ISOAMYLASE GENE FROM FLAVIOBACTERIUM SP., COMPOSITIONS CONTAINING IT AND METHODS USING IT

A method of producing plant products containing modified starch content, including higher ratios of amylose to amylopectin, increase in intermediate material, or amylopectin having fewer branches or altered branching pattern. Also provided are DNA constructs and transformed plant cells useful in that method. The preferred method uses isoamylase from a Flavobacterium sp., more preferably in combination with a gene encoding ADPglucose pyrophosphorylase. Also disclosed are the gene from Flavobacterium sp. and transformed bacterial and plant cells containing a derivative thereof.
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ISOAMYLASE GENE FROM FLAVIOBACTERIUM SP., COMPOSITIONS CONTAINING IT AND METHODS USING IT

BACKGROUND OF THE INVENTION

The majority of plant starches are composed of two polysaccharide fractions, amylopectin and amylose. Depending on the plant source, starch amylopectin content typically ranges from 70-85%, while amylose is at a corresponding 15-30%. Amylopectin, which is highly branched and bush shaped, consists of linear chains of α-1,4-linked D-glucose residues, frequently branched together, on average every 20-25 D-glucose residues, by α-1,6-D-glucosidic linkages. The amylose fraction is composed of linear α-1,4-linked D-glucose polymers and averages one α-1,6-D-glucosidic bond for every 1000 linear D-glucose residues. This essentially linear nature of amylose allows it to form a helical structure which complexes with fatty acids, low molecular weight alcohols, and iodine. While amylopectin has a molecular weight of approximately 10^7, with a degree of polymerization of several thousand or more, amylose has a molecular weight of approximately 10^5 with a degree of polymerization which ranges from about 900 for corn starch to 4000 for potato starch. In addition, starch from some plant varieties contains a lightly-branched, third fraction referred to as intermediate material. Although not extensively characterized, this intermediate fraction resembles both amylopectin and amylose, in that it has a has a degree of polymerization less than 1000, and may consist of only four or five branches with a chain length of 50 or more glucose residues.

Mutants are known in many plant species in which the ratio of amylose to amylopectin is increased. The starch products of such mutants are commercially valuable, but production is limited due to many problems, including low yields of total starch in the major crops in which such mutants are found, i.e., corn and rice. There are three maize endosperm mutants, amyllose extender (ae), dull (du-1), and sugary (su-1), which contain increased amounts of amylose in their granules, but have significantly lower starch yields (Preiss, J. in The Biochemistry of Plants, v. 14. New York: Academic Press, 1988). A variety of potato (ND860-2) having increased amylose content is also known to have improved cold storage properties.
High amylose starches have unique properties for film formation and gelling, mainly by imparting a dramatic increase in gel strength due to amylose retrogradation. Advantages can include a) reduced oil pick-up in fried batter coatings, b) more rapid gelation (reduced drying time), and enhanced structure and texture in candies and soft jelly gums, and c) prevention of sogginess and increased crispiness in microwave foods. Furthermore, increasing the amylose content in foods such as potato and corn can bring about significant reductions in the caloric values of fried potato or corn products, mainly through a decrease in oil absorption. Finally, increasing the content of amylose has positive implications for non-food uses of starch, such as for the sizing of paper and board, for wet-end additives to pulp, and for paper lamination of corrugated boards, in the paper, packaging, and textile industries.

Isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) hydrolyzes the α-1,6-D-glucosidic linkages of starch, glycogen, and derived oligosaccharides, and is considered a direct debranching enzyme in that it attacks unmodified glycogen and starch, as opposed to indirect debranching enzymes which require initial polymer modification by a previous enzyme(s). Isoamylase is differentiated from the other major starch debranching enzyme, pullulanase, by its ability to cleave all the α-1,6 linkages of glycogen but not those of pullulan, whereas pullulanase completely hydrolyzes pullulan to maltotriose but has limited debranching activity on glycogen. Bacteria known to produce isoamylase include *Pseudomonas* sp. SMP1, *Pseudomonas amylofera* sp. SB-15, *Flavobacterium* sp., *Cytophaga* sp. (*Lyso- bacter* sp.), *Bacillus amyloliquefaciens*, and an alkalophilic strain of *Bacillus*.

While higher plants have been considered to have only debranching enzymes of the pullulanase type, a putative isoamylase from potato tuber has been described by Ishizaki et al., *Agric. Biol. Chem.* 47:771-779, 1983; Lee and Whelan, “Glycogen and starch debranching enzymes,” p. 191-234 *In* P. D. Boyer (ed.), *The Enzymes*, vol 5, 1971.) Two different pullulanases were also described from the same tuber extract. The potato isoamylase had substrate specificities similar to the *Pseudomonas* isoamylase, but had a higher pH optimum (5.5-6.0), and consisted of a dimer with a tentative molecular weight of 178,000 Kd. The physiological role of the potato
isoamylase is not well understood although it is speculated that it is involved in the enzymatic breakdown of starch during sprouting. One potato debranching enzyme activity has been reported to be pullulanase-like (by Kossman, J., et al. at the 1st Intl. Conf. on Plant Polysaccharide Engineering, Trondheim, Norway, June 1994).

It is an object of the present invention to provide a method of producing higher amylose starches. It is another object of the present invention to provide structurally modified starches having fewer α-1,6-D-glucosidic branches or an altered pattern of branching, thus having improved function properties for food and non-food uses. It is a further object of the present invention to provide DNA constructs for use in that method and plant cells containing those constructs. It is a still further object of the present invention to provide food crops having improved processing capabilities due to a structural modification of the starch content. It is a still further object of the present invention to provide transformed bacteria capable of producing an isoamylase having an improved pH optimum. It is a still further object of the present invention to provide a method of debranching starch using that isoamylase.

SUMMARY OF THE INVENTION

The present invention provides a gene for an isoamylase from Flavobacterium sp. as shown in SEQ ID NO:1. The invention further provides a DNA construct comprising in operative order:

a) a promoter which functions in plant cells to cause the production of an RNA sequence;

b) a structural coding sequence that encodes for an isoamylase; and
c) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

wherein said promoter is heterologous with respect to the structural coding sequence and wherein said promoter is operatively linked with said structural coding sequence, which is in turn operably linked with said nontranslated region. Preferably the structural coding sequence further comprises a sequence which encodes for a plastid targeting sequence. The invention further provides plant cells transformed to contain said DNA construct. The preferred isoamylase has an optimum pH from about 5 to about 8,
and the most preferred isoamylase has the sequence shown in SEQ ID NO:11.

Optionally plants transformed with the DNA construct of the present invention may contain other heterologous DNA constructs which provide other improvements. Such improvements may be unrelated to starch production, for example, herbicide resistance, disease resistance, or insect resistance. Alternatively, genes related to the production of starch in plant sink tissues may be used. Examples include granule bound starch synthases, starch branching enzymes, soluble starch synthases, and ADP-glucose pyrophosphorylases. Expression of a native starch branching enzyme may also be down-regulated by using an inverted sequence (antisense) or other means in order to effect other modifications in the starch molecule. It is preferred that the plants of the present invention also contain a gene which expresses a heterologous or foreign ADPglucose pyrophosphorylase, more preferably the E. coli glgC16 gene for ADPglucose pyrophosphorylase. Its use in plants and plants transformed therewith are disclosed in WO 91/19806 (Kishore) which is equivalent to U.S. Serial Number 08/120,703, filed July 13, 1993, which is incorporated herein by reference.

The invention further provides methods of producing starch having a higher amylose content or a structurally modified starch content comprising transforming plant cells to contain said DNA construct, regenerating whole plants, multiplying said plants, harvesting materials therefrom, and extracting the starch therefrom.

The invention further provides novel plant crops having modified starch content comprising transforming plant cells to contain said DNA construct, regenerating whole plants, and harvesting the starch containing crop. Such plants crops may include the tubers of potatoes, the roots of cassava or sweet potato, and the seeds of corn, wheat, rice, or barley.

The invention further provides transformed organisms containing a gene which encodes an isoamylase having substantially the amino acid sequence shown in SEQ ID NO:11. The invention further provides the isolated isoamylase produced by such transformed bacteria which may be used in debranching starch.
As used herein, the term "isoamylase" means an enzyme capable of hydrolyzing the α-1,6-D-glucosidic linkages of starch, glycogen, and derived oligosaccharides.

As used herein, the term "modified starch content" means containing starch having amylpectin with fewer branches or an altered pattern of branching, increased intermediate material, and/or a higher ratio of amylose to amylpectin. As used herein, the term "modified starch structure" means having amylpectin with fewer branches or an altered pattern of branching, increased intermediate material, and/or a higher ratio of amylose to amylpectin. As used herein, the term "structurally modified starch" means starch having amylpectin with fewer branches or an altered pattern of branching, increased intermediate material, and/or a higher ratio of amylose to amylpectin. In all cases, the modified starch is compared to that produced by plants of the same genotype except for the introduced isoamylase gene.

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises a new method of producing plant starches having a structurally modified starch structure, preferably including a higher ratio of amylose to amylpectin, by transforming a starch producing plant with a gene for a polypeptide having the properties of an isoamylase. The gene may be derived from a variety of sources including plants and microorganisms which exhibit isoamylase activity. The preferred isoamylase has an optimum pH from about 5 to about 8, which is near the pH of the interior of an amyloplast. Such an isoamylase is provided.

For brevity of disclosure, the following detailed description of the invention will be limited to the isoamylase of Flavobacterium sp. Those skilled in the art will readily recognize that the methods described herein can be employed to utilize isoamylase genes from other sources.

Flavobacterium sp. Isoamylase

The isoamylase used in the following experiments is naturally produced by an organism, which has been characterized as a Flavo-
bacterium species, disclosed in Sato, H.H. and Park, Y.K. Starch 32:132-
136, 1980. *Flavobacterium* sp. was grown aerobically at 28 °C on Luria-Bertani (LB) agar, or in LB broth with shaking at 200 rpm.

For purification of isoamylase, *Flavobacterium* sp. was grown in 500 ml of 0.1% tryptone, 0.2% yeast extract, 0.1% casamino acids, and 0.8% maltose, with shaking at 225 rpm in 2 liter flasks at 30 °C for 20 hr. The cells were removed by centrifugation at 15,000 x g, and the supernatant was filtered through a 0.2μm filter. The cell-free supernatant was concentrated to 20 ml in an Amicon stirred cell concentrator with YM-10 membrane, and then to 2 ml in an Amicon Centriprep-30 centrifugal concentrator. The retentate was applied to an amylose/agarose affinity matrix column (2.5 cm x 5 cm) pre-equilibrated with 100 mM sodium phosphate, pH 6.5 (buffer A). The column was washed with 250 ml of buffer A at 1.5 ml/min, or until no detectable protein eluted in the wash. Isoamylase was eluted from the column with 25% maltose in buffer A at 1.5 ml/min. Active one ml fractions were pooled, concentrated to 250 μl, and re-equilibrated with buffer A to remove the maltose, using Amicon Centriprep-30 and Centricon-30 centrifugal concentrators. Glycerol was added to 20% final concentration, and the isoamylase was stored at 4 °C until evaluation for purity by SDS-PAGE.

The isoamylase has a molecular weight in the range of 80-85 kD, and co-purified with a second minor band having a molecular weight in the range of 70-75 kD as determined by SDS-PAGE. It has a specific activity of approximately 50,000 units/mg protein, as determined by the standard iodine assay. [A mixture is prepared with 1 ml of 1% amylopectin, 0.2 ml of 0.2 M sodium phosphate (pH 6.5), 0-0.2 ml of enzyme preparation, and water to a final volume of 1.4 ml. Upon addition of enzyme, the reaction mixture is incubated at 40 °C. At various time intervals, 0.2 ml of the reaction mixture is added to 0.2 ml of 0.2% I₂, 2.0% KI, 0.2% H₂SO₄ , and diluted to 10 ml with water. After 15 min at room temperature, the absorbance is read at 610 nm. One unit of isoamylase activity is defined as the amount of enzyme causing a 610 nm absorbance increase of 0.01 in one hour.]

These results differ from those reported by Sato and Park (molecular weight of 121,000 as determined by size exclusion chromatography and a final specific activity of 11,110 units/mg protein). The difference in molecular weight is probably attributable to a more accurate
determination using SDS-PAGE. Furthermore, the difference in specific activity may be attributable to a protein purity difference, since purification of isoamylase to homogeneity as determined by SDS-PAGE was not presented in the original report.

N-terminal Sequencing

The purified _Flavobacterium_ sp. enzyme (75 µg) was sequenced directly from 75 µl (3 x 25 µl loadings) of a 200 µM sodium phosphate, pH 6.5, solution. An Applied Biosystems, Inc. model 470A gas phase sequencer was employed for amino terminal, automated Edman degradation chemistry, using the standard sequencer cycle, 03RPTH. The respective PTH-aa derivatives were identified by RP-HPLC analysis in an on-line fashion employing an Applied Biosystems, Inc., Model 120A PTH Analyzer fitted with a Brownlee 2.1 mm I.D. PTH-C18 column. The following N-terminal sequence was determined: AIDAQQQLGARYDAQAANLAFRVYSS-RATXVEXFLYKNP (SEQ ID NO:3). This sequence is 55% identical to the N-terminal sequence of the processed, mature _Pseudomonas_ sp. isoamylase (Tognoni et al., _J. Gen. Microbiol._ 135:37-45, 1989), and indicates the probable cleavage of a signal peptide from the _Flavobacterium_ sp.

Isoamylase purified to homogeneity from _Flavobacterium_ sp. culture was also blotted onto a PVDF membrane (Immobilon, Millipore Corp.), and both major (upper) and minor (lower) bands were cut out. Samples blotted onto PVDF were sequenced directly in an N-terminal fashion using the sequencer cycle, 01RPVD. The PVDF-blotted N-terminal sequences of both bands were identical and corresponded to SEQ ID NO:3, which indicates a probable C-terminal truncation of the native enzyme _in vivo_ or during purification.

Gene Sequencing

All basic DNA manipulations such as PCR, agarose electrophoresis, restriction digests, ligations, _E. coli_ transformations, blue-white colony screens, colony lifts, and Southern blots were performed by standard protocols as described in Sambrook et al., _Molecular cloning: A laboratory manual_, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y, 1989.
The cell pellet from a 24 hr, 100 ml 

*Flavobacterium* sp. culture was resuspended in 3.5 ml of 10 mM EDTA, 25 mM Tris (pH 8.0), 5% glycerol, and 400 μl of 10% SDS was added. The suspension was incubated on ice for 10 min, and then frozen on dry ice for 10 min. The suspension was thawed and heated with occasional gentle rocking in a 70 °C water bath, for 5 min or until the cells lysed. The lysate was gently extracted twice with 4 ml of phenol-chloroform (1:1), then once with chloroform-isoamyl alcohol (24:1), and the DNA was precipitated by the addition of 10 ml of absolute ethanol. Without centrifugation, the supernatant was decanted, and the DNA precipitate was dissolved in 2 ml of 10 mM Tris, pH 8.0, 1.0 mM EDTA (TE8 buffer). The DNA was precipitated without centrifugation with 225 μl of 3 M sodium acetate, pH 5.2, and 6.0 ml of ethanol. The precipitate was redissolved in 0.5 ml of TE8, treated with 25 μl (300 units) of DNase-free RNase (United States Biochemical) for 20 min at 37 °C, and then extracted with an equal volume of phenol-chloroform. The DNA was precipitated, redissolved in TE8 buffer, and dialyzed against TE8 buffer (4 x 1.0 l) for 18 hr.

A DNA probe for isoamylase was generated by PCR amplification from *Flavobacterium* sp. chromosomal DNA. Degenerate PCR primers were designed from two highly conserved, consensus amino acid sequences among bacterial glucanases. These are SEQ ID NO:8 and SEQ ID NO:9. Specifically, the enzyme sequences used were for *Pseudomonas* sp. isoamylase, *Klebsiella pneumoniae* pullulanase, and *Bacillus stearothermophilus* neopullulanase. The oligonucleotide primers were also designed with a high GC content to reflect the high GC content of the *Flavobacterium* genome.

The PCR cycling conditions were as follows: 94 °C, 3'; 60 °C, 2'; 72 °C, 3' (5 cycles); 94 °C, 30s; 60 °C, 2'; 72 °C, 3' (30 cycles). The 260 bp PCR product was gel purified, ligated into the EcoRV site of pBSSK+, and clones were isolated by a blue-white screen as described in Sambrook et al.

The nucleotide sequence of the 260 bp PCR product, generated from *Flavobacterium* sp. chromosomal DNA, is SEQ ID NO:4. Sequence analysis shows it to be 56% identical at the amino acid level to the same region within the *Pseudomonas* sp. isoamylase as published by Tognoni et al. This 260 bp fragment was random prime-labelled with digoxigenin according to the Genius™ Nonradioactive DNA Labelling System.
Flavobacterium sp. genomic DNA was individually digested to completion with BamHI, BglII, ClaI, EcoRI, NotI, PstI, SacI, and XhoI. The digests were Southern blotted onto Zeta-Probe membranes (Bio-Rad), baked under vacuum at 80 °C for 1 hr, probed with the digoxigenin-labelled 260 bp fragment, and detected as recommended by the Genius™ Nonradioactive Detection System. Hybridization was overnight at 68 °C, and final washings were with 0.2 x SSC, 0.1% SDS at 68 °C. A single probe-positive band was obtained for each digest, with BglII, NotI, and PstI giving a band of approximately 10 kb, 9 kb, and 5 kb, respectively. Genomic BglII fragments (9-12 kb), NotI fragments (7-10 kb), and PstI fragments (4-6 kb) were isolated by preparative agarose gel electrophoresis, and ligated into their respective, dephosphorylated sites in pBSSK+. The ligations were transformed into E. coli DH5α, and transformant colonies were lifted onto Hybond™-N+ nylon membranes. The membranes were laid colony-side up on fresh LB-ampicillin plates and incubated at 37 °C for 4 hours. Colonies were lysed by placing membranes for 5 min, consecutively, on Whatman 3MM paper saturated with 10% SDS, followed by 0.5 M NaOH, 1.5 M NaCl, followed by 1 M Tris-HCl, pH 8.0, 1.5 M NaCl. Cell debris was completely removed by washing with 5 X SSC, and the membranes were baked under vacuum at 80 °C for 1 hr.

Hybridization of the same 260 bp probe (SEQ ID NO:4) and detection were essentially as described for Southern blots. Putative probe-positive colonies were prepared for plasmid DNA, and verified for probe-positive inserts by Southern blot hybridization. In order to screen for isoamylase activity, E. coli SR193 containing pGP1-4 was individually transformed with two NotI probe-positive clones, three PstI probe-positive clones, and pBSSK+ as control. E. coli SR193 contains a temperature sensitive, excision-defective lambda prophage. When colonies grown at 28 °C are transferred and incubated at 37 °C, the lambda lytic genes are induced and colonies become “porous” due to cell wall degradation. pGP1-4 is a ColE1-based plasmid which contains the bacteriophage T7 RNA polymerase under control of the temperature inducible lambda P_L promoter, and the gene for the heat-sensitive lambda repressor, c1857 (Tabor et al., Proc. Natl. Acad. Sci. USA, 82:1074-1078, 1985). Transformations were plated on LB agar containing amylopectin (1%), ampicillin,
and kanamycin, and assayed for isoamylase expression using the plate detection assay. [Isoamylase plate detection assay: After an initial incubation at 28 °C for 20 hr, or until colonies were 1 mM in diameter, the plates were transferred to 37 °C for an additional 20 hr to induce cell wall leakiness (lambda lytic genes) and T7 RNA polymerase transcription. The plates were inverted over several drops of a 2% I2, 1% KI, 25% ethanol solution, and isoamylase activity was detected by a blue halo surrounding the colonies (due to the formation of amyllose from amylopectin).]

The NotI clones did not show isoamylase activity; however, the PstI clones exhibited an intense blue halo surrounding the colonies due to the release of active isoamylase. One active isolate with a 4.9 kb PstI insert was chosen for all subsequent analyses, and was designated pMON17481.

Restriction fragments from the PstI insert of pMON17481 were subcloned in both orientations in pBSSK+ and pBSKS+. Single-stranded DNA was prepared from E. coli JM101 carrying pMON17481 derivatives, after superinfection with M13K07 helper phage (Bio-Rad). Single-stranded DNA was purified using the ssPhage™ DNA Isolation Kit from Bio 101, and sequenced by the dideoxy chain termination method using the TAQuence™ Version 2.0 DNA Sequencing Kit from United States Biochemical. Custom synthesized primers were used to sequence in both directions, with and without 7-deaza-dGTP, the Flavobacterium sp. isoamylase open reading frame and the 5' and 3' untranslated regions. Sequence analysis was performed using the University of Wisconsin GCG Sequence Analysis Software Package.

The nucleotide sequence thus determined is shown in SEQ ID NO:1. Sequence analysis revealed a 2334 bp open reading frame that encodes a 777 amino acid pre-enzyme. This translation (SEQ ID NO:2) has a predicted molecular weight of 84,340.

**Expression in E. coli**

Using pMON17481 as template, PCR mutagenesis was performed to remove the microbial signal sequence from the cloned gene. The following primers were designed to add a methionine residue to the beginning of the processed mature isoamylase (alanine-33) to allow translational initiation, and an additional stop codon (TAA) 3' adjacent to the endogenous
TGA codon. The primers also included N-terminal NcoI and C-terminal SacI and EcoRI sites.

N-terminal: 5'-GGGGCCATGGCCATCGATGCGCAGCTCGG-CGCGCGCTACGAC-3' (SEQ ID NO:5)

C-terminal: 5'-CCCCGAATTCGAGCTCTTATCATTCTCGAGATCACGAGCGA -3' (SEQ ID NO:6).

PCR reactions required either 5% formamide or 10% DMSO, final concentration, due to the high GC content of the coding sequence. PCR cycling conditions were as follows: 96 °C, 3'; 65 °C, 2'; 72 °C, 3' (5 cycles);

an additional 5 units of Taq polymerase were added per reaction, followed by 94 °C, 30s; 65 °C, 2'; 72 °C, 3'+20 sec/cycle (30 cycles). The 2.3 kb PCR product (SEQ ID NO:10) was gel purified and cloned into the NcoI-EcoRI sites of a pUC-based E. coli cloning vector in which a modified polylinker is located within the lacZ α-peptide gene and expression is driven by the lac promoter. Clones with the 2.3 kb insert (SEQ ID NO:10) were identified in E. coli JM101 using a blue-white screen. The protein expressed has the sequence shown in SEQ ID NO:11.

Isoamylase was also prepared from liquid cultures of E. coli JM101 transformants [25 ml of LB-ampicillin broth, 200 rpm, at 37 °C, and inducing expression with 0.5 mM IPTG, at optical density (610 nm) of 0.5]. Cells were pelleted at 3 hr post-induction, resuspended in 500 µl of 100 mM sodium phosphate, pH 6.5, and sonicated (Heat Systems-Ultrasonics, Inc., Model W-375) on ice for 2 x 10 sec bursts at 30% relative maximum output. The crude lysate was centrifuged at 12,000 x g for 5 min, and debranching activity was measured in the supernatant by the standard iodine assay described above.

The isolate producing the highest level of isoamylase activity, pMON17408, was chosen and the 2.3 kb insert (SEQ ID NO:10) was subcloned into the NcoI-EcoRI sites of an E. coli expression vector to give pMON17409. This vector contains the pACYC replicon and the ribosome binding site of the synthetic G10 leader sequence in which expression is driven by the tac promoter. High level isoamylase expression from pMON-17409 in E. coli JM101 was achieved by induction with IPTG of a 500 ml LB-kanamycin culture, as described above. At times 0, 1, 2, and 3 hr, post-induction, 1 ml aliquots were removed from the culture, pelleted, resuspended in a volume (µls) of Laemmli sample buffer that corresponded
numerically to the optical density in Klett units, and boiled for 8 min. The appearance of a new, highly-expressed 80-85 Kd protein was determined by SDS-PAGE. The remaining cells were pelleted at 3 hr post-induction, resuspended in 8 ml of 100 mM sodium phosphate, pH 6.5, sonicated on ice for 2 x 20 sec bursts, and centrifuged at 15,000 x g for 5 min.

Isoamylase from the intracellular crude supernatant from a 500 ml culture was purified to homogeneity as described above. The purified recombinant isoamylase was verified for cross reactivity with anti-

*Flavobacterium* sp. isoamylase polyclonal antibodies by western blot analysis. Interestingly, the minor 70-75 Kd band seen with extracellular isoamylase purified from *Flavobacterium* sp. was not detected, and suggests that the C-terminal truncation of the donor enzyme was due to cleavage by a *Flavobacterium* protease. The purified recombinant enzyme (SEQ ID NO:11) had a specific activity of approximately 50,600 Units/mg, which is similar to that determined for purified donor enzyme, and had the same molecular weight as determined by SDS-PAGE.

**Other Hosts for Fermentation**

The isoamylase of the present invention (SEQ ID NO:11) can be produced in and prepared from a variety of host organisms. The gene for producing this enzyme in a host organism may be the gene (SEQ ID NO:1) for the immature sequence having a signal sequence for secretion or the gene (SEQ ID NO:10) for the mature sequence. Alternatively, a secretion signal sequence native to the host organism may be used.

*E. coli* transformed as described above may be used to prepare the isoamylase by fermentation. Alternatively, a *Bacillus* sp. may be used as described in *Molecular Biological Methods for Bacillus*, Harwood et al. (eds.), New York: Wiley-Interscience, 1990. See also F.G. Priest, *Aspects of Microbiology*, 9, *Extracellular Enzymes*, Washington: American Society for Microbiology, 1984.

Yeast species are also useful in producing heterologous polypeptides. One of skill in the art will be familiar with the mechanism for transformation and fermentation of yeasts. General directions may be found in (1) *Biotechniques*, 13(1): 18-19, 1992; and (2) *Current Opinion in Biotechnology*, 3:486-496, 1992.
Alternatively, insect cells can be made to produce a desired peptide, e.g., *Flavobacterium* sp. isoamylase, by infecting cell cultures with transformed baculovirus particles. See Summers, et al. *Texas Exp. Station Bull.* 1555:1-57.

### Use in Debranching Starch

The isoamylase produced by recombinant *E. coli* or other organisms can be used in the production of various products from plant starches. Debranching is one step in the production of high fructose corn syrup from corn starch. The properties of this particular debranching enzyme make it particularly suitable for those applications where a near neutral pH would be advantageous. Currently, *Pseudomonas* isoamylase, having pH optimum of 3 - 4, is the only commercial isoamylase available for starch structure analysis and starch processing for food use. Recently PCT application WO 94/13792 reported the discovery of an amylase from *Bacillus licheniformis* having a pH optimum of 4.0 - 5.5. Its use in “liquefying corn starch” to produce glucose without adjusting the pH was stated to be an advantage. Therefore, use of *Flavobacterium* sp. isoamylase could provide a still further improvement in this process because it will function at a pH even closer to neutral.

The pH optimum for purified, pMON17409-produced isoamylase was determined under standard iodine assay conditions in 50 mM acetate, 50 mM MES, 100 mM Tris buffer, over the pH range of 4 - 9.5. Initial linear velocities were used to calculate relative activities (% of maximum).

For pH stability profile, the purified, pMON17409 isoamylase was re-equilibrated to 8 mM sodium phosphate, pH 6.5, 20% glycerol, by an Amicon centricron-30 concentrator. To 3.5 µl of enzyme, 2.0 µl of a 175 mM acetate, 175 mM MES, 350 mM Tris stock buffer was added, to give a final concentration of 64 mM acetate, 64 mM MES, 127 mM Tris. The enzyme was then incubated at 40 °C for 30 min, over a pH range of 4.5 - 9.5. Residual isoamylase activity of the 5.5 µl mixture was then determined by the standard iodine assay, in 50 mM acetate, 50 mM MES, 100 mM Tris, pH 6.5, final concentration.

The results indicated a pH optimum of 6.0 - 7.0, which is similar to the previously reported value of 6.3 for the donor enzyme (Sato and Park). The optimum stability is at pH 6.5 - 7.0. However, when assayed at 22 °C,
recombinant isoamylase displayed broader activity and stability optima of pH 5.0 - 8.0.

Substrate specificities for purified, recombinant isoamylase from pMON17409 were determined for oyster glycogen, rabbit liver glycogen, corn amylopectin, rice starch, potato starch, and pullulan. Specific activities were determined from initial rates of hydrolysis at pH 6.5. The relative rates of debranching of various branched polysaccharides by purified recombinant isoamylase are shown in Table 1. The enzyme had highest substrate specificity for glycogen, but scarcely hydrolyzed pullulan, which corroborates that the purified enzyme is an isoamylase.

Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster glycogen</td>
<td>182</td>
</tr>
<tr>
<td>Rabbit liver glycogen</td>
<td>174</td>
</tr>
<tr>
<td>Corn amylopectin</td>
<td>172</td>
</tr>
<tr>
<td>Rice starch</td>
<td>154</td>
</tr>
<tr>
<td>Potato starch</td>
<td>120</td>
</tr>
<tr>
<td>Pullulan</td>
<td>3</td>
</tr>
</tbody>
</table>

* S.A. = μmoles reducing groups released/min/mg protein.

Transformation of Plants

The expression of a plant gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the promoter. The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding complimentary strand.

Promoters which are useful in the present invention are those which will initiate transcription in tissues in which starch is produced. Such promoters may be derived from the plant species to be transformed or may be heterologous to such plant. Examples of promoters useful in the
present invention are those which will function in potato tubers. These include the promoters for granule bound starch synthases, soluble starch synthases, ADPglucose pyrophosphorylases, patatins (Class I), sucrose synthases, branching enzymes, debranching enzymes, and tuber polyphenol oxidases (GenBank® Accession Numbers M95196 and M95197).

Promoters which will cause the production of an isoamylase in a seed are useful in the present invention. From any plant seed, one may obtain the genes for enzymes involved in the production of starch and use those promoters in the DNA constructs of described herein. Thus native promoters for corn, wheat, rice, and barley may be obtained and used in the present invention.

Examples of promoters which will function in the seeds of corn include the zein promoters. The zeins are a group of storage proteins found in maize endosperm. Genomic clones for zein genes have been isolated and published, and the promoters from these clones, including the 15 kD, 16 kD, 19 kD, 22 kD, 27 kD, and gamma genes, could also be used to express an isoamylase gene in the seeds of corn and other plants. Other promoters known to function in corn include the promoters for the following genes: waxy, brittle 2, shrunken 2, branching enzymes I and II, starch synthases, debranching enzymes, oleosins, glutelins, and sucrose synthases.

Examples of promoters suitable for expression of an isoamylase gene in wheat include those for the genes for the ADPGPP subunits, for the granule bound and soluble starch synthases, for the branching and debranching enzymes, for the embryogenesis-abundant proteins, for the gliadins, and for the glutenins. Examples of such promoters in rice include those for the genes for the ADPGPP subunits, for the granule bound and soluble starch synthases, for the branching enzymes, for the debranching enzymes, for the sucrose synthases, and for the glutelins. Examples of such promoters for barley include those for the genes for the ADPGPP subunits, for the granule bound and soluble starch synthases, for the branching enzymes, for the debranching enzymes, for the sucrose synthases, for the hordeins, for the embryo globulins, and the aleurone specific proteins.

The plants which are amenable to transformation and use in the present invention are many. Examples include, but are not limited to, corn, potato, rice, barley, sweet potato, cassava, and wheat. Each of these
plants may be transformed by one of ordinary skill in the art using known methods.

**Introns**

In general, optimal expression in monocotyledonous plants is obtained when an intron sequence is located between promoter and coding sequences. Examples of such intron sequences are Adh1 and Hsp70 introns. The Hsp70 intron described in WO 93/19189 is preferred.

**Plastid Targeting Sequence**

The DNA constructs of the present invention may optionally contain a plastid targeting sequence. If they do not contain such a sequence, the enzyme translated from the structural coding sequence will be generally found in the cytoplasm of the cell. Such cytosolic enzyme would be useful in the production of food products because it would lead to debranching of starch during processing of the product from the raw plant part. For example, cutting potato tubers disrupts the starch granules contained therein, making the starch available for debranching by cytosolic isoamylase. The processed potato product would then have the improved properties noted above, such as reduced oil pick-up and improved texture.

Optionally, the DNA construct may be prepared with a plastid targeting sequence. Such a targeting sequence causes the protein to be imported into the plastid; the targeting sequence is removed during importation. It is believed that chloroplast and amyloplast targeting sequences will each function to move a protein into either type of plastid. The use of both types is within the present invention.

The preferred targeting sequence is a modified chloroplast targeting sequence (CTP) derived from the sequence reported by Timko et al. in “Genetic Engineering of Nuclear Encoded Components of the Photosynthetic Apparatus in Arabidopsis” in Impact of Chemistry on Biotechnology: A Multidisciplinary Discussion, ed. by Phillips, et al., pp 279-295, 1988. This modified targeting sequence (CTP1) was reported by Stark et al., “Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase,” Science 258:287-292, 1992, and is shown in SEQ ID NO:7.
A fusion of the selected plastid targeting sequence and an isoamyrase gene may be made by standard procedures and used in the present invention.

5 **Polyadenylation Signal**

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylated signal of *Agrobacterium* the tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene.

15 **Isoamyrase Gene Sources**

The isoamyrase gene used in the DNA constructs for plant transformation of the present invention may be any isoamyrase gene. It is not limited to the *Flavobacterium* sp. gene described above, although it is preferred. If the enzyme is not targeted to the plastid as described above, the pH optimum requirements may not be as strict. An isoamyrase gene from a *Pseudomonas* or a *Bacillus* may be used, for example. Genes already in use for the commercial production of isoamyrases may be moved into plasmids containing plant-appropriate regulatory sequences and used in the present invention.

Another possibility is the use of a gene for a plant isoamyrase, for example, a native potato isoamyrase, which is engineered behind a promoter which will cause production of the isoamyrase during starch metabolism in a sufficient amount to cause production of modified starch. Thus, the potato isoamyrase enzyme activity could be moved from sprouting to starch production. Assays described here can be used for complementation cloning of isoamyrase genes from plant sources from expression libraries and the gene thus obtained can be substituted for a bacterial isoamyrase gene. Alternatively, the isoamyrase gene from plant sources can be obtained by purification of the protein, amino acid sequencing, and gene isolation using well known techniques.
In order to isolate such genes, the DNA primers shown below as
SEQ ID NO:8 and SEQ ID NO:9, or related primers with more or less bias
for genes with lower or higher G+C percent content, may be used with PCR
techniques to clone an isoamylase gene. Alternatively, SEQ ID NO:4,
which is an internal region of the Flavobacterium sp. gene described above,
may be used as a probe to isolate other isoamylase genes.

The following examples use the isoamylase gene from Flavobacterium sp. (hereafter iam), but should not be interpreted in any way to
limit the scope of the present invention. Those skilled in the art will
recognize that various other genes as well as modifications, truncations,
etc., can be made to the methods and genes described herein while not
departing from the spirit and scope of the present invention. For example,
mutagenesis and screening may be employed to produce mutant deriva-
tives of the other known isoamylases discussed above which will have pH
optima closer to the preferred 5 - 8 range.

Expression in Potato

In order to obtain tuber specific expression, a vector (pMON16953)
was constructed to contain a ~1.0 kb portion of the tuber-specific class I
patatin promoter (hereafter, "Ppatatin1.0, described by Bevan et al.,
Nucleic Acids Res. 14:4625:4638, 1986), the 3'-nontranslated polyadenyla-
tion region of the nopaline synthase gene (NOS 3'), and spectinomycin resis-
tance for selection in E. coli and Agrobacterium tumefaciens. The iam gene
was isolated as an NcoI-EcoRI fragment from pMON17409. The modified
chloroplast transit peptide (CTP1) gene (SEQ ID NO:7) as a BglII-NcoI
fragment was fused to the translation initiation site of the iam gene by a
triple ligation into the BglII-EcoRI sites of pMON16953, to give pMON-
17411. The Ppatatin1.0/CTP1-iam/NOS3' NotI expression cassette from
pMON17411 was ligated into the unique, dephosphorylated NotI site of
pMON17227 and pMON17320, to give pMON17418 and pMON17419,
respectively. pMON17227 is a Ti plasmid vector disclosed and described
by Barry et al. in WO 92/04449 (1991), incorporated herein by reference,
which contains the FMV/CP4 construct for glyphosate selection in plant
transformation and regeneration. pMON17320 is a pMON17227 deriva-
tive which also contains a Ppatatin 1.0/CTP1-glgC16 cassette. The CTP1-
glgC16 fusion encodes a modified ADPglucose pyrophosphorylase as described by Kishore in WO 91/19806.

Both pMON17418 and pMON17419 constructs were screened in E. coli MM294 for clockwise orientation to FMV/CP4. Both pMON17418 and pMON17419 were mobilized into a disarmed ABI Agrobacterium tumefaciens strain by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al. 1980).

To transform Russet Burbank potato using glyphosate as a selectable marker, the appropriate Agrobacterium was grown overnight in 2 ml of LB broth supplemented with 75 µg/ml of spectinomycin, 75 µg of kanamycin, and 50 µg/ml of chloramphenicol. The overnight culture was diluted 1:10 with MSO, or until an optical density 600 nm of 0.2-0.33 was established. Leaves were removed from the stems of potato plants that had been grown under sterile conditions for three weeks on PM media supplemented with 25 mg/ml ascorbic acid. The stems were cut into 3-5 mm segments and inoculated with diluted bacteria for 15 min in square petri dishes. Explants were placed onto co-culture plates which contained 1/10 MSO with 1.5 ml of TxD (tobacco feeder) cells overlain with wetted filter paper. About 50 explants were placed per plate. After 2 days co-culture period, explants were placed onto callus induction media, which contained 5.0 mg/l Zeatin Riboside, 10 mg/l AgNO₃, and 0.1 mg/l naphthaline acetic acid, for 2 days. Explants were subsequently transferred onto callus induction media which contained 0.025 mM glyphosate for selection. After 4 weeks, explants were placed onto shoot induction media which contained 5.0 mg/l Zeatin Riboside, 10 mg/l AgNO₃, 0.3 mg/l gibberellic acid, and 0.025 mM glyphosate. Shoots began to appear at 8 weeks. Explants were transferred to fresh shoot induction media every 4 weeks for 12 weeks. Shoots were excised and placed on PM media for about 2 weeks or until they were large enough to be placed into soil. Growth chamber conditions the first 2 months included a 14 hr photoperiod with light intensity of 600 µE, 60% relative humidity, fertilization, and 25°C day/19°C night incubation. The third month conditions were modified to a 12 hr photoperiod, no fertilization, and 25 °C day/12 °C night incubation, after which tubers were harvested.
Analysis of Tubers from Transformed Potatoes

Out of 24 lines transformed with pMON17419, several showed an increase in tuber specific gravity similar to that previously reported for potatoes containing the glgC16 gene.

Mature, greenhouse-grown tubers, both control and transformed, were processed, without extraction buffer, through a Braun high speed juicer, followed by immediate centrifugation at 2000 x g for 5 min to pellet the starch granules. The supernatant was frozen on dry ice and stored at -80 °C. Western analysis of the supernatant of the juiced tuber extract indicated the presence of new bands that had lower molecular weights than Flavobacterium sp. isoamylase, perhaps due to proteolysis. This occurred even in the presence of protease inhibitors.

The pelleted starch granules were washed and centrifuged six times with several volumes of cold (4 °C) water, followed by two cold (-20 °C) acetone washes. The granules were air dried and stored at 4 °C.

For western blot analysis, 1.0 g of these granules was vortexed for 3 min in 10 ml of 1.0% SDS, 50 mM Tris-HCl, pH 7.5, to strip granule surface proteins, and centrifuged at 2000 x g for 5 min. The procedure was repeated, the granules were washed 5 times in 10 ml of water, and then washed twice with 5 ml of acetone. Proteins internally-embedded within the granule were extracted from SDS-washed granules by boiling 100 mg granules in 1.0 ml of Laemmli sample buffer for 6 min. The Laemmli-starch gel was macerated in the microfuge tube with a 200 µl pipette tip, and centrifuged at 12,000 x g for 10 min. The supernatant (approximately 0.4 µg of protein loaded per lane) underwent SDS-PAGE, followed by western blot to determine the isoamylase protein level within the granule. Western analysis of these SDS-washed granules extracted with Laemmli sample buffer showed that 18 out of 23 lines transformed with pMON-17419 contained Flavobacterium sp. isoamylase within the granules, at the same molecular weight as the E. coli-expressed isoamylase. Isoamylase levels, estimated by western blot, ranged between 0.5-3.0% of total granule-extracted protein. While isoamylase appeared to be proteolytically degraded during tuber extraction, western analysis of proteins extracted from SDS-washed granules showed isoamylase to be protected from proteolysis. The results demonstrated that isoamylase was not only imported within the amyloplast but was embedded inside the granule.
matrix. The presence of isoamylase during starch biosynthesis probably allowed the enzyme to become entrapped within the starch network as the growing starch molecules crystallized.

Starch granules stored at 4 °C were analyzed by light microscopy using a Nikon Diaphot-TMD inverted microscope. Granules (50 mg) were suspended in 2 ml of water, and 100 μl of the suspension was visualized under cover slip at 100X, 200X, and 400X magnification. pMON17419 granules containing isoamylase displayed gross morphological alterations. Several pMON17419 lines produced isoamylase-containing granules that were elongated, cylindrical, spindle-like, angular, less spheroidal, less symmetrical, and highly irregular in shape. However, the expression level of isoamylase, as determined by western blot, did not always correlate perfectly with the degree of granule irregularity. In comparison, wild type lines, or lines transformed only with gIC16, produced granules which were oval, spheroidal, and symmetrical.

Particle size distribution was determined for starch granules using a Coulter LS 130 Series Particle Size Analyzer with the Micro Volume Module. Granule suspensions (40 mg/10 ml of water) were sonicated 1 min followed by rapid mixing with a magnetic stir bar (3.5 setting) for 1 min. Preliminary analysis determined that a 1 min sonication time had no effect on particle size. Duplicate size determinations were obtained for 800 μl aliquots from the sonicated suspensions.

Table 2 summarizes the expression level estimated by western blot and the subjective degree of irregularity in granule shape for all pMON17419 lines, and the particle size analysis for selected lines. A scale of 0 to 10 was used for granule irregularity with 0 being normal and 10 being extremely deformed. The particle size analysis was not adjusted for the different shape of the transgenic granules. The data indicate smaller granules for lines 3, 19, and 25.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Irregular (0-10 scale)</th>
<th>Isoamylase by Western</th>
<th>Vol. Diameter (μM) Mean</th>
<th>Medal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type-1</td>
<td>0</td>
<td>Neg</td>
<td>35.06</td>
<td>35.37</td>
</tr>
<tr>
<td>Wild type-2</td>
<td>0</td>
<td>Neg</td>
<td>34.50</td>
<td>34.32</td>
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<tr>
<td>Wild type-3</td>
<td>0</td>
<td>Neg</td>
<td>34.76</td>
<td>35.10</td>
</tr>
<tr>
<td>17419-1</td>
<td>8</td>
<td>High</td>
<td>35.30</td>
<td>35.45</td>
</tr>
<tr>
<td>ID</td>
<td>Value</td>
<td>Type</td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>17419-2</td>
<td>5</td>
<td>Med</td>
<td>30.88</td>
<td>30.84</td>
</tr>
<tr>
<td>17419-3</td>
<td>2</td>
<td>Med</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-4</td>
<td>6</td>
<td>High</td>
<td>35.34</td>
<td>34.60</td>
</tr>
<tr>
<td>17419-5</td>
<td>2</td>
<td>Med</td>
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<td></td>
</tr>
<tr>
<td>17419-6</td>
<td>10</td>
<td>High</td>
<td>34.74</td>
<td>33.64</td>
</tr>
<tr>
<td>17419-12</td>
<td>8</td>
<td>Med</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-13</td>
<td>5</td>
<td>Med</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-14</td>
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<td>High</td>
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<td></td>
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<tr>
<td>17419-15</td>
<td>7</td>
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<td>17419-16</td>
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<td>17419-17</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>17419-18</td>
<td>0</td>
<td>Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-19</td>
<td>7</td>
<td>High</td>
<td>32.92</td>
<td>32.22</td>
</tr>
<tr>
<td>17419-20</td>
<td>3</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-21</td>
<td>10</td>
<td>High</td>
<td>34.30</td>
<td>33.82</td>
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<td>17419-22</td>
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<tr>
<td>17419-23</td>
<td>3</td>
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<tr>
<td>17419-25</td>
<td>0</td>
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</tr>
<tr>
<td>17419-26</td>
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<td>Med</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-27</td>
<td>0</td>
<td>Neg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-29</td>
<td>5</td>
<td>Low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17419-30</td>
<td>9</td>
<td>High</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of the modifications caused by *iam* on the thermo-gelation properties of starch was studied with differential scanning calorimetry using a Perkin-Elmer model DSC-7 instrument. Starch samples (3.0 +/- 0.1 mg) were weighed into Perkin-Elmer aluminum volatile sample pans and 10 µl of distilled deionized water was added. The sample pans were hermetically sealed and reweighed. Samples were immediately scanned from 20 °C to 90 °C at 10 °C per minute. Samples were reweighed afterwards to ensure there was no weight loss. Temperatures and enthalpy values were determined using Perkin Elmer System 7 software, except for the extrapolated final temperature (T_m) values which had to be determined manually. The results are shown in Table 3. The values are the mean ± the standard deviation. For Wild type-1, n=4; Wild type-2 and type-3, n=2; Lines 6, 21, and 30, n=3.
Table 3
Endothermic transitions(°C)          Enthalpy of
gelation (Joules/gm)

<table>
<thead>
<tr>
<th>Source</th>
<th>Onset temp.</th>
<th>Peak temp.</th>
<th>End temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type-1</td>
<td>63.9±0.27</td>
<td>67.1±0.21</td>
<td>70.5±0.25</td>
</tr>
<tr>
<td>Wild type-2</td>
<td>66.3±0.07</td>
<td>69.5±0.07</td>
<td>73.4±0.07</td>
</tr>
<tr>
<td>Wild type-3</td>
<td>63.5±0.14</td>
<td>66.6±0.14</td>
<td>71.4±0.0</td>
</tr>
<tr>
<td>pMON17419</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Line 6</td>
<td>63.0±0.35</td>
<td>66.1±0.29</td>
<td>69.5±0.35</td>
</tr>
<tr>
<td>10-Line 21</td>
<td>60.9±0.25</td>
<td>65.0±0.20</td>
<td>69.2±0.10</td>
</tr>
<tr>
<td>-Line 30</td>
<td>61.6±0.21</td>
<td>65.9±0.06</td>
<td>70.0±0.06</td>
</tr>
</tbody>
</table>

The gelation properties of the transgenic granules, as determined by DSC, corroborate the changes in granule morphology. The lower gelatinization temperatures for the iam-glfC16 granules suggest a higher amount of intermediate material due to an alteration of the elongation to branching ratio. Furthermore, starch granules with higher gelatinization temperatures typically have a higher degree of crystallinity, which provides structural and thermal stability to the granules. The DSC results also indicate the transgenic granules have 1) an overall less-highly ordered crystalline structure, and/or 2) fewer crystalline regions that are thermally and structurally more stable, and/or 3) less-stable amorphous regions. Therefore, a perturbation of the starch biosynthetic machinery by isoamylase activity may have created intermediate material that is less able to form a high degree of crystallinity.

High performance anion exchange chromatography (HPAEC) was performed with a Dionex system consisting of a GP40 gradient pump, an eluant organizer pressurized with helium, an ED40 pulsed amperometric detector (PAD-II), a post-column pneumatic controller, and an AS3500 autosampler (Thermo Separations Products). The PAD-II utilized a gold working electrode and a silver-silver chloride reference electrode. Data integration and analysis were performed using the Dynamax® MacIntegrator™, ChromPic™, and Dynamax® Compare Modules software package from Rainin Instrument Co.

Starch samples from lines 6, 21, and 30 (lines having the highest degrees of granule shape irregularity) were prepared for HPAEC by
dissolving 75 mg of purified starch granules in 5 ml of 0.1 N sodium hydroxide, adding 7.5 ml of water, and boiling 15 min. The solution was adjusted to approximately pH 4.0 with 2.5 ml of 1.0 M sodium acetate, pH 3.5. One ml was removed; 200 units (10-30 μl) of Pseudomonas sp. isoamylase (desalted by Amicon centricron-30) were added; and the reaction incubated at 37 °C for 4 hr, or until no further increase in reducing groups was determined by the Somogyi-Nelson method (Hodge and Hofreiter, in Methods in carbohydrate chemistry. New York: Academic Press, 1962). A 500 μl aliquot of the starch solution was desalted through a Quick Spin™ G-25 sephadex column previously equilibrated with water. The eluant was adjusted to pH 12 with 3 μl of 50% sodium hydroxide, and filtered through a 0.45 μm membrane. Sample injection volume was 25 μl. Chain length distribution was determined using a CarboPac PA-1 column (Dionex, 4 X 250 mm) with an in line filter (5 μm, 35 μm). Eluent A was 150 mM sodium hydroxide, and eluent B was 150 mM sodium hydroxide containing 500 mM sodium acetate. The sodium acetate elution gradient was as follows: 30% of eluent B at 0 min; 40% at 2.0 min; 60% at 20 min; 80% at 50.0 min; 80% at 55.0 min. The PAD-II pulse potentials (volts) and durations (s) were as follows: $E_1$ 0.05 ($t_1$ 0), $E_2$ 0.05 ($t_2$ 0.2), $E_3$ 0.05 ($t_3$ 0.5), $E_4$ 0.6 ($t_4$ 0.51), $E_5$ 0.6 ($t_5$ 0.59), $E_6$ -0.6 ($t_6$ 0.6), $E_7$ -0.6 ($t_7$ 0.65). High purity malto-oligosaccharides (G2-G7) (Hayashibara Co.) were used to construct a standard response curve.

Chain length distribution, as determined by HPAEC, indicated distinct differences between starch extracted from wild type lines and transgenic lines. Chromatograms of starch from pMON17419 lines 6, 21, and 30 showed a significantly higher percentage of long branch chains having a degree of polymerization of 30 and higher, in comparison to wild type lines. The results indicate that the transgenic starch contains longer distances between branch points and/or an increase in intermediate material. This is in agreement with the lower gelatinization values obtained by DSC which suggested an increase in intermediate material due to an alteration in elongation/branching ratio.

Starch isolated from tubers of lines transformed with pMON17419 was analyzed for amylose content by the method of Williams, et al. (1970) Cereal Chemistry, 47(7): 411-420, with the following minor modifications. Exactly 100 mg of starch was dispersed in 50 ml of 0.5 N KOH. The
starch was added with rapid stirring with a magnetic stir bar in order to prevent clumping during gelatinization. Exactly 10 ml of the starch-KOH solution was transferred in duplicate to 100 ml volumetric flasks and diluted to 100 ml with water. Exactly 20 ml of the diluted starch solution was transferred to a new 100 ml volumetric flask and 10 ml of 0.1 N HCl was added, followed by 1.0 ml of iodine reagent B. The volume was diluted to 100 ml and after five minutes the absorbance was measured at 625 nm. A standard curve was constructed by combining different volumes (totalling 10 ml) of stock solutions of potato amylose (100 mg/50 ml KOH) and potato amylpectin (100 mg/ 50 ml KOH) and diluting to 100 ml with water.

Duplicate analysis of starch from control (nontransformed) tubers contained approximately 23.6% amylose, while starch from pMON17419 lines 6, 12, 14, 21, 30 consisted of 31.0, 25.5, 31.3, 28.1, and 27.6% amylose, respectively. While amylose has a high affinity for iodine, intermediate material and abnormal amylpectin that contains long branch chains each possess high degrees of iodine binding capacity. Thus some of the increase in percent amylose may be attributable to longer distances between certain amylpectin branch points and/or an increase in intermediate material.

The results on the amylose content agree with the HPAEC and the DSC results which showed that starch from pMON17419-lines 6, 21, and 30 had longer distances between branch points. In addition, the increase in amylose content correlates with the altered granule morphology of starch from pMON17419 lines. The irregular, angular, cylindrical shape of the starch from the pMON17419 lines resembles the granule morphology of amylose extender starch from maize, which also has elevated levels of amylose and intermediate material. (Jane, J., et al., 1994, Starch/Stärke 46:121-129; Katz, F., et al., 1993, Carb. Polymers 21:133-136.)

Expression in Monocots

Using methods known to those in the art, a gene for an isoamylase may be stably transformed into a monocot cell and plants regenerated therefrom. See for example, U.S. Serial Number 08/275,929 (Armstrong et al.) which corresponds to EP 586 355 A for methods of transforming and
regenerating corn. To test the capability of the *iam* gene to be expressed in corn and other monocots, the following experiments were performed.

The CTP1-*iam* fusion was isolated as a *Bgl*II-*EcoRI* fragment from pMON17411 and subcloned into the *BamHI*-*EcoRI* sites of a pUC-based vector to give pMON17431. The CTP-*iam* fragment in pMON17431 was 3' to the constitutive CaMV 35S promoter and HSP70 intron, and 5' to the NOS3' transcriptional terminator (i.e., E35S/HSP70/CTP1-*iam*/NOS3'). A second vector, pMON17482, was similarly prepared but placed the CTP-*iam* fragment behind the glutelin 1 promoter in order to obtain expression in the endosperm of the corn kernel following stable transformation of regenerable corn tissues.

pMON17431 and empty control vector were purified from *E. coli* and electroporated in duplicate (100μg DNA each) into protoplasts prepared from corn leaves. Approximately 24 h later, fractions from the electroporated cells were disrupted by sonication in 100mM sodium phosphate, pH 6.5, and analyzed by western blot. Transient isoamylase expression in corn leaf protoplasts was estimated at 0.1% of total extracted protein.

pMON17431 was also cotransformed with the plasmid EC9 (described by Fromm et al., *Biotechnology* 8:833-839, 1990) into Black Mexican Sweet (BMS) corn callus tissue by particle gun bombardment. pEC9 contains the maize mutated ALS cDNA which confers chlorsulfuron resistance and is driven by the E35S promoter. Protein was isolated from chlorsulfuron resistant callus tissue by grinding tissue in 2% SDS, 15% glycerol, 75 mM Tris, pH 7.4, and analyzed by western blot. Isoamylase expression in BMS callus tissue was estimated at 0.025-0.05% of total extracted protein.

The CTP1-*iam*/NOS3' cassette was isolated as a *Bgl*II-*Not*I fragment from pMON17411, and subcloned into the *BamHI*-*Not*I sites of another pUC-based vector give pMON17482. The CTP-*iam*/NOS3' fragment in pMON17482 was 3' to the endosperm-specific rice glutelin promoter (P-osgt1) and HSP70 intron (i.e., P-osgt1/HSP70/CTP1-*iam*/NOS3'). Approximately 1 mg pMON17482 was purified from two liters of *E. coli* using cesium chloride density gradient centrifugation.

Supercoiled plasmid DNA was used to transform corn cells by particle gun bombardment (see, e.g., EP 586 355 A, Armstrong et al.). Individual corn
seed from R0 plants were ground to a fine powder on liquid nitrogen. Protein was extracted from the powder by vortexing in 0.1 ml of 100 mM Tris, 10 mM EDTA, 35 mM KCl, 20% glycerol, pH 7.5, and analyzed by Western blot. Isoamylase expression in the endosperm of mature kernels was detected in five of eighteen transformed lines. The line with the highest expression of the enzyme contained isoamylase at slightly higher than 0.2% of the total extractable protein. Structural modification of the starch would be expected from expression of the enzyme in this range.

Similarly, an isoamylase gene may be transformed into wheat using known methods, such as that of Vasil et al., U.S. Patent 5,405,765, incorporated herein by reference. Promoters which would be useful in expression of an isoamylase gene such as iam are discussed above. The HSP70 intron would also be used in vectors for wheat transformation.

From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with advantages which are obvious and which are inherent to the invention. It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims. Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth is to be interpreted as illustrative and not in a limiting sense.

All publications and patents mentioned in this specification are herein incorporated by reference as if each individual publication or patent was specifically and individually stated to be incorporated by reference.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:
(A) NAME: Monsanto Company
(B) STREET: 800 North Lindbergh Boulevard
(C) CITY: St. Louis
(D) STATE: Missouri
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 63167
(G) TELEPHONE: (314)694-3131
(H) TELEFAX: (314)694-5435

10 (ii) TITLE OF INVENTION: Novel Isoamylase Gene, Compositions Containing It and Methods of Using Isoamylases

15 (iii) NUMBER OF SEQUENCES: 11

20 (iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25 (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/281902
(B) FILING DATE: 28-JUL-1994

30

(2) INFORMATION FOR SEQ ID NO:1:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2334 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

45 ATGGACCCACACGCGCCGCCGGCAAAGAAACGAGCGGAGGGCACTTGGGGGGCCCTCCCTCTG 60
GCGCGCTGAGCTGCGGGCTGAGCCGCGGCAATGCGAGCGGCAAGCCGTGGGGGTCTATTTCTCG 120
GCGGCTAAGCACGCGGCGGCGCGCGAACCCTGGTCTCGGGGTCTATTTCTCGGCGCGGCGGGGCC 180
CGGCGTGAGGCTGTTCTCTGAAGAAGCGCGCTGCGAGCGGAGAAGGGGAGGCTGGGCTGG 240
CTGAGGCAAGGACCGGCGGCGCCAGGAGGTGAGGCTCGCTGCGGCGCGAAGGACAGTCGAAG 300
AACACCTACG GCATCAACGG GGCGGTCTC TACTACGTTACC GGGGCTTGGGG CCGCGAACTGG 380
CCCTACGATG CGCGCTTGGAC CAAAGGCCAGC GACCGCGGCT GCAGTCGAAGA CAGATGACGC 480
GGCAAGCGA ACAAGCGGAC TGCCGCGCAG GCGCAGATCT AGCGCGAGGC CCGCGCGCAG 540
CGCAAAAGCG ACAACGGGGCT GTTGGCCGAGC AAGGGCATTG CGCGCGCCGC GGAACGCGACC 680
TGGTGGGAGCA GCGAGCCAAGC CGCGCGGCTC AAGGAGAGAG TTATCTACGA AGTGCAGCGT 880
CGGGGCCTGA CGCCGCAAGA CAGACAGTG TGGCAGGCGG AAGGGCGGAC CTACACAGG 720
GGCGCGGCGCA AAGCGCGCCTGC GTTTGCCGCG CGCCGCGGTCG CGCGGCTGAGA GTTCTCAGG 780
GTGCAAGAAA CCCAGAAAGC CACAGAAAGAT GCGATCCCAA ATTCACGCC GGGCGACAAC 840
TACTGGGGCT ACATGACCT CAACACTTTC GCACCGGAACC GCGCTCATGC GCTACGACAAG 900
TGGCGCGCGG CGCGCGGCGG CGAATGGAAG GCGATGCGAT CGCGCTTCGA CGACGCGGGC 980
ATCAAGGTCT ACATCGAGCT GGCTCATTAC CACAGCAGCGG GAAAGCGGCCC GTGGAAGGAGGC 1020
ACCGAAGCGGC TCGCGCGTCTA CAACCTCTCTG GCCTGACGCAA CGGGCGCCTAC 1080
TACTCGCTGA GCAGCGATTA CAAGTATCGG TGCGCAACAA CGCGCGTGGG CGGCAACTAC 1140
AACACCGCGG ATCCATCGGC CGAGAAACCTG ATCGTTCAGCT CGCTGGCGTA CGCGCGAGGAC 1200
GGCGCTGGGG TGGAGGGTTT CGCGCTTGGG ATCGGCGCTGG CGCGCGCGCA CAGCGGCGAG 1280
CAAGGCGCTGCTG TCAACCTGGA CGAGAAGCGAC TGCGCGCCAC CGCCTCAAACG CATCGGCGCC 1320
GAGCTGCCGCG CGCGCGGCGG CGCGGCGGCG CGCGCGGCGCG CGCGCGCGCG GACTGGACGG 1380
GGATCGGGGC GCGACTCTTA CCGGCTCGGC CGCGCGCGCG CGCGCGCGCG GAGGCTGGAAAC 1440
GGTCCTTACC GCCGAGCGCGGT CGCGCAAGAG CAGAAACAGGC TGGCGCTGGG AAGGGCGTACCC 1500
CCCGCCAGCG CCGCGGCGTGCT CGCGCGCGCT GCGGCGACACCG TGACCGCGGG CGACGCGCGC 1580
AAGCCGTTGG AGTGTGCAAG GCTCGTGGGCT GCAGCGAAGG GCAGTCACCG CTAACGACCTC 1620
TACGCTTACA ACGCAACAGCA GAACAAACAG CCGGCGCGCT ACGCGCGCTG CGACGCGCGG 1680
GAGGAAACCA AACTGGAGCTG GACAGCCGGGC GCGATGTGGG GGAGCGCGAC CAAGCGCGCGG 1740
CGCACGGCGACTGCGGCTTGGC GATGCTCGAGCGCGCGGGTCG CGATGATCCAC CGCGCGGCGAC 1800
GAGGCGCTTG GCACGCGGAGT TGGCAACACG AACACCTACA ACGCGTCGAGCGCGCGG 1860
TGGCTGTACT GGAGCGCGAG CGCGCTGAGG CGAGCGACGC AAGAATCACAC GAACGCGCGG 1920
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 777 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Pro His Ala Pro Gin Arg Gln Arg Ser Gly Gin Arg Leu Arg

1 5 10 15

Ala Leu Ala Leu Ala Leu Ala Cys Ala Leu Ser Pro Ala His Ala

20 25 30

Ala Ile Asp Ala Gin Gln Leu Gly Ala Arg Tyr Asp Ala Ala Gln Ala

35 40 45

Asn Leu Ala Phe Arg Val Tyr Ser Ser Arg Ala Thr Arg Val Glu Val

50 55 60

Phe Leu Tyr Lys Asn Pro Thr Gly Ser Gin Glu Val Ala Arg Leu Ala

65 70 75 80

Leu Ser Lys Asp Pro Ala Thr Gln Val Trp Ser Leu Ser Leu Pro Thr

85 90 95

Ser Thr Ile Lys Asn Thr Tyr Gly Ile Thr Gly Ala Val Tyr Gly

100 105 110

Tyr Arg Ala Trp Gly Pro Asn Trp Pro Tyr Asp Ala Ala Trp Thr Lys

115 120 125

Gly Ser Ala Thr Gly Phe Val Ser Asp Val Asp Asn Ala Gly Asn Arg

130 135 140
Phe Asn Pro Asn Lys Leu Leu Leu Leu Asp Pro Tyr Ala Arg Glu Ile Ser
145  150  155  160
Gln Asp Pro Asn Thr Ala Thr Cys Ala Asp Gly Thr Ile Tyr Ala Thr
165  170  175
Gly Ala Ala His Arg Asn Lys Ser Gly Leu Cys Ala Ser Lys Gly
180  185  190
Ile Ala Leu Ala Ala Asp Ala Thr Ser Val Gly Ser Lys Pro Thr Arg
195  200  205
Ala Leu Lys Asp Glu Val Ile Tyr Glu Val His Val Arg Gly Leu Thr
210  215  220
Arg Asn Asp Asp Ser Val Pro Ala Ala Glu Arg Gly Thr Tyr Lys Gly
225  230  235  240
Ala Ala Arg Lys Ala Ala Leu Ala Ala Leu Val Thr Ala Val
245  250  255
Glu Phe Leu Pro Val Gln Glu Thr Gln Asn Asp Gln Asn Asp Val Asp
260  265  270
Pro Asn Ser Thr Ala Gly Asp Asn Tyr Trp Gly Tyr Met Thr Leu Asn
275  280  285
Tyr Phe Ala Pro Asp Arg Arg Tyr Ala Tyr Asp Lys Ser Ala Gly Gly
290  295  300
Pro Thr Arg Glu Trp Lys Ala Met Val Lys Ala Phe His Asp Ala Gly
305  310  315  320
Ile Lys Val Tyr Ile Asp Val Val Tyr Asn His Thr Gly Glu Gly Gly
325  330  335
Pro Trp Ser Gly Thr Asp Gly Leu Ser Val Tyr Asn Leu Leu Ser Phe
340  345  350
Arg Gly Leu Asp Asn Pro Ala Tyr Tyr Ser Leu Ser Ser Asp Tyr Lys
355  360  365
Tyr Pro Trp Asp Thr Gly Val Gly Gly Asn Tyr Asn Thr Arg His
370  375  380
Pro Ile Ala Gln Asn Leu Ile Val Asp Ser Leu Ala Tyr Trp Arg Asp
385  390  395  400
Ala Leu Gly Val Asp Gly Phe Arg Phe Asp Leu Ala Ser Val Leu Gly
405  410  415
Asn Ser Cys Gln His Gly Cys Phe Asn Phe Asp Lys Asn Asp Ser Gly
420  425  430
Asn Ala Leu Asn Arg Ile Val Ala Glu Leu Pro Pro Arg Pro Ala Ala 435 440 445
Gly Gly Ala Gly Ala Asp Leu Ile Ala Glu Pro Trp Ala Ile Gly Gly 450 455 460
Asn Ser Tyr Gln Val Gly Gly Phe Pro Ala Gly Trp Ala Glu Trp Asn 465 470 475 480
Gly Leu Tyr Arg Asp Ala Leu Arg Lys Gln Asn Lys Leu Gly Val 485 490 495
Glu Thr Val Thr Pro Gly Thr Leu Ala Thr Arg Phe Ala Gly Ser Asn 500 505 510
Asp Leu Tyr Gly Asp Asp Gly Arg Lys Pro Trp His Ser Ile Asn Phe 515 520 525
Val Val Ala His Asp Gly Phe Thr Leu Asn Asp Leu Tyr Ala Tyr Asn 530 535 540
Asp Lys Gln Asn Asn Gln Pro Trp Pro Tyr Gly Pro Ser Asp Gly Gly 545 550 555 560
Glu Asp His Asn Leu Ser Trp Asn Gln Gly Gly Ile Val Ala Glu Gln 565 570 575
Arg Lys Ala Ala Arg Thr Gly Leu Ala Leu Leu Met Leu Ser Ala Gly 580 585 590
Val Pro Met Ile Thr Gly Gly Asp Glu Ala Leu Arg Thr Gln Phe Gly 595 600 605
Asn Asn Asn Thr Tyr Asn Leu Asp Ser Ala Ala Asn Trp Leu Tyr Trp 610 615 620
Ser Arg Ser Ala Leu Glu Ala Asp His Glu Thr Tyr Thr Lys Arg Leu 625 630 635 640
Ile Ala Phe Arg Lys Ala His Pro Ala Leu Arg Pro Ala Asn Phe Tyr 645 650 655
Ser Ala Ser Asp Thr Asn Gly Asn Val Met Glu Gln Leu Arg Trp Phe 660 665 670
Lys Pro Asp Gly Ala Gln Ala Asp Ser Ala Tyr Phe Asn Gly Ala Asp 675 680 685
Asn His Ala Leu Ala Trp Arg Ile Asp Gly Ser Glu Phe Gly Asp Ser 690 695 700
Ala Ser Ala Ile Tyr Val Ala Tyr Asn Gly Trp Ser Gly Ala Val Asp 705 710 715 720
Phe Lys Leu Pro Trp Pro Gly Thr Gly Lys Gln Trp Tyr Arg Val Thr
725  730  735
Asp Thr Ala Thr Trp Asn Glu Gly Pro Asn Ala Val Ala Leu Pro Gly
740  745  750
Ser Glu Thr Leu Ile Gly Gly Glu Thr Val Tyr Gty Met Gin Ala
755  760  765
Arg Ser Leu Leu Leu Leu Ile Ala Lys
770  775

(2) INFORMATION FOR SEQ ID NO:3:

15  (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 38 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

20  (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ile Asp Ala Gin Gin Leu Gly Ala Arg Tyr Asp Ala Ala Gin Ala
1  5  10  15
Asn Leu Ala Phe Arg Val Tyr Ser Ser Arg Ala Thr Xaa Val Glu Xaa
20  25  30
Phe Leu Tyr Lys Asn Pro
35  35

(2) INFORMATION FOR SEQ ID NO:4:

35  (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 260 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

40  (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

45  ATGAGCTGGTCTACAACCAACACGGGCGGACTGGGCGGGGACTGGGCTC  60
AGCAGCTACAACCGTGCTTCGTTCGGCGGCCTGGACACACCACGGGGTACTCTACTGCGTGAGC  120
AGCGATTACAAGTATCGGTGGGAAACAACCACGGGGTGGCCGCAACTACCAACACCGGGCGCAT  180
50  CCCATCGGCGAAGAACTGTAGCTGGACTCTGGGCTACTGGGCGGACGGGGGCGTGGGCTA  240
GACGGGTTCGCCTCGATCT  260
(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGCCATGG CCATCGATGC GCAGCACTGC GGGGCGGGT AOGAC

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGCAATTC GAGCTCTTAT CACTTCGCGA TCAGCAACAG CACGGA

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 355 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 91/19806
(I) FILING DATE: 07-JUN-1991
(J) PUBLICATION DATE: 26-DEC-1991
(K) RELEVANT RESIDUES IN SEQ ID NO:7: FROM 1 TO 355

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGCTTGTTC TCATTTGTGT TATCATTATA TATAGATGAC CAAAGCACTA GACCAAACCT

CAGTCACACA AAGAGTAAGG AAGAAACATTG GCTTCTCTCA TGCTCTCTTC CGCTACATG

GTGGCTCTCC GCGCTCAGGC CACTATGGTC GCTCTCTTCCA ACGGACTTAA GTCTCTCGCT

GCTTTCAGG CGACCGGCAA GGCTAACACA GACATTACTT GCATCAAAAG CAAACGGCGGA
AGAGTTAAGT GCATGCAGGT GTGGOCTOOG ATGGAAAGA AGAAGTTTGA GACTCTCTCTT 300

TAOCTTCCTG ACCTTAOGA TTOCGGTGGT CGCGTCAACT GCATGCAGGC CATGG 355

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(x) SEQUENCE DESCRIPTION: SEQ ID NO:8:
Asp Val Val Tyr Asn His

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(x) SEQUENCE DESCRIPTION: SEQ ID NO:9:
Gly Phe Arg Phe Asp Leu Ala Ser Val

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2244 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2241

(x) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATG GCC ATC TGC GCG CAG CAG CTC GGC GCG CTC GAC GCC GCC CAG 48
Met Ala Ile Asp Ala Gln Gln Leu Gly Ala Arg Tyr Asp Ala Ala Gln

GCC AAC CTC GCG TTC GCG GTC TAT TOC TCG GC GCG ACC GGC GTC GAG 96
Ala Asn Leu Ala Phe Arg Val Tyr Ser Ser Arg Ala Thr Arg Val Glu
20 25 30

GTG TTC CTG TAC AAG AAC CGG ACC GCC TGC TAC GAA GTC GCG CGG CTG
Val Phe Leu Tyr Lys Asn Pro Thr Gly Ser Gin Glu Val Ala Arg Leu
35 40 45

GCG CTG AGC AAG GAC CGG GCG ACC CAG GTG TGG TGC CTG TGC CTG CGG
Ala Leu Ser Lys Asp Pro Ala Thr Gin Val Trp Ser Leu Ser Leu Pro
50 55 60

ACC AGC ACG ATC AAG AAC ACC TAC GGC ATC ACC GGC GCC GTCT TAC TAC
Thr Ser Thr Ile Lys Thr Tyr Gly Ile Thr Gin Val Tyr Val Tyr
65 70 75 80

GGT TAC CGC GCC TGC GGC CGG AAC CTG GCC ACC TAC GAT GCC GCC TGC TGACC
Gly Tyr Arg Ala Trp Gly Pro Asn Trp Pro Tyr Asp Ala Ala Trp Thr
85 90 95

AAG GGC AGC GCC ACC GCC TTC GTG TGC AGC GAC GTC GAC AACC GCC GCC AAC
Lys Gly Ser Ala Thr Gly Phe Val Ser Asp Val Asp Ala Gly Asn
100 105 110

CGT TTC AAT CGC AAC AAG CTG CTG CTG GCC CAC CCC TAC GCC GCC GCG GAG ATC
Arg Phe Asn Pro Asp Lys Leu Leu Leu Asp Pro Tyr Ala Arg Glu Ile
115 120 125

AGC CAG GAC CGG AAC ACC GCC GCC TGC GCC GCC GAC GCC ACC ATC TAC GCC
Ser Gin Asp Pro Asn Thr Ala Thr Cys Ala Asp Gly Thr Ile Tyr Ala
130 135 140

ACC GGC GCC GCC AAC AAG GAC AGC GGC ATC TGC GCC GCC AAG GCC AGC AAG
Thr Gly Ala Ala His Arg Asp Gin Ser Gin Cys Ala Ser Lys
145 150 155 160

GGC ATC GCC ATG GCC GCC GCC ACC CTG GCC GCC AGC GCC AAG CCG ACC
Gly Ile Ala Leu Ala Ala Asp Ala Thr Ser Val Gly Ser Lys Pro Thr
165 170 175

CGC GCC ATC AAG GCC AAC GTG ATC TAC GAA GTG CAC GTG GCC GCC CTG
Arg Ala Leu Lys Asp Glu Val Ile Tyr Glu Val His Val Arg Gly Leu
180 185 190

ACC GCC AAC GCC AAC GCC GCC GCC GCC GCC GCC GCC GCC GCC ACC TAC AAG
Thr Arg Asn Asp Ser Val Pro Ala Ala Glu Arg Gly Thr Tyr Lys
195 200 205

GGC GCC GCC GCC AAG GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC TCC
Gly Ala Ala Arg Asp Ala Ala Ala Ala Ala Ala Ala Val Thr Ala
210 215 220

GTC GAG TTC CTG CGG GTG CAG GAA ACC CAG AAC GAC CAG AAC GAT GTC
Val Glu Phe Leu Pro Val Gin Glu Thr Gin Asn Gin Asn Gin Asp Val
225 230 235 240
GAT CCC AAT TCC ACC GCG GGC GAC AAC TAC TGG GGC TAC ATG ACC CTC  768
Asp Pro Asn Ser Thr Ala Gly Asp Asn Tyr Trp Gly Tyr Met Thr Leu
  245    250    255
5
AAT TAC TTC GCC CGG ACC CGC GAC TAC TCC TGC AAC AAG TCG GCC GGC  816
Asn Tyr Phe Ala Pro Asp Arg Arg Tyr Ala Tyr Asp Lys Ser Ala Gly
  260    265    270
10
GGG CCG ACC CGC GAA TGG AAG GCG ATG GTC AAG GCC TTC CAC GAC GCC  864
Gly Pro Thr Arg Glu Trp Lys Ala Met Val Lys Ala Phe His Asp Ala
  275    280    285
GCC ATC AAG GTC TAC ATC GAC GTG GTC TAC AAC CAC ACC GCC GAA GGC  912
Gly Ile Lys Val Tyr Ile Asp Val Val Tyr Asn His Thr Gly Glu Gly
  290    295    300
GCC CGG TGG AGC GGC ACC GAC GGG CTC AGC GTC TAC AAG CTG CTC TG  960
Gly Pro Trp Ser Gly Thr Asp Gly Leu Ser Val Tyr Asn Leu Leu Ser
  305    310    315    320
TTC CGC GGC CTC GAC AAC CGG GCC TAC TAC TCG CGC AGC AGC GAT TAC 1008
Phe Arg Gly Leu Asp Ser Pro Ala Tyr Tyr Ser Leu Ser Ser Asp Tyr
  325    330    335
25
AAG TAT CCG TGG GAC AAC ACC GGC GTC GCC GCC AAC TAC AAC ACC CGC 1056
Lys Tyr Pro Trp Asp Asn Thr Gly Val Gly Gly Asn Tyr Asn Thr Arg
  340    345    350
30
CAT CCC ATC GCC CAG AAC CTG ATC GTC GAC TCG CTG GCG TAC TGG CGC 1104
His Pro Ile Ala Gin Asn Leu Ile Val Asp Ser Leu Ala Tyr Trp Arg
  355    360    365
GAC GGC CTC GCC GTA GAC GGT TTC CGC TTC GAT CTG GCC TCG GTG CTC 1152
Asp Ala Leu Gly Val Asp Gly Phe Arg Phe Asp Leu Ala Ser Val Leu
  370    375    380
40
GAC AAC AGC TGC CAG CAC GCC TGC TTC AAC TTC GAC AAG AAC GAC TG 1200
Gly Asn Ser Cys Gin His Gly Cys Phe Asp Asn Phe Lys Asn Asp Ser
  385    390    395    400
GAC AAC GCG CTC AAC GGC ATC GTC GCC GAG CTG CGG CGC CGC CGC CAC 1248
Gly Asn Ala Leu Asn Arg Ile Val Ala Glu Leu Pro Pro Arg Pro Ala
  405    410    415
45
GGC GCC GGC GCC CGG GCG GAC CTG ATC GCC GAA CCC TGG GCG ATC GCC 1296
Ala Gly Ala Gly Ala Asp Leu Ile Ala Glu Pro Trp Ala Ile Gly
  420    425    430
50
GAC AAT CCC TAC CAC GTC GCC GGC TCC CGG GCC GCC TGG GCC GAC TGG 1344
Gly Asn Ser Tyr Gin Val Gly Gly Phe Pro Ala Gly Trp Ala Glu Trp
  435    440    445
AAC GGC CTC TAC CGC GAC GCG CTG CGC AAC AAG CAG AAC AAG CTC GCC 1392
Asn Gly Leu Tyr Arg Asp Ala Leu Arg Lys Gln Asn Lys Leu Gly 450 455 460

GTG GAA ACG GTC ACC CCC GGC ACC CTG GCC ACG GCC TTC GCC GCC TCC 1440
Val Glu Thr Val Thr Pro Gly Thr Leu Ala Thr Arg Phe Ala Gly Ser 465 470 475 480

AAC GAC CTG TAC GGC GAC GAC GCC CCG AAG CGG TGG CAT TGG ATC AAC 1488
Asn Asp Leu Tyr Gly Asp Asp Gly Arg Lys Pro Trp His Ser Ile Asn 485 490 495

TTC GTG GTC GCC CAC GAC GGC TTC ACC CTC AAC GAC CTG TAC GCC TAC 1536
Phe Val Val Ala His Asp Gly Phe Thr Leu Asp Leu Tyr Ala Tyr 500 505 510

AAC GAC AAG CAG AAC AAG CAG CGG TGG CGG TAC GGG CGG TCC GAC GGC 1584
Asn Asp Lys Gin Asn Asn Gin Pro Trp Pro Tyr Gly Pro Ser Asp Gly 515 520 525

GGC GAG GAC CAC AAC CTG ACG TGG AAC CAG GCC GGC ATC GTC GCC GAG 1632
Gly Glu Asp His Asn Leu Ser Trp Asn Gin Gly Gly Ile Val Ala Glu 530 535 540

CAG CGC AAG GCC GCG GCC ACC GGA CTG GCC TTC ATG ATC ATC AGC GCC 1680
Gln Arg Lys Ala Ala Arg Thr Gly Leu Ala Leu Leu Met Leu Ser Ala 545 550 555 560

GGC GTG CGG ATG ATC ACC GCC GGC GAC GAC GCG CGC CTG ACC CAG TTC 1728
Gly Val Pro Met Ile Thr Gly Asp Glu Ala Leu Arg Thr Gin Phe 565 570 575

GGC AAC AAC ACC TAC AAC CTG GAT TCG GCC GCC AAG TGG CTG TAC 1776
Gly Asn Asn Asn Thr Tyr Asn Leu Asp Ser Ala Ala Asn Trp Leu Tyr 580 585 590

TGG AGC GGC AGC GCC CTC GAG GCC GAC CAC GAG ACC TAC ACC AAG CGC 1824
Trp Ser Arg Ser Ala Leu Glu Ala Asp His Glu Thr Tyr Thr Lys Arg 595 600 605

CTG ATC GCG TTC CGC AAG CGG CAC CGG GCG CTG GCC CGG CGG AAC TTC 1872
Leu Ile Ala Phe Arg Lys Ala His Pro Ala Leu Arg Pro Ala Asn Phe 610 615 620

TAT TGG GCC AGC GAC ACC AAC GGC AAC ATG GTG ATG GAG CAG TTG CGT TGG 1920
Tyr Ser Ala Ser Asp Thr Asn Gly Asn Val Met Glu Gin Leu Arg Trp 625 630 635 640

TTC AAG CCC GCC GGC GCG CAG GCC GAC GCC AGC GCC TAC TTG AAC GCC GGC 1968
Phe Lys Pro Asp Gly Ala Glu Ala Asp Ser Ala Tyr Phe Asn Gly Ala 645 650 655

GAC AAC CAC GGC CTG GCC TGG CGC ATC GAC GCC AGC GAG TTC GCC GAC 2016
Asp Asn His Ala Ala Trp Arg Ile Asp Gly Ser Glu Phe Gly Asp 660 665 670
AGC GCC AGC GCG ATC TAC GTC GCC TAC AAC GGC TGG TCC GGC GCG GTC 2064
Ser Ala Ser Ala Ile Tyr Val Ala Tyr Asn Gly Trp Ser Gly Ala Val
675 680 685
5
GAC TTC AAG CTG CCG TGG CCG GGC ACC GGC AAG CAG TGG TAC CGG GTC 2112
Asp Phe Lys Leu Pro Trp Pro Gly Thr Gly Lys Gln Trp Tyr Arg Val
690 695 700
10
ACC GAT ACC GGC ACC TGG AAC GAA GGC CCC AAC GCG GTG CGC CTG CCC 2160
Thr Asp Thr Ala Thr Trp Asn Glu Gly Pro Asn Ala Val Ala Leu Pro
705 710 715 720
GCG AGC GAG ACC CTG ATC GGC GCC GAG AAC ACC GTC TAC GGC ATG CAG 2208
Gly Ser Glu Thr Leu Ile Gly Gly Asn Thr Val Tyr Gly Met Gln
725 730 735
GCG CGC TCG CTG CTG TGG ATC GTC GCG AAG TGATAA 2244
Ala Arg Ser Leu Leu Leu Leu Ile Ala Lys
2250 2255

(2) INFORMATION FOR SEQ ID NO:11:

25  (i) SEQUENCE CHARACTERISTICS:
30  (A) LENGTH: 746 amino acids
30  (B) TYPE: amino acid
30  (D) TOPOLOGY: linear

30  (ii) MOLECULE TYPE: protein

50  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ala Ile Asp Ala Gln Gln Leu Gly Ala Arg Tyr Asp Ala Ala Gln 35
1 5 10 15
Ala Asn Leu Ala Phe Arg Val Tyr Ser Ser Arg Ala Thr Arg Val Glu
20 25 30
40  Val Phe Leu Tyr Lys Asn Pro Thr Gly Ser Gln Glu Val Ala Arg Leu
35 40 45
Ala Leu Ser Lys Asp Pro Ala Thr Gln Val Trp Ser Leu Ser Leu Pro
50 55 60
45  Thr Ser Thr Ile Lys Asn Thr Tyr Gly Ile Thr Gly Ala Val Tyr Tyr
65 70 75 80
Gly Tyr Arg Ala Trp Gly Pro Asn Trp Pro Tyr Asp Ala Ala Trp Thr
85 90 95
50  Lys Gly Ser Ala Thr Gly Phe Val Ser Asp Val Asp Asn Ala Gly Asn
100 105 110
Arg Phe Asn Pro Asn Lys Leu Leu Leu Asp Pro Tyr Ala Arg Glu Ile
115 120 125
Ser Gln Asp Pro Asn Thr Ala Thr Cys Ala Asp Gly Thr Ile Tyr Ala
5 130 135 140
Thr Gly Ala Ala His Arg Asn Lys Asp Ser Gly Leu Cys Ala Ser Lys
145 150 155 160
10 Gly Ile Ala Leu Ala Ala Asp Ala Thr Ser Val Gly Ser Lys Pro Thr
165 170 175
Arg Ala Leu Lys Asp Glu Val Ile Tyr Glu Val His Val Arg Gly Leu
180 185 190
15 Thr Arg Asn Asp Asp Ser Val Pro Ala Ala Glu Arg Gly Thr Tyr Lys
195 200 205
Gly Ala Ala Arg Lys Ala Ala Ala Leu Ala Ala Leu Gly Val Thr Ala
210 215 220
Val Glu Phe Leu Pro Val Gln Glu Thr Gln Asn Asp Gln Asn Asp Val
225 230 235 240
25 Asp Pro Asn Ser Thr Ala Gly Asp Asn Tyr Trp Gly Tyr Met Thr Leu
245 250 255
Asn Tyr Phe Ala Pro Asp Arg Tyr Ala Tyr Asp Lys Ser Ala Gly
260 265 270
30 Gly Pro Thr Arg Glu Trp Lys Ala Met Val Lys Ala Phe His Asp Ala
275 280 285
Gly Ile Lys Val Tyr Ile Asp Val Val Tyr Asn His Thr Gly Glu Gly
329 295 300
35 Gly Pro Trp Ser Gly Thr Asp Gly Leu Ser Val Tyr Asn Leu Leu Ser
305 310 315 320
40 Phe Arg Gly Leu Asp Pro Ala Tyr Tyr Ser Leu Ser Ser Asp Tyr
325 330 335
Lys Tyr Pro Trp Asp Thr Gly Val Gly Gly Asn Tyr Asn Thr Arg
340 345 350
45 His Pro Ile Ala Gln Asn Leu Ile Val Asp Ser Leu Ala Tyr Trp Arg
355 360 365
Asp Ala Leu Gly Val Asp Gly Phe Arg Phe Asp Leu Ala Ser Val Leu
370 375 380
Gly Asn Ser Cys Gln His Gly Cys Phe Asn Phe Asp Lys Asn Asp Ser
385 390 395 400
Gly Asn Ala Leu Asn Arg Ile Val Ala Glu Leu Pro Pro Arg Pro Ala
405 410 415

Ala Gly Gly Ala Gly Ala Asp Leu Ile Ala Glu Pro Trp Ala Ile Gly
5 420 425 430

Gly Asn Ser Tyr Gln Val Gly Gly Phe Pro Ala Gly Trp Ala Glu Trp
435 440 445

10 Asn Gly Leu Tyr Arg Asp Ala Leu Arg Lys Lys Gln Asn Lys Leu Gly
450 455 460

Val Glu Thr Val Thr Pro Gly Thr Leu Ala Thr Arg Phe Ala Gly Ser
465 470 475 480

15 Asn Asp Leu Tyr Gly Asp Asp Gly Arg Pro Trp His Ser Ile Asn
485 490 495

Phe Val Val Ala His Asp Gly Phe Thr Leu Asn Asp Leu Tyr Ala Tyr
500 505 510

20 Asn Asp Lys Gln Asn Asn Gln Pro Trp Pro Tyr Gly Pro Ser Asp Gly
515 520 525

25 Gly Glu Asp His Asn Leu Ser Trp Asn Gln Gly Gly Ile Val Ala Glu
530 535 540

Gln Arg Lys Ala Ala Arg Thr Gly Leu Ala Leu Leu Met Leu Ser Ala
545 550 555 560

30 Gly Val Pro Met Ile Thr Gly Gly Asp Glu Ala Leu Arg Thr Gln Phe
565 570 575

Gly Asn Asn Thr Tyr Asn Leu Asp Ser Ala Ala Asn Trp Leu Tyr
35 580 585 590

Trp Ser Arg Ser Ala Leu Glu Ala Asp His Glu Thr Tyr Thr Lys Arg
595 600 605

40 Leu Ile Ala Phe Arg Lys Ala His Pro Ala Leu Arg Pro Ala Asn Phe
610 615 620

Tyr Ser Ala Ser Asp Thr Asn Gly Asn Val Met Glu Gin Leu Arg Trp
625 630 635 640

45 Phe Lys Pro Asp Gly Ala Gln Ala Asp Ser Ala Tyr Phe Asn Gly Ala
645 650 655

Asp Asn His Ala Leu Ala Trp Arg Ile Asp Gly Ser Glu Phe Gly Asp
660 665 670

50 Ser Ala Ser Ala Ile Tyr Val Ala Tyr Asn Gly Trp Ser Gly Ala Val
675 680 685
Asp Phe Lys Leu Pro Trp Pro Gly Thr Gly Lys Gln Trp Tyr Arg Val
690 695 700

Thr Asp Thr Ala Thr Trp Asn Glu Gly Pro Asn Ala Val Ala Leu Pro
705 710 715 720

Gly Ser Glu Thr Leu Ile Gly Gly Glu Asn Thr Val Tyr Gly Met Gln
725 730 735

Ala Arg Ser Leu Leu Leu Leu Ile Ala Lys
740 745
Claims:

1. An isolated DNA molecule which encodes the protein of SEQ ID NO:2.

5 2. An isolated DNA molecule of SEQ ID NO:10.

3. A transformed cell comprising a heterologous DNA sequence encoding a polypeptide having substantially the sequence of SEQ ID NO:11, wherein said cell is selected from E. coli, Bacillus sp., and yeast.

10 4. A DNA construct comprising operatively linked in the 5' to 3' direction:

a) a promoter which functions to cause the production of an RNA sequence; and

b) the structural coding sequence of SEQ ID NO:10;

wherein said promoter is heterologous to said structural coding sequence.

5. A method of producing an isoamylase comprising the steps of fermenting a culture of organisms containing the DNA construct of Claim 4 and extracting isoamylase therefrom.

20 6. A DNA construct comprising operatively linked in the 5' to 3' direction:

a) a promoter which functions in selected starch-producing plant cells to cause the production of an RNA sequence;

b) a structural coding sequence that encodes for an isoamylase;

and

c) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

wherein said promoter is heterologous with respect to the structural coding sequence.

7. The DNA construct of Claim 6 wherein said structural coding sequence further comprises a plastid targeting sequence.

8. The DNA construct of Claim 7 wherein said plastid targeting sequence is the CTP-1 plastid targeting sequence SEQ ID NO:7.

35 9. The DNA construct of Claim 6 wherein said isoamylase is SEQ ID NO:11.
10. A transformed plant cell comprising a DNA construct comprising operatively linked in the 5' to 3' direction:
   a) a promoter which functions in selected starch-producing plant cells to cause the production of an RNA sequence;
   b) a structural coding sequence that encodes for an isoamylase; and
   c) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

wherein said promoter is heterologous with respect to the structural coding sequence.

11. The transformed plant cell of Claim 10 wherein said structural coding sequence further comprises a plastid targeting sequence and wherein said isoamylase has a pH optimum of 5 - 8.

12. The transformed plant cell of Claim 11 wherein said plastid targeting sequence is the CTP-1 plastid targeting sequence SEQ ID NO:7.

13. The transformed plant cell of Claim 12 wherein said isoamylase is SEQ ID NO:11.

14. The transformed plant cell of Claim 13 wherein said isoamylase is encoded by SEQ ID NO:10.

15. The transformed plant cell of Claim 10 wherein said plant cell is from potato, corn, wheat, barley, sweet potato, cassava, or rice.

16. The transformed plant cell of Claim 10 further stably transformed with a foreign ADPglucose pyrophosphorylase gene.

17. A method of producing starch having a modified structure comprising
   a) transforming plant cells to contain a DNA construct comprising operatively linked in the 5' to 3' direction:
      i) a promoter which functions in selected starch-producing plant cells to cause the production of an RNA sequence;
      ii) a structural coding sequence that encodes for an isoamylase; and
iii) a 3' non-translated region which functions in said plant
cells to cause the addition of polyadenylate nucleotides
to the 3' end of the RNA sequence;

wherein said promoter is heterologous with respect to the
structural coding sequence;

b) regenerating whole plants;
c) multiplying said plants;
d) harvesting plant materials; and
e) extracting the starch therefrom.

18. The method of Claim 17 wherein said structural coding sequence
further comprises a plastid targeting sequence and wherein said
isoamylase has a pH optimum of 5 - 8.

19. The method of Claim 18 wherein said structural coding sequence
comprises the CTP-1 plastid targeting sequence SEQ ID NO:7.

20. The method of Claim 19 wherein said isoamylase is SEQ ID NO:11.

21. The method of Claim 20 wherein said isoamylase is encoded by
SEQ ID NO:10.

22. The method of Claim 21 wherein said plant cell is from potato, corn,
wheat, barley, sweet potato, cassava, or rice.

23. The method of Claim 17 wherein said plant cells further comprise a
stably transformed, foreign ADPglucose pyrophosphorylase gene.


25. A plant comprising transformed plant cells of Claim 10 and
containing structurally modified starch.

26. The plant of Claim 25 wherein said plant is potato, corn, wheat,
barley, sweet potato, cassava, or rice.

27. The plant of Claim 25 wherein said structurally modified starch
comprises a higher ratio of amyllose to amylopectin.