Proteases Involved in Generation of \( \beta \)- and \( \alpha \)-Amylases from a Large Amylase Precursor in Bacillus polymyxa

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The genes for extracellular neutral protease (Npr) and intracellular serine protease (Isp) were cloned from Bacillus polymyxa in order to elucidate the process involved in the generation of multiple \( \beta \)-amylases and an \( \alpha \)-amylase from a large amylase precursor. The npr gene was composed of 1,770 bp and 570 amino acids, while the isp gene was composed of 978 bp and 326 amino acids. Both proteases produced by E. coli cleaved the amylase precursor to generate \( \beta \)- and \( \alpha \)-amylases. Furthermore, several other proteases produced the same products from the precursor. A 130-kDa amylase precursor has two large domain structures responsible for the generation of \( \beta \)- and \( \alpha \)-amylases. The junction region of approximately 200 amino acids may be exposed on the surface of the molecule and susceptible to proteolytic enzymes, which results in the formation of multiple amylases.

Bacillus polymyxa produces multiform \( \beta \)-amylases with approximate molecular masses of 70, 56, and 42 kDa and a 48-kDa \( \alpha \)-amylase (3, 29). A large precursor protein with an approximate molecular mass of 130 kDa was detected transiently in the culture broth at an early phase of enzyme production and also when B. polymyxa was grown in the presence of protease inhibitors (3). The amylase gene comprises 3,588 nucleotides, which encode a mature amylase of 1,161 amino acids with a molecular weight of 127,314, and contains in-phase \( \beta \)- and \( \alpha \)-amylase-coding sequences in its 5' and 3' regions, respectively (29). The 130-kDa amylase exhibits both \( \beta \)- and \( \alpha \)-amylase activities and was proposed to be proteolytically processed, resulting in the generation of multiform \( \beta \)-amylases and an \( \alpha \)-amylase (29). This is the first demonstration of a single precursor protein for two enzymes in a procaryote. This type of DNA arrangement is also rare in eucaryotes. Only two nonviral polyprotein precursors have previously been reported, one for two mitochondrial enzymes in Neurospora crassa (31) and the other for yolk proteins in the nematode Caenorhabditis elegans (20).

Many species of the genus Bacillus produce a variety of extracellular and intracellular proteases. The major extracellular proteolytic enzymes are alkaline serine proteases, such as subtilisin produced by B. subtilis (25), and neutral (metallo-) proteases, such as thermolysin produced by B. thermoproteolyticus (28). Furthermore, many genes encoding extracellular proteases have been cloned from various Bacillus species and well characterized (2, 5, 15, 17, 22–24, 27, 30, 32, 33). The presence of a pro sequence is common to all the secreted proteases from bacilli. On the other hand, only one intracellular serine protease produced within B. subtilis cells, designated as Isp-1, has been studied in detail (4, 21).

To characterize enzymes involved in the proteolytic processing of the amylase precursor into \( \beta \)- and \( \alpha \)-amylases, two protease genes were cloned from B. polymyxa and sequenced: the extracellular neutral protease gene (npr) and the intracellular serine protease gene (isp). Both proteases produced in Escherichia coli were shown to process the amylase precursor into \( \beta \)- and \( \alpha \)-amylases.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation. The bacterial strains used were B. brevis HPD31 (26), B. polymyxa 72 (14), and E. coli HB101 and JM103 (9). B. brevis HPD31 and B. polymyxa 72 were grown at 37 and 30°C, respectively, as described previously (29). E. coli was grown at 37°C in L-broth (11). When required, ampicillin was added at a concentration of 50 \( \mu \)g/ml. E. coli HB101 was used as the cloning host, and JM103 was used for DNA manipulation and the growth of M13 phages. The pBR322 and pUC plasmids were used as cloning vectors. pYN520 containing the B. polymyxa amylase gene was used to produce the 130-kDa amylase in B. brevis HPD31 (29). Transformation of E. coli was performed as described by Lederberg and Cohen (8).

Isolation and analysis of DNAs. B. polymyxa chromosomal DNA was isolated as described by Saito and Miura (18). Plasmid DNAs were isolated from E. coli as described by Birnboim (1). Treatment of DNAs with restriction enzymes and ligation were carried out under the conditions specified by the supplier, and DNA fragments were analyzed by electrophoresis in 0.7% agarose gels and 5% polyacrylamide gels (9). DNA sequencing was carried out by the dideoxychain termination method of Sanger et al. (19) after subcloning of appropriate restriction fragments into derivatives of bacteriophage M13.

Enzyme assay. Protease-producing clones identified by halo formation around the clones, were detected on L-broth plates supplemented with 1% skim milk. Protease activity was determined by two methods. For the azocasein method (12), 0.1 ml of a 0.5% azocasein solution in 0.2 M Tris-HCl buffer (pH 7.5) was incubated with 0.1 ml of the enzyme solution for 60 min at 37°C. The reaction was stopped by adding 0.2 ml of a 10% trichloroacetic acid solution. After standing for 20 min at 25°C, the mixture was centrifuged (15,000 rpm, 15 min, 25°C) and the supernatant was mixed with 0.4 ml of 0.5 N NaOH. The A440 was determined. One unit was arbitrarily defined as the activity giving an increase

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in $A_{440}$ of 1 in 60 min at 37°C. In the second method, we assayed processing activity toward the 130-kDa amylase. The 130-kDa amylase was isolated from $B.\ polymyxa$ carrying pYN520 as described previously (29) and used as a substrate for proteases. The 130-kDa amylase, approximately 1 $\mu$g/7 $\mu$L, was mixed with 9 $\mu$L of the enzyme solution and incubated for 60 min at 37°C. The reaction was stopped by heating for 5 min at 100°C in the presence of 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol, and the preparation was subjected to electrophoresis on SDS-polyacrylamide gels (10%) as described by Laemmli (7). Amylase activity bands were detected in situ as described by Lacks and Springhorn (6).

Purification and amino-terminal amino acid sequence analysis of proteases. $E.\ coli$ carrying pST2 grown at 37°C for 24 h in L broth was harvested by centrifugation (8,000 rpm, 15 min, 4°C), suspended in 1/100 the original volume of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl$_2$, and then disrupted with a French press operating at 400 kg/cm$^2$. After centrifugation (15,000 rpm, 20 min, 4°C), the supernatant was dialyzed at 4°C against the same buffer. The dialyzed sample was applied to a column of DEAE-cellulose (3 by 8 cm) equilibrated with the same buffer. The enzyme was eluted from the column at a flow rate of 20 mL/h with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The protease was eluted between 0.3 and 0.4 M NaCl. The fractions containing the protease were pooled. The enzyme was precipitated with ammonium sulfate (80% saturation), dissolved in and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl$_2$ and 0.2 M NaCl, and then subjected to gel filtration on a column of Toyopearl HW-55 (2.2 by 60 cm) with the same buffer at a flow rate of 20 mL/h. The fractions containing the protease were pooled and kept at 4°C. The yield of protease up to this step corresponded to approximately 25% of the initial total activity. The protease purified gave a single band corresponding to a molecular mass of 35 kDa on SDS-polyacrylamide gel electrophoresis. The protease produced extracellularly by $B.\ polymyxa$ was partially purified as follows. The protease was precipitated by the addition of ammonium sulfate to the culture fluid to 80% saturation. The precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl$_2$ and then dialyzed against the same buffer. After removal of the precipitate by centrifugation (10,000 rpm, 10 min, 4°C), the dialyzed sample was applied to a column of DEAE-cellulose (2.9 by 8 cm) that had been equilibrated and washed extensively with the same buffer to elute amylases produced simultaneously by $B.\ polymyxa$. The protease was eluted from the column with the same buffer containing 0.5 M NaCl. The amino-terminal amino acid sequence of the protease was determined with an ABI 477A-120A protein sequencer after extensive dialysis against distilled water.

Other methods. Protein was determined by means of the standard Bio-Rad protein assay, as outlined by the supplier, with bovine plasma gamma globulin as a standard. Protease type IX (0.9 U/mg [solid]) isolated from $B.\ polymyxa$, purchased from Sigma Chemical Co. (St. Louis, Mo.), was purified further by SDS-polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidene difluoride membrane for sequence determination (10). The a-amylase-specific inhibitor S-A1 (13) was a gift from S. Murao and M. Arai, University of Osaka Prefecture, and was used to inhibit the 48-kDa a-amylase as described previously (29).

TABLE 1. Enzymatic properties of proteases produced in $E.\ coli$ carrying pST1 and pST2

| Strain | Protease activity (U/mg of protein) | % Inhibition of protease activity by: |
|--------|------------------------------------|-------------------------------------|
|        | 5 mM EDTA | 2 mM DFP |
| $E.\ coli$(pST1) | 0.7 | 100 | 5 |
| $E.\ coli$(pST2) | 0.6 | 100 | 92 |
| $E.\ coli$(pBR322) | 0.07 | ND | ND |

* $E.\ coli$ grown overnight at 37°C in L broth supplemented with ampicillin (50 $\mu$g/mL) was harvested by centrifugation (10,000 rpm, 10 min, 4°C), suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl$_2$, and then sonicated three times for 1-min intervals in an ice bath. After centrifugation (10,000 rpm, 15 min, 4°C), protease activity in the supernatant was determined with azocasein as a substrate. ND, not determined.

RESULTS AND DISCUSSION

Cloning of genes encoding neutral and serine proteases involved in processing of amylase precursor into $\beta$- and $\alpha$-amylases. Chromosomal DNA from $B.\ polymyxa$ 72 was partially digested with HindIII and then ligated to pBR322 cleaved with HindIII. The ligated molecules were used to transform $E.\ coli$ HB101 to ampicillin resistance. The protease-producing transformants were detected on skim milk-containing plates by observation of halo formation. Approximately 10$^4$ transformants were examined, and three halo-forming clones (ST1, ST2, and ST3) were obtained after 2 to 3 days of incubation. Two of them, ST2 and ST3, were found to carry the identical recombinant DNA, as described below. ST3 was not characterized further.

The clones carrying pST1 and pST2 produced approximately 10 times more protease than did the clone carrying pBR322. The protease encoded on pST1 was inhibited by EDTA but not by diisopropylfluorophosphate (DFP). The protease encoded on pST2 was inhibited by both EDTA and DFP (Table 1). This finding suggests that the enzymes encoded on pST1 and pST2 are neutral protease and a serine protease, respectively. To further characterize these proteases with respect to specific cleavage of the amylase precursor, the 130-kDa amylase was incubated with the enzymes, and then amylase activity bands were detected in situ in the presence and absence of an $\alpha$-amylase inhibitor, S-A1 (Fig. 1C and D). The protease prepared from $E.\ coli$ carrying pBR322 cleaved the 130-kDa amylase rather randomly, producing several amylase activity bands (Fig. 1C, lane 3). Both neutral and serine proteases, however, cleaved the 130-kDa amylase, two distinct 70- and 48-kDa amylase activity bands being observed (Fig. 1C, lanes 1 and 2; Fig. 1D, lanes 1 and 2). Furthermore, the 48-kDa amylase activity band was due to $\alpha$-amylase activity, since it was not observed after treatment with S-A1 (Fig. 1D, lanes 3 and 4). The same processing activity was detected with protease(s) partially purified from the culture broth of $B.\ polymyxa$ (Fig. 1B).

Restriction analysis and deletion mapping of neutral and serine protease genes. pST1 contained a 15-kb HindIII insert, while pST2 and pST3 contained 5-kb HindIII inserts that were shown to be identical upon further restriction analysis. To reduce the size of the insert on pST1, pST1 was digested with EcoRI and the resultant fragments were ligated. All of the protease-producing clones carried at least a 5-kb EcoRI fragment. One of these clones was designated ST11. The restriction maps of pST11 and pST2 are presented in Fig. 2. Further deletion analysis revealed that the neutral and serine
protease genes were located within a 3.2-kb \textit{BglII}-EcoRI fragment on pST11 and a 3.4-kb EcoRI-SalI fragment on pST2, respectively.

\textbf{Nucleotide sequence of the npr gene.} The nucleotide sequences of the \textit{B. polymyxa} npr gene and its flanking regions were determined for the approximately 2.3 kb from the \textit{BglII} site to downstream of the PstI site on pST11 shown in Fig. 2A (Fig. 3). Only one large open reading frame was found, which was composed of 1,770 bp and 590 amino acids. The first 23 amino acids show characteristics typical of signal peptides of secretory precursors (16). \textit{B. polymyxa} protease type IX was analyzed as to its NH\textsubscript{2}-terminal amino acid sequence. Except for the first three amino acids, Ala-Thr-Gly, the NH\textsubscript{2}-terminal amino acid sequence, Thr-Gly-Lys-Val-Leu-Gly-Asp-X-Lys-Ser-Phe, was in complete agreement with that deduced from the nucleotide sequence starting at ACA (nucleotides 1210 to 1212; Thr-290) (Fig. 3). The reason for the difference in the first three amino acids is unclear; however, these results clearly indicate that Npr should also be synthesized as a preproenzyme with a rather long pro sequence of 264 amino acids. If there is no additional processing, the mature Npr enzyme would be composed of 304 amino acids with a molecular weight of 32,477, which is in reasonable agreement with the molecular weight of the protease type IX of 35,000, as determined by SDS-polyacrylamide gel electrophoresis. The putative pro sequence of \textit{B. polymyxa} Npr showed no significant homology with sequences of any previously characterized \textit{Bacillus} proteases. In contrast, the \textit{B. polymyxa} Npr sequence from Asn-287 to Gly-590 showed considerable homology with sequences of neutral metalloproteases such as thermolysin (54.8\%) from \textit{B. thermoproteolyticus} (28).

\textbf{Nucleotide sequence of the isp gene.} The nucleotide sequences of the \textit{B. polymyxa} isp gene and its flanking regions were determined for the approximately 1.2 kb from 150 bp upstream of the \textit{Aval} site to downstream of the PstI site on pST2 shown in Fig. 2B (Fig. 4). A single open reading frame was found, which was 978 bp in size and encoded a polypeptide of 326 amino acid residues with a molecular weight of 35,173. This size was in good agreement with the molecular weight of 35,000 of the protease (Isp) purified from \textit{E. coli} carrying pST2, as judged by SDS-polyacrylamide gel electrophoresis. Furthermore, the 20-residue NH\textsubscript{2}-terminal amino acid sequence of the purified Isp determined chemically, Glu-Gln-Gln-Val-Asn-Glu-Ile-Pro-Arg-Gly-Val-Glu-
B. POLYMYXA AMYLASE-PROCESSING PROTEASES

The amylase gene of *B. polymyxa* is over 3,588 nucleotides and is homologous to *B. subtilis* Isp-1 (4), exhibiting the same processing activity. These data clearly demonstrate that various types of proteases are involved in the formation of multiple amylases in *B. polymyxa*.

Met-Ile-Gln-Ala-Pro-X-Val-Trp, was in complete agreement with that deduced from the nucleotide sequence starting at GAG (nucleotides 85 to 87; Glu-16) (Fig. 4). The preceding 15-residue NH$_2$-terminal amino acid sequence is quite different from that of a typical signal peptide, which suggests that the enzyme should be an intracellular enzyme. The 15 amino acids from the primary translation product could be removed as a consequence of some artifact of the purification, as proven for *B. subtilis* Isp-1 (21). Therefore, the amylase encoded on pST2 was designated the *B. polymyxa* Isp. *B. polymyxa* Isp showed 62% homology with *B. subtilis* Isp (4).

Despite the apparent differences in their catalytic properties, both Npr and Isp cleaved the amylase precursor to generate β- and α-amylases (Fig. 1). This finding suggests that site-specific proteolytic cleavage may not be required for the formation of multiple amylases. This, in turn, prompted us to determine the processing activities of various proteases, such as trypsin, chymotrypsin, and subtilisin. All enzymes examined processed the precursor into β- and α-amylases, as did the *B. polymyxa* proteases, when assayed under optimal conditions. Furthermore, *B. subtilis* Isp, the gene for which was cloned from *B. subtilis* BD104 and which is 98% homologous to *B. subtilis* Isp-1 (4), exhibited the same processing activity. These data clearly demonstrate that various types of proteases are involved in the formation of multiple amylases in *B. polymyxa*.

![FIG. 3. Nucleotide and deduced amino acid sequences of the neutral protease gene on pST11. A probable Shine-Dalgarno (SD) sequence and the putative promoter (~35 and ~10 regions) are indicated by solid lines under the nucleotide sequence. The asterisk indicates a stop codon. A possible transcription terminator is indicated by arrows. The 15-residue NH$_2$-terminal amino acid sequence of *B. polymyxa* neutral protease type IX determined by the Edman method is given in italics. The first three amino acids that differ from the deduced ones are underlined. X indicates an amino acid not assigned.](image_url)
FIG. 4. Nucleotide and deduced amino acid sequences of the intracellular serine protease gene on pST2. A probable Shine-Dalgarno (SD) sequence is underscored. The asterisk indicates a stop codon. The 17-residue NH₂-terminal amino acid sequence of the protease isolated from E. coli carrying pST2, determined by the Edman method, is overlined. X indicates an amino acid not assigned.

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