# (12) UK Patent (19) GB (11) 2 169 902 (13) B

(54) Title of Invention

## Polypeptide possessing cyclomaltodextrin glucanotransferase activity

(51) INT CL⁴; C12N 15/00, C13K 13/00


(21) Application No
8529759.6

(22) Date of filing
3 Dec 1985

(30) Priority data

(31) 59255339
60228169

(32) 3 Dec 1984
14 Oct 1985

(33) JP

(43) Application published
23 Jul 1986

(45) Patent published
6 Sep 1989

(52) Domestic classification
(Edition J)
C3H HB7E H642 H650 H651
H724
C2S SBB S220
U1S S1075

(56) Documents cited
GB 1459654 A
GB 1447492 A
GB 1442480 A
GB 1414235 A
GB 4317881 A

(58) Field of search

As for published application
2169902 A *viz:*
UK CL (Edition J) C3H
INT CL⁴ C12N
updated as appropriate



(73) Proprietor
Kabushiki Kaisha Hayashibara
Seibutsu Kagaku Kenkyujo

(Incorporated in Japan)

2-3 1-chome Shimoishii
Okayama-shi
Okayama
Japan

(72) Inventors
Toshiyuki Sugimoto
Michio Kubota
Shuzo Sakai

(74) Agent and/or
Address for Service
Page White & Farrer
5 Plough Place
New Fetter Lane
London
EC4A 1HY
United Kingdom

FIG. 1

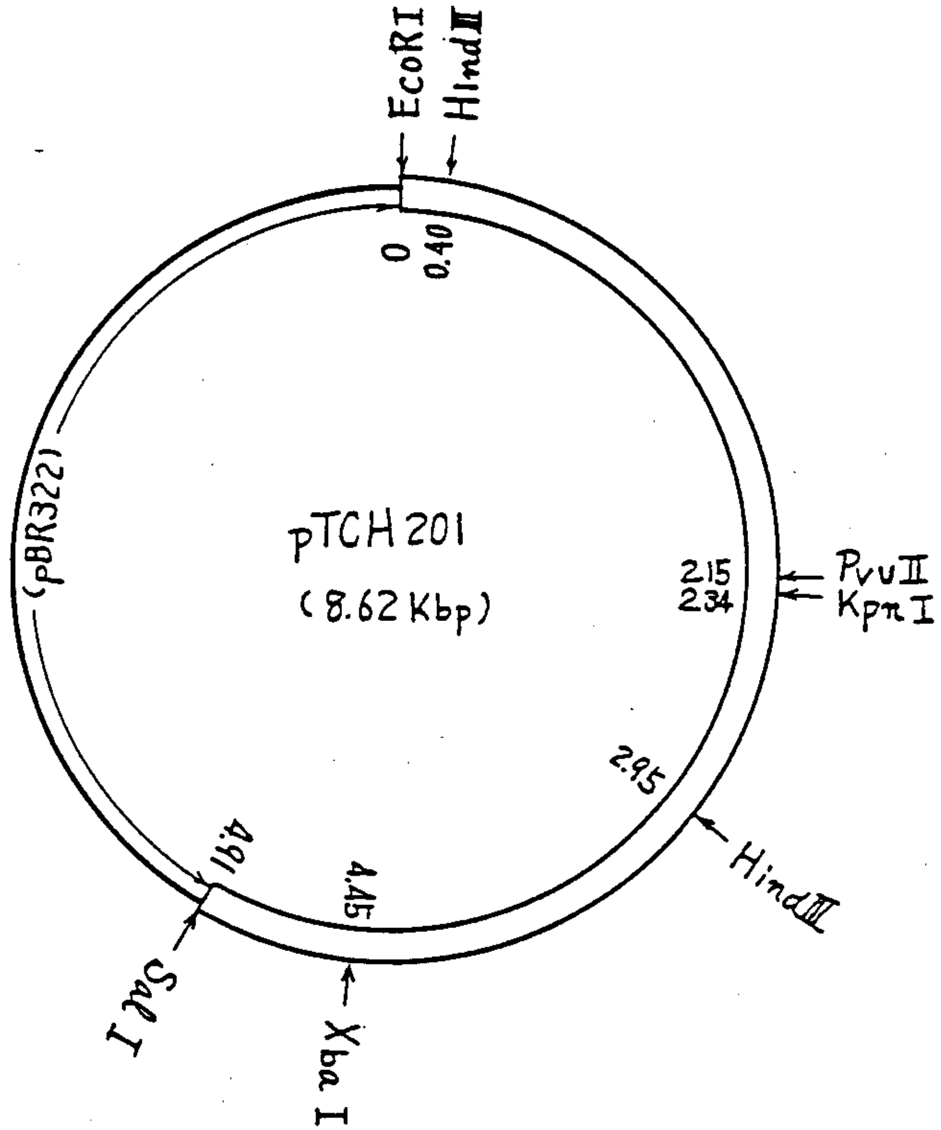


FIG. 2

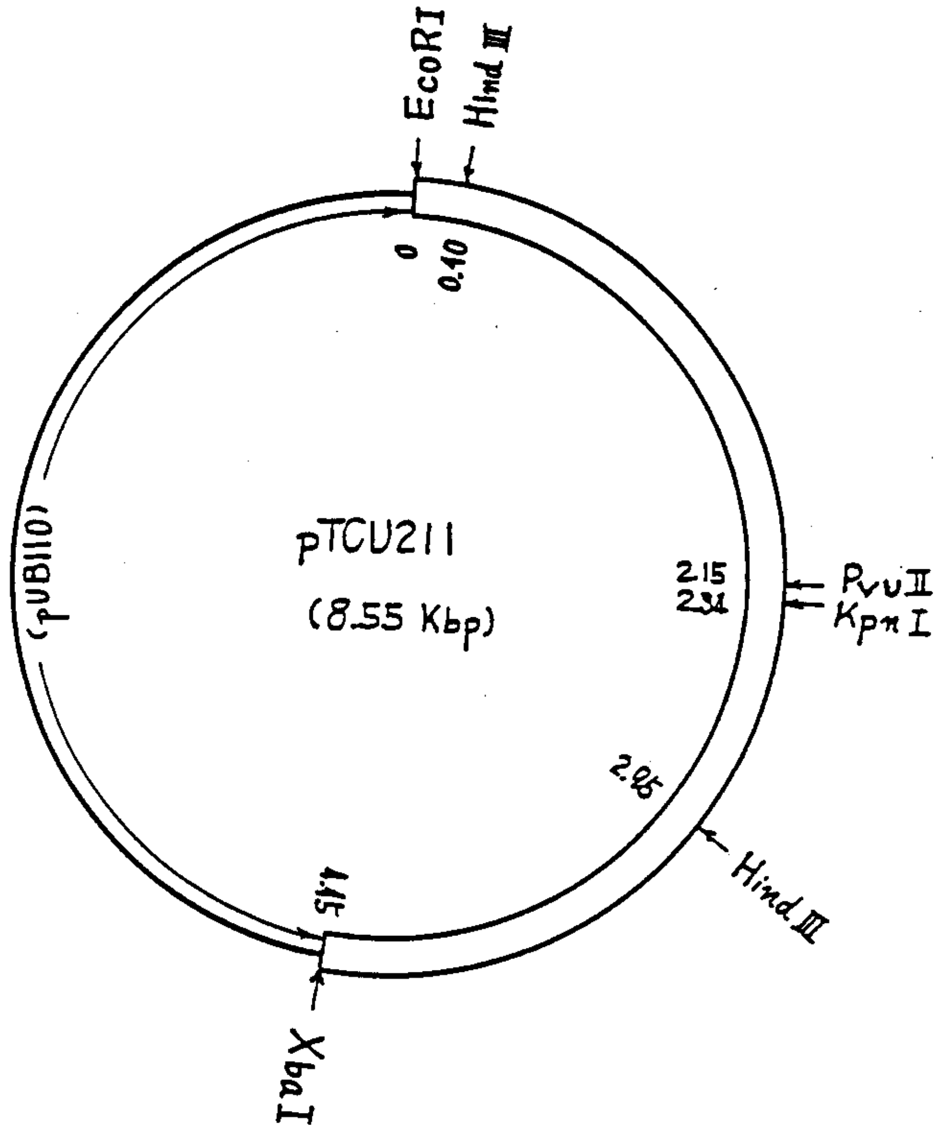


FIG. 3

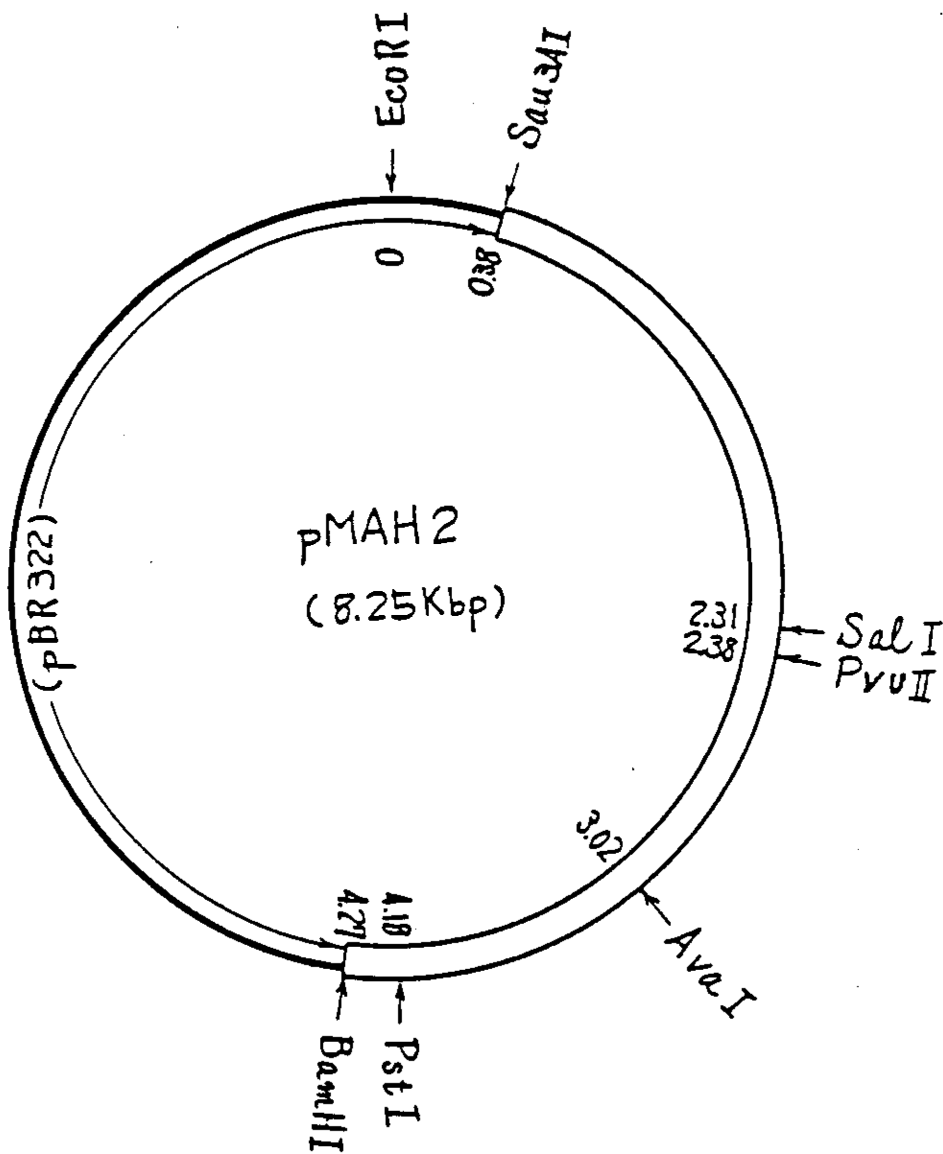
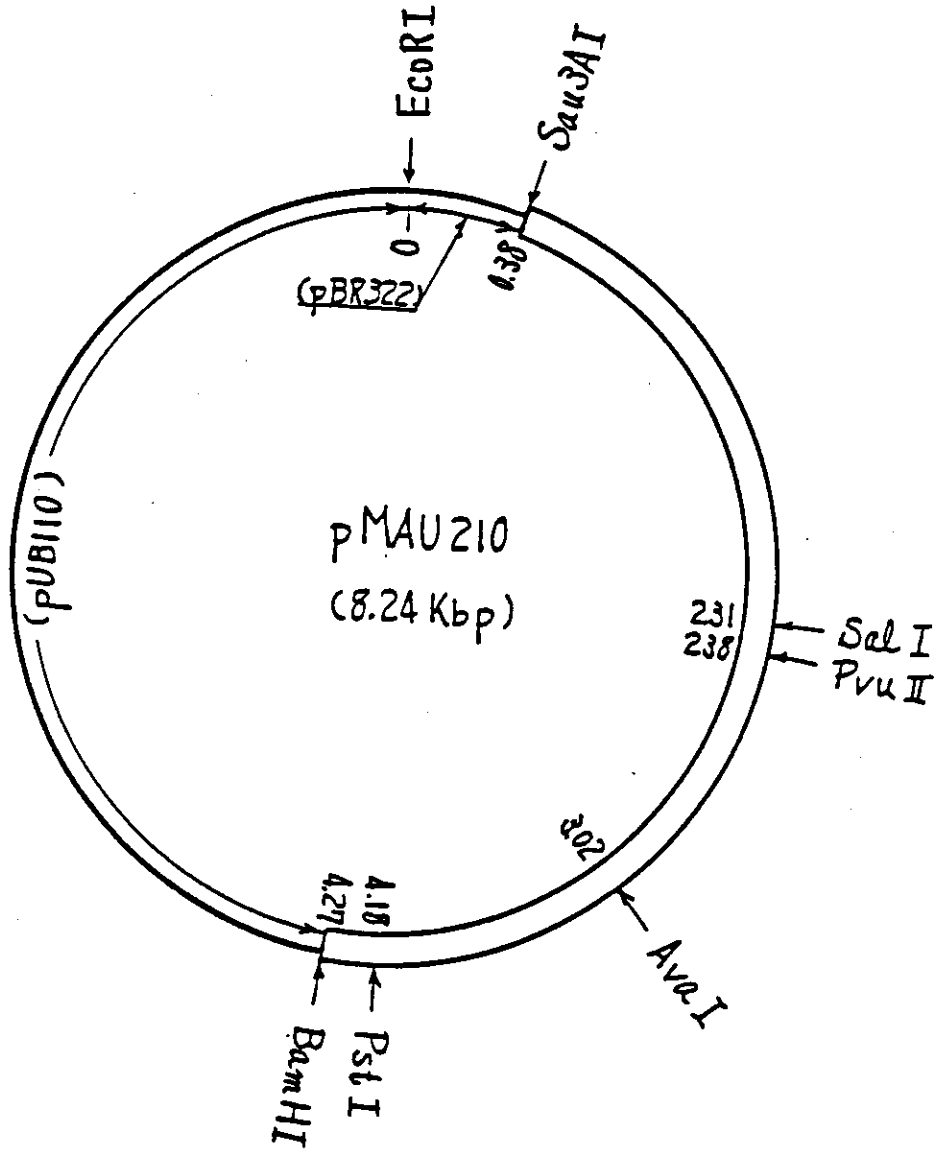


FIG. 4



2169902

# POLYPEPTIDE POSSESSING CYCLOMALTODEXTRIN GLUCANOTRANSFERASE ACTIVITY

The present invention relates to a polypeptide, and more particularly to a polypeptide possessing cyclomaltodextrin glucanotransferase activity.

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, deoxycytidin 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 CGTase, cyclomaltodextrin glucanotransferase.

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".

CGTase, or macerans amylase, has been known for sometime as any enzyme produced by Bacillus macerans.

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.

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.

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 susceptive 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".

In order to meet these demands, there is an urgent need to provide a stable CGTase supply. This requires determination of the amino acid sequence of the polypeptide that possesses CGTase activity.

The amino acid sequence of CGTase has not been known hitherto.

The Applicants have performed investigations to determine the amino acid sequence of the recombinant polypeptide possessing CGTase activity. According to the present invention there is provided a polypeptide possessing 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; and said polypeptide having an isoelectric point of $5.0 \pm 0.1$.

More particularly, these partial amino acids sequences (a), (b), (c), (d) and (e) are located in sequence of nearness to the N-terminal end of polypeptide.

In a further aspect of the invention there is provided a recombinant DNA having a Pvull restriction cleavage site and carrying CGTase gene, said recombinant DNA comprising:

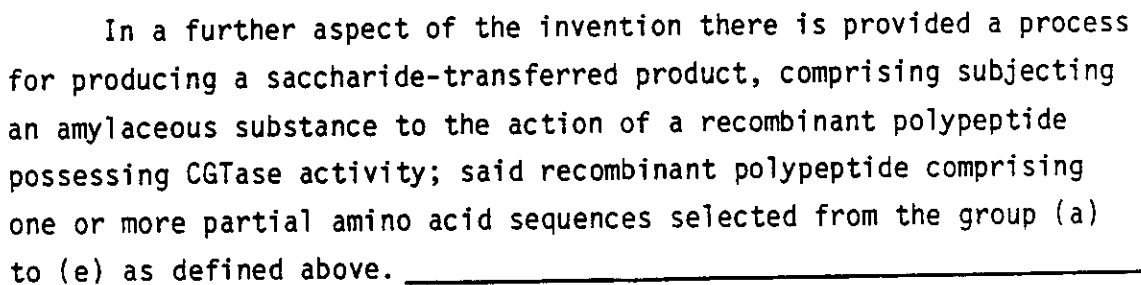
a DNA fragment, obtained by digesting the DNA of a donor microorganism capable of producing CGTase in which the CGTase has the one or more partial amino acid sequences as defined above, with a restriction enzyme *in vitro*; and

a vector fragment, obtained by cleaving a vector with the restriction enzyme, these fragments being ligated.

In a further aspect of the invention there is provided a biologically-pure culture of a recombinant microorganism in which has been introduced a recombinant DNA as defined above.

In a further aspect of the invention there is provided a process for producing CGTase comprising culturing with a nutrient culture medium a recombinant microorganism in which has been introduced a recombinant DNA as defined above.

In a further aspect of the invention there is provided a process for producing a saccharide-transferred product, comprising subjecting an amylaceous substance to the action of a recombinant polypeptide possessing CGTase activity; said recombinant polypeptide comprising one or more partial amino acid sequences selected from the group (a) to (e) as defined above.



The polypeptide is characterized by the fact that it forms cyclodextrin from soluble starch; that it exhibits a molecular weight of 70,000$\pm$10,000 daltons on SDS-polyacrylamide electrophoresis; and that it has a specific activity of 200$\pm$30 units/mg protein.

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 regulate secretion of the polypeptide from producer microorganisms.

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.

The amino acid sequence containing N-terminal end is determined by analyzing a highly-purified polypeptide with a gas phase protein sequencer.

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 means or restriction enzymes), and a vector fragment (obtained by cleaving a vector in the same 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 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 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 ß-glucanase, 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 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, SalI, SlaI, XmaI, 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 λ*gt*·λ*C* and λ*gt*·λ*B* may be used, while ρ11, φ1 and φ105 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 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 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 autonomically and consistently proliferates to express its characteristics. Specifically, microorganisms 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 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 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 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.

Sequence of the polypeptide gene

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 recombinant 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 aearation-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 $2x10^{-3}$ 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 were deposited on November 2, 1984 with the Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305 Japan, and have received depository numbers of FERM BP-2109, 2110, 2111 and 2112 respectively.

In the accompanying drawings:-

FIG. 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.

FIG. 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.

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

FIG. 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.

Example 1

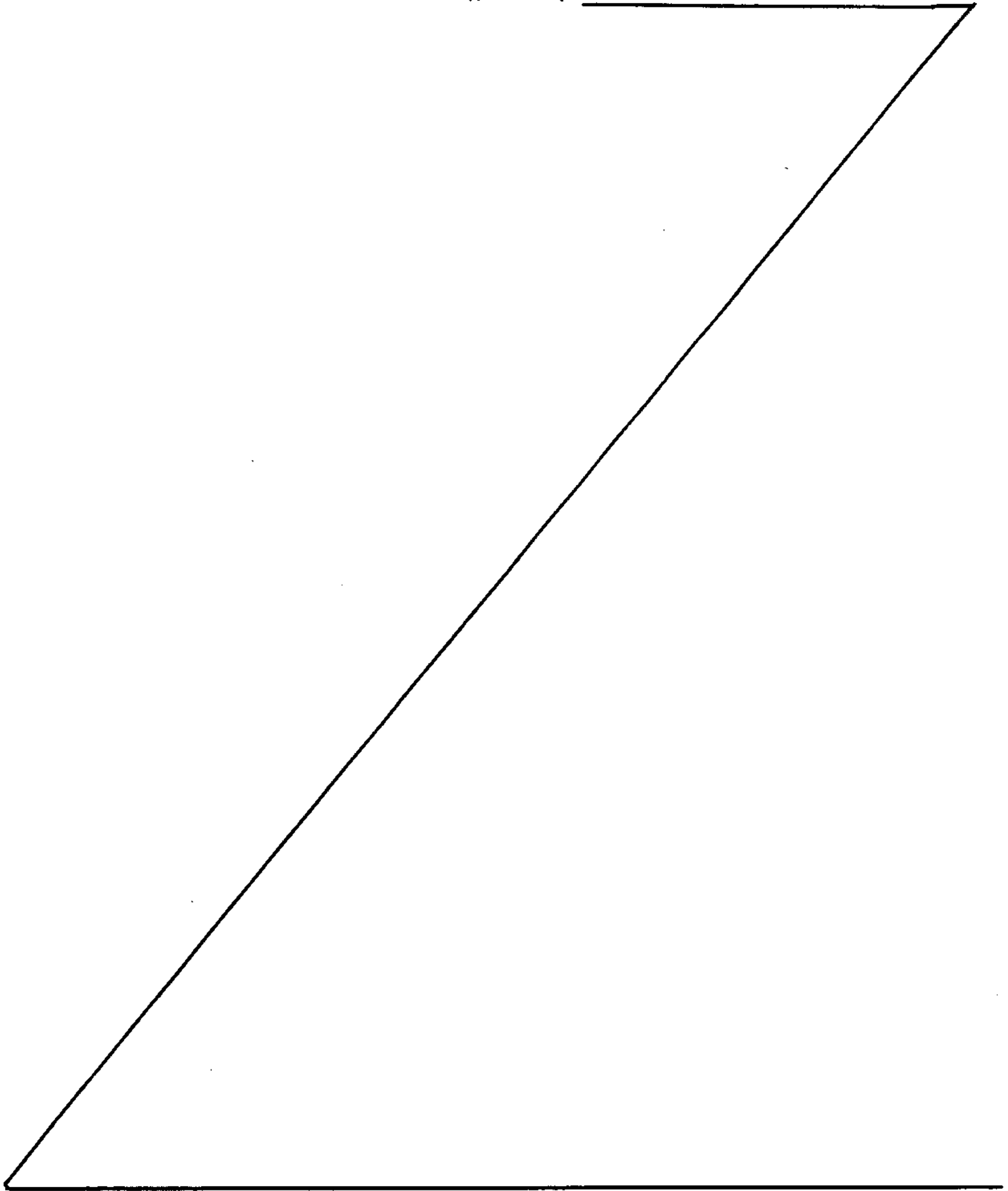
Cloning of Bacillus stearothermophilus polypeptide gene into Escherichia ccli

Example 1-(1)

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

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 Biophisica 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 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 Tris-aminomethane, hydrochloric acid, sodium lauryl sulfate and sodium

chloride, 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 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.

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 (1976).

Example 1-(3)

Preparation of recombinant DNA carrying polypeptide gene

The purified chromosome DNA carrying heat-resistant-polypeptide gene, prepared in Example 1-(1), was partially digested with restriction enzyme MboI, purchased from Nippon Gene Co., Ltd., Toyama, 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 the plasmid fragment as well as to dephosphorize the 5'-terminal end of the fragment.

Both fragments were then ligated by subjecting them to $T_4$ DNA ligase, purchased from Nippon Gene Co., Ltd., at 4°C overnight to obtain a

recombinant DNA.

Example 1-(4)

Introduction of recombinant DNA into Escherichia coli

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 $T_4$ 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 SalI, 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 pol· -peptide gene.

One of these microorganisms and its recombinant DNA were named as "Escherichia coli TCH201 (FERM BP-2109)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 AccI, 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 BP-2109) 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 Bacteri-

ology, 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 $T_4$ 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 microorganisms in which the re-

combinant DNA carrying heat-resistant-polypeptide gene had been introduced were selected by the method in Example 1-(4). One of these microorganisms and its recombinant DNA were named as "Bacillus subtilis TCU211 (FERM BP-2112)"and "pTCU211" respectively.

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, but not by EcoRI, BamHI, PstI, XhoI, BglII, AccI or XbaI.

Example 3

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

Example 3-(1)

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 sulfate to obtain a polypeptide fraction which was then 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.

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

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

Example 3-(2)

Partial amino acid sequence of polypeptide containing N-terminal end

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.

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

Example 4

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

Example 4-(1)

Preparation of plasmid pUC18

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

Example 4-(2)

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 cleaving a pUC18 specimen, prepared by the method in Example 4-(1), in the same manner, were subjected to $T_4$ DNA

ligase to obtain a recombinant DNA.

Example 4-(3)

Introduction of recombinant DNA into Escherichia coli

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

The recombinant DNA was introduced into this 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 selected.

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 the obtained cell was then treated with the alkaline mini-preparation method to obtain a recombinant DNA.

Example 4-(5)

Sequence of recombinant DNA

The recombinant DNA was sequenced by the dideoxy chain terminator method.

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, ($\alpha$-$^{32}$P) 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.

The dehydrated gel was then autographed, and the polypeptide gene was determined by analyzing the base bands on the radioautogram.

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.

The results were as shown in Table 1-2.

Example 4-(6)

Amino acid sequence of polypeptide

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.

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.

Example 5

Preparation of polypeptide with recombinant microorganism

Polypeptides were prepared with recombinant microorganisms Escherichia coli TCH201 (FERM BP-2109) and Bacillus subtilis TCU211 (FERM BP-2112) both in which recombinant DNA carrying heat-resistant-polypeptide gene derived from Bacillus stearothermophilus had been introduced.

The polypeptide productivities of these recombinant microorganisms were compared with those of host microorganism and donor Bacillus stearo-

Table 1-1

```
        10         20         30         40         50         60
GCTGGAAATC TTAATAAGGT AAACTTTACA TCAGATGTTG TCTATCAAAT TGTAGTGGAT

        70         80         90        100        110        120
CGATTTGTGG ATGGAAATAC 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 GCATCCTATC ATGGTTATTG GGCGCGCGAT

       310        320        330        340        350        360
TTCAAAAAGC CAAACCCGTT TTTTGGTACC CTCAGTGATT TCCAACGTTT AGTTGATGCC

       370        380        390        400        410        420
GCACATGCAA AAGGAATAAA GGTAATTATT GACTTTGCCC CCAACCATAC TTCTCCTGCT

       430        440        450        460        470        480
TCAGAAACGA ATCCTTCTTA TATGGAAAAC GGACGACTGT ACGATAATGG GACATTGCTT

       490        500        510        520        530        540
GGCGGTTACA CAAATGATGC CAACATGTAT TTTCACCATA ACGGTGGAAC AACGTTTTCC
```

Table 1-1 continued (1)

```
       550        560        570        580        590        600
AGCTTAGAGG ATGGGATTTA TCGAAATCTG TTTGACTTGG CGGACCTTAA CCATCAGAAC

       610        620        630        640        650        660
CCTGTTATTG ATAGGTATTT AAAAGATGCA GTAAAAATGT GGATAGATAT GGGGATTGAT

       670        680        690        700        710        720
GGTATCCGTA TGGATGCGGT GAAGCACATG CCGTTTGGAT GGCAAAAATC TCTGATGGAT

       730        740        750        760        770        780
GAGATTGATA ACTATCGTCC TGTCTTTACG TTTGGGGAGT GGTTTTTGTC AGAAAATGAA

       790        800        810        820        830        840
GTGGACGCGA ACAATCATTA CTTTGCCAAT GAAAGTGGAA TGAGTTTGCT CGATTTTCGT

       850        860        870        880        890        900
TTCGGACAAA AGCTTCGTCA AGTATTGCGC AATAACAGCG ATAATTGGTA TGGCTTTAAT

       910        920        930        940        950        960
CAAATGATTC AAGATACGGC ATCAGCATAT GACGAGGTTC TCGATCAAGT AACATTCATA

       970        980        990       1000       1010       1020
GACAACCATG ATATGGATCG GTTTATGATT GACGGAGGAG ATCCGCGCAA GGTGGATATG

      1030       1040       1050       1060       1070       1080
GCACTTGCTG TATTATTGAC ATCCCGTGGC GTACCGAATA TTTACTATGG TACAGAGCAA
```

Table 1-1 continued (2)

```
      1090       1100       1110       1120       1130       1140
TACATGACCG GTAACGGCGA TCCAAACAAT CGTAAGATGA TGAGTTCATT CAATAAAAAT

      1150       1160       1170       1180       1190       1200
ACTCGCGCGT ATCAAGTGAT TCAAAAACTA 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 TTACTCGATT

      1330       1340       1350       1360       1370       1380
ACTGGCTTAT TTACAGCTTT ACCAGCAGGA ACATATACGG ATCAGCTTGG CGGTCTTTTA

      1390       1400       1410       1420       1430       1440
GACGGAAATA CAATTCAAGT CGGTTCAAAT GGATCAGTTA ATGCATTTGA CTTAGGACCG

      1450       1460       1470       1480       1490       1500
GGGGAAGTCG GTGTATGGGC ATACAGTGCA ACAGAAAGCA CGCCAATTAT TGGTCATGTT

      1510       1520       1530       1540       1550       1560
GGACCGATGA TGGGGCAAGT CGGTCATCAA GTAACCATTG ATGGCGAAGG ATTCGGAACA

      1570       1580       1590       1600       1610       1620
AATACGGGCA CTGTGAAGTT CGGAACGACA GCTGCCAATG TTGTGTCTTG GTCTAACAAT
```

Table 1-1 continued (3)

```
      1630       1640       1650       1660       1670       1680
CAAATCGTTG TGGCTGTACC AAATGTGTCA CCAGGAAAAT ATAATATTAC CGTCCAATCA

      1690       1700       1710       1720       1730       1740
TCAAGCGGTC AAACGAGTGC GGCTTATGAT AACTTTGAAG TACTAACAAA TGATCAAGTG

      1750       1760       1770       1780       1790       1800
TCAGTGCGGT TTGTTGTTAA TAACGCGACT ACCAATCTAG GGCAAAATAT ATACATTGTT

      1810       1820       1830       1840       1850       1860
GGCAACGTAT ATGAGCTCGG CAACTGGGAC ACTAGTAAGG CAATCGGTCC AATGTTCAAT

      1870       1880       1890       1900       1910       1920
CAAGTGGTTT ACTCCTATCC TACATGGTAT ATAGATGTCA GTGTCCCAGA AGGAAAGACA

      1930       1940       1950       1960       1970       1980
ATTGAGTTTA AGTTTATTAA AAAAGACAGC CAAGGTAATG TCACTTGGGA AAGTGGTTCA

      1990       2000       2010       2020       2030       2040
AATCATGTTT ATACGACACC AACGAATACA ACCGGAAAAA TTATAGTGGA TTGGCAGAAC
```

Table 1-2

```
         10         20         30         40         50         60
ATGAGAAGAT GGCTTTCGCT AGTCTTGAGC ATGTCATTTG TATTTAGTGC AATTTTTATA

         70         80         90        100
GTATCTGATA CGCAGAAAGT CACCGTTGAA GCA
```

Table 2-2

|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1> | Met | Arg | Arg | Trp | Leu | Ser | Leu | Val | Leu | Ser | Met | Ser | Phe | Val | Phe |
| 16> | Ser | Ala | Ile | Phe | Ile | Val | Ser | Asp | Thr | Gln | Lys | Val | Thr | Val | Glu |
| 31> | Ala |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Table 2-1

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

Table 2-1 continued

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

thermophilus microorganism in relation to their CGTase activity.

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 each 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 microorganisms are advantageously usable in industrial-scale production of polypeptide because these microorganisms 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

Table 3

| Microorganism | CGTase activity (units/ml) | | |
|---|---|---|---|
| | Supernatant | Cell | Total |
| Escherichia coli TCH201 (FERM BP-2109) | 0.8 | 13.5 | 14.3 |
| Bacillus subtilis TCU211 (FERM BP-2112) | 46.7 | 20.5 | 67.2 |
| Escherichia coli HB101 | 0 | 0 | 0 |
| Bacillus subtilis 715A | 0 | 0 | 0 |
| Bacillus stearothermophilus FERM-P No.2225 | 8.5 | 0.3 | 8.8 |

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.

Example 6

Cloning of Bacillus macerans polypeptide gene into Escherichia coli

Example 6-(1)

Preparation of chromosome DNA carrying Bacillus macerans polypeptide gene

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

Example 6-(2)

Preparation of recombinant DNA carrying polypeptide gene

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.

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 dephosphorized by the method in Example 1-(3). The fragments thus obtained were ligated in accordance with the method in Example 1-(3) to obtain a recombinant DNA.

Example 6-(3)

Introduction of recombinant DNA into Escherichia coli

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.

Separately, a plasmid pBR322 specimen, obtained by the method Example 1-(2), was completely cleaved with restriction enzyme BamHI, and the 5'-terminal end of the resultant product was dephosphorized 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.

One of these recombinant microorganisms and its recombinant DNA were named as "Escherichia coli MAH2 (FERM BP-2110)" 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.

FIG.3 shows that this recombinant DNA is cleaved by either restriction enzyme PvuII, SalI, 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 BP-2110)in accordance with the method in Example 1-(2).

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.

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.

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.

One of the recombinant microorganisms and its recombinant DNA were named as "Bacillus subtilis MAU210 (FERM BP-2111)" 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 DNA is cleaved by either restriction enzyme PvuII, SalI, AvaI or PstI, but not by EcoRI, HindIII, KpnI, BamHI, XbaI, XhoI or SmaI.

Example 8

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

Example 8-(1)

Preparation of polypeptide

The polypeptide was produced by culturing *Bacillus subtilis* MAU210 (FERM BP-2111) with a liquid culture medium similarly as in Example 10, and then purified in accordance with the 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±10,000 daltons and a specific activity of 200±30 units/mg protein.

Example 8-(2)

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).

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

Example 9

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

Example 9-(1)

Preparation of recombinant DNA carrying polypeptide gene

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 4-(2), in the same manner, were ligated with $T_4$ DNA ligase to obtain a recombinant DNA.

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 Escherichia coli JM83 as the host microorganism to obtain a recombinant microorganism.

Example 9-(3)

Preparation of recombinant DNA from recombinant microorganism

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

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.

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

The results were as shown in Table 4-2.

Example 9-(5)

Amino acid sequence of polypeptide

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.

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

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

as well as 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.

Table 4-1

```
         10         20         30         40         50         60
TCCCCGGATA CGAGCGTGAA CAACAAGCTC AATTTTAGCA CGGATACGGT TTACCAGATT
         70         80         90        100        110        120
GTAACCGACC GGTTTGTGGA CGGCAATTCC GCCAACAACC CGACCGGAGC AGCCTTCAGC
        130        140        150        160        170        180
AGCGATCATT CCAACCTGAA GCTGTATTTC GGGGGCGACT GGCAGGGGAT CACGAACAAA
        190        200        210        220        230        240
ATCAACGACG GCTATCTGAC CGGAATGGGC ATCACCGCCC TCTGGATCTC GCAGCCGGTT
        250        260        270        280        290        300
GAGAACATCA CCGCCGTCAT CAATTATTCG GGCGTCAACA ATACAGCTTA CCACGGTTAC
        310        320        330        340        350        360
TGGCCTCGCG ACTTCAAGAA GACCAATGCC GCGTTCGGCA GCTTCACCGA CTTCTCCAAT
        370        380        390        400        410        420
TTGATCGCCG CAGCGCATTC 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
ACCGATTTCT CGACGACTGA AAGCGGTATC TACAAGAACC TGTACGATCT CGCGGATATC

       610        620        630        640        650        660
AATCAGAACA ACAACACCAT CGACTCGTAT CTCAAGGAAT CGATCCAGCT GTGGCTGAAT

       670        680        690        700        710        720
CTCGGAGTCG ACGGCATCCG CTTCGACGCC GTGAAGCATA TGCCTCAGGG CTGGCAGAAG

       730        740        750        760        770        780
AGCTACGTCT CGTCGATCTA CAGCAGCGCC AATCCGGTGT TCACCTTCGG TGAATGGTTC

       790        800        810        820        830        840
CTCGGCCCCG ACGAAATGAC CCAGGACAAC ATCAACTTCG CGAATCAGAG CGGCATGCAC

       850        860        870        880        890        900
CTGCTGGACT TTGCGTTTGC GCAGGAAATC CGTGAAGTGT TCCGCGACAA GTCGGAGACG

       910        920        930        940        950        960
ATGACCGACC TGAACTCGGT GATCTCCAGC ACCGGCTCCA GCTATAATTA CATCAACAAC

       970        980        990       1000       1010       1020
ATGGTTACGT 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 ACAACCGCGG CATGATGACC
      1150       1160       1170       1180       1190       1200
GGCTTCGATA CGAACAAGAC 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 AGCGCTTACT GCTCTGCCAA ACGGAACGTA TACCGACGTT
      1390       1400       1410       1420       1430       1440
CTCGGCGGCC TGCTTAATGG CAATTCAATT ACCGTTAACG GCGGCACGGT CAGCAACTTT
      1450       1460       1470       1480       1490       1500
ACACTTGCAG CGGGCGGTAC GGCAGTCTGG CAGTACACGA CGACGGAATC CTCGCCGATT
      1510       1520       1530       1540       1550       1560
ATCGGCAACG TCGGCCCGAC TATGGGCAAG CCCGGCAACA CCATCACGAT CGACGGACGC
      1570       1580       1590       1600       1610       1620
GGCTTCGGTA CTACGAAGAA CAAAGTTACT TTCGGTACGA CAGCCGTTAC CGGCGCGAAC
```

Table 4-1 continued (3)

```
      1630       1640       1650       1660       1670       1680
ATCGTGAGCT GGGAAGATAC CGAAATCAAG GTCAAAGTTC CGAACGTGGC CGCCGGCAAC

      1690       1700       1710       1720       1730       1740
ACGGCCGTTA CGGTAACGAA CGCCGCCGGC ACTACCAGCG CAGCGTTCAA CAACTTTAAC

      1750       1760       1770       1780       1790       1800
GTACTGACTG CCGATCAGGT CACTGTCCGC TTCAAAGTCA ACAATGCCAC CACGGCCCTG

      1810       1820       1830       1840       1850       1800
GGACAAAACG TCTACCTGAC CGGTAACGTC GCCGAGCTTG GCAACTGGAC AGCCGCCAAC

      1870       1880       1890       1900       1910       1920
GCAATCGGTC CGATGTACAA CCAGGTAGAA GCCAGCTATC CGACTTGGTA CTTCGACGTC

      1930       1940       1950       1960       1970       1980
AGCGTTCCGG CCAACACGGC GCTGCAATTC AAGTTCATCA AAGTGAACGG CTCGACAGTG

      1990       2000       2010       2020       2030       2040
ACTTGGGAAG GCGGCAACAA CCACACCTTC ACCTCGCCTT CGAGCGGCGT TGCGACCGTA

      2050       2060
ACGGTCGATT GGCAGAAC
```

<u>Table 4-2</u>

```
        10         20         30         40         50         60
ATGAAAAAGC AAGTCAAATG GTTGACGTCG GTGTCGATGT CCGTAGGGAT CGCACTCGGC

        70         80         90
GCGGCGCTGC CTGTATGGGC A
```

<u>Table 5-2</u>

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1> | Met | Lys | Lys | Gln | Val | Lys | Trp | Leu | Thr | Ser | Val | Ser | Met | Ser | Val |
| 16> | Gly | Ile | Ala | Leu | Gly | Ala | Ala | Leu | Pro | Val | Trp | Ala | | | |

Table 5-1

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

Table 5-1 continued

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

Example 10

Preparation of polypeptide with recombinant microorganism

Polypeptides were prepared with Escherichia coli MAH2 (FERM BP- ) and Bacillus subtilis MAU210 (FERM BP-2111) 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.

Escherichia coli MAH2 was inoculated to the liquid culture medium additionally containing 50 μg/ml of ampicillin, while Escherichia coli HB101 was inoculated to the liquid culture medium without addition of antibiotic. In each case, microorganism was cultured at 35°C for 24 hours under vigorous shaking conditions.

Bacillus subtilis MAU210 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 macerans 17A was cultured with the liquid culture medium at 28°C for 72 hours without addition of antibiotic.

Each culture was treated similarly as in Example 5, and its CGTase activities was then determined. The results were as shown in Table 6.

These evidences clearly show that the recombinant microorganisms are advantageously usable in industrial-scale production of polypeptide because they have an improved polypeptide productivity.

Table 6

| Microorganism | CGTase activity (units/ml) | | |
|---|---|---|---|
| | Supernatant | Cell | Total |
| *Escherichia coli* MAII2 (FERM BP-2110) | 0.6 | 11.8 | 12.4 |
| *Bacillus subtilis* MAU210 (FERM BP-2111) | 54.6 | 0.3 | 54.9 |
| *Escherichia coli* IIB101 | 0 | 0 | 0 |
| *Bacillus subtilis* 715A | 0 | 0 | 0 |
| *Bacillus macerans* 17A | 7.5 | 0.4 | 7.9 |

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 suitable 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-, ß- 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 confectionery 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-cariogenic 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.

The words "Toyopearl" and "Amberlite" used in this specification are registered Trade Marks.

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;

the said polypeptide having an isoelectric point of 5.0 $\pm$ 0.1.

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±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:

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

391> Ser Ser Leu Arg Arg Asn Asn Pro Ala Leu Ala Tyr Gly Asp Thr

406> Glu Gln Arg Trp Ile Asn Gly Asp Val Tyr Val Tyr Glu Arg Gln

421> Phe Gly Lys Asp Val Val Leu Val Arg Val Asn Arg Ser Ser Ser

436> Ser Asn Tyr Ser Ile Thr Gly Leu Phe Thr Ala Leu Pro Ala Gly

451> Thr Tyr Thr Asp Gln Leu Gly Gly Leu Leu Asp Gly Asn Thr Ile

466> Gln Val Gly Ser Asn Gly Ser Val Asn Ala Phe Asp Leu Gly Pro

481> Gly Glu Val Gly Val Trp Ala Tyr Ser Ala Thr Glu Ser Thr Pro

496> Ile Ile Gly His Val Gly Pro Met Met Gly Gln Val Gly His Gln

511> Val Thr Ile Asp Gly Glu Gly Phe Gly Thr Asn Thr Gly Thr Val

526> Lys Phe Gly Thr Thr Ala Ala Asn Val Val Ser Trp Ser Asn Asn

541> Gln Ile Val Val Ala Val Pro Asn Val Ser Pro Gly Lys Tyr Asn

556> Ile Thr Val Gln Ser Ser Ser Gly Gln Thr Ser Ala Ala Tyr Asn

571> Asn Phe Glu Val Leu Thr Asn Asp Gln Val Ser Val Arg Phe Val

586> Val Asn Asn Ala Thr Thr Asn Leu Gly Gln Asn Ile Tyr Ile Val

601> Gly Asn Val Tyr Glu Leu Gly Asn Trp Asp Thr Ser Lys Ala Ile

616> Gly Pro Met Phe Asn Gln Val Val Tyr Ser Tyr Pro Thr Trp Tyr

631> Ile Asp Val Ser Val Pro Glu Gly Lys Thr Ile Glu Phe Lys Phe

646> Ile Lys Lys Asp Ser Gln Gly Asn Val Thr Trp Glu Ser Gly Ser

661> Asn His Val Tyr Thr Thr Pro Thr Asn Thr Thr Gly Lys Ile Ile

676> Val Asp Trp Gln Asn

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 N-terminal side of said polypeptide.

7. The polypeptide in accordance with claim 4, whose partial amino acid sequence containing 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 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1> | Ser | Pro | Asp | Thr | Ser | Val | Asn | Asn | Lys | Leu | Asn | Phe | Ser | Thr | Asp |
| 16> | Thr | Val | Tyr | Gln | Ile | Val | Thr | Asp | Arg | Phe | Val | Asp | Gly | Asn | Ser |
| 31> | Ala | Asn | Asn | Pro | Thr | Gly | Ala | Ala | Phe | Ser | Ser | Asp | His | Ser | Asn |
| 46> | Leu | Lys | Leu | Tyr | Phe | Gly | Gly | Asp | Trp | Gln | Gly | Ile | Thr | Asn | Lys |
| 61> | Ile | Asn | Asp | Gly | Tyr | Leu | Thr | Gly | Met | Gly | Ile | Thr | Ala | Leu | Trp |
| 76> | Ile | Ser | Gln | Pro | Val | Glu | Asn | Ile | Thr | Ala | Val | Ile | Asn | Tyr | Ser |
| 91> | Gly | Val | Asn | Asn | Thr | Ala | Tyr | His | Gly | Tyr | Trp | Pro | Arg | Asp | Phe |
| 106> | Lys | Lys | Thr | Asn | Ala | Ala | Phe | Gly | Ser | Phe | Thr | Asp | Phe | Ser | Asn |
| 121> | Leu | Ile | Ala | Ala | Ala | His | Ser | His | Asn | Ile | Lys | Val | Val | Met | Asp |
| 136> | Phe | Ala | Pro | Asn | His | Thr | Asn | Pro | Ala | Ser | Ser | Thr | Asp | Pro | Ser |
| 151> | Phe | Ala | Glu | Asn | Gly | Ala | Leu | Tyr | Asn | Asn | Gly | Thr | Leu | Leu | Gly |
| 166> | Lys | Tyr | Ser | Asn | Asp | Thr | Ala | Gly | Leu | Phe | His | His | Asn | Gly | Gly |
| 181> | Thr | Asp | Phe | Ser | Thr | Thr | Glu | Ser | Gly | Ile | Tyr | Lys | Asn | Leu | Tyr |
| 196> | Asp | Leu | Ala | Asp | Ile | Asn | Gln | Asn | Asn | Asn | Thr | Ile | Asp | Ser | Tyr |
| 211> | Leu | Lys | Glu | Ser | Ile | Gln | Leu | Trp | Leu | Asn | Leu | Gly | Val | Asp | Gly |
| 226> | Ile | Arg | Phe | Asp | Ala | Val | Lys | His | Met | Pro | Gln | Gly | Trp | Gln | Lys |
| 241> | Ser | Tyr | Val | Ser | Ser | Ile | Tyr | Ser | Ser | Ala | Asn | Pro | Val | Phe | Thr |
| 256> | Phe | Gly | Glu | Trp | Phe | Leu | Gly | Pro | Asp | Glu | Met | Thr | Gln | Asp | Asn |
| 271> | Ile | Asn | Phe | Ala | Asn | Gln | Ser | Gly | Met | His | Leu | Leu | Asp | Phe | Ala |
| 286> | Phe | Ala | Gln | Glu | Ile | Arg | Glu | Val | Phe | Arg | Asp | Lys | Ser | Glu | Thr |
| 301> | Met | Thr | Asp | Leu | Asn | Ser | Val | Ile | Ser | Ser | Thr | Gly | Ser | Ser | Tyr |
| 316> | Asn | Tyr | Ile | Asn | Asn | Met | Val | Thr | Phe | Ile | Asp | Asn | His | Asp | Met |
| 331> | Asp | Arg | Phe | Gln | Gln | Ala | Gly | Ala | Ser | Thr | Arg | Pro | Thr | Glu | Gln |
| 346> | Ala | Leu | Ala | Val | Thr | Leu | Thr | Ser | Arg | Gly | Val | Pro | Ala | Ile | Tyr |
| 361> | Tyr | Gly | Thr | Glu | Gln | Tyr | Met | Thr | Gly | Asn | Gly | Asp | Pro | Asn | Asn |
| 376> | Arg | Gly | Met | Met | Thr | Gly | Phe | Asp | Thr | Asn | Lys | Thr | Ala | Tyr | Lys |

391> Val Ile Lys Ala Leu Ala Pro Leu Arg Lys Ser Asn Pro Ala Leu

406> Ala Tyr Gly Ser Thr Thr Gln Arg Trp Val Asn Ser Asp Val Tyr

421> Val Tyr Glu Arg Lys Phe Gly Ser Asn Val Ala Leu Val Ala Val

436> Asn Arg Ser Ser Thr Thr Ala Tyr Pro Ile Ser Gly Ala Leu Thr

451> Ala Leu Pro Asn Gly Thr Tyr Thr Asp Val Leu Gly Gly Leu Leu

466> Asn Gly Asn Ser Ile Thr Val Asn Gly Gly Thr Val Ser Asn Phe

481> Thr Leu Ala Ala Gly Gly Thr Ala Val Trp Gln Tyr Thr Thr Thr

496> Glu Ser Ser Pro Ile Ile Gly Asn Val Gly Pro Thr Met Gly Lys

511> Pro Gly Asn Thr Ile Thr Ile Asp Gly Arg Gly Phe Gly Thr Thr

526> Lys Asn Lys Val Thr Phe Gly Thr Thr Ala Val Thr Gly Ala Asn

541> Ile Val Ser Trp Glu Asp Thr Glu Ile Lys Val Lys Val Pro Asn

556> Val Ala Ala Gly Asn Thr Ala Val Thr Val Thr Asn Ala Ala Gly

571> Thr Thr Ser Ala Ala Phe Asn Asn Phe Asn Val Leu Thr Ala Asp

586> Gln Val Thr Val Arg Phe Lys Val Asn Asn Ala Thr Thr Ala Leu

601> Gly Gln Asn Val Tyr Leu Thr Gly Asn Val Ala Glu Leu Gly Asn

616> Trp Thr Ala Ala Asn Ala Ile Gly Pro Met Tyr Asn Gln Val Glu

631> Ala Ser Tyr Pro Thr Trp Tyr Phe Asp Val Ser Val Pro Ala Asn

646> Thr Ala Leu Gln Phe Lys Phe Ile Lys Val Asn Gly Ser Thr Val

661> Thr Trp Glu Gly Gly Asn Asn His Thr Phe Thr Ser Pro Ser Ser

676> Gly Val Ala Thr Val Thr Val Asp Trp Gln Asn

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 recombinant microorganism in which a recombinant DNA carrying CGTase gene has been introduced.

11. The polypeptide in accordance with claim 1, which originates from a microorganism of the species *Bacillus stearothermophilus*.

12. A recombinant DNA having a *PvuII* restriction cleavage site and carrying CGTase gene, said recombinant DNA comprising:

a DNA fragment, obtained by digesting the DNA of a donor microorganism capable of producing CGTase in which the CGTase has the one or more partial amino acid sequences as defined in claim 1 or 2, with a restriction enzyme *in vitro*; and

a vector fragment, obtained by cleaving a vector with the restriction enzyme, these fragments being ligated.

13. The recombinant DNA in accordance with claim 12, wherein said donor microorganism is of genus *Bacillus*.

14. The recombinant DNA in accordance with claim 12, wherein said donor microorganism is a member selected from the group consisting of *Bacillus stearothermophilus* and *Bacillus macerans*.

15. The recombinant DNA in accordance with claim 12, which has restriction cleavage sites as shown in FIG. 1, 2, 3 or 4.

16. A biologically-pure culture of a recombinant microorganism in which has been introduced a recombinant DNA as defines in claim 12 or claim 15.

17. The culture in accordance with claim 16, wherein said recombinant DNA has restriction cleavage sites as shown in FIG. 1, 2, 3 or 4.

18. The culture in accordance with claim 16, wherein said recombinant microorganism is of genus Escherichia or Bacillus.

19. The culture in accordance with claim 16, wherein said recombinant microorganism is a member selcted from the group consisting of Escherichia coli TCH201 (FERM BP-2109) or Escherichia coli MAH2 (FERM BP-2110).

20. The culture in accordance with claim 16, wherein said recombinant microorganism is a member selected from the group consisting of Bacillus subtilis MAU210 (FERM BP-2111) and Bacillus subtilis TCU211 (FERM BP-2112).

21. A process for producing CGTase, comprising:

culturing with a nutrient culture medium a recombinant microorganism, in which has been introduced a recombinant DNA as defined in claim 12 or claim 15; and

recovering the accumulated CGTase.

22. The process in accordance with claim 21, wherein said recombinant microorganism is of genus Escherichia or Bacillus.

23. The process in accordance with claim 21, wherein said recombinant microorganism is a member selected from the group consisting of Escherichia coli TCH201 (FERM BP-2109) or Escherichia coli MAH2 (FERM BP-2110).

24. The process in accordance with claim 21, wherein said recombinant microorganism is a member selected from the group consisting of Bacillus subtilis MAU210 (FERM BP-2111) and Bacillus subtilis TCU211 (FERM BP-2112).

25. A process for producing a saccharide-transferred product, comprising subjecting an amylaceous substance to the action of a recombinant polypeptide possessing CGTase activity; said recombinant polypeptide comprising one or more partial amino acid sequences selected from the group (a) to (e) as defined in claim 1.

26. The process in accordance with claim 25, wherein said saccharide-transferred product is cyclodextrin.

27. The process in accordance with claim 25, wherein said amylaceous substance is subjected to the action of said recombinant polypeptide in the presence of a saccharide acceptor.

28. The process in accordance with claim 25, wherein said amylaceous substance is selected from the group consisting of starch, amylose, cyclodextrin, dextrin, and mixtures thereof.

29. The process in accordance with claim 27, wherein said saccharide acceptor is a member selected from the group consisting of saccharide sweetener, glycoside, and mixtures thereof.

30. The process in accordance with claim 27, wherein the saccharide-transferred product is a member selected from the group consisting of $\alpha$-glycosylsucrose, $\alpha$-glycosyl stevioside, and $\alpha$-glycosyl ginsenoside.

31. The process in accordance with claim 27, wherein said saccharide-transferred product is used as sweetener.

32. A polypeptide according to claim 1, substantially as hereinbefore described with reference to any one of the foregoing Examples 1 to 10.

33. A process according to claim 21, substantially as hereinbefore described with reference to any one of the foregoing Examples 1 to 10.

34. A process according to claim 25, substantially as hereinbefore described with reference to any one of the foregoing Exmples 11 to 14.

35. Cyclomaltodextrin Glucanotransferase whenever obtained by a process as claimed in any one of claims 21 to 24 and 33.

36. A saccharide transferred product whenever obtained by a process as described in any one of claims 25 to 31 and 34.

---

REGISTER ENTRY FOR GB2169902

Form 1 Application No GB8529759.6 filing date 03.12.1985

Priorities claimed:
03.12.1984 in Japan - doc: 59255339
14.10.1985 in Japan - doc: 60228169

Title POLYPEPTIDE POSSESSING CYCLOMALTODEXTRIN GLUCANOTRANSFERASE ACTIVITY

Applicant/Proprietor
KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO, Incorporated in Japan, 2-3 1-chome Shimoishii, Okayama-shi, Okayama, Japan
[ADP No. 00864728002]

Inventors
TOSHIYUKI SUGIMOTO, 7-10-301 1-chome Okuda, Okayama-shi, Okayama, Japan
[ADP No. 03687779001]

MICHIO KUBOTA, 190-3 Nakai, Okayama-shi, Okayama, Japan
[ADP No. 03687787001]

SHUZO SAKAI, 3-41 1-chome Ejiri-asahigaoka, Seto-cho, Akaiwa-gun, Okayama, Japan
[ADP No. 02924793001]

Classified to
C3H C2S U1S
C12N C13K

Address for Service
PAGE WHITE & FARRER, 5 Plough Place, New Fetter Lane, LONDON, EC4A 1HY, United Kingdom
[ADP No. 00001255001]

Publication No GB2169902 dated 23.07.1986

Examination requested 15.01.1987

Patent Granted with effect from 06.09.1989 (Section 25(1)) with title
POLYPEPTIDE POSSESSING CYCLOMALTODEXTRIN GLUCANOTRANSFERASE ACTIVITY

---

**** END OF REGISTER ENTRY ****

RENEWAL DETAILS

PUBLICATION NUMBER GB2169902

PROPRIETOR(S)

Kabushiki Kaisha Hayashibara Seibutsu Kagaku Kenkyujo, Incorporated in Japan, 2-3 1-chome Shimoishii, Okayama-shi, Okayama, Japan

| | |
|---|---|
| DATE FILED | 03.12.1985 |
| DATE GRANTED | 06.09.1989 |
| DATE NEXT RENEWAL DUE | 03.12.1993 |
| DATE NOT IN FORCE | |
| DATE OF LAST RENEWAL | 01.12.1992 |
| YEAR OF LAST RENEWAL | 08 |
| STATUS | PATENT IN FORCE |