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Cloning of proline-specific endopeptidase gene from *Flavobacterium meningosepticum*: expression in *Escherichia coli* and purification of the heterologous protein*

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Abstract. Proline-specific endopeptidase (PSE) (EC 3.4.21.26) from *Flavobacterium meningosepticum* was subjected to partial amino acid sequencing. According to the peptide sequences obtained, oligonucleotides were used to amplify a PSE-specific DNA fragment of 930 bp from *F. meningosepticum* genomic DNA, employing the polymerase chain reaction technique. This fragment served as a molecular probe to isolate the respective gene. DNA sequencing revealed that the PSE gene consists of 2118 bp coding for a 78,634 Da protein of 705 amino acids. The coding region was cloned in different expression vectors of *Escherichia coli*. Transformed *E. coli* cells overproduce an active prolyl endopeptidase of 75,000 relative molecular mass, which is delivered to the bacterial periplasmic space. Up to 1.6 units of active prolyl endopeptidase were obtained from 1 mg *E. coli* cells. Furthermore, the efficient purification of active prolyl endopeptidase from the periplasm of recombinant *E. coli* cells is described.

Introduction

Proteolytic enzymes are involved in a multitude of important physiological processes. Referring to cleavage mechanism and substrate specificity, they can be assigned to different families. Even within these families, enzymes with quite different functions are found (Greer 1990; Siezen et al. 1991). Some of these enzymes are helpful tools in protein sequencing, protein domain structure analysis, the study of configuration of peptide bonds, the detection of amino acid exchanges in site-directed mutagenesis, fingerprint analyses and peptide synthesis. Especially serine and thiol endoproteases that cleave specifically on the carboxyl side of defined amino acid residues are gaining in significance for these types of biochemical analysis (Svendsen and Breddam 1992; Morihara 1987).

Post-proline-specific endopeptidases were isolated and characterized from animals (Moriyama et al. 1988; Andrews et al. 1980; Orlowski et al. 1979; Blumenberg et al. 1980), plants (Yoshimoto et al. 1987) and microorganisms (Yoshimoto et al. 1980; Szwajcer-Dey et al. 1992; Sattar et al. 1990). Recently the respective genes from porcine brain and *Flavobacterium meningosepticum* have been cloned and sequenced (Rennex et al. 1991; Yoshimoto et al. 1991; Chevallier et al. 1992). The amounts of proline-specific endopeptidase (PSE) produced by the original sources, however, are very low (Sommer 1992; Yoshimoto et al. 1980). Therefore it would be of advantage to establish an expression system that will facilitate the subsequent steps of protein purification.

To achieve this we have cloned and sequenced the PSE gene from *F. meningosepticum*. In the present paper, we report the expression of the PSE gene in *Escherichia coli*, the processing of the endopeptidase and its secretion to the periplasmic space. Furthermore the purification of the active heterologous enzyme is described.

Materials and methods

Amino acid analysis and sequence determination. F. meningosepticum PSE (EC 3.4.21.26) was purchased from Seikagaku Kogyo (Tokyo, Japan). Since the N-terminus of the enzyme was blocked, amino acid sequence determination according to standard methods (Shively 1986) was carried out on internal peptides generated by tryptic digests and cyanogen bromide cleavage.

Molecular cloning and DNA sequencing. Polymerase chain reactions (PCR) and most of the methods used for molecular cloning were done as described in Sambrook et al. (1989). As plasmid hosts were used the *E. coli* strains BMH71-18 *Δ[lac-proAB] supE thi F' lacI^{q}ZΔM15 proAB* (Messing et al. 1977), XL1-BLUE *recA1 lac$^-$ endA1 gyrA96 thi hsdR17 supE44 relA1 F' proAb lacI^{q}ZΔM15* Tn*10* (Bullock et al. 1987), JM105 *supE endA*

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*The DNA sequence reported in this paper has been submitted to the Gene Bank data base under the accession no. X63674

sbcB15 hsdR4 rpsL thi Δ[lac-proAb] F′traD36 proAB lac I^{q}ZΔM15 (Yanisch-Perron et al. 1985), SG13009 *F′ his pyrD Δlon-100 rpsL* (Gottesman et al. 1981) hosting plasmid pUHA1 (Grimm 1990). Plasmid pBLUESCRIPT II KS(−) was purchased from Stratagene (Heidelberg, Germany), pBTac1 (de Boer et al. 1983) from Boehringer (Mannheim, Germany), pIH902 (New England Biolabs 1991) from New England Biolabs (Schwalbach, Germany), and pDS56/RBSII-2 (Stueber et al. 1984) from Diagen (Düsseldorf, Germany).

Restriction enzymes and other nucleic-acid-modifying enzymes were purchased from Boehringer, Pharmacia LKB Biotechnology (Freiburg, Germany), Stratagene and United States Biochemical (Bad Homburg, Germany). Radionucleotides were purchased from Amersham (Braunschweig, Germany). Linearized DNA fragments were labelled by the random primer procedure of Feinberg and Vogelstein (1983). DNA sequencing was carried out by the dideoxy chain termination method (Sanger et al. 1977; Tabor and Richardson 1987). DNA and polypeptide sequence analysis and alignment were performed using the programmes of the HIBIO DNASIS and PROSIS software package.

F. meningosepticum cells (strain ATCC 13253) were obtained from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Chromosomal DNA from *F. meningosepticum* was isolated by the method of Grimberg et al. (1989).

Expression of the gene for PSE. E. coli strains XL1-BLUE, BMH71-18, JM105 or SG13009 carrying recombinant plasmids were cultured overnight at 37°C in LB (Luria-Bertani) medium (Sambrook et al. 1989) supplemented with the appropriate antibiotics (ampicillin, 200 μg/ml; tetracycline, 20 μg/ml; kanamycin, 50 μg/ml). The cells were inoculated (2%) in the same medium and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.4. Crude extract preparations from culture aliquots taken at $OD_{600}=0.4$ served as controls of "not induced" cells. For inducing PSE synthesis isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM and the cells were routinely cultured at 37°C for 2 h. The cells were harvested by centrifugation and lysed by the osmotic lysis procedure described by Osborn et al. (1972). The lysate was centrifuged for 10 min at 12000 *g*, 4°C, and the prolyl endopeptidase activity in the supernatant was measured.

Separation of cytoplasmic and periplasmic fractions. Recombinant *E. coli* cells were grown and induced for PSE synthesis as described above. Aliquots were taken at different time points and cells were harvested by centrifugation at 4000 *g* for 10 min. Periplasmic fractions were prepared by the cold osmotic shock procedure (Neu and Heppel 1965). For subsequent release of cytoplasmic proteins the resulting pellet was resuspended in lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 0.25% (v/v) Tween 20, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.0; 4 ml for each 0.1 g cell wet weight), frozen in liquid nitrogen and thawed in cold water. Cell disruption was achieved by repeated sonication on ice using a Bandelin Sonopuls HD70 sonicator at 70% power. After centrifugation at 9000 *g* for 20 min the cytoplasmic fraction was obtained in the supernatant.

Purification of PSE from E. coli. Periplasmic fractions were concentrated in an Amicon ultrafiltration apparatus using a PM-30 membrane. The concentrated enzyme solution was adjusted to 50 mM phosphate buffer, pH 7.2, and 0.15 M NaCl and subjected to gel filtration on a Sephacryl S300 column (16×100 cm, 200-ml gel bed volume, flow rate 10 ml/min) equilibrated with the same buffer. Fractions (5 ml) were collected and tested for PSE activity. PSE-containing fractions were pooled, concentrated by ultrafiltration and kept at 4°C. The efficiency of purification was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; see below).

Determination of enzyme activity. PSE activities were determined via a colorimetric assay using substrate Z-Gly-Pro-pNA (Bachem, Heidelberg, Germany) as described recently (Chevallier et al. 1992). Activity of β-lactamase was assayed with Nitrocefin (Oxoid, Wesel, Germany) at 390 nm as described previously (Roggenkamp et al. 1981). For measuring specific enzyme activities [units (U)/mg protein] the protein concentration was measured by the method of Bradford (1976) using bovine gamma globulin as standard.

Polyacrylamide gel electrophoresis. SDS-PAGE according to Laemmli et al. (1970) was carried out as described previously (Studier 1973). Prior to electrophoresis, proteins were precipitated from diluted fractions with 5% (v/v) trichloroacetic acid (TCA) on ice for 30 min or samples from concentrated crude extracts were taken directly and boiled for 10 min with an equal volume of twofold concentrated sample buffer (see above).

Results

Nucleotide sequence

Based on the data obtained from partial amino acid sequencing of flavobacterial PSE (see Materials and methods) various oligonucleotide primers were synthesized. Employing one specific primer pair, a 930-bp DNA fragment was amplified via PCR from genomic *F. meningosepticum* DNA and used as a screening probe for isolating the PSE gene from an *F. meningosepticum* gDNA library.

The established DNA sequence revealed an open reading frame consisting of 2118 nucleotides and starting with an ATG codon. The enzyme is composed of 705 amino acid residues and has a calculated molecular mass of 78.634 kDa, being in good agreement with its experimentally determined mass of 76 kDa (Yoshimoto et al. 1980).

Homology search

The active sites of most of the mammalian serine proteases and serine esterases known so far share the consensus sequence Gly-X-Ser-X-Gly (Brenner 1988). Indeed, in the deduced amino acid sequence of the isolated PSE gene this characteristic could be identified within a Gly-Arg-Ser-Asn-Gly-Gly-Leu region, surrounding the reactive site serine (Fig. 1). The putative active site seryl residue derived from our sequence could be found in a homologous region with respect to another prolyl endopeptidase isolated from porcine brain (Rennex et al. 1991). Amino acid sequence comparison between the two peptidases exhibits 38% identity. The most homologous parts of these two sequences comprise the N-terminal and C-terminal regions (Yoshimoto et al. 1991; Chevallier et al. 1992).

Sequence analysis revealed that flavobacterial PSE shares a homologous region with another peptidase of different substrate specificity, the aminopeptidase pII of *E. coli* (Kanatani et al. 1991). By including aminopeptidase pII, which has not been considered by Rawlings et al. (1991), we draw a slightly different alignment

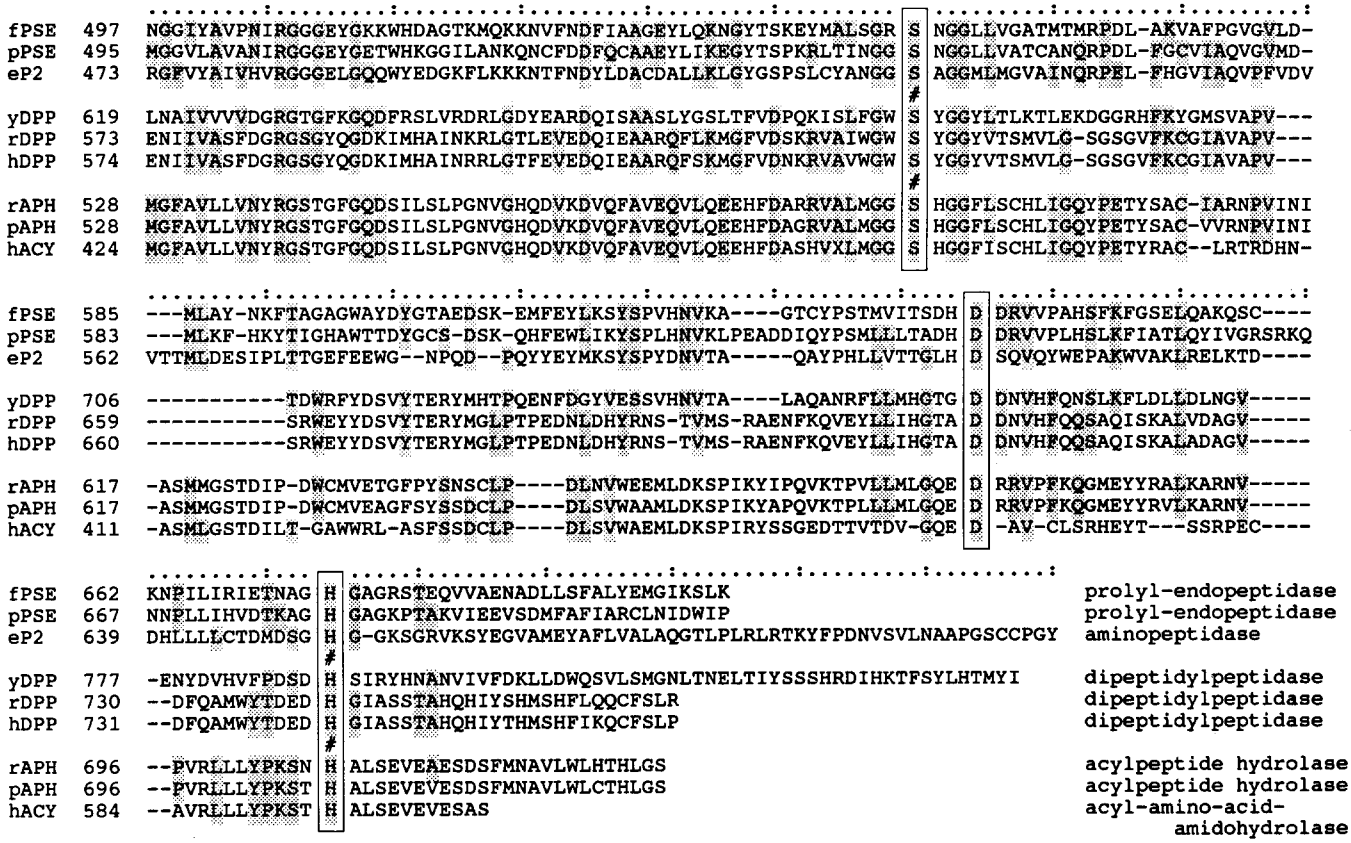

Fig. 1. Alignment of the C-terminal segments of *Flavobacterium meningosepticum* proline specific endopeptidase (PSE) and related oligopeptidases. The sequences are: fPSE, *F. meningosepticum* PSE (this work); pPSE, pig brain PSE (Rennex et al. 1991); eP2, *Escherichia coli* protease pII (Yoshimoto et al. 1991); yDPP, *Saccharomyces cerevisiae* dipeptidyl aminopeptidase (Roberts et al. 1989); rDPP, rat dipeptidyl aminopeptidase IV (Ogata et al. 1989); hDPP, human dipeptidyl aminopeptidase IV (Misumi et al. 1992); rAPH, rat liver acylaminoacyl-peptidase (Kobayashi et al. 1989); pAPH, pig liver acylaminoacyl-peptidase (Mitta et al. 1989); hACY, human acylase (Naylor et al. 1989). Amino acid residues with positional identity in at least four sequences are indicated by *shading;* residues that are proposed to belong to the catalytic triad are *boxed;* # denotes those residues (S_{556}, H_{675} in *F. meningosepticum* PSE) that have been implied in catalysis by biochemical evidence (Rennex et al. 1991; Stone et al. 1991)

(Fig. 1). Referring to the C-terminal 250 amino acid residues, *F. meningosepticum* PSE exhibits moderate similarity to both X-Pro specific dipeptidyl aminopeptidases (EC 3.4.14.5) from yeast and rat, acylaminoacyl peptidases from pig and rat liver, and to human acylase. Resemblances are mainly restricted to the C-terminal third.

Plasmid constructions

To achieve efficient expression of flavobacterial PSE in *E. coli* different strategies and expression vector systems were employed. Plasmid pIH902 was used to express PSE as a fusion protein, which should allow for its efficient purification. Two other vectors (pBTac1, pDS56/RBSII-2) were chosen to express the PSE gene under the control of different strong promoters.

For cloning of the 2118-bp coding region of the PSE gene, appropriate restriction sites were created by two synthetic oligonucleotides using the PCR technique. Plasmid pPSE-g (Fig. 2) was used as the DNA template for the PCR reaction. The amplified DNA fragment was recloned (pPSE-a, Fig. 2) and sequence-verified. Plasmid pPSE-a was digested with *Hin*cII and *Pst*I or *Hin*cII and *Bam*HI. The 2118-bp DNA fragment containing the PSE gene was isolated and used to construct the plasmids pHD33, pVW7 and pTD1.

Expression plasmid pHD33 was constructed from pIH902 digested with *Stu*I and *Bam*HI. The isolated 2118-bp DNA fragment from plasmid pPSE-a digested with *Hin*cII and *Bam*HI was religated in the linearized pIH902 vector. Plasmid pVW7 was constructed from pDS56-2/RBSII-2. The vector was digested with *Bam*HI and the sticky ends were filled in using Klenow polymerase. After phosphatase treatment the plasmid was digested with *Pst*I and ligated with the isolated *Hin*cII/*Pst*I PSE-DNA fragment (see above). The same PSE-DNA fragment was ligated into pBTac1 digested with *Sma*I and *Pst*I, resulting in plasmid pTD1.

Expression of active PSE

E. coli strains XL1-BLUE, BMH71-18, SG13009 and JM105 were transformed with either plasmid pTD1, pVW7 or pHD33. Transformed cells were grown under selective conditions. PSE activity could be detected in

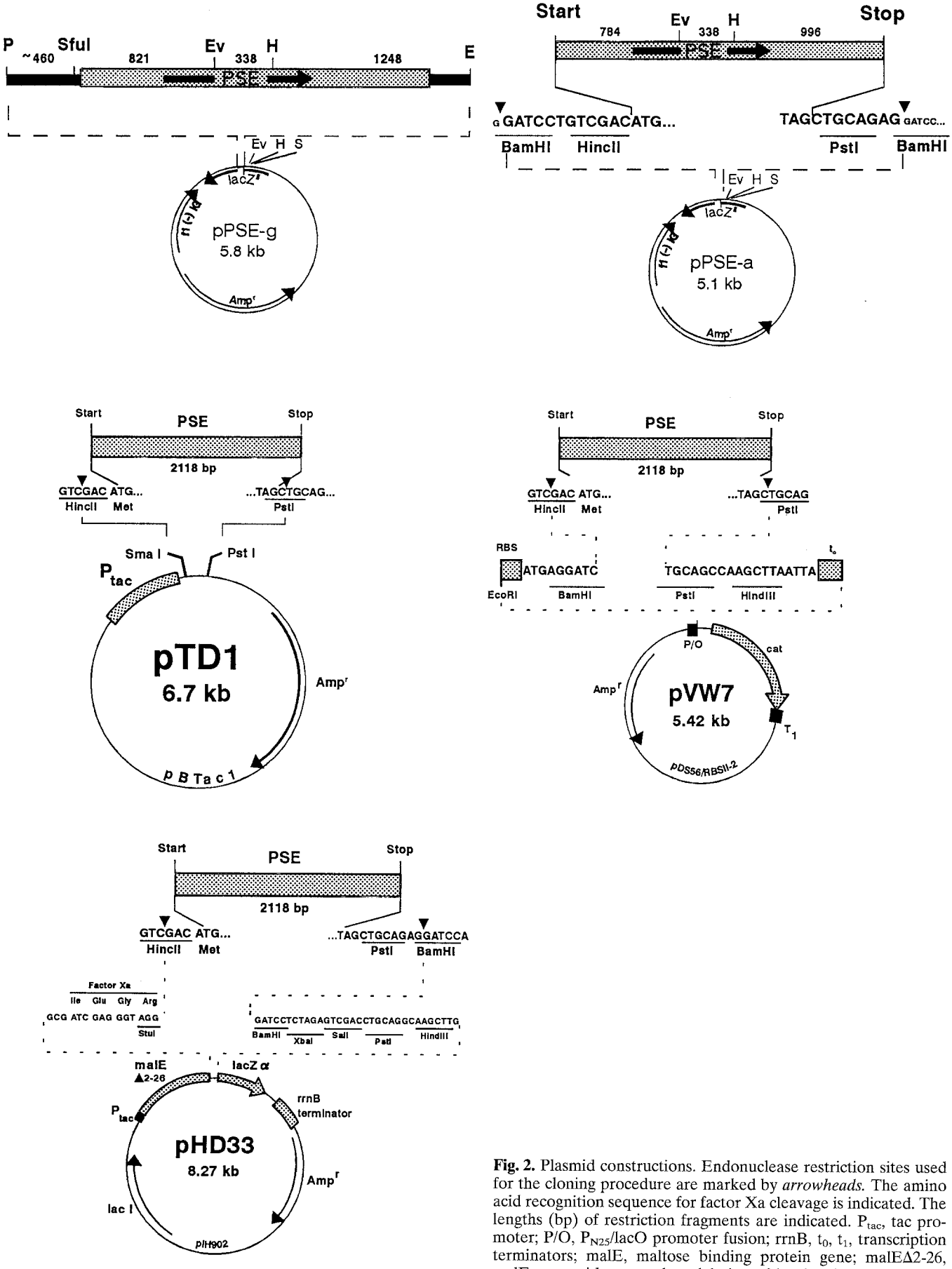

Fig. 2. Plasmid constructions. Endonuclease restriction sites used for the cloning procedure are marked by *arrowheads.* The amino acid recognition sequence for factor Xa cleavage is indicated. The lengths (bp) of restriction fragments are indicated. P_{tac}, tac promoter; P/O, P_{N25}/lacO promoter fusion; rrnB, t_0, t_1, transcription terminators; malE, maltose binding protein gene; malEΔ2-26, malE gene with a complete deletion of its signal sequence; lacI, lac repressor gene; Amp^r, ampicillin-resistance gene

Table 1. Proline specific endopeptidase (PSE) activities in *Escherichia coli* transformants

Strain	Plasmid	Activity (U/ml)	
		Not induced	Induced
XL1-BLUE	pTD1	0.061	0.523
BMH71-18	pTD1	0.240	0.393
SG13009	pTD1	0.081	0.274
JM105	pTD1	0.045	0.700
XL1-BLUE	pVW7	0.040	0.448
SG13009	pVW7	0.031	0.345
XL1-BLUE	pHD33	0.064	0.572
SG13009	pHD33	0.014	0.151

None of the corresponding non-recombinant control transformants showed any PSE activity; U, units

the crude extracts of all transformants carrying the PSE gene (Table 1). Depending on the plasmids and host strains used, IPTG-induced PSE gene expression reached different levels. The resulting peptidase activities ranged from 0.15 to 0.7 U/ml. High enzyme activities (approx. 0.6 and 0.7 U/ml) were obtained as a result of *tac*-promoted expression with plasmids pHD33 and pTD1, respectively (Table 1).

The crude extracts of *E. coli* transformants were further analysed by SDS-PAGE. Upon Coomassie brilliant blue staining the homogenates of induced transformed cells showed clearly distinct bands of heterologous proteins (data not shown).

E. coli cells transformed with plasmid pHD33 were expected to express an maltose binding protein (MBP)/PSE fusion protein of nearly 120 kDa. However, SDS-PAGE analysis of the protein homogenate revealed two protein bands with apparent molecular masses of 44 and 75 kDa (data not shown). The high enzyme activity and comigration of a 75-kDa protein with native flavobacterial PSE suggests that processing has occurred in *E. coli* by specific cleavage of the MBP/PSE-fusion protein at the C-terminal side of the experimentally determined signal sequence (see below). The observed 44-kDa peptide can be interpreted as the fusion of the maltose binding protein (42 kDa) and the PSE signal peptide (approx. 2 kDa). The 75-kDa protein band would then represent the processed enzymatically active PSE.

The apparent molecular masses of the heterologous proteins were about 75 kDa in transformants harbouring plasmids pVW7 (data not shown) and pTD1 (c.f. Fig. 3), which is in good agreement with the experimentally determined mass of 76 kDa for native flavobacterial PSE.

Localization of expressed PSE in E. coli

Among all PSE-expressing transformants, JM105{pTD1} turned out to be the best candidate (Table 1) for fermentation and purification of the heterologous peptidase. As a prerequisite for developing an efficient and simple purification procedure the localization of PSE in JM105{pTD1} under different growth conditions was studied. Transformants were induced for PSE synthesis by IPTG and aliquots were taken at different time points. The cells were separated into periplasmic and cytoplasmic fractions (see Materials and methods). In each cell compartment PSE enzyme activity was detected (Table 2). Relative to the non-induced transformant culture a 79-fold increase in PSE activity was obtained after 3 h of IPTG induction. We estimated, that the amount of PSE produced by these

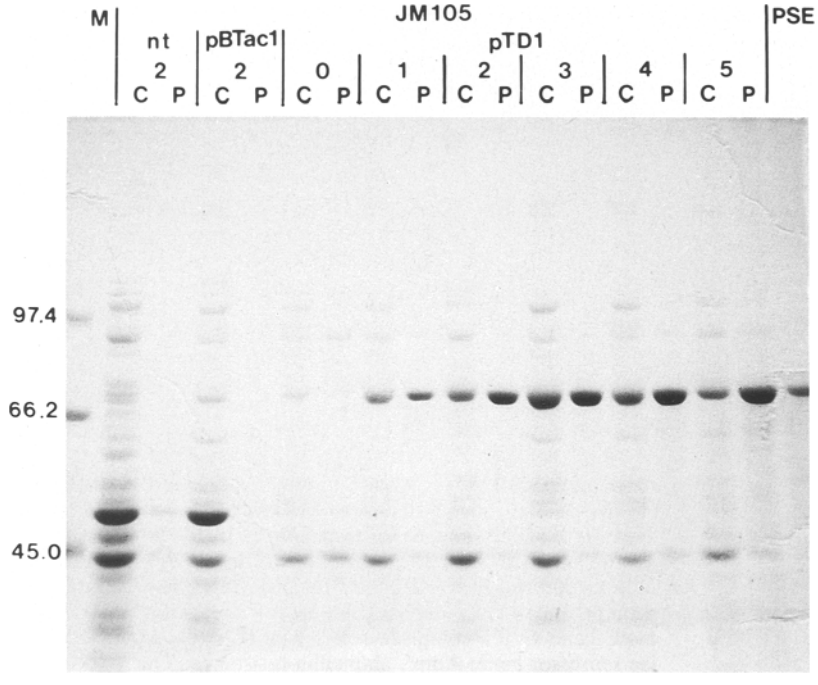

Fig. 3. Localization of PSE in *E. coli*. *E. coli* JM105 carrying plasmid pTD1 was cultured and expression of the PSE gene was induced for 0–5 h (0–5) with isopropyl-β-D-thiogalactopyranoside (IPTG). Cytoplasmic (C) and periplasmic (P) fractions were prepared as described (Materials and methods). After trichloroacetic acid (TCA, 5%) precipitation the proteins in each fraction were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% acrylamide) and stained with Coomassie brilliant blue. Non-transformed (nt) JM105 host cells and cells carrying the non-recombinant vector (pBTac1), that were cultured in parallel and induced with IPTG for 2 h served as controls, respectively. PSE, authentic *F. meningosepticum* PSE; M, protein standard with molecular masses given in kDa

Table 2. Cytoplasmic and periplasmic PSE activities in *E. coli* JM105 conferred by pTD1

	Cytoplasm[a]			Periplasm[a]		
		PSE			PSE	
Induction time (h)	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Total protein (mg)	Total activity (u)	Specific activity (u/mg)
0	1.9	0.8	0.4	0.8	1.2	1.5
1	4.2	27.6	6.6	0.6	30.8	51.3
2	3.1	41.3	13.3	0.7	64.6	92.3
3	5.9	66.2	11.2	0.8	84.4	105.5
4	5.8	74.0	12.7	0.9	84.0	93.3
5	2.4	37.3	15.5	1.1	82.0	74.5

Values given in this table refer to 35-ml aliquots of a 250-ml bacterial culture

[a] For assessing the efficiencies of spheroplast formation the activity distribution of the periplasmic marker enzyme β-lactamase was assayed

transformants constitutes up to 15% of the total cellular protein. This evaluation is based on activity and protein measurements (Table 2), which are consistent with observations from SDS-PAGE (Fig. 3). After 4 h of induced PSE synthesis, a maximum of total enzyme activity (158 U) was obtained, which corresponds to 1.6 U/mg of *E. coli* cells.

Sequence data suggest that in *F. meningosepticum* the PSE protein is expressed as a precursor molecule of 78.6 kDa with an N-terminal signal sequence. Our fractionation experiments revealed that PSE is secreted, to a considerable extent, to the periplasmic space of *E. coli* (Fig. 3).

N-Terminal amino acid sequencing of periplasmically located PSE revealed that for secretion in *E. coli* the peptidase precursor protein is specifically processed at two alternative cleavage sites, thus supporting the existence of the aforementioned signal sequence. It turned out that the PSE precursor molecule is cleaved between either residues Ser_{19} and Ala_{20} or Ala_{20} and Gln_{21}, respectively, with the latter cleavage site being utilized eight times more frequently than the first one.

After 3 h of induction, PSE enrichment in the periplasmic fraction reached its maximum whereas residual enzyme activity (about 40%, Table 2) could still be found in the cytoplasm. These results indicate a limited translocation of the heterologous protein through the inner bacterial membrane, suggesting that the flavobacterial signal sequence can function in *E. coli.*

Protein purification

The high specific PSE activity found in the periplasmic fraction after 3 h of induction and the dominating band observed in SDS-PAGE revealed that PSE in this cell compartment accounts for most of the protein. This specific accumulation of active PSE enzyme in the periplasmic space of *E. coli* was utilized to purify the enzyme to homogeneity by a combination of periplasmic preparations and gel chromatography (Fig. 4). Periplasmic proteins from *E. coli* cells were released by a gentle osmotic shock procedure (see Materials and methods). In order to purify the PSE, gel filtration on Sepharyl S-300 was performed. PSE-activity-containing fractions were pooled and analysed by SDS-PAGE. By Coomassie brilliant blue staining, only a single protein band was detectable representing homogeneous PSE (Fig. 4). Up to 1.25 mg of pure active protein could be isolated from a 1-l culture of *E. coli.*

Discussion

We have cloned and sequenced the gene coding for PSE from *F. meningosepticum.* The open reading

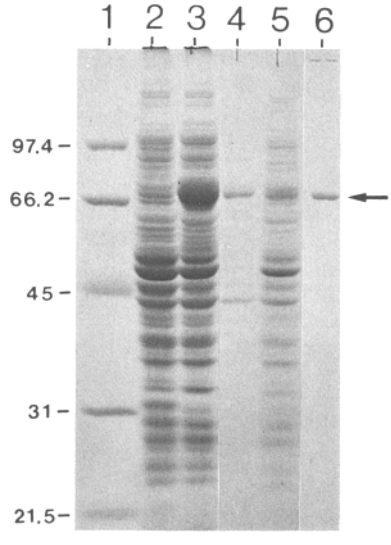

Fig. 4. Purity of the heterologous PSE. *E. coli* JM105 carrying plasmid pTD1 was cultured (1 l) and expression of the PSE gene was induced for 3 h with IPTG. Protein samples were fractionated by SDS-PAGE on a 10% acrylamide gel and stained with Coomassie brilliant blue: *lane 1,* molecular mass standards; *lane 2,* cell-free extract (not induced); *lane 3,* cell-free extract (IPTG-induced); *lane 4,* periplasmic fraction; *lane 5,* cytoplasmic fraction; *lane 6,* Sephacryl S-300 purified PSE

frame of the PSE gene consists of 2118 bp and encodes a 705-amino-acid protein. For isolating the PSE gene we used the same strain (ATCC 13253) as that employed by Yoshimoto et al. (1991). Accordingly, our deduced amino acid sequence is almost identical with the PSE sequence reported by this group. Only one amino acid exchange could be found. If the sequence is compared to that of another PSE gene recently published by Chevallier et al. (1992), however, fifty amino acid exchanges were detected. As the *F. meningosepticum* strain (ATCC 33958) used by this group was different from ours, the observed sequence discrepancies probably reflect genetic variations.

Sequence comparison indicates that PSE from *F. meningosepticum*, in association with aminopeptidase pII from *E. coli*, can be regarded as represantatives of a new serine type peptidase family, to which porcine brain PSE has already been assigned (Rawlings et al. 1991). The catalytic triad of the well-studied trypsin and subtilisin families is characterized by the spatial arrangement His, Asp, Ser and Asp, His, Ser, respectively (Kraut 1977), with the active site serine residue always located nearest to the respective stop codon (Polgár 1992). In contrast, the third position of the catalytic triad in the peptidases belonging to the new family is characterized by a histidyl residue (Rawlings et al. 1991). However, the exact position of the putative aspartic acid residue of the catalytic triad has not been determined so far. Considering amino acid sequence comparison data (Fig. 1), the two aspartic acid residues at either positions 531 (D_{531}) or 640 (D_{640}) could be a member of the catalytic triad. The fact that the catalytic triad of four carboxypeptidases (carboyxypeptidase Y from yeast, carboxypeptidase I and II from malt, carboxypeptidase from wheat bran) is characterized by a Ser, Asp, His arrangement (Breddam et al. 1987) would favour D_{640}. These carboxypeptidases probably employ the catalytic mechanism of the serine endopeptidases (Breddam 1986).

The most efficient synthesis of heterologous PSE in *E. coli* was achieved using plasmid construct pTD1, which is based on the expression vector pBTac1. Localization studies revealed that PSE is released to a considerable extent into the periplasmic space of *E. coli*. From the nucleotide sequence data of the PSE gene it is suggested that in *F. meningosepticum* the PSE is synthesized as a precursor molecule, characterized by an N-terminal signal sequence. This is supported by ion-spray mass spectrometry studies (Chevallier et al. 1992) and by our finding that the PSE protein is secreted to the periplasmic space in *E. coli* undergoing the expected processing as indicated by direct amino acid sequence analysis. The additional detection of cytoplasmic PSE protein, however, indicates a limited translocation through the inner bacterial membrane.

The establishment of an efficient expression system for the production of PSE protein in *E. coli* enables us to develop a simple method for purification of the enzyme. In the *F. meningosepticum* strain that is used for the commercial production of prolyl endopeptidase, only 34.5 U/g *F. meningosepticum* cells (wet weight) were obtained (Yoshimoto et al. 1980). In contrast, JM105{pTD1} transformants produce up to 558 U PSE/g *E. coli* cells (wet weight), which represents a 16-fold increase in PSE production with respect to *F. meningosepticum*.

We found that in *E. coli* PSE accumulates in the periplasmic space (up to 60%). We could replace the complex PSE purification procedures described by Yoshimoto et al. (1991) and Chevallier et al. (1992) with a fast two-step purification method that is suitable to achieve pure enzyme. A gel chromatographic purification of the PSE enriched in the periplasmic fraction results in 0.5 mg pure enzyme/g *E. coli* cells (wet weight) with a specific activity of 84 U/mg. Sephacryl S-300 gel filtration can be replaced by anion exchange chromatography on Sepharose-Q yielding also pure enzyme preparations (data not shown).

The high amount of pure active heterologous PSE now allows us to investigate its potential in kinetically controlled peptide synthesis.

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