JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 375–382
0021-9193/89/010375-08$02.00/0

Vol. 171, No. 1

A Single Gene Directs Synthesis of a Precursor Protein with β- and α-Amylase Activities in *Bacillus polymyxa*

NOBUYUKI UOZUMI, KYOKO SAKURAI, TAKUJI SASAKI, SHIRO TAKEKAWA, HIDEO YAMAGATA, NORIHIRO TSUKAGOSHI,* AND SHIGEZO UDAKA

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan

Received 6 July 1988/Accepted 10 October 1988

The *Bacillus polymyxa* amylase gene comprises 3,588 nucleotides. The mature amylase comprises 1,161 amino acids with a molecular weight of 127,314. The gene appeared to be divided into two portions by the direct-repeat sequence located at almost the middle of the gene. The 5′ region upstream of the direct-repeat sequence was shown to be responsible for the synthesis of β-amylase. The 3′ region downstream of the direct-repeat sequence contained four sequences homologous with those in other α-amylases, such as Taka-amylase A. The 48-kilodalton (kDa) amylase isolated from *B. polymyxa* was proven to have α-amylase activity. The amino acid sequences of the peptides generated from the 48-kDa amylase showed complete agreement with the predicted amino acid sequence of the C-terminal portion. The *B. polymyxa* amylase gene was therefore concluded to contain in-phase β- and α-amylase-coding sequences in the 5′ and 3′ regions, respectively. A precursor protein, a 130-kDa amylase, directed by a plasmid, pYN520, carrying the entire amylase gene, had both β- and α-amylase activities. This represents the first report of a single protein precursor in procaryotes that gives rise to two enzymes.

More than one polypeptide can be produced from a single gene as a consequence of either a unique DNA arrangement, as in the case of overlapping genes, or the processing of precursor polyproteins. Overlapping genes are relatively common in bacteriophages, viruses, and mitochondria as well as in bacteria (28). The processing of precursor polyproteins is very rare, the main examples being viral polyproteins and polypeptide hormones in eucaryotes (41). Recently, a single precursor protein for two mitochondrial enzymes, acetylglutamate kinase and acetylglutamyl-phosphate reductase, was demonstrated in *Neurospora crassa* (47). We report here that the *Bacillus polymyxa* amylase is synthesized as a precursor protein with β- and α-amylase activities that gives rise to β- and α-amylases.

β- and α-amylases hydrolyze the α-1,4-glucosidic linkages in exo and endo fashions, respectively, from the nonreducing ends of starch-type substrates. β-Amylase (EC 3.2.1.2) produces maltose and is present in certain bacteria, such as *B. polymyxa* (21, 25), *Bacillus cereus* (39, 43), and *Clostridium thermosulfurogenes* (8), as well as in plants (2, 13). α-Amylase (EC 3.2.1.1) produces a series of maltooligosaccharides and is widely distributed in microorganisms as well as in plants and animals (2). Many microbial and eucaryotic α-amylase genes have been cloned and well characterized (4, 18, 27, 29, 33, 37, 45, 49, 50).

We reported that *B. polymyxa* 72 produces multiform β-amylases with approximate molecular masses of 70, 56, and 42 kilodaltons (kDa) and one unclassified amylase with an approximate molecular mass of 48 kDa (11). 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. To elucidate the structures of the *B. polymyxa* β-amylases and the regulation of their formation and secretion, the *B. polymyxa* β-amylase gene was cloned, and a partial nucleotide sequence of the gene has been reported (5, 11, 32).

* Corresponding author.

In this paper, we report the sequencing of the 3′ region of the β-amylase gene, the features of the unclassified amylase, and evidence that the *B. polymyxa* β-amylase gene contains an in-phase α-amylase-coding sequence in the 3′ region. Furthermore, a large precursor protein was shown to possess both β- and α-amylase activities.

MATERIALS AND METHODS

Materials. All restriction enzymes, T4 DNA ligase, and a dideoxy sequencing kit were purchased from Takara Shuzo Co., Ltd. The universal translation terminator 5′-GCT TAATTAATTAAGC-3′ was from Pharmacia Fine Chemicals, Piscataway, N.J. [α-^{32}P]dCTP (400 mCi/mmol) was from Amersham International Ltd. Biodyne A membranes were from PALL Ultrafine Filtration Corp. *Achromobacter* lysylendopeptidase (EC 3.4.21.50) was from Wako Pure Chemical Industries Ltd. *Bacillus licheniformis* α-amylase (Termamyl) was a gift from Novo Industri Japan Ltd. Amylose was from Sigma Chemical Co., St. Louis, Mo. α- and β-cyclodextrins and oligosaccharide mixtures were gifts from Hayashibara Biochemical Laboratories Inc. S-AI, an oligosaccharide produced by *Streptomyces diastaticus* which inhibits α-amylases of animal, plant, and microbial origins (24), was a gift from S. Murao and M. Arai of the University of Osaka Prefecture. α-EPG (2,3-epoxypropyl α-D-glucopyranoside), which inhibits β-amylases of plant and microbial origins (10), was a gift from Y. Nitta of the University of Osaka Prefecture. All other reagents were of reagent grade.

Bacterial strains, plasmids, media, and transformation. The bacterial strains used were *B. polymyxa* 72 (25), *Escherichia coli* HB101, JM83, JM103, and DH20 (20), and *Bacillus brevis* HPD31 (H. Takagi, K. Kadowaki, and S. Udaka, submitted for publication). *B. polymyxa* 72 was grown at 30°C as described previously (11). *B. brevis* HPD31 was grown at 37°C in T_2 medium (48). *E. coli* was grown at 37°C in L broth (23). When required, ampicillin and erythromycin were added at concentrations of 50 and 10 μg/ml, respectively. Transformation of *E. coli* and *B. brevis* was per-

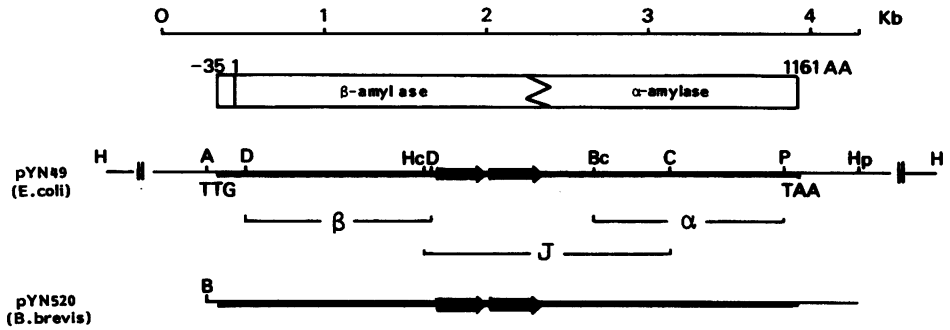

FIG. 1. Diagramatic representation of the amylase gene. At the top is a schematic diagram of the β- and α-amylase-coding regions. Beneath are shown the positions of the restriction enzyme sites used to construct pYN520 and to prepare DNA fragments. The origins of the three DNA probes used in this study, β, J and α, are indicated below the restriction map. The direct-repeat sequences are indicated by arrows. The start codon (TTG) and stop codon (TAA) are also indicated. At the bottom is the DNA fragment subcloned onto pRU100. Abbreviations for restriction enzyme sites: A, *Acc*I; B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; D, *Dra*I; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*II; P, *Pvu*II.

formed by the methods of Lederberg and Cohen (17) and Takahashi et al. (42), respectively. Amylase-positive clones were detected by staining the plates with a 1.7 mM I_2-KI solution as described previously (45).

Plasmid pYN49, containing the *B. polymyxa* β-amylase gene, was described previously (11). pRU100 was constructed as follows: a 55-base-pair (bp) *Hin*dIII-*Eco*RI fragment, the multicloning sequence, on pUC19 was inserted into pHW1 (7) after the *Pvu*II and *Hin*dIII sites on pHW1 had been converted to *Hin*dIII and *Eco*RI sites, respectively.

Isolation and analysis of DNAs. *B. polymyxa* chromosomal DNA was isolated as described by Saito and Miura (34). Plasmid DNAs were isolated from *E. coli* and *B. brevis* as described by Birnboim (3). 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 (20). Southern blot analysis of the *B. polymyxa* chromosomal DNA was performed by using Biodyne A membranes as described by Southern (40). The β (1,149-bp *Dra*I-*Dra*I), J (1,521-bp *Hin*cII-*Cla*I), and α (1,180-bp *Bcl*I-*Pvu*II) fragments were isolated from pYN49, labeled by nick translation with [α-^{32}P]dCTP, and then used as hybridization probes (Fig. 1). DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (36) after subcloning of appropriate restriction fragments into derivatives of bacteriophage M13.

Purification of 48-kDa amylase. *B. polymyxa* 72 was grown at 30°C for 40 h in the medium described above. The following steps were performed at 4°C. The amylase was precipitated by addition of ammonium sulfate to 80% saturation to the culture fluid. The precipitate was dissolved in a small volume of 10 mM Tris hydrochloride buffer (pH 8) and then dialyzed extensively against the same buffer. After removal of the precipitate by centrifugation (10,000 × *g*, 10 min), the dialyzed sample was applied to a column of DEAE-cellulose (2.9 by 15 cm) that was equilibrated and eluted at a flow rate of 18 ml/h with 10 mM Tris hydrochloride buffer (pH 8.0). Both the 48-kDa amylase and β-amylase were recovered in the flowthrough fraction, precipitated by addition of ammonium sulfate to 80% saturation, and then dissolved in and dialyzed against 50 mM Tris hydrochloride buffer (pH 7.5). The dialyzed sample was subjected to gel filtration on a column of Sephadex G-75 (2.5 by 40 cm) with the same buffer at a flow rate of 18 ml/h. The fractions containing the 48-kDa amylase were visualized in situ in sodium dodecyl sulfate (SDS)-polyacrylamide gels after electrophoresis as described below and then pooled. The enzyme was precipitated with ammonium sulfate (80% saturation) and dissolved in and dialyzed against 10 mM acetate buffer (pH 6.0). The dialyzed sample was applied to a column of carboxymethyl cellulose (2 by 10 cm) previously equilibrated with the same buffer. The enzyme was eluted at a flow rate of 18 ml/h from the column with a linear gradient of 0 to 0.3 M NaCl in the same buffer. The 48-kDa amylase was eluted at 0.15 M NaCl.

Production and isolation of 130-kDa amylase. To produce the 130-kDa amylase, a plasmid, pYN520, was constructed as described below, using *B. brevis* HPD31 as the host. First, the *Acc*I site situated in the 5′ region of the β-amylase gene (Fig. 1) was converted to a *Bam*HI site, followed by digestion with *Pvu*II. The resultant *Bam*HI-*Pvu*II fragment (3.6 kilobases) was isolated. Second, the *Pvu*II-*Hpa*II fragment (0.45 kilobases) containing the 3′ region of the gene (Fig. 1) was also isolated. Third, the *Bam*HI-*Pvu*II and *Pvu*II-*Hpa*II fragments were ligated to pRU100, which had been cleaved with *Bam*HI and *Acc*I and then used to transform *B. brevis* HPD31 to erythromycin resistance. The transformants were assayed for amylase production on T_2 plates supplemented with 0.5% soluble starch, 10 μg of erythromycin per ml, and 1.5% agar, as described above. One of the amylase-positive clones contained pYN520 (Fig. 1). *B. brevis* HPD31 carrying pYN520 was grown at 30°C for 24 h in T_2 medium supplemented with 0.5% soluble starch and 10 μg of erythromycin per ml. The following steps were performed at 4°C. The amylase was precipitated with ammonium sulfate (80% saturation). The precipitate was dissolved in a small volume of 10 mM Tris hydrochloride buffer (pH 8) containing 1 mM EDTA and then dialyzed against the same buffer. After removal of the precipitate by centrifugation (10,000 × *g*, 10 min), the dialyzed sample was applied to a column of DEAE-cellulose (2.9 by 8 cm) equilibrated with the same buffer. The enzyme was eluted from the column at a flow rate of 18 ml/h with a linear gradient of 0 to 1 M NaCl in the same buffer. The fractions containing the 130-kDa amylase were visualized in situ in the SDS-polyacrylamide gels as described below and pooled. The enzyme was precipitated with ammonium sulfate (80% saturation), dissolved in and dialyzed against 100 mM Tris hydrochloride buffer (pH 7.5), and then subjected to gel filtration on a column of Toyopearl HW-55 (2.2 by 65 cm) with the same buffer containing 0.5 M NaCl, at a flow rate of 18 ml/h. The fractions containing the 130-kDa amylase were visualized, pooled, concentrated

with ammonium sulfate (80% saturation), and then dialyzed against 50 mM Tris hydrochloride buffer (pH 7.5).

Amino acid sequence analysis. A 500-μg sample of the 48-kDa amylase in 10 mM acetate buffer (pH 6.0) was extensively dialyzed at 4°C against 50 mM triethylamine acetate buffer (pH 9.0), digested at 37°C for 6 h with 0.1 U of lysylendopeptidase, and then lyophilized. The lysylendopeptidase digest was dissolved in formic acid and then subjected to high-performance liquid chromatography with a Shimadzu LC-6A system equipped with a reverse-phase Toso ODS-120T column (0.46 by 25 cm). Elution was performed with a linear gradient of acetonitrile (5 to 60%) in 0.1% trifluoroacetic acid for 90 min at a flow rate of 0.5 ml/min. The peptides were monitored by measuring A_{220}. The amino acid sequences were determined with a JEOL JAS-47K sequence analyzer as described previously (46). The N-terminal amino acid sequence of the 130-kDa amylase was determined with an ABI 477A-120A protein sequencer.

Assaying of amylase activities. The β- and α-amylase activities were determined at 37 and 45°C with soluble starch as a substrate by the methods of Murao et al. (25) and Saito (35), respectively. One unit of β- and α-amylase was defined as the activity causing the formation of 1 μmol of maltose from soluble starch in 1 min and the hydrolysis of 0.1 mg of soluble starch in 10 min, respectively. The hydrolysis products from soluble starch and cyclodextrin were analyzed by paper chromatography with *n*-propanol–H_2O (7:3, vol/vol) as the solvent. Three ascents of approximately 20 cm each were completed on each chromatogram at room temperature. After drying, the chromatograms were developed by the silver nitrate dip method of Robyt and French (30). Blue-value-reducing-value curves were determined at 37°C, with 0.5% amylose as a substrate, by the method of Robyt and French (31). Amylase activity bands were detected in situ after electrophoresis as described by Lacks and Springhorn (15). Amylases were heated at 100°C for 5 min in the presence of 2% SDS and 5% 2-mercaptoethanol and then electrophoresed on SDS-polyacrylamide gels (10%) as described by Laemmli (16). The gels were briefly rinsed with water and washed twice at room temperature by gentle shaking in 200 ml of 40 mM Tris hydrochloride buffer (pH 7.5) for 1 h each, followed by incubation for 1 h with 100 ml of 0.5% soluble starch in 100 mM Tris hydrochloride buffer (pH 7.5). Finally, the gels were stained with a 1.7 mM I_2-KI solution.

Other methods. Protein was determined by the method of Lowry et al. (19), with bovine serum albumin as a standard. The 70-kDa β-amylase was purified from *B. polymyxa* as described previously (11).

RESULTS

Nucleotide sequence and characterization of the amylase gene. The nucleotide sequence for the 5′ region of the amylase gene up to the *Cla*I site on the insert of pYN49 (Fig. 1) has been reported (11). The nucleotide sequence for the 3′ region of the gene downstream of the *Cla*I site was determined and is shown along with a part of the 5′ sequence in Fig. 2. The amylase gene comprises 3,588 nucleotides and appears to be divided into two portions by a large direct repeat (nearly 300 bp long). The predicted amino acid sequence comprises a putative 35-amino-acid signal sequence and a 1,161-amino-acid amylase sequence. The mature amylase should therefore be synthesized as a protein with a molecular weight of 127,134.

To determine the similarity of the deduced amino acid sequence of the *B. polymyxa* amylase with those of amylases of various origins, the amino acid sequences of the latter were optimally aligned with the *B. polymyxa* sequence. The *B. polymyxa* amylase contained sequences homologous with sequences in other β- and α-amylases in the N- and C-terminal portions, respectively. However, the N- and C-terminal portions of the *B. polymyxa* amylase showed no significant similarity with those of the other α- and β-amylases. Interestingly, these two portions were separated by the direct-repeat sequence described above.

In the N-terminal portion upstream of the direct-repeat sequence, three highly conserved sequences (Fig. 2) were recognized at similar intervals among the β-amylases derived from *B. polymyxa*, *C. thermosulfurogenes* (12), and barley (13) (Ile-77 to Pro-94, Gly-159 to Pro-171, and Leu-319 to Ser-329 of the *B. polymyxa* β-amylase), which were suggested to comprise the active site (12). In the C-terminal portion downstream of the direct-repeat sequence, the *B. polymyxa* amylase contained four sequences homologous at similar intervals with sequences in Taka-amylase A (44) (Fig. 2). X-ray crystallographic analysis of Taka-amylase A has shown that His-210 and Asp-297 are located in the active center cleft and that His-122, His-296, and Asp-297 participate in substrate binding (22). Three of the four homologous sequences (I, II, and IV) observed in the *B. polymyxa* amylase should therefore function as active centers of α-amylase. Further comparison with other procaryotic (*B. licheniformis* and *Bacillus stearothermophilus*) and eucaryotic (*Aspergillus oryzae* and human) amylases showed almost the same homologous sequences in the same regions as demonstrated previously (9, 26). This finding suggests that the *B. polymyxa* amylase gene encodes a single translation product that possesses two enzymatic activities.

We next examined whether the *B. polymyxa* genome contains a DNA fragment identical in size to the plasmid segment, since it is conceivable that the insert on pYN49 resulted from artificial DNA rearrangement during propagation of the plasmid in *E. coli*. Three DNA probes were prepared from the insert on pYN49: β probe (1,149-bp *Dra*I-*Dra*I fragment within the β-amylase-coding region), J probe (1,521-bp *Hin*cII-*Cla*I fragment at the junction of the β- and α-amylase-coding sequences), and α probe (1,180-bp *Bcl*I-*Pvu*II fragment within the α-amylase-coding region). Both the *B. polymyxa* chromosomal and plasmid DNAs were cleaved with restriction enzymes *Eco*RI and *Pvu*II and probed with the ^{32}P-labeled β, J, and α DNA fragments. All three probes hybridized to DNA fragments of the same sizes generated from the chromosomal and plasmid DNAs with different restriction enzymes (Fig. 3). The *B. polymyxa* genome therefore appears to contain an amylase-coding fragment of the same size as plasmid pYN49 and thus presumably contains in-phase β- and α-amylase-coding sequences.

Characterization and amino acid sequence analysis of 48-kDa amylase. The 48-kDa amylase and 70-kDa β-amylase were purified to homogeneity from the culture broth of *B. polymyxa* (Fig. 4). To determine what type the 48-kDa amylase was, the action pattern of the 48-kDa amylase on soluble starch was compared with those of the 70-kDa β-amylase and the *B. licheniformis* α-amylase (Fig. 5). The 48-kDa amylase produced a series of maltooligosaccharides ranging from maltose to maltopentaose, maltose being the most prominent species (Fig. 5A, lane 2, and Fig. 6A, lane 1). The *B. licheniformis* α-amylase also produced a series of maltooligosaccharides, with glucose as the main product (Fig. 5A, lane 5). The 70-kDa β-amylase, on the other hand, yielded only maltose (Fig. 5A, lane 3, and Fig. 6A, lane 2).

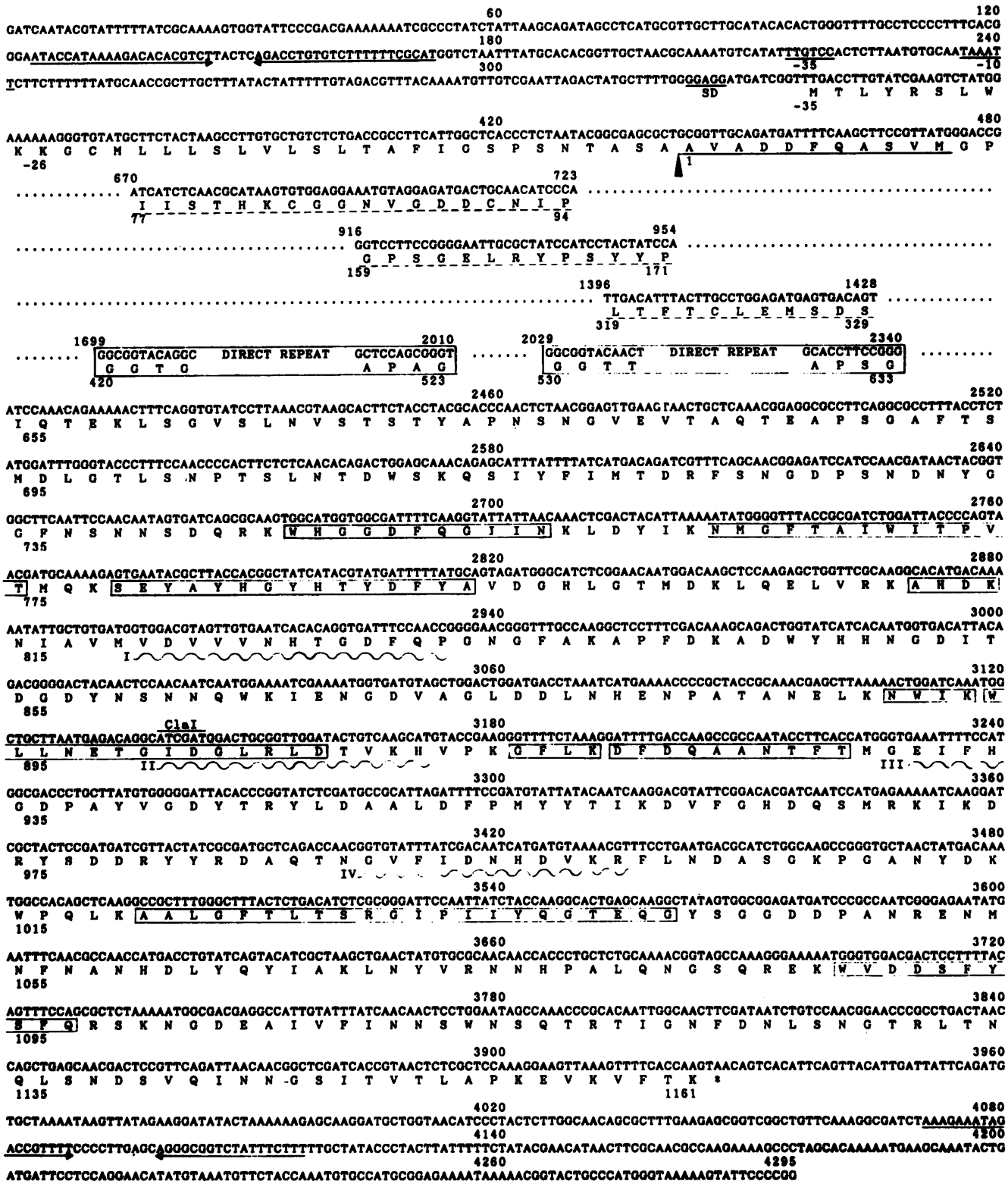

FIG. 2. Nucleotide and amino acid sequences of the 3′ region of the amylase gene with portions of those at the 5′ region. As the nucleotide sequence up to the *Cla*I site (3,145 nucleotides) has already been published (11), only essential portions of the 5′ region are shown. The omitted portions are indicated by dotted lines. The underlined sequences marked −35 and −10 refer to the possible RNA polymerase-binding and recognition sites, respectively. The possible ribosome-binding site (SD) is also underlined. The cleavage site of the signal sequence is indicated by an arrowhead. The 11-residue NH_2-terminal amino acid sequence of the 130-kDa amylase is underlined. Three sequences showing homology with those in other β-amylases are underlined with broken lines. Direct-repeat sequences and amino acid sequences of the 10 peptides prepared from the 48-kDa amylase are boxed. The four sequences showing homology with those in other α-amylases are underlined with wavy lines. The stop codon is indicated by an asterisk. Palindromic sequences in the 5′ and 3′ flanking regions are indicated by arrows. The amino acids are numbered, from 1, from the N terminus of the mature amylase. The last digit is aligned with the corresponding amino acid or nucleotide. *Cla*I site is underlined.

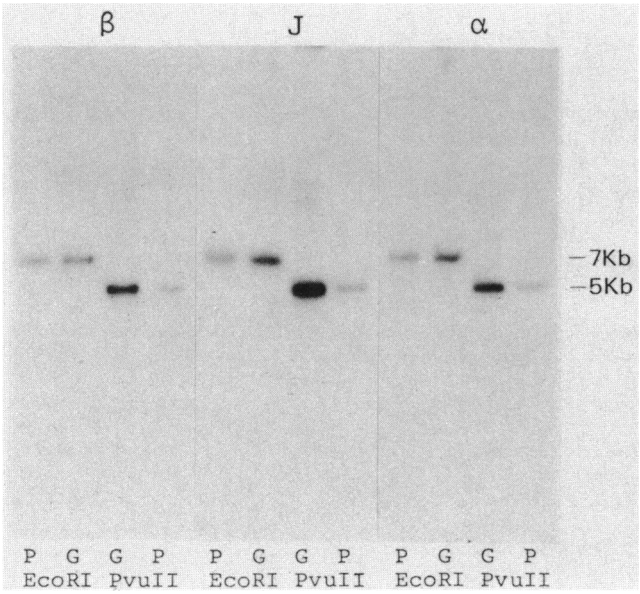

FIG. 3. Comparison of the amylase-coding region in the genome and plasmid pYN49. G and P represent genomic and plasmid DNAs, respectively. A 3-μg sample of DNA from *B. polymyxa* and 0.1 μg of plasmid DNA were completely digested with *Pvu*II or *Eco*RI and then processed for Southern blot hybridization with the β, J, and α probes as described in Materials and Methods.

The 48-kDa amylase was able to hydrolyze β-cyclodextrin (Fig. 5C, lane 1) but showed no detectable activity toward α-cyclodextrin. The blue-value-reducing-value curve of the 48-kDa amylase was similar to that of the *B. licheniformis* α-amylase but quite different from that of the 70-kDa β-amylase (data not shown). Furthermore, an α-amylase specific inhibitor, S-AI, inhibited the 48-kDa amylase activity (Fig. 5B, lane 2) but not the 70-kDa β-amylase activity (Fig. 5B, lane 1). On the basis of the data described above, the 48-kDa amylase is regarded as having alpha-type amylase activity and to act in an endo fashion.

To determine the amino acid sequence of the 48-kDa amylase, the enzyme was digested with lysylendopeptidase and the resulting peptides were isolated by high-performance liquid chromatography, since the N terminus of the enzyme is blocked. The amino acid sequences of 10 representative peptides determined by Edman degradation are shown in Fig. 2. The agreement found between the sequences of the 10 representative peptides and some nucleotide sequences in the 3′ part of the gene suggests that the rest of the predicted amino acid sequence of the protein is in agreement with the nucleotide sequence. This clearly indicates that the 3′ region of the amylase gene codes for the 48-kDa α-amylase.

Characterization of 130-kDa amylase. As the isolation of 130-kDa amylase from *B. polymyxa* was not successful because of the rapid degradation of this enzyme, *B. brevis* HPD31 was used as a host to produce the 130-kDa amylase since *B. brevis* HPD31 has a large capacity to produce extracellular protein (20 g/liter) and produces extracellular protease(s) at almost undetectable levels as determined with casein or bovine serum albumin as a substrate. The 130-kDa amylase was thus isolated from *B. brevis* HPD31 carrying pYN520 (Fig. 4). The 130-kDa amylase had the same N-terminal amino acid sequence as did the major β-amylases produced by *B. polymyxa* (Fig. 2).

The type of the 130-kDa amylase was determined as follows. The 130-kDa amylase produced maltose, maltotriose, and maltotetraose (maltose being the main product) from soluble starch and hydrolyzed β-cyclodextrin (Fig. 6A and B, lanes 3). Although the action pattern of the 130-kDa amylase on soluble starch was generally similar to that of the 48-kDa amylase, the former appeared unable to degrade β-cyclodextrin into small oligosaccharides (Fig. 6B, lane 3).

The amylase inhibitors S-AI and α-EPG were used to separately determine the β- and α-amylase activities of the 130-kDa amylase. Under conditions in which the α-amylase inhibitor, S-AI, inhibited the 48-kDa amylase (Fig. 5B, lane 2), the 130-kDa amylase produced only maltose from soluble starch (Fig. 6B, lane 2) and showed no hydrolysis activity toward β-cyclodextrin (Fig. 6B, lane 1), whereas the 70-kDa β-amylase was not affected (Fig. 5B, lane 1). Under conditions in which the β-amylase inhibitor, α-EPG, inhibited the 70-kDa β-amylase (Fig. 6C, lane 3), the 130-kDa amylase produced a series of maltooligosaccharides from soluble starch (Fig. 6C, lane 1). All of these data indicate that the 130-kDa amylase is a bifunctional enzyme, possessing both β- and α-amylase activities.

DISCUSSION

The cloned *B. polymyxa* amylase gene appears to have an amylase-coding fragment of the same size as the *B. polymyxa* genome and to comprise 3,588 nucleotides, which code for a mature amylase with a molecular weight of 127,314. A large enzyme protein with an approximate molecular weight of 130 kDa was transiently detected in the culture broth of *B. polymyxa*; this protein disappeared upon prolonged culture, generating multiple β-amylases of 70, 56, and 42 kDa as the main enzymes. All three β-amylases had exactly the same N-terminal amino acid sequences, which

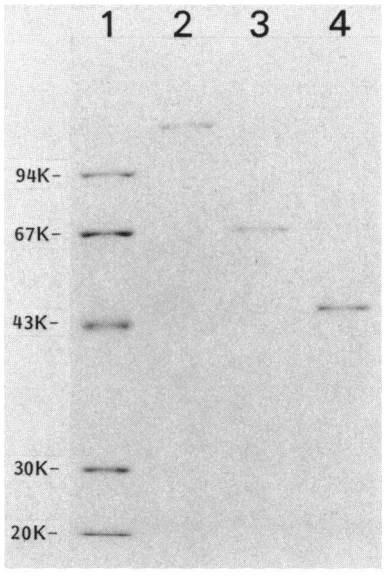

FIG. 4. SDS-polyacrylamide gel electrophoretic profiles of the purified amylases. The 130-kDa amylase (lane 2; 0.2 μg of protein) was purified from *B. brevis* HPD31 carrying pYN520. The 70-kDa β-amylase (lane 3; 0.2 μg of protein) and 48-kDa amylase (lane 4; 0.2 μg of protein) were purified from *B. polymyxa*. Molecular weight marker proteins (phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and α-lactoalbumin, 14,400) were simultaneously electrophoresed (lane 1). The gel was stained with Coomassie brilliant blue R-250.

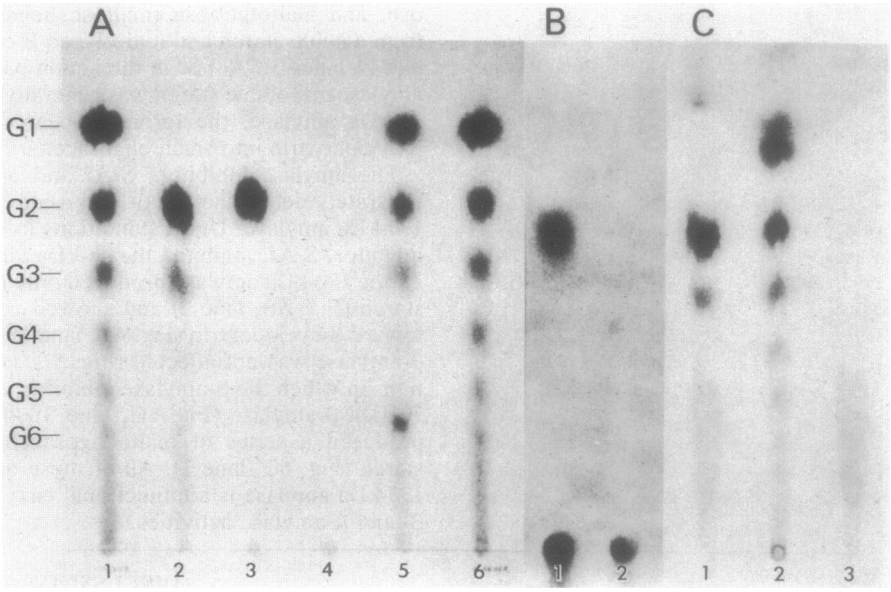

FIG. 5. Paper chromatographic analysis of the products of the reaction of the 48-kDa amylase, 70-kDa β-amylase, and crystalline *B. licheniformis* α-amylase with soluble starch (A and B) and β-cyclodextrin (C). To 99 μl of 0.5% soluble starch or β-cyclodextrin in 50 mM Tris hydrochloride buffer (pH 7.5) was added 1 μl of diluted enzyme (0.1 μg of protein each), followed by incubation at 25°C for 20 h. A 20-μl amount of the hydrolysate was applied to the chromatograms. (A) Oligosaccharide mixture used as a standard (lanes 1 and 6). Other lanes show products after reaction with the 48-kDa amylase (lane 2), 70-kDa β-amylase (lane 3), and *B. licheniformis* α-amylase (lane 5). In lane 4, soluble starch was used as a substrate. (B) S-AI was included, at a concentration of 200 μg/ml, in the reaction mixture. Shown are products after reaction with the 70-kDa β-amylase (lane 1) and 48-kDa amylase (lane 2). (C) Products after reaction with the 48-kDa amylase (lane 1). Oligosaccharide mixture (lane 2) and β-cyclodextrin (lane 3) were used as substrates.

coincided with the deduced amino acid sequence (11). This finding ruled out the possibility of overlapping reading of the gene. Therefore, we previously proposed that the 130-kDa enzyme might be proteolytically cleaved to produce multiform β-amylases after secretion. This further suggests that the N-terminal portion of the 130-kDa protein is responsible for the β-amylase activity. To minimize the size of the β-amylase at the N-terminal side, a universal translation

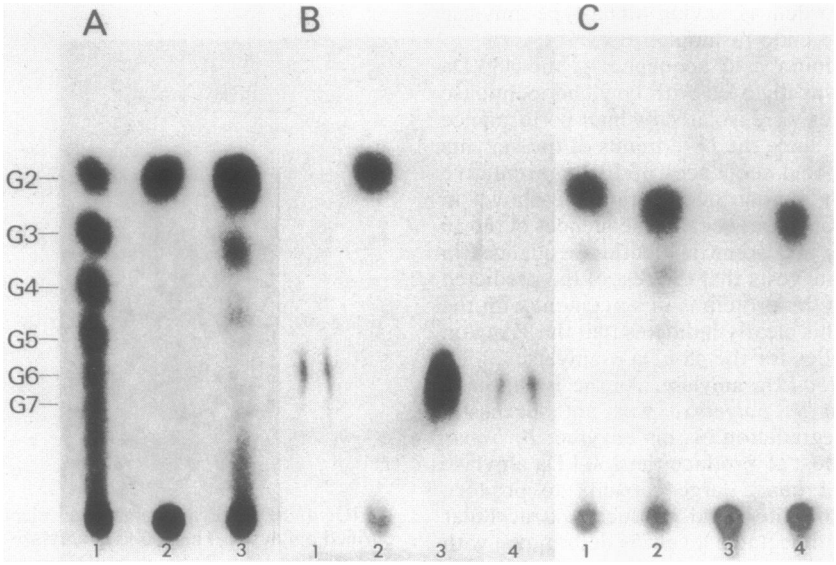

FIG. 6. Paper chromatographic analysis of products after reaction of the 130-kDa amylase with soluble starch and β-cyclodextrin. The enzyme reaction and processing were carried out as described in the legend to Fig. 5. (A) Soluble starch used as a substrate. Shown are products after reaction with the 48-kDa amylase (lane 1), 70-kDa β-amylase (lane 2), and 130-kDa amylase (lane 3). (B) Products after reaction of the 130-kDa amylase with β-cyclodextrin (lanes 1 and 3) and soluble starch (lane 2). S-AI was included, at 200 μg/ml, in the reaction mixture (lanes 1 and 2). In lane 4, β-cyclodextrin used as a substrate. (C) Soluble starch used as a substrate. Shown are products after reaction with the 130-kDa amylase in the presence (lane 1) and absence (lane 2) of α-EPG (1 mg/ml) and after reaction with the 70-kDa β-amylase in the presence (lane 3) and absence (lane 4) of α-EPG (1 mg/ml).

terminator, 5′-GCTTAATTAATTAAGC-3′, was inserted into various restriction enzyme sites in the 5′ region of the insert on pYN49. One of the clones, containing the terminator at the *Hin*cII site (nucleotide 1620; Val-393) exhibited β-amylase activity. Furthermore, the 5′ sequence up to the *Hin*cII site contained three sequences homologous with those in other β-amylases described above, which might comprise the active sites of the enzyme. This clearly indicates that only the approximately one-third of the gene in the 5′ region is required for synthesis of the functional β-amylase.

In the 3′ region downstream of the direct-repeat sequence, four sequences were found to be homologous with sequences in α-amylases of various origins (9, 26). This prompted us to search for enzymes showing α-amylase activity in the culture broth of *B. polymyxa*. The 48-kDa amylase was proven to have α-amylase activity and to act in an endo fashion. Although the N-terminal amino acid sequence of the 48-kDa α-amylase was not established, the amino acid sequences of peptides generated on lysylendopeptidase digestion showed complete agreement with the nucleotide sequence in the 3′ region. The 48-kDa α-amylase is thus concluded to be encoded by the amylase gene. This raises the possibility that the *B. polymyxa* amylase gene directs the synthesis of a bifunctional protein with both β- and α-amylase activities. The 130-kDa enzyme isolated from *B. brevis* carrying the gene on a plasmid had the same N-terminal amino acid sequence as did three major β-amylases produced by *B. polymyxa* and exhibited both β- and α-amylase activities. All of the results presented here indicate that the *B. polymyxa* amylase gene contains in-phase β- and α-amylase-coding sequences in the 5′ and 3′ regions, respectively, and directs the synthesis of a bifunctional enzyme, which gives rise to β- and α-amylases. To our knowledge, this is the first demonstration of a single precursor protein for two enzymes in procaryotes. This type of DNA arrangement is also rare in eucaryotes. Only two nonviral polyprotein precursors have been reported, one for two mitochondrial enzymes in *N. crassa* (47) and another for yolk proteins in the nematode *Caenorhabditis elegans* (38). The production of a bifunctional amylase appears to be advantageous for *B. polymyxa* in at least two respects: more efficient utilization of starch-type substrates and reduction of so-called shipping costs (14) for secretion.

Recently, another β-amylase gene was cloned from *C. thermosulfurogenes*. The cloned gene comprised 1,653 bp and showed no similarity with other α-amylases (12). This finding also indicates that the *B. polymyxa* amylase gene is quite unique and worth further investigation. It will be of interest to determine whether the *B. polymyxa* amylase gene is an ancestral gene for β- and α-amylases or is generated as a consequence of DNA rearrangement. It is tempting to suggest that the direct-repeat sequence may mediate the in-phase fusion of the two genes through an as yet unknown mechanism, since the gene appears to be divided into two functional units by the sequence. Although such sequences are reminiscent of transposable elements, these sequences were found only in the amylase gene of *B. polymyxa* and not in genomes prepared from other bacilli as determined by Southern blot analysis with the direct-repeat sequence (194 bp; nucleotides 2100 to 2293) as a probe. The existence of similar repeat sequences has been demonstrated in two cellulase genes cloned from *Clostridium thermocellum* (1) and alkalophilic *Bacillus* sp. strain N-4 (6). These sequences, however, showed no similarity with that of the *B. polymyxa* amylase gene. At present, the role of such a direct-repeat sequence within a structural gene is not known.

LITERATURE CITED

1. **Beguin, P., P. Cornet, and J.-P. Aubert.** 1985. Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum*. J. Bacteriol. **162**:102–105.
2. **Bernfeld, P.** 1955. Amylases, α and β. Methods Enzymol. **1**: 149–158.
3. **Birnboim, H. C.** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100**:243–255.
4. **Boer, P. H., and D. A. Hickey.** 1986. The α-amylase gene in *Drosophila melanogaster*: nucleotide sequence, gene structure and expression motifs. Nucleic Acids Res. **14**:8399–8411.
5. **Friedberg, F., and C. Rhodes.** 1986. Cloning and characterization of the beta-amylase gene from *Bacillus polymyxa*. J. Bacteriol. **165**:819–824.
6. **Fukumori, F., N. Sashihara, T. Kudo, and K. Horikoshi.** 1986. Nucleotide sequences of two cellulase genes from alkanophilic *Bacillus* sp. strain N-4 and their strong homology. J. Bacteriol. **168**:479–485.
7. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
8. **Hyum, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable extracellular β-amylase from *Clostridium thermosulfurogenes*. Appl. Environ. Microbiol. **49**: 1162–1167.
9. **Ihara, H., T. Sasaki, A. Tsuboi, H. Yamagata, N. Tuskagoshi, and S. Udaka.** 1985. Complete nucleotide sequence of a thermophilic α-amylase gene: homology between prokaryotic and eukaryotic α-amylases at the active sites. J. Biochem. **98**:95–103.
10. **Isoda, Y., S. Asanami, K. Takeo, and Y. Nitta.** 1987. Attempt at affinity labeling of α- and β-amylases by α- and β-D-glucopyranosides and α- and β-maltooligosaccharides with 2,3-epoxypropyl residues as aglycone: specific inactivation of β-amylase. Agric. Biol. Chem. **5**:3223–3229.
11. **Kawazu, T., Y. Nakanishi, N. Uozumi, T. Sasaki, H. Yamagata, N. Tsukagoshi, and S. Udaka.** 1987. Cloning and nucleotide sequence of the gene coding for enzymatically active fragments of the *Bacillus polymyxa* β-amylase. J. Bacteriol. **169**:1564–1570.
12. **Kitamoto, N., H. Yamagata, T. Kato, N. Tsukagoshi, and S. Udaka.** 1988. Cloning and sequencing of the gene encoding thermophilic β-amylase of *Clostridium thermosulfurogenes*. J. Bacteriol. **170**:5848–5854.
13. **Kreis, M., M. Williamson, B. Buxton, J. Pywell, J. Hejgaard, and I. Svendsen.** 1987. Primary structure and differential expression of β-amylase in normal and mutant barleys. Eur. J. Biochem. **169**:517–525.
14. **Kurjan, J., and I. Herskowitz.** 1982. Structure of a yeast pheromone gene (MFα): a putative α-factor precursor contains four tandem copies of mature α-factor. Cell **30**:933–943.
15. **Lacks, S. A., and S. S. Springhorn.** 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J. Biol. Chem. **255**:7467–7473.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**:1072–1074.
18. **Long, C. M., M.-J. Virolle, S.-Y. Chang, S. Chang, and M. J. Bibb.** 1987. α-Amylase gene of *Streptomyces limosus*: nucleotide sequence, expression motifs, and amino acid sequence homology to mammalian and invertebrate α-amylase. J. Bacteriol. **169**:5745–5754.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Marshall, J. J.** 1974. Characterization of *Bacillus polymyxa* amylase as an exo-acting (1→4)-α-D-glucan maltohydrolase. FEBS Lett. **46**:265–275.
22. **Matsuura, Y., M. Kusunoki, W. Harada, and M. Kakudo.** 1984. Structure and possible catalytic residues of Taka-amylase A. J. Biochem. **95**:697–702.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Murao, S., and K. Ohyama.** 1975. New amylase inhibitor (S-AI) from *Streptomyces diastaticus* var. *amylostaticus* no. 2476. Agric. Biol. Chem. **39**:2271–2273.
25. **Murao, S., K. Ohyama, and M. Arai.** 1979. β-Amylases from *Bacillus polymyxa* no. 72. Agric. Biol. Chem. **47**:941–947.
26. **Nakajima, R., T. Imanaka, and S. Aiba.** 1986. Comparison of amino acid sequences of eleven different α-amylases. Appl. Microbiol. Biotechnol. **23**:355–360.
27. **Nakamura, Y., M. Ogawa, T. Nishide, M. Emi, G. Kosaki, S. Himeno, and K. Matsubara.** 1984. Sequences of cDNAs for human salivary and pancreatic α-amylases. Gene **28**:263–270.
28. **Normark, S., S. Bergstrom, T. Edlund, T. Grundstrom, B. Jaurin, F. P. Lindberg, and O. Olsson.** 1983. Overlapping genes. Annu. Rev. Genet. **17**:499–525.
29. **Palva, I.** 1982. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. Gene **19**:81–87.
30. **Robyt, J. F., and D. French.** 1963. Action pattern and specificity of an amylase from *Bacillus subtilis*. Arch. Biochem. Biophys. **100**:451–467.
31. **Robyt, J. F., and D. French.** 1967. Multiple attack hypothesis of α-amylase action: action of porcine pancreatic, human salivary, and *Aspergillus oryzae* α-amylases. Arch. Biochem. Biophys. **122**:8–16.
32. **Rodes, C., J. Strasser, and F. Friedberg.** 1987. Sequence of an active fragment of *B. polymyxa* beta amylase. Nucleic Acids Res. **15**:3934.
33. **Rogers, J. C., and C. Milliam.** 1983. Isolation and sequence analysis of a barley α-amylase cDNA clone. J. Biol. Chem. **258**: 8169–8174.
34. **Saito, H., and K. Miura.** 1963. Preparation of transforming DNA by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
35. **Saito, N.** 1973. A thermophilic extracellular α-amylase from *Bacillus licheniformis*. Arch. Biochem. Biophys. **155**:290–298.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Schibler, U., A. Pittet, R. A. Young, O. Hagenbuchle, M. Tosi, S. Gellman, and P. K. Wellauer.** 1982. The mouse α-amylase multi-gene family: sequence organization of members expressed in the pancreas, salivary gland and liver. J. Mol. Biol. **155**:247–266.
38. **Sharrock, W. J.** 1984. Cleavage of two yolk proteins from a precursor in *Caenorhabditis elegans*. J. Mol. Biol. **174**:419–431.
39. **Shinke, R., H. Nishira, and M. Mugibayashi.** 1974. Isolation of β-amylase producing microorganisms. Agric. Biol. Chem. **38**: 665–666.
40. **Southern, E.** 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. **68**:152–176.
41. **Steiner, D. F., Jr., P. S. Quinn, S. J. Chan, J. Marsh, and H. S. Trager.** 1980. Processing mechanisms in the biosynthesis of proteins. Ann. N.Y. Acad. Sci. **343**:1–16.
42. **Takahashi, W., H. Yamagata, K. Yamaguchi, N. Tsukagoshi, and S. Udaka.** 1983. Genetic transformation of *Bacillus brevis* 47, a protein-secreting bacterium, by plasmid DNA. J. Bacteriol. **156**:1130–1134.
43. **Takasaki, Y.** 1976. Production and utilization of β-amylase and pullulanase from *Bacillus cereus* var. *mycoides*. Agric. Biol. Chem. **40**:1515–1522.
44. **Toda, H., K. Kondo, and K. Narita.** 1982. The complete amino acid sequence of Taka-amylase A. Proc. Jpn. Acad. **58(B)**:208–212.
45. **Tsukagoshi, N., H. Ihara, H. Yamagata, and S. Udaka.** 1984. Cloning and expression of a thermophilic α-amylase gene from *Bacillus stearothermophilus* in *Escherichia coli*. Mol. Gen. Genet. **193**:58–63.
46. **Tsukagoshi, N., S. Iritani, T. Sasaki, T. Takemura, H. Ihara, Y. Idota, H. Yamagata, and S. Udaka.** 1985. Efficient synthesis and secretion of a thermophilic α-amylase by protein-producing *Bacillus brevis* 47 carrying the *Bacillus stearothermophilus* amylase gene. J. Bacteriol. **164**:1182–1187.
47. **Wandinger-Ness, A. U., and R. L. Weiss.** 1987. A single precursor protein for two separable mitochondrial enzymes in *Neurospora crassa*. J. Biol. Chem. **262**:5823–5830.
48. **Yamada, H., N. Tsukagoshi, and S. Udaka.** 1981. Morphological alterations of cell wall concomitant with protein release in a protein-producing bacterium, *Bacillus brevis* 47. J. Bacteriol. **148**:322–332.
49. **Young, M., A. Galizzi, and D. Henner.** 1983. Nucleotide sequence of the amylase gene from *Bacillus subtilis*. Nucleic Acids Res. **11**:237–249.
50. **Yuuki, T., T. Nomura, H. Tezuka, A. Tsuboi, H. Yamagata, N. Tsukagoshi, and S. Udaka.** 1985. Complete nucleotide sequence of a gene coding for heat- and pH-stable α-amylase of *Bacillus licheniformis*: comparison of the amino acid sequences of three bacterial liquefying α-amylases deduced from the DNA sequences. J. Biochem. **98**:1147–1156.