A glutamic acid-specific protease has been purified to homogeneity from *Bacillus licheniformis* ATCC 14580 utilizing Phe-Leu-OMe-Sepharose affinity chromatography and crystallized. The molecular weight of the protease was estimated to be approximately 25,000 by SDS-polyacrylamide gel electrophoresis. This protease, which we propose to call BLase (glutamic acid-specific protease from *B. licheniformis* ATCC 14580), was characterized enzymatically. Using human parathyroid hormone (13-34) and p-nitroanilides of peptidyl glutamic acid and aspartic acid, we found a marked difference between BLase and V8 protease, EC 3.4.21.9, although both proteases showed higher reactivity for glutamyl bonds than for aspartyl bonds. Disopropyl fluorophosphate and benzoxycarbonyl-Leu-Glu chloromethyl ketone completely inhibited BLase, whereas EDTA reversibly inactivated the enzyme. The findings clearly indicate that BLase can be classified as a serine protease. To elucidate the complete primary structure and precursor of BLase, its gene was cloned from the genomic DNA of *B. licheniformis* ATCC 14580, and the nucleotide sequence was determined. Taking the amino-terminal amino acid sequence of the purified BLase into consideration, the clones encode a mature peptide of 94 residues. The recombinant BLase was expressed in *Escherichia coli* and purified to homogeneity. Its key physical and chemical characteristics were the same as those of the wild-type enzyme. BLase was confirmed to be a protease specific for glutamic acid, and the primary structure deduced from the cDNA sequence was found to be identical with that of a glutamic acid-specific endopeptidase isolated from Alcalase* (7) (Svendsen, I., and Breddam, K. (1992) Eur. J. Biochem. 204, 165-171), being different from V8 protease and the Glu-specific protease of *Streptomyces griseus* which consist of 268 and 188 amino acids, respectively.

Microbial extracellular proteases generally have very wide ranges of side chain specificity. However, some investigations have shown the existence of proteases possessing high specificity in peptide bond hydrolysis. Three trypsin-like enzymes isolated from *Streptomyces* exhibit a specificity similar to mammalian pancreatic trypsin (1-3). The protease, which only catalyzes the cleavage of lysyl peptide bonds, was found in *Achromobacter lyticus* and has come to be called lysyl endopeptidase (4). Two other proteases which prefer acidic amino acid residues have also been purified and characterized. The enzymes from *Staphylococcus aureus* (5, 6), *Streptomyces griseus* (7), and *Bacillus subtilis* (8) cleave on the carboxyl side of either glutamic acid or aspartic acid. In contrast, a metalloendopeptidase which preferentially cleaves the peptide bonds on the amino-terminal side of either aspartic acid or cysteic acid residues was found in a mutant of *Pseudomonas fragi* (9). Proteases with highly restricted substrate specificity, particularly lysyl endopeptidase, EC 3.4.21.50, and a glutamic acid-specific endopeptidase from *S. aureus* strain V8, EC 3.4.21.9 (V8 proteinase), have been found to be very useful in the determination of the primary structures of proteins.

Very specific proteases also can be used in the production of biologically active peptides by the expression of a fusion protein in which the peptide is connected via an amino acid residue not present in the peptide, such as lysine in human atrial natriuretic peptide and glutamic acid in human glucagon, to the COOH terminus of highly expressed proteins in *Escherichia coli*. The fusion protein can then be treated with a lysyl endopeptidase or a glutamic acid-specific endopeptidase (10, 11). The search for such novel proteases possessing new and narrow specificities may be achieved by screening non-pathogenic microorganisms or by selective alteration of the substrate specificity of proteases by site-specific mutagenesis. Clariﬁcation of the structural characteristics of the substrate binding site can contribute to the production of useful recombinant peptides in large quantities.

Screening of some 500 kinds of non-pathogenic microorganisms, such as fungi, actinomycetes, and bacteria, did not reveal any proteases with novel specificities. However, we did find a glutamic acid-specific endopeptidase from *B. licheniformis* ATCC 14580. Glutamic acid-specific proteases are interesting because they may possess structural homologies in their substrate binding sites. This is of value in view of work presently in progress on the three-dimensional structure of V8 protease (12). Interest in the structural basis of the specificity of Glu-specific proteases prompted us to undertake purification of the Glu-specific protease from *B. licheniformis* ATCC 14580 (the enzyme is designated as BLase hereafter).1

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1 The abbreviations used are: BLase, glutamic acid-specific protease from *B. licheniformis* ATCC 14580; V8 protease, glutamic acid-specific protease from *S. aureus* V8 strain (EC 3.4.21.9); hPTH(1-34)
The present report describes studies on the purification of BLase, characterization of enzymatic activity, cloning of the gene encoding the enzyme, and determination of the nucleotide sequence and also demonstrates the expression of the enzyme-coding gene in B. subtilis resulting in production of recombinant BLase identical with the wild-type mature enzyme. These data allow us to design BLase mutants and to prepare large amounts of the enzyme by means of genetic engineering. BLase was confirmed to be a Glu-specific protease having a different protein structure from those of the other proteases with the similar substrate specificity. Finally, the primary structure deduced from the nucleotide sequence of the enzyme is discussed in relation to the active site.

**EXPERIMENTAL PROCEDURES**

**Materials**

Protease Type XVII-B from *S. aureus* strain V8 (V8 protease) and DFP were from Sigma. Human parathyroid hormone (residues 13-34), hPTH(13-34), was from Peptide Institute Inc. (Osaka, Japan). S-Phosphorase and CH-Phosphorase 4B were from Pharmacia LKB Biotechnology Inc., and Amberlite CG-50 was from Organco Co. (Tokyo, Japan). The protein Assay Kit was from Bio-Rad, and the protein molecular weight marker “RAINBOW”™ were from Amersham Corp. The high performance liquid chromatography (HPLC) column “Protein C4” was from Vydac. Benzyloxycarbonyl-Phe-Leu-Glu-p-nitroanilide (Z-Phe-Leu-Glu-pNA) was from Boehringer Mannheim, and Ac-Asp p-nitroanilide and t-butyloxycarbonyl-Ala-Ala-Ala p-nitroanilide (Boc-Ala-Ala-pNA) were from Bachem. Other p-nitroanilide substrates than those were synthesized by us. We also synthesized Phe-Leu-p-Glu-OMe as a ligand in affinity chromatography, Z-X-Leu-Ala-Ala (X: Ghu, Asp, Ala, Leu, Phe, Tyr, and Lys), and chloromethyl ketones of Z-Leu-Glu, Z-Phe-Leu-Glu, and Boc-Ala-Ala-Asp-pNA were from Bachem. Other p-nitroanilide substrates than those were synthesized by us. We also synthesized Phe-Leu-p-Glu-OMe as a ligand in affinity chromatography, Z-X-Leu-Ala-Ala (X: Ghu, Asp, Ala, Leu, Phe, Tyr, and Lys), and chloromethyl ketones of Z-Leu-Glu, Z-Phe-Leu-Glu, and Boc-Ala-Ala-Ala.

**Methods**

**Assay of Enzymatic Activity**—Protease activity was routinely measured from the amount of p-nitroaniline liberated from Z-Phe-Leu-Glu p-nitroanilide (Z-Phe-Leu-Glu-pNA). The reaction was started by adding an appropriate amount of an enzyme solution to a solution composed of 0.2 mM Z-Phe-Leu-Glu-pNA, 50 mM Tris-HCl, 2 mM CaCl₂, and 2% dimethylformamide (DMF), pH 7.5. After 10 min at 37°C, the absorbance was measured at 410 nm. One unit of the enzyme was expressed as the enzymatic activity giving an absorbance of 1.0 under the above conditions. As for the other p-nitroanilides, the reaction was carried out for 1.0 mM p-nitroanilide in 0.1 M Tris-HCl, 2 mM CaCl₂, and 10% DMF at pH 7.5 and 37°C. With regard to hPTH(13-34), the peptide was dissolved in a buffer composed of 0.1 M Tris-HCl and 2 mM CaCl₂, pH 8.0, for a final concentration of 1.0 mg/ml. To this solution, the enzyme was added to equal 1/100 of the final ratio of enzyme to substrate, followed by incubation for 4 h at room temperature. Next, the CG-50 was packed into a column, washed with the acetate buffer, and eluted with 0.5 M sodium acetate, pH 8.5, containing 2 mM CaCl₂. The fractions having enzymatic activity eluted from the CG-50 were combined and dialyzed against water. After the conductivity and pH of the dialysate were adjusted to 1.90 ms/cm and 7.5, respectively, the fluid was loaded onto an S-Sepharose column equilibrated with 5 mM acetic acid buffer, pH 6.0, containing 2 mM CaCl₂. The column was then washed with the acetate and eluted with the buffer under a linear gradient of 0%–25% NaCl. Fractions containing the enzymatic activity were pooled and dialyzed against 2 mM CaCl₂, aqueous solution. The dialysate, adjusted to pH 7.5, was applied to an affinity column of Phe-Leu-p-Glu-OMe-CH-Sepharose 4B equilibrated with 5 mM Tris-HCl, pH 7.5, containing 2 mM CaCl₂. After the column was washed with the Tris buffer, the elution was carried out with a linear gradient from 0 to 0.7 M NaCl in the buffer, and the active fractions were pooled. The purified enzyme was dissolved in 5 mM HCl buffer, pH 7.5, room temperature to a concentration of 2%. Crystals of the enzyme were grown at 25°C from 3.4 M NaCl in 50 mM Tris-HCl buffer at pH 7.5 using the hanging drop technique.

**SDS-PAGE and Isoelectric Focusing**—SDS-PAGE was performed on a 12.5% gel containing 5% stacking gel and 10% gel, 2.5% polyacrylamide gel electrophoresis. The protein was measured at room temperature with a Jasco 5-40 spectrophotometer. The α-helical and β-sheet contents of the protein were calculated according to the method of Provencher et al. (14).

**Enzymatic Digestion**—The purified BLase was incubated in 5% 2-propanol solution containing 2.5 mM DFP for 1 h at 37°C. The inactive DFP-treated enzyme (DIP-BLase) was recovered by gel filtration on Sephadex G-25 equilibrated with 10 mM NH₄CO₃ and lyophilized. DIP-BLase was dissolved with 50 mM Tris-HCl, pH 9.0, containing 1 mM urea, and lysyl endopeptidase was added to yield 1:100 (w/w) as the final ratio of enzyme to substrate, followed by incubation for 5 h at 37°C. The resulting digests were fractionated by HPLC using a TSK-GEL ODS-120T column (4.6 × 250 mm), and the obtained peptides were subjected to sequence analysis. The amino acid sequence of five peptide fragments were determined.

In order to determine the positions of the disulfide bonds, BLase was digested with lysyl endopeptidase. To DIP-BLase dissolved with 0.05 M Tris-HCl, pH 8.0, lysyl endopeptidase was added at an enzyme/substrate ratio of 1:17 (w/w), and the mixture was incubated for 3 h at 37°C. The digest was fractionated by HPLC using a Vydac Protein C4 column (4.6 × 250 mm), and peaks were collected and subjected to Edman degradation. Five peptide fragments were subjected to sequence analysis. The disulfide-containing peptides were detected by reduction and cleavage with tritylthiophosphate and then coupling with SBD-F.
from the purified enzyme in a few days by the hanging drop vapor diffusion method (Fig. 1). The amino acid composition of BLase was analyzed (data not shown), and the amino-terminal sequence of BLase modified with DFP (DIP-BLase) was determined up to the 23rd residue.

**Enzymatic Properties**—The activity toward Ac-Glu-pNA showed a pH optimum around 8.0 either in Tris-HCl buffer or phosphate. The stable pH range of BLase was found to be 4.0–10.0 after preincubation for 24 h at various pH values in the presence of 2 mM CaCl₂ at 25 °C.

The enzyme activity of BLase was examined using seven tetrapeptides, Z-X-Leu-Ala-Ala (X: Glu, Asp, Ala, Leu, Phe, Tyr, and Lys) as substrate. When X was Glu or Asp, the enzyme cleaved the peptide bond of X-Leu, whereas with the other five peptides, no hydrolytic product was found. Kinetic parameters for the enzyme with the Glu- and Asp-peptide substrates were 13.7 and 0.04 μmol/min/mg, respectively. The reactivity of BLase with the latter amounted only to 0.3% of that of the Glu-peptide substrate, and this ratio did not depend on the kind of buffer used in the reaction system. In order to examine the specificity of BLase toward a long peptide, we compared in detail the activity of BLase and V8 protease on hPTH(13–34), a peptide fragment which consists of 22 amino acid residues and contains 2 glutamic acid residues and 1 aspartic acid residue. When the enzymes were incubated with hPTH(13–34) in 100 mM Tris-HCl, pH 8.0, at 37 °C for 40 s, BLase cleaved the peptide bond between Glu¹ and Arg² of the substrate in approximately 50% yield and the Glu¹-Trp¹¹ bond at 10%, whereas V8 protease cleaved only the Glu¹-Trp¹¹ bond in 20% yield. After 2 min, the Glu¹-Arg² bond was cleaved by BLase in 70% yield compared with 5% by V8 protease. On the other hand, the yields for cleavage of the Glu¹-Trp¹¹ bond with BLase and V8 protease were 30 and 50%, respectively. When the digestion had proceeded for 6 min, BLase produced three peptide fragments, Lys¹-Glu⁵, Arg⁶-Glu¹⁰, and Trp¹¹-Phe²², all in yields of approximately 80%, and V8 protease produced four fragments of which Lys¹-Glu⁵, Arg⁶-Glu¹⁰, and Lys¹-Glu¹⁰ were in yields of approximately 50%, and Trp¹¹-Phe²² in a yield of not less than 80%. These results indicated that the cleavage sites of both enzymes are at the same positions, at Glu¹-Arg² and Glu¹-Trp¹¹, but each of the enzymes showed clearly different cleavage patterns. Within 1 h, no cleavage of the Asp-bond was observed. When the reaction time was 3 h, production of two additional peptide fragments, Trp¹¹-Asp¹⁸ and Val¹⁹-Phe²², with both enzymes were observed at less than 10%. After 24 h, BLase and V8 protease cleaved the Asp-bond in approximately 75
and 40% yield, respectively. These findings are summarized in Fig. 2. The kinetic parameters for BLase- and V8 protease-catalyzed hydrolysis of various Z-peptidyl Glu-pNA substrates were determined and compared (Table II). The use of the $k_{cat}/K_m$ ratio for describing relative reactivity facilitates comparison of the substrate specificity for each reaction system. Thus, for the majority of substrates, BLase has a greater reactivity than V8 protease. Notably, the relative reactivity ($k_{cat}/K_m$) of BLase for Z-Ala-Leu-Glu-pNA is approximately six times higher than that of V8 protease. The activity of both enzymes was also examined using acetyl-Y-pNA (Ac-Y-pNA) and Boc-Ala-Ala-Y-pNA (Y: Glu and Asp) as substrates, and the apparent activities are given in Table III. Both enzymes showed very high reactivity toward the Glu substrate in comparison with the Asp substrates. The ratio of the yield of p-nitroaniline produced with BLase from the Asp substrates to that from the Glu substrates, which was defined as the relative reactivity for Asp substrate, was observed as 0.5 and 0.8%, whereas 1.3 and 2.5% were found for V8 protease (Table III).

DFP, a typical inhibitor of serine proteases, inhibited BLase and V8 protease, which were also inactivated by Z-peptidyl chloromethyl ketones containing a glutamic acid residue at position P1, Z-Leu-Glu-CH$_2$Cl, and Z-Phe-Leu-Glu-CH$_2$Cl. In inactivation by EDTA, BLase but not V8 protease was susceptible depending upon its concentration: 90% of the control at $10^{-5}$-10$^{-3}$ M EDTA, 60% at $10^{-2}$ M, and 40% at 10$^{-1}$ M. In the presence of 10$^{-4}$ M EDTA, $K_m$ and $k_{cat}$ for the enzyme-catalyzed hydrolysis of Z-Phe-Leu-Glu-pNA were 6.2 mM and 7.9 s$^{-1}$, respectively. When the enzyme inactivated with 10$^{-4}$ M EDTA was combined with 2 mM CaCl$_2$ or MgCl$_2$ or ZnCl$_2$, the enzyme recovered up to 90% of the activity of native BLase. The sequence of a peptide fragment obtained from the lysyl endopeptidase digest of DIP-BLase was Leu-Gln-Tyr-

![Fig. 2. Cleavage sites observed when human parathyroid hormone (13-34) was digested with BLase and V8 protease. The double arrow and single arrow indicate primary and secondary cleavage sites, respectively, and the dotted arrow indicates a very weak cleavage site.](image)

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BLase</th>
<th>V8 protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_1$</td>
<td>$P_1$</td>
<td>$P_1$</td>
</tr>
<tr>
<td>Z-Glu-pNA</td>
<td>1.8 (±0.1)</td>
<td>0.28 (±0.01)</td>
</tr>
<tr>
<td>Z-Ala-Glu-pNA</td>
<td>4.2 (±0.7)</td>
<td>5.9 (±0.4)</td>
</tr>
<tr>
<td>Z-Leu-Glu-pNA</td>
<td>5.5 (±0.2)</td>
<td>22.6 (±0.8)</td>
</tr>
<tr>
<td>Z-Ala-Glu-pNA</td>
<td>3.5 (±0.4)</td>
<td>40.9 (±1.6)</td>
</tr>
<tr>
<td>Z-Ala-Leu-Glu-pNA</td>
<td>1.5 (±0.1)</td>
<td>131.0 (±3.6)</td>
</tr>
<tr>
<td>Z-Phe-Leu-Glu-pNA</td>
<td>3.9 (±1.1)</td>
<td>40.9 (±9.5)</td>
</tr>
<tr>
<td>Z-Leu-Leu-Glu-pNA</td>
<td>2.8 (±0.6)</td>
<td>50.8 (±7.3)</td>
</tr>
<tr>
<td>Boc-Ala-Ala-Glu-pNA</td>
<td>4.9 (±0.5)</td>
<td>11.9 (±0.8)</td>
</tr>
<tr>
<td>Boc-Ala-Ala-Asp-pNA</td>
<td>11.6 (±3.4)</td>
<td>0.16 (±0.04)</td>
</tr>
</tbody>
</table>

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**Glu-specific Protease from B. licheniformis**

23785

Ala-Met-Asp-Thr-Tyr-Gly-Gly-Gln-(X)-Gly-Ser-Pro-Val-

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**Cloning of the BLase Gene**—To clone the BLase gene, PCR methodology (22) was adopted. Upstream and downstream primers were designed from the two known amino acid sequences at positions 12-19 of the NH$_2$-terminal sequence and one (Gly-Tyr-Pro-Gly-Asp-Lys) of the peptide fragments (unknown position), which were isolated from lysyl endopeptidase digests of DIP-BLase. A DNA fragment of about 370 bp was amplified by PCR with the sense and antisense primers described above. Sequence analysis of the 370-bp PCR product revealed that the deduced amino acid sequence contained other partial amino acid sequences determined by amino acid sequencing of BLase (Fig. 3), demonstrating that the amplified DNA fragment is derived from the BLase gene. The 370-bp fragment thus obtained was then used as a probe for Southern hybridization of the digest of _B. licheniformis_ genomic DNA with appropriate restriction enzymes (BamHI, EcoRI, HindIII, PstI, SfiI, and XbaI). The 11-kb BamHI, 8- kb EcoRI, 6- kb HindIII, 4.3-kb PstI, 3.1-kb Saff, and 20-kb XbaI fragments were detected with the probe (data not shown). The 3.1-kb Saff fragment was subsequently eluted from a gel slice and ligated with Saff-digested M13 mp11, and the resultant recombinant phages were transfected into _E. coli_ JM103. The transformants which did not turn blue on a 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside plate were screened by plaque hybridization with the BLase gene-specific 370-bp probe. Several positive clones were isolated and found to carry a gene coding for BLase.

**Nucleotide Sequence of the BLase Gene**—On the basis of the results described above, we determined the nucleotide sequence of the BLase gene involved in the 3.1-kb Saff fragment. The whole nucleotide sequence was determined by the chain termination method (29) in both orientations. The nucleotide sequence of the BLase gene and its flanking regions together with the amino acid sequence derived therefrom are shown in Fig. 3. Starting from the initiation codon TTG at nucleotide position 323 and terminating in the TAA codon at nucleotide position 1271, a single open reading frame was found: it was composed of 948 nucleotides (316 amino acid residues) in which all of the lysyl endopeptidase-digested peptide fragments are present (Fig. 3).

The translational initiation codon, TTG, is preceded by a
316 amino acids, from which the prepeptide and the 222 amino acids of the mature protein are produced by proteolytic processing. A transcription terminator-like inverted sequence was also found downstream from the termination codon (Fig. 3).

A computer-assisted search revealed that the mature protein sequence of BLase is identical with that of a glutamic acid-specific endopeptidase (designated GSE) recently isolated from Alcalase™ and protein-chemically determined by Svendsen and Breddam (23). No significant similarity of BLase was found to any other proteins in the available DNA databases, including V8 protease and the Glu-specific protease of S. griseus (24) which consist of 268 and 188 amino acids, respectively. There is slight homology sequence between BLase and V8 protease; i.e. no more than 21.6% similarity in amino acid sequences in the optimally matched alignment.

Expression of Recombinant BLase in B. subtilis—B. subtilis harboring a recombinant plasmid containing the cloned BLase gene secreted the enzymatically active Glu-specific protease into the culture medium, whose production level was compatible with that of the natural producing strain. The purified recombinant BLase thus obtained was indistinguishable from wild-type BLase on SDS-PAGE, in amino acid composition and NH2-terminal amino acid sequence as well as enzymatic properties (data not shown).

### Table III

Hydrolysis of glutamyl and aspartyl p-nitroanilide substrates with BLase and V8 protease

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BLase Activity (μmol/min/mg)</th>
<th>BLase Relative reactivity (%)</th>
<th>V8 protease Activity (μmol/min/mg)</th>
<th>V8 protease Relative reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Glu-pNA</td>
<td>118</td>
<td>109</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>Ac-Asp-pNA</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Boc-Ala-Ala-Glu-pNA</td>
<td>4,040</td>
<td>200</td>
<td>2,010</td>
<td>100</td>
</tr>
<tr>
<td>Boc-Ala-Ala-Asp-pNA</td>
<td>34</td>
<td>8</td>
<td>51</td>
<td>2.5</td>
</tr>
</tbody>
</table>

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![Fig. 3. Nucleotide sequence and deduced amino acid sequence of BLase.](image-url)
Secondary Structure of BLase—In the absence of reduction before labeling with a thiol-specific reagent, SBD-F, no peptide could be coupled with the reagent, and thus, absence of free cysteine in the enzyme was confirmed. Analyses of the lysyl endopeptidase digest of DIP-BLase followed by reduction and coupling with SBD-F showed that BLase contains two disulfide bonds, one between Cys35 and Cys48 and the other between Cys185 and Cys186, the same location as in GSE (29).

BLase was analyzed by circular dichroism. In the near UV region, the spectrum features a broad negative band centered near 280 nm (θ_{280} = -1.36 × 10^4 degree-cm^2/dmol) and a positive band at 250 nm (θ_{250} = + 7.7 × 10^4 degree-cm^2/dmol), suggesting the contribution of 17 tyrosine, 5 phenylalanine, and 4 tryptophan residues in the protein molecule. The far UV CD band, which usually indicates secondary structure, reflected unordered structure with a few β-sheets. When the α-helical and β-sheet contents were estimated by the method of Provencher et al. (14), the secondary structure of the BLase protein was found to be composed of approximately 11% of α-helix and 36% of β-sheet. Prediction of the secondary structure for BLase by the Chou-Fasman algorithm (25) indicates that BLase contains 9% of α-helix and 20% of β-sheet, which generally agrees with the data obtained by CD analysis.

DISCUSSION

In the present study, a glutamic acid-specific protease from B. licheniformis ATCC 14580 was purified by sequential chromatography. For convenience, we refer to the purified protease as BLase. We have cloned the gene coding for the enzyme, characterized and constructed an expression plasmid which directs the synthesis of recombinant BLase (26), and found that the primary structure deduced from cDNA sequence of BLase was identical with the amino acid sequence of GSE isolated from Alcalase™ (23). However, no gene cloning and kinetic analyses of the enzyme in that study were attempted. The present work emphasizes the importance of the relationship between the substrate specificity and the structure of the active site to design BLase mutants possessing novel specificities.

Several research groups have reported previously on the substrate specificity of acidic amino acid-specific proteases from S. aureus, S. griseus, B. subtilis, and B. licheniformis (5–8, 23). However, none of these enzymes has been quantitatively characterized for differences in reactivity in the cleavage at glutamic acid and at aspartic acid. In this study, we examined the substrate specificity of BLase using various short peptide substrates containing Glu or Asp and a long peptide, hPTH(13–34), which contains 2 Glu residues and 1 Asp residue. First, the reactivity of BLase toward Z-Asp-Leu-Ala-Ala-Ala amounted to only 0.3% of that toward Z-Glu-Leu-Ala-Ala. When the reactivity of the enzyme was examined using Ac-X-pNA and Boc-Ala-Ala-X-pNA (X, Asp and Glu) as substrates, the yield of p-nitroaniline from both p-nitroanilides of the Asp substrate was less than 1% of those from glutamyl p-nitroanilide substrates (Table III). The low reactivity toward Asp substrates can be attributed to inefficient hydrolysis of the substrate rather than binding affinity between the enzyme and the substrate. This is shown by the kinetic parameters for Boc-Ala-Ala-X-pNA (Table II). The difference in k_{cat} between the Glu substrate and the Asp substrate may indicate that the side chain of Glu contributes not only to the formation of a productive E-S complex, but also to effective catalysis in cleavage of the C-N bond on the carboxyl side of glutamic acid. From the results of BLase-catalyzed hydrolysis of hPTH(13–34), the enzyme also shows higher reactivity for cleavage at the glutamyl bond than at the aspartyl bond (Fig. 2). Although both the glutamyl bonds of hPTH(13–34) were cleaved by BLase at approximately 80% within a reaction time of up to 8 min, a reaction time of 24 h was necessary for cleavage of 75% of the aspartyl bond. These results led to the conclusion that the specificity of BLase for aspartic acid is very low but is high for glutamic acid, which is likely to be important in the physiological function of BLase in the cell.

It is noteworthy that BLase differs from V8 protease in several aspects of substrate specificity. As shown in Fig. 2, the reactivity of the glutamyl bond of hPTH(13–34) with both proteases results in different cleavage patterns. To examine this difference in detail, kinetic parameters of both enzymes for peptide p-nitroanilide substrates were estimated and compared. Although both enzymes show a higher relative reactivity for the Glu substrates than for the Asp substrates shown in Table III, the relative reactivity of Asp substrates with V8 protease was approximately three times higher than that of BLase. These findings thus suggest that the specificity for glutamic acid of BLase is higher than that of V8 protease. While K_m for the BLase-substrate complexes have values within the range 2–5 mM, K_m of V8 protease depends on peptide size, that is, the larger the size, the smaller the K_m (Table II). These observations led us to propose that the amino acid residues at the P_2 and P_3 positions in the substrate have little effect on the binding of substrate to BLase, but have a marked effect on that for V8 protease.

BLase is one of the extracellular proteases synthesized and secreted by B. licheniformis. Its nucleotide sequence analysis suggests that the gene product is initially synthesized as a 316-residue precursor protein, followed by processing to a mature enzyme consisting of 222 amino acid residues. Since the NH_2-terminal sequence consisting of 30 residues seems to be a signal peptide, the length of the pro-sequence is around 60 amino acid residues. The propeptide sequence has been implicated to mediate the folding of the protease. It has been well characterized in the case of subtilisin. Removal of either the entire pro-sequence or just the first 15 amino acids from the propeptide results in production of an inactive form of subtilisin (27). It is not uncommon in microorganisms such as bacilli and micrococci to find TTG or GTG in place of ATG as the initiation codon. For example, in Micrococcus luteus, GTG is used as an initiation codon in 10 out of 18 protein genes examined (28). A highly homologous sequence with a B. subtilis ribosome binding site (29) exists 10 nucleotides upstream from the putative translation initiation codon TTG. An AT-rich sequence located just upstream from the consensus sequence for the –10 and –35 regions of B. subtilis promoters is similar to those of σ70 gene (29) and a sporulation gene, spoVG (30), which may promote efficient utilization of promoters controlled by σ factors from B. subtilis.

Finally, we point out that in BLase, the structure of the active site, consisting of the catalytic site and the substrate binding site, should be analogous to those of the serine protease family, including chymotrypsin and trypsin. Based on alignment of the sequence around the catalytic site residues, Fig. 4 shows the catalytic site topographies of the three serine proteases possessing the same catalytic mechanism in what we call the “proton relay system” (31). The catalytic triad residues His^{67}, Asp^{105}, and Ser^{167} (chymotrypsinogen numbering) are found at the expected positions corresponding to His^{67}, Asp^{105}, and Ser^{167} in the BLase sequence. On the other hand, it would not be possible to predict the substrate binding site for BLase without knowledge of the three-dimensional
structure of the enzyme deduced from crystallographic study of the substrate- or inhibitor-enzyme complex. The crystal structure of trypsin bound to various inhibitors suggests that Asp$^{189}$ (chymotrypsin numbering) resides at the base of the substrate binding pocket and anchors the positively charged side chain of Lys or Arg (32-35). In BLase, the residue at position 189, which is a primary determinant of substrate specificity using the technique of site-directed mutagenesis, might closely agree with the segment corresponding to residues Ser-Trp-Gly-Ser-Ser in chymotrypsin.

References