextrin, and mixtures thereof. 65



32. The process in accordance with claim 30, wherein said saccharide acceptor is a member selected from the group consisting of saccharide sweetener, glycoside, and mixtures thereof.
33. The process in accordance with claim 30, wherein the saccharide-transferred product is a member selected from the group consisting of α -glycosylsucrose, α -glycosyl stevioside, and α -glycosyl ginsenoside. 5
34. The process in accordance with claim 30, wherein said saccharide-transferred product is used as a sweetener.
35. A polypeptide according to claim 1, substantially as hereinbefore described with reference to any one of the foregoing Examples 1 to 10.
- 10 36. A process according to claim 23, substantially as hereinbefore described with reference to any one of the foregoing Examples 1 to 10. 10
37. A process according to claim 28, substantially as hereinbefore described with reference to any one of the foregoing Examples 11 to 14.
- 15 38. Cyclomaltodextrin Glucanotransferase whenever obtained by a process as claimed in any one of claims 23 to 27 and 36. 15
39. A saccharide transferred product whenever obtained by a process as described in any one of claims 28 to 34 and 37.