Cloning and Nucleotide Sequence of the Gene (amyP) for Maltotetraose-Forming Amylase from Pseudomonas stutzeri MO-19

MASAYA FUJITA, †‡ KAKUJI TORIGOE, TETSUYA NAKADA, KEIJI TSUSAKI, MICHO KUBOTA, SHUZO SAKAI, AND YOSHIO TSUJISAKA

Hayashibara Biochemical Laboratories, Inc., 7-7 Amase-minami, Okayama 700, Japan

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The gene (amyP) coding for maltotetraose-forming amylase (exo-maltotetraohydrolase) of Pseudomonas stutzeri MO-19 was cloned. Its nucleotide sequence contained an open reading frame coding for a precursor (547 amino acid residues) of secreted amylase. The precursor had a signal peptide of 21 amino acid residues at its amino terminus. An extract of Escherichia coli carrying the cloned amyP had amylolytic activity with the same mode of action as the extracellular exo-maltotetraohydrolase obtained from P. stutzeri MO-19. A region in the primary structure of this amylase showed homology with those of other amylases of both procaroytic and eucaryotic origins. The minimum 5' noncoding region necessary for the expression of amyP in E. coli was determined, and the sequence of this region was compared with those of Pseudomonas promoters.

Under appropriate culture conditions, Pseudomonas stutzeri produces an extracellular amylase that forms maltotetraose (EC 3.2.1.60, exo-maltotetraohydrolase; abbreviated as G₄-amylase) (21). This enzyme is a unique amylase because it catalyzes the release of α-anomeric oligosaccharide (α-maltotetraose) exoglycolytically from the nonreducing ends of starch (23), whereas other exo-type amylases (glucoamylase and β-amylase) release β-anomeric products by exoglycolytic cleavage, and α-amylases hydrolyze starch endoglycolytically to produce α-malto-oligosaccharides. Thus, the G₄-amylase has unique activity for hydrolysis of starch intermediate between those of α-amylases and β- or gluco-amylase; G₄-amylase produces a similar product to α-amylases but hydrolyzes starch exoglycolytically like β- or gluco-amylase.

Robyt and Ackerman (21) reported that the G₄-amylase of P. stutzeri consists of isozymes. Multiple forms of this enzyme (seven isozymes) varying in molecular weight and isoelectric point were also reported by Schmidt and John (26). Sakano et al. (23, 24) purified two forms of enzyme (F-1 and F-2), each giving a single band on polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. The enzymatic properties of F-1 and F-2 were the same, but their isoelectric points were different. The reason for the multiple forms of the enzyme is still unknown.

G₄-amylase is commercially important for producing maltotetraose, which is used as a substrate of amylases in studies on their mode of action and as a highly sensitive substrate for detection of α-amylase when coupled with a chromogenic compound. Maltotetraose is also being tested for use as a food additive to improve the texture and moisture retention of foods.

As an initial step in understanding the molecular basis of the unique action mechanism of G₄-amylase and the genetic basis of its multiple forms and also for elucidating the regulation of the synthesis of this industrially important enzyme, we cloned and sequenced the G₄-amylase gene (amyP) from P. stutzeri. Then, we compared its amino acid sequence with those of other amylases of both procaroytic and eucaryotic origins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were P. stutzeri MO-19, isolated in this laboratory; P. stutzeri NRRRL B-3389, obtained from the Northern Regional Research Laboratory, Peoria, Ill.: Escherichia coli HB101 (F⁻ hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 λ⁻) (15) and E. coli JM83 [ara, Δ(lac-proAB) rpsL d80 lacZΔM15] (35). Plasmids pBR322 (15) and pUC18 (35) were used as vectors.

Media. P. stutzeri MO-19 was grown at 30°C in medium containing 50 g of dextrin, 0.5 g of peptone, 1 ml of corn steep liquor, 5 g of diaminon hydrogen citrate, 10 g of NH₄H₂PO₄, 1 g of KH₂PO₄, 0.5 g of CaCl₂ · 2H₂O, 5 mg of FeSO₄ · 7H₂O, 0.5 g of MgSO₄ · 7H₂O, and 1 mg of ZnSO₄ · 7H₂O per liter (pH 7.0). The E. coli strains were grown routinely in LB medium (15) at 37°C. Ampicillin was added at 50 μg/ml for selecting transformants carrying recombinant plasmids.

Purification of G₄-amylase produced by P. stutzeri. P. stutzeri MO-19 was grown for 20 h at 30°C with vigorous aeration in 2.5 liters of medium. Cells were separated from the culture fluid by centrifugation. Solid (NH₄)₂SO₄ was added to the fluid to 20% saturation, and the mixture was left to stand for 16 h at 4°C. The precipitate was removed by centrifugation (10,000 × g, 20 min), and the supernatant was then brought to 40% saturation of (NH₄)₂SO₄ and left to stand for 16 h at 4°C. The resulting precipitate was dissolved in 10 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer at 4°C. Insoluble material that appeared was removed by centrifugation, and the supernatant was dialyzed against 10 mM Tris hydrochloride (pH 8.0) at 4°C. The dialyzed enzyme was applied to a column of DEAE-Toyopearl 650S (Toyoda Soda, Tokyo, Japan), and material was eluted with a linear gradient of 0 to 0.5 M NaCl. G₄-amylase was eluted with a concentration of between 0.1 and 0.2 M NaCl. The purity of each fraction containing enzyme activity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14), and the fractions giving a single protein band were pooled and used for further studies.

Amino acid sequence analysis of the purified G₄-amylase.

* Present author.
† Present address: The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567, Japan.
The NH₂-terminal amino acid sequence of the purified G₄-amylase was determined by step-wise Edman degradation of 100 μg of the enzyme in an Applied Biosystems protein sequencer 470A fitted with a Senshu Pak AQUASIL SEQ-4(K) column (Senshu Scientific, Tokyo, Japan).

Preparation of chromosomal DNA from *P. stutzeri* and cloning of the *amyP* gene for G₄-amylase. Chromosomal DNA of *P. stutzeri* MO-19 was prepared (22), partially digested with restriction enzyme Sau3AI, and ligated to pBR322 DNA which had been treated with BamHI and bacterial alkaline phosphatase. The ligated DNA was used to transform *E. coli* HB101 by the conventional CaCl₂ procedure. Transformants were selected on LB plates supplemented with 50 μg of ampicillin per ml and 0.5% soluble starch. G₄-amylase-positive clones were detected by staining the plates with 10 mM KI-I₂ solution; the positive colonies had a semitransparent halo. DNA manipulations were done essentially as described by Maniatis et al. (15). Plasmids were isolated by the alkaline lysis procedure (1). Enzymes were obtained from commercial sources and used according to the recommendations of the suppliers.

Assay of G₄-amylase activity. A reaction mixture containing 0.2 ml of enzyme solution and 5 ml of 1% soluble starch buffered with 20 mM phosphate buffer (pH 7.0) was incubated for 20 min at 40°C. The reaction was stopped by mixing 1 ml of the reaction mixture with 2 ml of Somogyi reagent. Increase of reducing power was determined by the Somogyi-Nelson method (29). One unit of enzyme was defined as the activity forming 1 μmol of maltotetraose from soluble starch in 1 min.

Analysis of hydrolysis products from starch. A mixture of 5 ml of 5% soluble starch buffered with 20 mM phosphate buffer (pH 7.0) and 1 ml of enzyme solution (1 U) was incubated for 1 h at 40°C. Then an aliquot of the mixture was spotted onto Toyo filter paper (no. 50; Toyo Roshi, Tokyo, Japan). Paper chromatography was carried out by the ascending technique with a solvent system of n-butanol-pyridine-water (6:4:3) at 60°C. Then the chromatogram was dried and dipped in silver nitrate solution (32).

DNA sequence analysis. DNA sequencing was carried out by the dideoxy-chain termination method (25), with a slight modification using deoxy-7-deazaguanosine triphosphate (18) and pUC18 (5, 35).

Southern blot hybridization. Chromosomal DNA was digested with *SalI* and subjected to electrophoresis on a 1% agarose gel. The fragments separated were located by staining with ethidium bromide (0.5 μg/ml) and were transferred to a Zeta-probe membrane (Bio-Rad Japan, Tokyo, Japan). As a probe, the 3.0-kilobase-pair (kb) *SalI* DNA fragment prepared from pTPS618 was labeled with [α-³²P]dCTP (3,000 Ci/mmol) using a nick translation kit from Takara Shuzo (Kyoto, Japan). Hybridization was carried out as previously described (30).

RESULTS

Cloning of *amyP* in *E. coli*. A library of *P. stutzeri* MO-19 DNA was constructed in *E. coli* HB101 by using pBR322, and about 18,000 transformants were screened for G₄-amylase activity as described in Materials and Methods. One G₄-amylase-positive clone was obtained from this library. The recombinant plasmid from the positive clone was designated pTPS6. The restriction map of the 7.2-kb insert in pTPS6 is shown in Fig. 1. pTPS6 DNA was partially digested with *SalI* to reduce the size of the insert and then ligated and used to transform *E. coli* HB101. Several deletion plasmids conferring the G₄-amylase-positive character were obtained; one of these plasmids, designated pTPS618, lacked the C and D regions (Fig. 1). Thus, the *amyP* was found to be included in the A and B regions.

Homology between the cloned fragment and chromosomal DNA of *P. stutzeri*. Southern blot hybridization indicated that the labeled 3.0-kb *SalI* fragment (Fig. 1, B region) from pTPS618 hybridized with a 3.0-kb fragment of the chromo-

**FIG. 1.** Structures of plasmids pTPS6 and pTPS618 and the sequenced DNA fragment containing the *amyP* coding region. The box and thin line in the upper part represent the inserted DNA from *P. stutzeri* MO-19 and the vector pBR322 DNA, respectively. pTPS618 had deleted DNA regions (C and D) generated by partial digestion with *SalI*. Positions corresponding to the NH₂ terminus and COOH terminus of premature G₄-amylase are given (amino acid (aa) positions –21 and 526, respectively). □, Signal peptide (amino acid positions –21 to –1).
somal DNA of \textit{P. stutzeri} MO-19 digested with \textit{SalI}, whereas no hybridization band was detected with \textit{SalI}-digested chromosomal DNA from \textit{E. coli} HB101 (data not shown). The probe also hybridized with the same-size fragment from \textit{SalI}-digested chromosomal DNA of \textit{P. stutzeri} NRRL B-3389, which has been used in studies of \textit{G4} amylase by Robyt and Ackerman (21), Schmidt and John (26), and Sakano et al. (23, 24). Thus, these two \textit{P. stutzeri} strains seem to have homologous enzyme.

\textbf{Starch hydrolysis with \textit{G4} amylase produced by \textit{E. coli}.} Paper chromatography was used to determine whether the starch-hydrolyzing activity conferred by pTPS618 was due to the synthesis of \textit{G4} amylase. A cell extract prepared by sonication of \textit{E. coli} HB101 harboring pTPS618 was used as the crude enzyme because no amylolytic activity was detectable in the culture medium. The crude enzyme solution was incubated with soluble starch, and the products were analyzed by paper chromatography. The crude enzyme formed maltotetraose from soluble starch (Fig. 2), indicating that the cell extract from \textit{E. coli} HB101 harboring pTPS618 contained \textit{G4} amylase.

\textbf{DNA sequence of \textit{amyP}.} The nucleotide sequence of both strands of the 2.1-kb \textit{SalI}-\textit{Eco47III} segment (Fig. 1) was determined. The sequence contains a single open reading frame (1,641 base pairs) that encodes a protein of 547 amino acids corresponding to the \textit{amyP} product (Fig. 3). A protein with a calculated molecular weight of 59,736 could be translated from this open reading frame. No in-frame initiation codon could be found upstream of the open reading frame. The sequence GGAG, found 8-bp upstream of the first letter of the initiation codon is presumably the ribosome-binding site (27).

\textbf{Determination of the NH\textsubscript{2}-terminal amino acid sequence.} The sequence of the 20 NH\textsubscript{2}-terminal amino acids of the extracellular form of \textit{G4} amylase from \textit{P. stutzeri} MO-19 was determined with a peptide sequencer to be Asp-Gln-Ala-Gly-Lys-Ser-Pro-Asn-Ala-Val-Arg-Tyr-His-Gly-Gly-Asp-Glu-Ile-Leu. This sequence was identical to that deduced from the DNA sequence starting at position 64 (Fig. 3). These results suggest that the first 21 amino acid residues (Met-1 to Ala-21) constitute a signal peptide involved in secretion of the protein. The molecular weight of the mature \textit{G4} amylase calculated from the DNA sequence was 57,547, which is consistent with the value of 58,000 obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme. The amino acid composition calculated from the deduced amino acid sequence was essentially consistent with that of the purified enzyme (data not shown).

\textbf{Homology of \textit{G4} amylase with other \textit{\alpha}-amylases.} The \textit{G4} amylase sequence was compared with the primary structures of \textit{\alpha}-amylases from eucaryotes and prokaryotes. Four regions (I to IV) which are conserved in \textit{\alpha}-amylases (19) were also found in \textit{G4} amylase (Fig. 4). The homology of region III is limited, although the spacings of the four regions in the sequences of \textit{G4} amylase and \textit{\alpha}-amylases are similar. However, no homology was found between \textit{G4} amylase and \textit{\beta}- (13) or gluco-amylase (12; data not shown).

\textbf{Construction and characterization of deletion plasmids of the \textit{amyP} promoter region.} Plasmid pTPS618 was digested with \textit{Eco47III}, which has one site in the vector pBR322, ligated with pUC18 cleaved by \textit{Smal}, and then used to transform \textit{E. coli} HB101. One of the \textit{G4} amylase-positive clones was selected. Restriction analysis showed that this plasmid, designated pSE86R, carried the 2.2-kb \textit{Eco47III} fragment containing \textit{amyP}. The \textit{amyP} gene is in the opposite orientation to the \textit{lac} promoter on pUC18 (Fig. 5). The 2.2-kb \textit{Eco47III} fragment contains a 119-bp \textit{Eco47III}-\textit{BamHI} portion derived from pBR322. This 119-bp portion does not contain the promoter, since this portion is the middle part of the Tet' gene of pBR322. Thus, these results suggest that the \textit{amyP} is expressed in \textit{E. coli} by its own promoter.

To determine the location of the promoter, we constructed plasmids having a deletion in the 5' noncoding region of \textit{amyP}. pSE86R was linearized with \textit{BamHI} and \textit{PstI} and digested with exonuclease III to produce various extents of deletion of the \textit{amyP} promoter region. After treatment with mung bean nuclease and Klenow enzyme, the resultant blunt-ended fragments were ligated, and the ligated products were used to transform \textit{E. coli} HB101. The extents of deletion were determined by estimating the sizes of fragments generated by \textit{EcoRI} and \textit{HindIII} by 1% agarose gel electrophoresis. Five deletion plasmids were selected, and their \textit{G4} amylase activities in \textit{E. coli} were assayed as a function of the \textit{amyP} promoter (Fig. 5). The deletion endpoint of each plasmid was determined by DNA sequencing. The results revealed that the minimum 5' noncoding region required for \textit{G4} amylase activity in \textit{E. coli} is 77 nucleotides upstream from the initiation codon; this 77-nucleotide region is essential for the function of \textit{amyP} promoter in \textit{E. coli}. The endpoint of the region is located within an inverted repeat sequence (Fig. 3).

\textbf{DISCUSSION} We have cloned and analyzed the DNA fragment encoding the \textit{G4} amylase structural gene (\textit{amyP}) from \textit{P. stutzeri} MO-19. Its nucleotide sequence contained a single open reading frame of 1,641 bp, beginning from an ATG initiation codon at position 1 and ending with a TAG termination codon at position 1642 (Fig. 3). The deduced amino acid sequence corresponding to nucleotide positions 64 to 123 was identical to that of the NH\textsubscript{2}-terminal 20 amino acids of extracellular \textit{G4} amylase determined chemically. This finding suggests that the nucleotides from the initiation Met-1 codon to the Ala-21 codon at position 61 code for a signal
FIG. 3. Nucleotide sequence of amyP and its amino acid translation product. Only the sequence of the antisense strand is shown. The numbering of nucleotides begins at the first letter of the potential initiation codon. The cleavage site between the signal peptide and extracellular mature Gα-amylase is indicated by the upward arrow. The stem-and-loop structures upstream and downstream from the structural gene are indicated by horizontal arrows below the sequence. The sequence matching that obtained by Edman degradation of the NH2-terminal region of the purified Gα-amylase is underlined. A putative promoter is indicated by an overline. The line marked SD indicates the proposed Shine-Dalgarno (ribosome-binding) sequence. The upstream endpoint of the deletion, which is the minimum potential region for the promoter, is indicated by the downward arrow. The four sequences (I to IV) homologous to those of α-amylases are boxed.

GATCGCCCTTTCAGGAAAGTATAGACCTCTCTCCGCAGAATTTGTCGCCGCAAGTACAGCTCGGCGTACCCGAACTGGGACTGGCGCAACACCGCCAGCTGGCAGCAGATCATCAAGGACTGGTCCGACCGGGCC

AGCGCCAGGGCGCCGCGTACTCGGCAGGCCACTGGGAGTACCCGAACTGGGACTGGCGCAACACCGCCAGCTGGCAGCAGATCATCAAGGACTGGTCCGACCGGGCC

AGCGCCAGGGCGCCGCGTACTCGGCAGGCCACTGGGAGTACCCGAACTGGGACTGGCGCAACACCGCCAGCTGGCAGCAGATCATCAAGGACTGGTCCGACCGGGCC

AGCGCCAGGGCGCCGCGTACTCGGCAGGCCACTGGGAGTACCCGAACTGGGACTGGCGCAACACCGCCAGCTGGCAGCAGATCATCAAGGACTGGTCCGACCGGGCC
FIG. 4. Comparison of amino acid sequences of \( G_4 \)-amylase and various \( \alpha \)-amylases. Abbreviations: P. stu., \( P. \) \( stutzeri \); B. sub., \( B. \) subtilis; B. any., \( B. \) amylo-liquefaciens; A. ory., \( A. \) oryzae; Bar., barley; H. S. P., human saliva and pancreas. Sequences homologous to those of \( G_4 \)-amylase are underlined. Amino acid residues are numbered from the NH\(_2\) termini of the mature enzymes. N and C indicate the NH\(_2\) and COOH termini of \( G_4 \)-amylase, respectively.

The deduced amino acid sequence of this region, which has several positively charged amino acids near the NH\(_2\) terminus followed by a hydrophobic amino acid core and Ala at the COOH-terminal end, is similar to that typical of signal peptides of other secreted proteins (28, 33, 34).

We compared the primary structure of \( G_4 \)-amylase with those of \( \alpha \)-amylases from eucaryotes and prokaryotes and found that \( G_4 \)-amylase has regions homologous to the four regions (I to IV) conserved in the primary structures of \( \alpha \)-amylases (19; Fig. 4). A molecular model of Taka-amylase (\( \alpha \)-amylase from \( A. \) \( oryzae \)) proposed by Matsuura et al. (16) suggests that Glu-230 and Asp-297 in regions III and IV of this enzyme, respectively (Fig. 4), function as the catalytic residues. Corresponding residues, Glu-240 and Asp-314 were found in regions III and IV, respectively, of \( G_4 \)-amylase. These common regions may contain the amino acid residues responsible for cleaving 1,4-\( \alpha \)-D-glucosidic linkages. However, \( G_4 \)-amylase is unique in that it has Arg-217 and Gly-218 in region II; all other \( \alpha \)-amylases studied except barley \( \alpha \)-amylase, have Lys and His in this position (19). These amino acid residues found only in region II of \( G_4 \)-amylase may be related to the unique action of the enzyme in producing \( \alpha \)-maltotetraose from starch by an exomechanism. We mentioned previously that the mode of hydrolysis of starch by \( G_4 \)-amylase is intermediate between those of \( \alpha \)-amylases and \( \beta \)- or gluco-amylase. However, since no homologous regions were found in the primary structures of \( G_4 \)-amylase and \( \beta \)- or gluco-amylase, the evolutionary origin and catalytic mechanism of \( G_4 \)-amylase are closer to those of \( \alpha \)-amylases than to those of \( \beta \)- or gluco-amylase.

FIG. 5. \( G_4 \)-amylase activity in \( E. \) \( coli \) as a function of the \textit{amyP} promoter of pSE86R and its deletion derivatives. A 2.2-kb \textit{Eco}47III DNA fragment containing \textit{amyP} inserted into the \textit{Smal} site of pUC18 was designated pSE86R. The upper part of the figure shows the multiple cloning sites of pUC18. lacP indicates the \textit{lacZ} promoter. The construction of deletions in the 5’ noncoding region of \textit{amyP} is described in the text. □ and □. Noncoding and coding regions for \( G_4 \)-amylase, respectively; □, 119-bp \textit{Eco}47III-\textit{Bam}HI portion derived from pBR322; N and C, NH\(_2\) and COOH termini of \( G_4 \)-amylase, respectively. Numbers on the left indicate the nucleotide positions of deletion endpoints numbered from the first letter of the initiation Met codon. Crude cell extracts of \( E. \) \( coli \) harboring the plasmids were prepared, and their \( G_4 \)-amylase activities were assayed as described in Materials and Methods. Activities are shown on the right in milliunits per milliliter of culture.
Multiplicities in molecular weight or isoelectric point of \( G_4 \)-amylase have been reported by others (21, 23, 24, 26). We also observed two forms of the enzyme purified from our strain by gel electrophoresis (unpublished data). These multiplicities may be ascribed to limited proteolysis during culture of the organisms or purification of the enzyme, because Southern blot hybridization revealed that the \( G_4 \)-amylase gene (amyP) exists in a single locus of chromosomal DNA as a single copy and \( G_4 \)-amylase is primarily synthesized as a single polypeptide. However, the possibility that other nonhomologous genes for \( G_4 \)-amylase exist cannot be ruled out.

The minimum 5' noncoding region necessary for the expression of amyP in \( E. coli \) has been mapped by deletion analysis. This region was within 77-bp upstream from the initiation codon. Several characteristics of this region can be pointed out. (i) It contains an inverted repeat which has a high potential free energy \(-17.2 \text{ kcal} \) as calculated by the method of Tinoco et al. (31; Fig. 3). (ii) It does not show extensive homology to the consensus sequence recognized by the RNA polymerase holoenzyme \( \alpha \beta^\prime \beta \sigma^\prime \) in \( E. coli \) (17, 20). (iii) It has weak homology to the consensus sequence for \( ntr/\alpha\) promoters recognized by \( \alpha\) in \( E. coli \) (6, 7) and the sequences of several \( Pseudomonas \) promoters found to have homology with the consensus sequence (2) (Fig. 6). These observations suggest that this region may act as a native promoter in \( P. stutzeri \) MO-19 and may be involved in regulation of gene expression.

Determination of the transcription start site in \( P. stutzeri \) is necessary in order to clarify these suggestions. The total \( G_4 \)-amylase activity in \( E. coli \) is 0.05% of that in \( P. stutzeri \) (data not shown). Since the nucleotide sequence preceding the initiation codon for amyP is complementary to the 3' end of the 16S rRNA not only in \( Pseudomonas aeruginosa \) but also in \( E. coli \) (27), the mRNA of amyP is expected to be translated in \( E. coli \). Therefore, the weak expression of amyP in \( E. coli \) may be due to inefficient recognition of the amyP promoter by \( E. coli \) RNA polymerase. A similar reason for the weak expression of \( Pseudomonas \) genes in \( E. coli \) has been suggested by others (3, 4, 8–11). There have been few studies on \( Pseudomonas \) RNA polymerase, and further work is needed to elucidate its interaction with the promoter in \( Pseudomonas \) spp.
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