Artificial insertion of peptides between signal peptide and mature protein: effect on secretion and processing of hybrid thermostable α -amylases in *Bacillus subtilis* and *Escherichia coli* cells

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To study the effect of inserted peptides on the secretion and processing of exported proteins in *Bacillus subtilis* and *Escherichia coli*, pBR322-derived DNA fragments coding for small peptides were inserted between the DNA coding for the 31 amino acid *B. subtilis* α -amylase signal peptide and that coding for the mature part of the extracellular thermostable α -amylase of *B. stearothermophilus*. Most of the inserted peptides (21 to 65 amino acids) decreased the production of the enzyme in *B. subtilis* and *E. coli*, the effect of each peptide being similar in the two strains. In contrast, with one peptide (a 21 amino acid sequence encoded by the extra DNA in pTUBE638), the production of α -amylase was enhanced more than 1.7-fold in *B. subtilis* in comparison with that of the parent strain. The molecular masses of the thermostable α -amylases in the periplasm of the *E. coli* transformants varied for each peptide insert, whereas those in the culture supernatants of the *B. subtilis* transformants had molecular masses similar to that of the mature enzyme. Based on the NH₂-terminal amino acid sequence of the hybrid protein from pTUBE638, it was shown that in *E. coli*, the NH₂-terminally extended thermostable α -amylase was translocated and remained in the periplasm after the 31 amino acid signal sequence was removed. In the case of *B. subtilis*, after the removal of a 34-amino acid signal sequence, the hybrid protein was secreted and processed to the mature form.

Introduction

Secreted proteins are usually synthesized as precursors with a short NH₂-terminal extension called the signal peptide which promotes the initiation of secretion and which is removed by a specific enzyme (Blobel & Dobberstein, 1975; Davis & Tai, 1980). Some precursors also contain propeptides, which are usually located between the signal peptide and the mature enzyme, and which are removed after secretion. The presence of propeptides has been reported in *Bacillus subtilis* α -amylase (Takase *et al.*, 1988), *B. subtilis* alkaline and neutral proteases (Wong & Doi, 1986; Yang *et al.*, 1984; Ikemura *et al.*, 1987), *B. licheniformis* penicillinase (Nielsen & Lampen, 1982), *Staphylococcus aureus* nuclease A (Shortle, 1983), *Saccharomyces cerevisiae* α -factor (Fuller *et al.*, 1988), and mammalian proteases and polypeptide hormones (Neurath & Walsh, 1976;

Steiner *et al.*, 1980). The role, if any, of these propeptides in protein translocation is not certain; however, in the case of subtilisin the propeptide plays an important role in the modulation of enzyme activity (Ikemura *et al.*, 1987; Ikemura & Inouye, 1988). The amino acid sequences and lengths of the propeptides differ widely among proteins.

The precursor protein of *B. subtilis* α -amylase is synthesized as a preproenzyme (Yamazaki *et al.*, 1983). A comparison of the amino acid sequence deduced from the DNA nucleotide sequence of the α -amylase gene (*amyE*) and the NH₂-terminal amino acid sequence of the extracellular mature α -amylase (Mäntsälä & Zalkin, 1979) indicated that the precursor contains an NH₂-terminal extension of 41 amino acid residues. However, recent experiments using deletion mutants (Sasamoto *et al.*, 1989) and analysis of intermediates in the processing of extracellular α -amylase suggested that the first 33 amino acid residues from the NH₂-terminus constitute the signal peptide of this enzyme in *B. subtilis* (Takase *et al.*, 1988).

In order to investigate the effect of artificially inserted peptides between the signal sequence and the mature

Abbreviations: DTT, dithiothreitol; *p*-APMSF, (*p*-amidinophenyl)-methanesulphonyl fluoride hydrochloride; *amyE*, α -amylase gene of *Bacillus subtilis*; *amyT*, thermostable α -amylase gene of *Bacillus stearothermophilus* A631.

protein on the secretion of exported proteins, and to analyse alteration of signal peptide cleavage sites in *B. subtilis* and *E. coli* cells, we constructed artificial *amyE'*-pBR322'-*amyT* fusion genes. In these fusion genes various small DNA fragments obtained from the *E. coli* plasmid pBR322 were inserted between the DNA encoding the first 31 amino acid residues of the *amyE* signal peptide and the DNA encoding the mature part of thermostable α -amylase (*amyT*) from *B. stearothermophilus* A631 (Sohma *et al.*, 1987). The 31 amino acid sequence was used because the response to the modification of the amino acid sequence around the *amyE* signal peptide cleavage site in the two strains can be easily analysed. The thermostable α -amylase was used as the marker enzyme because the *amyT* product does not contain a natural propeptide. Also, the structural gene for the mature thermostable α -amylase is expressed well from the *amyE* promoter, and the *amyE* signal peptide is functional both in *E. coli* and in *B. subtilis*. The expression of *amyE* in *E. coli* was, however, only 3% of that obtained in *B. subtilis* (Sasamoto *et al.*, 1989).

Methods

Bacterial strains, plasmids and culture conditions. *Escherichia coli* HB101 (F⁻ *hdsS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 sup44* λ^-) and *Bacillus subtilis* 207-25 (*m*₁₆₈ *hsrM recE4 amyE07 aro1906 lys-21 leuA8*), an α -amylase deficient strain (Yamazaki *et al.*, 1983), were used as host cells.

The *B. subtilis*-*E. coli* shuttle plasmid pTUBE627 contains the *amyE'*-*amyT* fused gene, in which the DNA sequence encoding the *B. subtilis* α -amylase promoter and the 31 amino acids of its signal peptide (*amyE'*) is fused to the DNA sequence encoding the extracellular mature thermostable α -amylase (*amyT*), using *Hind*III linker DNA. The *amyT* gene, which lacks its own promoter and signal peptide coding region, was derived from the chromosomal DNA of *B. stearothermophilus* A631 (Sohma *et al.*, 1987). For the construction of pTUBE627, *B. subtilis* plasmid pUB110 containing the replication origin of pBR322 (*Dra*I-PvuII 1.4 kb fragment) was used as the vector. *E. coli* and *B. subtilis* cells were grown with shaking at 37 °C in L-broth [1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl] as described previously (Yamane *et al.*, 1982).

Isolation and analysis of plasmid and chromosomal DNAs. Plasmid DNA was prepared by the rapid alkaline method of Birnboim & Doly (1979). It was purified by CsCl equilibrium centrifugation in the presence of ethidium bromide, followed by agarose gel electrophoresis and subsequent recovery using hydroxyapatite (Tabak & Flavell, 1978). DNA fragments were recovered from gels using DE81 paper (Dretzen *et al.*, 1981). Restriction fragments to be sequenced were ligated into the *Bam*HI site of the M13 mp19 phage (Yanisch-Perron *et al.*, 1985), and the DNA sequences were determined by the dideoxy chain-termination method of Sanger *et al.* (1977). Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I and bacterial alkaline phosphatase were obtained from Takara Shuzo Co. or BRL, and *Hind*III linker DNA (5' GGAAGCTTCC3') from Pharmacia.

Transformation of *E. coli* and *B. subtilis*. *E. coli* HB101 was transformed by the method of Lederberg & Cohen (1974), and *B. subtilis* 207-25 by the protoplast transformation method of Chang & Cohen (1979).

Assay of thermostable α -amylase activity. Amylase activity was measured at 60 °C by a slight modification of the method of Fuwa (1954), using 0.5% soluble starch as the substrate. Hydrolysis of 0.1 mg starch in 1 min was defined as 1 unit (U) of enzyme activity.

Detection of thermostable α -amylases by SDS-PAGE. α -Amylases in the periplasmic fractions of *E. coli* cells and in the culture supernatants of *B. subtilis* cells were precipitated with 6.25% (w/v) TCA, dissolved in 0.1% SDS, dialysed against 25 mM-Tris/HCl buffer (pH 7.5) containing 192 mM-glycine and 0.1% SDS, and boiled for 3 min in 1% SDS/1% mercaptoethanol. The samples were subjected to SDS-PAGE using an 8.5% (w/v) running gel (Laemmli, 1970). After electrophoresis, thermostable α -amylases in the gels were renatured *in situ* by the method of Lacks & Springhorn (1980), and then visualized by incubation of the gels in 1% soluble starch solution (pH 6.2) at 37 °C for 2 h followed by dipping in 0.01 M-KI/I₂ solution. The molecular mass markers used were: bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and cytochrome *c* (12.3 kDa).

Purification of NH₂-terminally extended thermostable α -amylases. NH₂-terminally extended thermostable α -amylases from the periplasm of 24 h *E. coli* cultures and from the culture supernatants of 12 and 36 h *B. subtilis* cultures were purified by the method described previously (Takase *et al.*, 1988). To minimize proteolysis during the purification steps, crude α -amylase preparations were treated with 0.2 mM-p-APMSF and passed through columns (1.2 × 5 cm) of Toyopearl HW55 (Toso Co.) in the presence of ammonium sulphate (25% saturation) and 0.1 mM-DTT. All steps were performed at 4 °C. The preparations were then electrophoresed on SDS-polyacrylamide gels (8.5%, w/v) and the bands corresponding to the thermostable α -amylases were blotted onto polybrene-coated glass filters (Whatman, GF/C) by the method of Vandekerckhove *et al.* (1985). The purified extended thermostable α -amylase preparation obtained from the 24 h culture of *E. coli* HB101(pTUBE638) was designated pTUBE638- α -amylase (E24). The purified enzyme preparations from the 12 and 36 h culture supernatants of *B. subtilis* 207-25(pTUBE638) were designated pTUBE638- α -amylase (B12) and pTUBE638- α -amylase (B36), respectively.

Amino acid sequencing. An Applied Biosystems 470A sequencer was used for NH₂-terminal amino acid sequence determination by automatic Edman degradation.

DNA-DNA hybridization. This was done in the presence of 50% (v/v) formamide at 38 °C using as probe ³²P-labelled pBR322 DNA from which the *Dra*I-PvuII 1.4 kb fragment, containing the replication origin, had been removed.

Results

Construction and analysis of plasmids pTUBE631 to pTUBE638 containing the *amyE'*-pBR322'-*amyT* fusion genes

To insert segments of pBR322 DNA between the *amyE'* and *amyT* DNA region, pBR322 DNA was digested with *Hap*II and *Hae*III, and with *Hap*II and *Sau*3AI. *Hind*III linker DNA was added to the resulting mixture, which, following *Hind*III digestion, was inserted into the unique *Hind*III site of pTUBE627. The recombinant plasmids were introduced into *E. coli* HB101, and DNA-DNA hybridization positive colonies were isolated. Eight representative transformants differing in

Fig. 1. Amino acid sequences of the inserted peptides, deduced from the DNA nucleotide sequences. ■, The 31 amino acid sequence from the NH₂-terminal f-Met in the *B. subtilis* α -amylase signal peptide. ▨, The amino acid sequence for the extracellular mature thermostable α -amylase originating from *B. stearothermophilus* A631, in which the NH₂-terminal amino acid (Ala) corresponds to position 36 from the NH₂-terminal f-Met in the precursor protein from the *amyT* gene in pTUBE627 (Sohma *et al.*, 1987). Thick lines indicate the amino acids encoded by the *Hind*III linker DNA.

the production of amylase were selected and their plasmids designated pTUBE631 to pTUBE638. The nucleotide sequences at and near the inserted fragments of pBR322 were determined and the amino acid sequences deduced (Fig. 1). Plasmids pTUBE631 and pTUBE634 contained two, different, fragments of pBR322 while all the others contained a single fragment. All of the small DNA fragments were derived from the ampicillin resistance or tetracycline resistance genes, except for the fragment in pTUBE638, in which a part of the 3' flanking region of the tetracycline resistance gene was inserted. In all of these constructions, between 21 and 65 amino acid residues were added between the signal peptide and the mature enzyme.

Effect of the inserted peptides on the production of thermostable α -amylases in *E. coli* and *B. subtilis* cells

The parental *B. subtilis* 207-25(pTUBE627) produced approximately 800 U of extracellular thermostable α -amylase ml⁻¹ after 36 h of culture, and *E. coli* HB101-(pTUBE627) produced 2000 U ml⁻¹ of the periplasmic enzyme after 24 h of culture (Fig. 2a, f). As summarized in Fig. 2 and Table 1, in most cases the additional amino acid residues reduced the amount of enzyme produced. The degree of reduction varied with each peptide; *E. coli* HB101(pTUBE637), for example, produced only 1% of

the amount of the enzyme produced by *E. coli* HB101(pTUBE627). In contrast, the quantity of the enzyme produced by *B. subtilis* 207-25(pTUBE638) was more than 1.7 times that produced by *B. subtilis* 207-25(pTUBE627), even though pTUBE638 had little or no enhancing activity in *E. coli*. The proportion of thermostable α -amylases detected in the intracellular fractions of *B. subtilis* 207-25 carrying pTUBE635, pTUBE636 and pTUBE637, and *E. coli* HB101 carrying pTUBE636 and pTUBE637 appeared to be increased, presumably due to the decrease in the total amount of α -amylases synthesized and unaltered levels of the activity retained in cells. Therefore, no increased accumulation of the extended enzymes was observed in the intracellular fractions of either *B. subtilis* or *E. coli*. These results indicate that the addition of specific amino acid sequences immediately after the signal peptide cleavage site may enhance the production of α -amylase, although in most cases these sequences decreased the amount of enzyme secreted.

The specific activity of the thermostable α -amylases (units per mg protein) in the periplasm of *E. coli* cells and in the culture supernatants of *B. subtilis* cells was similar to the activity of the parent when it was measured using a rabbit antiserum against thermostable α -amylase produced by *B. subtilis*. Growth of the *E. coli* and *B. subtilis* transformants and copy number of the plasmids per cell were unaffected by the insertion mutations. There was

Fig. 2. Activity of thermostable α -amylases in the extracellular fractions of *B. subtilis* 207-25 transformants carrying pTUBE627 (a), pTUBE631 (b), pTUBE633 (c), pTUBE637 (d) and pTUBE638 (e), and in the periplasm of the *E. coli* HB101 transformants carrying pTUBE627 (f), pTUBE631 (g), pTUBE633 (h), pTUBE637 (i) and pTUBE638 (j). The cells were cultured in L-broth containing 10 μ g kanamycin ml⁻¹ at 37 °C and the cultures were fractionated. ●—●, α -Amylase activity; ○---○, growth.

Table 1. Production and localization of thermostable α -amylases from the mutated fusion genes in *E. coli* and *B. subtilis* transformants

Plasmid	Length of added peptide*	<i>E. coli</i> †					<i>B. subtilis</i> ‡			
		Total activity (U ml ⁻¹)	Localization (%) in			Ratio of enzyme production§	Total activity (U ml ⁻¹)	Localization (%) in		Ratio of enzyme production§
			E	P	C			E	C	
pTUBE627	4	2022	1	98	1	1.00	808	99	1	1.00
pTUBE631	65	1226	1	79	20	0.61	451	99	1	0.58
pTUBE632	42	335	4	75	21	0.17	178	99	1	0.22
pTUBE633	34	266	2	82	17	0.13	166	99	1	0.21
pTUBE634	40	208	11	67	22	0.10	104	93	7	0.13
pTUBE635	46	133	6	67	27	0.07	65	85	15	0.07
pTUBE636	39	94	1	22	75	0.05	48	80	20	0.06
pTUBE637	46	27	2	9	89	0.01	38	74	26	0.05
pTUBE638	21	2018	1	96	3	1.00	1443	99	1	1.79

* Number of amino acid residues encoded in the inserted pBR322 DNA fragments and the *Hind*III linker DNA.

† The *E. coli* cells harbouring each of the plasmids were cultured in L-broth containing 10 μ g kanamycin ml⁻¹ at 37 °C for 24 h, and the cultures were fractionated into extracellular (E), periplasmic (P) and intracellular (C) fractions by the osmotic shock method of Chan *et al.* (1981).

‡ Details as for *E. coli*, except that cultures were harvested after 36 h and fractionated into extracellular (E) and intracellular (C) fractions.

§ Ratio of total amylase activity in *B. subtilis* or *E. coli* transformants to that of the respective parental strain.

no obvious direct relation between the lengths of the inserted peptides and enzyme production (Table 1). The smallest peptide, in pTUBE638, enhanced the extracellular production of the enzyme by *B. subtilis*. The effect of each inserted peptide on the production of the enzyme was similar in *E. coli* and *B. subtilis* cells, except for that encoded by pTUBE638.

Processing of the NH₂-terminally extended thermostable α -amylases in *E. coli* and *B. subtilis*

To analyse the processing system in *E. coli* and *B. subtilis* cells, the molecular masses of the exported thermostable α -amylases were estimated by SDS-PAGE. After electrophoresis, the bands corresponding to the amylases were

Fig. 3. (a) Different molecular masses of the periplasmic thermostable α -amylases in *E. coli* HB101 containing pTUBE631 (A), pTUBE632 (B), pTUBE633 (C), pTUBE634 (D) or pTUBE638 (E). (b) Identical molecular masses of the extracellular thermostable α -amylase from *B. subtilis* 207-25 containing pTUBE631 (A), pTUBE632 (B), pTUBE633 (C), pTUBE634 (D) or pTUBE638 (E). M, extracellular mature thermostable α -amylase obtained from the culture supernatant of the parental *B. subtilis* 207-25(pTUBE627). The *E. coli* and *B. subtilis* cells were cultured in L-broth containing $10 \mu\text{g}$ kanamycin ml^{-1} for 36 h at 37°C . The enzymes in the periplasmic fractions of *E. coli* cells and in the culture supernatant fractions from *B. subtilis* cells were analysed.

Fig. 4. (a) Identical molecular masses of the NH_2 -terminally extended thermostable α -amylases in the periplasm of *E. coli* HB101(pTUBE638), and (b) variations in the molecular mass of the extended enzyme in the culture supernatant of *B. subtilis* 207-25(pTUBE638) during culture. The cultures were sampled at the time indicated, fractionated, and analysed. M, see Fig. 3.

visualized by their starch-hydrolysing activity (Fig. 3a, b). The molecular masses of the NH_2 -terminally extended thermostable α -amylases in the periplasmic fractions of *E. coli* HB101 carrying pTUBE631, pTUBE632, pTUBE633, pTUBE634 or pTUBE638 after 36 h of culture ranged from 61 to 64 kDa. In contrast, the molecular mass of each of the extracellular enzymes from *B. subtilis* 207-25 carrying these plasmids after 36 h of culture was estimated to be 61 kDa, a value corresponding to that of the mature extracellular enzyme from *B. subtilis* 207-25(pTUBE627) and from *B. stearo-*

thermophilus A631 (Sohma *et al.*, 1987). The electrophoretic analysis of the α -amylase preparations from the strains carrying pTUBE635, pTUBE636 and pTUBE637 was not possible due to their low activity.

The apparent molecular mass of the extended thermostable α -amylase encoded by pTUBE638 in the periplasm of *E. coli* did not change throughout the 12 to 36 h period of culture. In contrast, the molecular mass of the extracellular enzyme from *B. subtilis* 207-25(pTUBE638) decreased from 63 kDa in the early stage of culture (6 to 18 h) to 61 kDa in the later stages (24 to 36 h) (Fig. 4a, b). The same result was observed by immunoblot analysis after the gel electrophoresis. These results suggest that the extended pTUBE638-encoded enzyme from the *B. subtilis* cells may have been processed to the mature form after it had been secreted into the culture medium, whereas the enzyme in the *E. coli* strain was translocated into the periplasm and remained there without removal of the inserted peptide.

In order to test this assumption, pTUBE638- α -amylase (E24), pTUBE638- α -amylase (B12) and pTUBE638- α -amylase (B36) were prepared and the amino acid sequences of their NH_2 -terminal regions were determined (Table 2). The sequence of pTUBE638- α -amylase (E24) was found to be Ser-Leu-Ala-Thr-Gly-Ala-His, in which the NH_2 -terminal amino acid corresponds to position 32 from the NH_2 -terminal f-Met. In contrast, the NH_2 -terminal amino acid sequence of pTUBE638- α -amylase (B12) was Thr-Gly-Ala-His-Asp-Arg-Ala, indicating that the sequence started from position 35. Two NH_2 -terminal amino acid sequences (Ala-Ala-Ala-Pro-Phe-Asn-Gly and Ala-Ala-Pro-Phe-Asn-Gly-Thr)

Fig. 5. Schematic representation of the cleavage of the signal peptide and processing of the precursor protein encoded by pTUBE638 in *E. coli* and *B. subtilis*. ∇ , Signal peptide cleavage site in *E. coli*; $\blacktriangledown$, signal peptide cleavage site in *B. subtilis*. ∇ , $\blacktriangledown$, processing sites proposed based on the NH₂-terminal amino acid sequences of the pTUBE638- α -amylase (B36) preparation, which was purified from the culture supernatant of *B. subtilis* 207-25(pTUBE638) after 36 h of culture. Numbers under the amino acid residues indicate the positions from the NH₂-terminal f-Met.

Table 2. NH₂-terminal amino acid sequences of the pTUBE638- α -amylase (E24) preparation from *E. coli*, and the pTUBE638- α -amylase (B12) and (B36) preparations from *B. subtilis*

Numbers in the parentheses indicate the amount (pmol) of amino acid residues recovered in each cycle.

Cycle	<i>E. coli</i>		<i>B. subtilis</i>	
	pTUBE638- α -amylase (E24)	pTUBE638- α -amylase (B12)	pTUBE638- α -amylase (B36)	
1	Ser (11-15)	Thr (7-14)	Ala (120-74)	Ala (120-74)
2	Leu (41-75)	Gly (29-38)	Ala (111-86)	Ala (111-86)
3	Ala (51-14)	Ala (13-65)	Ala (96-54)	Pro (24-53)
4	Thr (12-61)	His (1-79)	Pro (79-25)	Phe (20-60)
5	Gly (48-59)	Asp (9-09)	Phe (64-64)	Asn (14-81)
6	Ala (45-35)	Arg (3-85)	Asn (41-98)	Gly (32-67)
7	His (1-50)	Ala (9-54)	Gly (61-78)	Thr (3-84)
8	Asp (16-84)	Pro (11-02)	Thr (7-41)	Met (8-37)
9	Arg (4-15)	Val (5-10)	Met (29-30)	Met (29-30)
10	Ala (32-23)	Val (6-64)	Met (31-62)	Gln (33-60)

were found in the pTUBE638- α -amylase (B36), in which the amino acid sequences started at positions 52 and 53, respectively. Since the molar ratios of the recovered Pro, Phe, and Asn residues in the former and the latter cycles of the amino acid sequence analysis of pTUBE638- α -amylase (B36) were approximately 1:3 (Table 2), we considered that the pTUBE638- α -amylase (B36) preparation consists of the two thermostable α -amylases starting at positions 52 and 53 in the ratio of 3:1. The sequence Ala-Ala-Pro-Phe-Asn, starting at position 53, was found at the NH₂-terminus of the extracellular mature thermostable α -amylase from *B. stearothermophilus* A631 (Sohma *et al.*, 1987). These results indicate that the precursor expressed from pTUBE638 in the *E. coli* cells was cleaved between Ala-31 and Ser-32 by a signal peptidase, and that the resulting extended thermostable α -amylase was translocated into the periplasm, where it remained without removal of the inserted peptide. In contrast, the precursor synthesized in *B. subtilis* was first cleaved between Ala-34 and Thr-35, the extended thermostable α -amylase was secreted into the culture medium and then the additional amino acid residues from positions 35 (Thr) to 52 (Ala) were processed as illustrated in Fig. 5. The pTUBE638- α -amylase (B36) starting from position 52 (Ala) may be considered as an intermediate. Based on the analyses of the molecular masses and NH₂-terminal amino acid sequences of the extended and the mature thermostable α -amylases from pTUBE638, it can be assumed that the conversion from the NH₂-terminally extended form of the enzyme to the mature one proceeds in a stepwise manner. However, the number of steps involved in the processing of the enzymes remained to be determined.

Discussion

The deduced amino acid sequence around the signal peptide cleavage site of the precursor encoded by pTUBE638 is shown in Figs 1 and 5. On the basis of the analysis of the NH₂-terminal amino acid sequences in the pTUBE638- α -amylases (E24) and (B12), the signal peptide cleavage site of the precursor in *E. coli* was found to be located between Ala-31 and Ser-32, and in *B. subtilis* between Ala-34 and Thr-35. The amino acids at positions 33 and 34 in this precursor were Leu and Ala, and the peptide bond between them was not cleaved in *B. subtilis* cells, although the signal peptide cleavage site of the *B. subtilis* α -amylase precursor was found to be located between Ala-33 and Glu-34 by Takase *et al.* (1988) and Sasamoto *et al.* (1989). This result indicates that the signal peptide cleavage site is alterable in *B. subtilis* cells. In these cells, the precursor of

pTUBE638 could have been first cleaved between Ala-31 and Ser-32 as in the case of *E. coli* and in a previous paper (Sohma *et al.*, 1987), and then immediately three amino acids at positions 32 (Ser), 33 (Leu) and 34 (Ala) could have been removed by some protease(s) or peptidase(s). However, none of the amino acids involved were present in detectable amounts, suggesting that the formation of a cleavage product with a NH₂-terminal amino acid corresponding to Ser-32 is unlikely. This implies a difference in the cleavage site of the precursor protein encoded by pTUBE638 in *B. subtilis* and *E. coli*. The difference may be related to the finding that the signal peptides of *B. subtilis* extracellular enzymes were 8 to 10 amino acid residues longer than those of periplasmic and outer-membrane proteins in *E. coli* (Watson, 1984; Von Heijne & Abrahamsen, 1989). A different processing site for *B. stearothermophilus* α -amylase in *E. coli* was also reported by Suominen *et al.* (1987).

After the removal of the signal peptides, the resulting NH₂-terminally extended thermostable α -amylases were located in the *E. coli* periplasm and were secreted into the culture medium by *B. subtilis*. Based on the molecular masses of the extended α -amylases and the NH₂-terminal amino acid sequences of pTUBE638-encoded amylases from *E. coli* and *B. subtilis*, it appears that in *E. coli* each of the inserted peptides was processed differently, while in *B. subtilis* all the extended enzymes were processed to the mature form. The results suggest that in *B. subtilis* the processing of the extended enzymes into the mature form is done by extracellular protease(s) or peptidase(s) which have a wide substrate specificity like subtilisin.

In summary, artificially inserted peptides between the signal peptide and mature thermostable α -amylase were able to modulate the production of thermostable α -amylases in *E. coli* and *B. subtilis*. The NH₂-terminally extended enzymes were translocated into the periplasm of *E. coli* and were secreted into the culture medium by *B. subtilis* after the signal peptide was removed. The extended propeptides were rapidly processed in *B. subtilis*. The signal peptide cleavage site of a precursor protein was different in *E. coli* and *B. subtilis*.

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