Bacillopeptidase F is an extracellular serine protease that is expressed at the beginning of the stationary phase. To study its structure, regulation of expression, and physiological roles, we have cloned and characterized the structural gene (bpf) encoding this protease from Bacillus subtilis. DNA sequence analysis suggests this protease is synthesized as a preproenzyme (Mf = 92,000). Through processing at both the NH2 and COOH termini, it is gradually converted into various forms with molecular mass ranging from 80 to 48 kDa. Shortening the 3′ end of bpf demonstrates that at least 290 amino acid residues from the COOH-terminal portion of the enzyme were defrayed in part by the payment of page charges. This article has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J06400.

Bacillus subtilis produces several extracellular proteases after the end of the exponential growth. These proteases are the neutral protease (encoded by structural gene npr),1 subtilisin or alkaline protease (encoded by apr), extracellular protease (encoded by epr), and bacillopeptidase F (encoded by bpf). The structural genes for the first three proteases (l-3) have been cloned and sequenced. Further characterizations demonstrate that these three proteases are synthesized as preproenzymes (4, 5). In the case of extracellular protease, the COOH-terminal portion of the enzyme was also processed (3). The expression of both apr and npr can be further enhanced by a family of regulatory factors such as the gene products of sacQ (6), sacU (7), prrR (8), and senN (9). Bacillopeptidase F differs from all other extracellular proteases in that it has a relatively high esterolytic activity (10–12). Thus, it has been named esterase. It is also referred to as enzyme C (13) and acidic protease (14). Although it has been purified and characterized (15), its structure and physiological role are not well studied. Furthermore, this protease may account for the residual proteolytic activity observed for the double (DB104 with apr and npr inactivated; 16) and triple protease-deficient strain (GP216 with apr, npr, and epr inactivated; 3). This residual protease activity severely affects the stability of the secreted foreign proteins when using B. subtilis as an expression system (17). To study its physiological role(s), bacterial genetic manipulation, regulation of expression, and processing of the precursor, we describe in this paper the cloning and characterization of bpf from B. subtilis. A triple protease-deficient strain (WB300 with apr, npr, and bpf inactivated) has been constructed. The growth and efficiency of sporulation of this strain were examined.

MATERIALS AND METHODS

RESULTS

Purification of Bacillopeptidase F—Fig. 1 shows the purification of protease at various stages. After chromatofocusing on a Mono P column, only one of the protein peaks showed proteolytic activity. When the fractions from this peak were analyzed on a 12% SDS-polyacrylamide gel, one minor and two major protein bands of 68, 50, and 48 kDa, respectively, were detected (Fig. 1, lane 2). To determine whether these proteins are proteases or not, samples from this peak were resolved on a preparative nondenaturing acrylamide gel. Part of the gel was excised and briefly stained to facilitate the localization of the protein bands. As shown in Fig. 2A, two major protein bands of 68, 50, and 48 kDa, respectively, were detected (Fig. 1, lane 2). To determine whether these proteins are proteases, half was used for esterase activity assay and the other half for SDS-PAGE analysis. Protein bands labeled a and b in the nondenaturing gel correspond to the 50- and 48-kDa proteins observed in the SDS-PAGE (Fig. 2A). Both of them had esterase activity and thus, are proteases. To determine whether these 48-kDa protease is a degradation product

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* Portions of this paper (including "Materials and Methods" and Figs. 1-4 and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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Cloning of Bacillopeptidase F Gene

derived from the 50-kDa protease, antibody was prepared from each of the purified proteins. By Western blot analysis, antibody against the 50-kDa protease was shown to be cross-reacting with the 48-kDa protease (Fig. 2B) and vice versa (data not shown). These results demonstrate that both proteins are actually derived from the same protease.

NH2-terminal Sequence Determination—As reported by Roitsch and Hageman (15), three different forms of bacillopeptidase F with molecular mass of 45, 65, and 75 kDa, could be detected. The biggest one could be isolated only when B. subtilis was cultured in a medium containing 2.5 mM PMSF. Thus, from our purification, both the 50- and 48-kDa proteases represent the degraded form of bacillopeptidase F. They might have microheterogeneity at the NH2-terminus and would not be appropriate for sequencing. To overcome this problem, we decided to isolate an internal peptide for sequencing. The purified, 50-kDa protease was cleaved by cyanogen bromide (32) and the peptides were resolved by reverse-phase protein liquid chromatography. Four peaks were detected. One of them was well separated from the others and was reapplied to the column for further purification. This purified internal peptide and the 50-kDa protease were subjected to NH2-terminal sequence determination. The sequence of the first 14 amino acid residues from the NH2-terminus of the internal peptide was determined: Ala-Gly-Pro-His-Val-Ser-Ala-Val-Val-Leu-Leu-lys-Gln. No microheterogeneity was detected in the 50-kDa protease and the sequence for the first 33 amino acids was determined: Ala-Thr-Asp-Gly-Val-Glu-Trp-Asn-Val-Asp-Ala-Pro-lys-ala-Tyr-Asp-Gly-Thr-Gly-Thr-Val-Val-Ser-Ile-Asp.

Cloning of bpf—The sequence from positions 5 to 17 in the 50-kDa protease was selected to design a 38-nucleotide oligonucleotide probe for hybridization (see "Materials and Methods"). Southern blot analysis of B. subtilis chromosomal DNA indicated that the probe was selectively hybridized to a 6.5-kb PstI fragment (data not shown). A B. subtilis genomic library with enrichment of the 6.5-kb PstI fragments was constructed (see "Materials and Methods"). Eight positive clones were identified through colony hybridization. All carry the same 6.5-kb PstI insert. One of these plasmids was named pEBPF and was used for further characterizations.

Confirmation of the Cloning of bpf—The cloned 6.5-kb PstI fragment from pEBPF was ligated to pUB18 (9) to generate pBBPF. Compared with the control (DB104[pUB18]), DB104[pBBPF] showed a bigger halo on a SG plate containing 1% gelatin and 10 μg/ml kanomycin (Fig. 3A). Fig. 3B shows Western blot analysis of the supernatants from DB104[pBBPF] and DB104[pUB18] with anti-50-kDa protease antibody. Cells carrying pBBPF overproduced a 80-kDa protein. This protein could be detected easily if the cells were cultured in a medium containing 2.5 mM PMSF. In the absence of PMSF, several other cross-reacting bands with sizes of 68, 50, 48 kDa were observed (data not shown). The 80-kDa protein was electroblotted to a polyvinylidine difluoride membrane (Immobilon) for sequence determination. The following sequence was observed: Ala-Ile-Lys-Ala-Thr-Asp-Gly-Val-Glu-Trp-Asn-Val-Asp-Gln-Ile (see Fig. 5). The sequence from the third position to the end was found to match exactly with the NH2-terminal sequence of the 50-kDa protease.

Characterization of bpf—A detailed restriction map of this 6.5-kb PstI fragment was determined and is shown in Fig. 4. To determine the location of the protease gene within this fragment, different fragments were subcloned into pUB18 and the transformants were checked for the capability of produc-

Fig. 5. Nucleotide sequence of bpf. The open reading frame for bacillopeptidase F extends from base 342 to base 2061. Ribosome-binding sites are double-underlined. The COOH-terminal portion of ftsZ and the inverted repeat sequence which may function as the transcription terminator for the ftsZ operon are shown upstream of bpf. Another potential open reading frame downstream from bpf is also indicated. Amino acid sequences underlined by a straight line, a dotted line, and a wavy line indicate that the sequences were determined through amino acid sequencing from the 50- and 80-kDa forms of bacillopeptidase F and the internal peptide generated by CNBr treatment of 50-kDa bacillopeptidase F, respectively.
showed blue colonies on the SG/5-bromo-4-chloro-3-indoxyl β-d-galactoside plate. Expression of β-galactosidase was further confirmed by Western blot analysis of the cell extract with an anti-β-galactosidase monoclonal antibody (data not shown). This result suggested that bpf was translated from the predicted start site.

Characterization of the Protease—Since one of the major characteristics of bacillopeptidase F is its relatively high esterolytic activity, both the 80- and 50-kDa proteases were purified by non-denaturating PAGE (29) and electrophoresis. The esterolytic and proteolytic activities of these proteases were determined and compared with that of subtilisin. As shown in Table I, they showed relatively high esterolytic activity, as reported for bacillopeptidase F (10–12). The pI of the 80-kDa protease was determined to be 5.4 and has the same value as reported for the 75-kDa form of bacillopeptidase F by Roitsch and Hageman (15). Furthermore, the pI optimum of the 45-kDa form of bacillopeptidase F was reported to be in the pH range 8–9 by using benzoyl-L-tyrosine ethyl ester as substrate. Using the purified 50-kDa protease for assay, the pI optimum around 9.0 was observed. The activity of both the 80- and 50-kDa proteases could be completely inhibited by 1 mM PMSP and partially inhibited by soybean trypsin inhibitor and turkey egg white trypsin inhibitor. At final concentrations up to 10 mM EDTA, 1,10-phenanthroline, and 1-tosylamido-2-phenylethyl chloromethyl ketone showed no inhibitory effect. Based on the high esterase activity, similarity in molecular mass, pI, pH optimum, and sensitivity to serine protease inhibitors, we concluded that the cloned protease gene encodes bacillopeptidase F.

Homology with Other Serine Proteases—Since bacillopeptidase F is a serine protease, homology with other serine proteases was compared. This protease showed sequence similarity with thermitase (26%) from Thermoactinomyces vulgaris (34), proteinase K (20%) from Tritirachium album (35), atherotrypsin (34), and proteinase from Thermus aquaticus (36), and three B. subtilis proteases including subtilisin (31%), intracellular serine protease-1 (24%; 37), and extracellular protease (19%). Using the sequences around the three catalytic residues (Asp-227, His-274, and Ser-452) in bacillopeptidase F could be tentatively assigned.

Effect of Shortening the 3' End of bpf on the Catalytic Activity of the Enzyme and the Secretion of Protease—To determine whether the COOH-terminus region of bacillopeptidase F is required for catalytic activity and secretion, clones encoding proteases with shortened COOH termini (pBH, pBF, and pBN) were constructed. As shown in Figs. 3a and 4, even when missing 290 amino acids from the COOH terminus of bacillopeptidase F, active protease activity could still be detected from DB104[pBP]. Thus, these 290 amino acid residues are not essential for protease activity or secretion.

TABLE I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units of casein hydrolytic activity</th>
<th>Units of BTEE hydrolytic activity</th>
<th>Relative activity</th>
<th>Relative activity proteases/subtilisin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin</td>
<td>0.055</td>
<td>0.01</td>
<td>0.18</td>
<td>1.0</td>
</tr>
<tr>
<td>80-kDa protease</td>
<td>0.0557</td>
<td>0.054</td>
<td>9.7</td>
<td>53.8</td>
</tr>
<tr>
<td>50-kDa protease</td>
<td>0.069</td>
<td>2.16</td>
<td>31.5</td>
<td>175.0</td>
</tr>
</tbody>
</table>
With removal of 425 residues from the COOH terminus detected from DB104[pBN]. This result agrees with the prediction that extracellular proteases are in a monocistronic arrangement. This organization is quite unusual since all three other structural genes encoding for the protein. A transcription terminator-like structure (AG' = -22) is located at 135° on the B. subtilis genetic linkage map, bpf should be located at the same locus on the chromosome.

Inactivation of the Chromosomal Copy of bpf—To determine whether bacillopeptidase F plays any essential roles in cell growth and sporulation, a deletion was made from DraI (base 310 in Fig. 5) to EcoRV (base 1554) within the structural gene. This deletion removes sequence including the ribosome-binding site, signal sequence, and part of the coding region for the mature 50-kDa protease. The deleted gene was exchanged with the chromosomal copy in DB104 by gene conversion as described by Kawamura (16). The exchange was confirmed by Southern hybridization with the 6.5-kb PstI fragment as probe (Fig. 7). This triple protease-deficient strain was found to grow and sporulate normally in both nutrient broth and minimal medium (data not shown). Thus, bpf is a nonessential gene for growth and sporulation.

**DISCUSSION**

Based on the high esterolytic activity, pH profile, sensitivity to PMSF, PI, and molecular weight determined by SDS-PAGE, the cloned protease gene in the 6.5-kb PstI fragment was shown to code for bacillopeptidase F.

Judging from the DNA sequence of the gene, the primary product of this protease should have a molecular weight of 92,000. However, even when the cells were cultured in the presence of 2.5 mM PMSF, the largest form of protease that could be detected in the medium has a molecular weight of 80,000. NH2-terminal sequence determination indicated that this 80-kDa protease begins with an Ala at position 195. Since inactivation of the protease gene in the chromosome at 135° on the B. subtilis genetic linkage map, bpf should be located at the same locus on the chromosome.
derived from protein degradation. As _B. subtilis_ has the potential to be an expression host for secreting and producing foreign proteins, construction of the triple protease-deficient strain (WB300) will be ideal for this application.

REFERENCES

Cloning of Bacillopeptidase F Gene

Production of Bacillopeptidase F from the cloned gene was carried out by raising polyclonal, pho, ppp, and pho antibodies in rabbits. The production of Bacillopeptidase F was determined by Western blot analysis using the anti-Bacillopeptidase F antibodies in 50% methanol.

Production of proteins: Specific antisera

A 45-kDa, specific for the Bacillopeptidase F gene, was detected by the Western blot analysis. The antisera was used to detect the Bacillopeptidase F gene in the plasmid and in the bacterial cell.

Fig. 1. Purification of Bacillopeptidase F. Protein fractions during various stages of purification were analyzed on a SDS-PAGE gel and Coomassie Blue. Lane 1 shows the molecular mass standards (in kilodaltons). Lanes 2-3 show the protein profile of samples prepared after washing with glutathione-Sepharose 4B affinity resin. Lanes 4-5 show the protein profile of samples prepared after elution with Bacillopeptidase F. Lane 6 shows the protein profile of samples prepared after elution with 6M guanidine.

Fig. 2. Characterization of the cloned Bacillopeptidase F gene. The top panel of the figure shows the electrophoretic analysis of the parental DNA in a preparative non-denaturing gel. Lanes 1-6 show the protein bands on the gel. Lanes 7-8 show the protein bands on the gel after elution with Bacillopeptidase F. Lanes 9-10 show the protein bands on the gel after elution with 6M guanidine. Lanes 11-12 show the protein bands on the gel after elution with 6M guanidine.

Fig. 3. Production of Bacillopeptidase F. The production of Bacillopeptidase F was determined by Western blot analysis. Lanes 1-2 show the protein profile of the purified Bacillopeptidase F. Lanes 3-4 show the protein profile of the purified Bacillopeptidase F after treatment with 6M guanidine.

Fig. 4. Restriction of the cloned Bacillopeptidase F gene. The restriction map of the cloned Bacillopeptidase F gene is shown in the figure. Lanes 1-2 show the restriction map of the cloned Bacillopeptidase F gene. Lanes 3-4 show the restriction map of the cloned Bacillopeptidase F gene after treatment with 6M guanidine.

Fig. 5. Inhibition of the spontaneous activity of Bacillopeptidase F by Bacillopeptidase F. The spontaneous activity of Bacillopeptidase F was inhibited by Bacillopeptidase F. Lanes 1-2 show the spontaneous activity of Bacillopeptidase F. Lanes 3-4 show the spontaneous activity of Bacillopeptidase F after treatment with 6M guanidine.

Fig. 6. Inhibition of the specific activity of Bacillopeptidase F by Bacillopeptidase F. The specific activity of Bacillopeptidase F was inhibited by Bacillopeptidase F. Lanes 1-2 show the specific activity of Bacillopeptidase F. Lanes 3-4 show the specific activity of Bacillopeptidase F after treatment with 6M guanidine.

Fig. 7. Inhibition of the specific activity of Bacillopeptidase F by Bacillopeptidase F. The specific activity of Bacillopeptidase F was inhibited by Bacillopeptidase F. Lanes 1-2 show the specific activity of Bacillopeptidase F. Lanes 3-4 show the specific activity of Bacillopeptidase F after treatment with 6M guanidine.

Fig. 8. Inhibition of the spontaneous activity of Bacillopeptidase F by Bacillopeptidase F. The spontaneous activity of Bacillopeptidase F was inhibited by Bacillopeptidase F. Lanes 1-2 show the spontaneous activity of Bacillopeptidase F. Lanes 3-4 show the spontaneous activity of Bacillopeptidase F after treatment with 6M guanidine.
Cloning, genetic organization, and characterization of a structural gene encoding bacillopeptidase F from Bacillus subtilis.
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