Identification of autodigestion target sites in *Bacillus subtilis* neutral proteinase

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Autocatalytic degradation of purified *Bacillus subtilis* neutral proteinase was examined under various conditions. At elevated temperatures, under non-inhibitory conditions, mature protein was rapidly degraded, but no accumulation of specific breakdown products occurred. However, by incubating purified neutral proteinase on ice during extended periods of time, specific peptides accumulated. These peptides were analysed by SDS/PAGE and Western blotting, and the *N*-terminal sequences were determined for the four major peptides, which had sizes of 30, 22, 20 and 15 kDa. Sequence data identified five fission sites in the neutral proteinase, three of which were identical with autodigestion target sites in thermolysin, a thermostable neutral proteinase. Comparison of the identified fission sites of the *B. subtilis* neutral proteinase with the known substrate-specificity of the enzyme indicated that they were in agreement, showing a preference for the generation of fissions at the *N*-terminal side of large hydrophobic residues, such as leucine, isoleucine and methionine. These results suggest a high degree of similarity in the three-dimensional structures of *B. subtilis* neutral proteinase and thermolysin.

INTRODUCTION

Some neutral proteinases are metallo-endopeptidases (EC 3.4.24.4), which have their optimum activity at neutral pH. Several species belonging to the genus *Bacillus*, showing considerable differences in optimum growth temperature, are known to secrete neutral proteinases (Keay, 1969). These enzymes also show a broad range in temperature optima and thermostability (Endo, 1962; Tsuru *et al.*, 1965; Priest, 1977; Fuji *et al.*, 1983; Sidler *et al.*, 1986). For this reason we adopted this class of enzymes as a model system for the study of mechanisms that determine the thermostability of proteins. Thermostability of proteins is determined by several factors, e.g. structural stability or resistance to chemical degradation processes such as oxidation, deamidation and hydrolysis (Ahern & Klibanov, 1985). In the case of proteinases, resistance to autocatalytic degradation presumably plays a major role in their stability, in particular at elevated temperatures. Several authors have demonstrated a correlation between the thermostability of proteins and their susceptibility towards proteolysis (McLendon & Radany, 1978; Parsell & Sauer, 1989). Parsell & Sauer (1989) constructed variants of the *N*-terminal domain of lambda repressor exhibiting a wide range of melting temperatures, and observed a higher susceptibility to proteolytic breakdown in the thermolabile mutants. They concluded that thermal stability is a major determinant of the proteolytic susceptibility of this protein *in vivo*.

Fontana *et al.* (1986) studied the correlation between the sites of chain breakage in limited proteolysis and the segmental mobility in thermolysin, the thermostable neutral proteinase from *Bacillus thermoproteolyticus*. Using digestion with subtilisin, autodigestion at 55 °C and autodigestion in the presence of the chelating agent EDTA, which removes bound Ca²⁺ ions present in neutral proteinases, they identified a limited number of peptide bonds highly susceptible to proteolysis. Comparison with the three-dimensional structure of thermolysin, for which a highly refined structure at 0.16 nm (1.6 Å) is available (Matthews *et al.*, 1974; Holmes & Matthews, 1982), showed that these peptide bonds were all located in exposed surface segments of the polypeptide chain, characterized by high mobility.

The neutral proteinase from *Bacillus subtilis* shows 47.2% sequence identity with thermolysin at the amino acid level, but is significantly more thermolabile. By using synthetic oligopeptides it was shown that the substrate-specificities of both proteinases are very similar (Mori-hara & Tsuzuki, 1970). Crystallographic studies of thermolysin-inhibitor complexes suggested a catalytic mechanism in which the Zn²⁺ ion and a limited number of residues play a crucial role (Pangburn & Walsh, 1975; Hangauer *et al.*, 1984; Tronrud *et al.*, 1986). These residues are conserved in the *Bacillus* neutral proteinases. Toma *et al.* (1989) replaced two of these residues in the *B. subtilis* neutral proteinase by other amino acids. Their results strongly supported the assumption that the catalytic mechanisms in both enzymes are highly similar.

To examine the role of autodigestion in the stability of neutral proteinases, we decided to determine the major autodigestion target sites in the neutral proteinase from *B. subtilis*. To this end purified enzyme was subjected to limited autodigestion, and the major breakdown products were identified. The positions of the fissions were determined by *N*-terminal sequencing, and are compared with the sites determined in thermolysin (Fontana *et al.*, 1986).

MATERIALS AND METHODS

Materials

Chemicals for electrophoresis were obtained from Bio-Rad Laboratories, Watford, Herts., U.K. Azocoll, phosphoramidon [*N*-(α -rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl]-L-tryptophan] and chloramphenicol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Alkaline-phosphatase-conjugated antibodies were from Promega Corp., Madison, WI, U.S.A.

Methods

Bacterial strains and plasmids. *B. subtilis* DB104 (Kawamura & Doi, 1984) was used as a host for the chloramphenicol-resistance plasmid pGS1, carrying the neutral-proteinase gene (*npr* gene) from *B. subtilis* 1A40. pGS1 was constructed by cloning a 3 kb *Bc*II fragment, containing the *npr* gene, from plasmid pNP10

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(kindly provided by Gist-brocades N.V., Delft, The Netherlands) in pTZ12 (Oaki *et al.*, 1987; V. G. H. Eijssink, B. van den Burg & B. K. Stulp, unpublished work). *B. subtilis* DB104 (pGS1) produced large quantities of neutral proteinase. Bacteria were grown in TY broth (Biswal *et al.*, 1967), containing 10 µg of chloramphenicol/ml, at 30 °C.

Purification of neutral proteinase. Neutral proteinases were isolated from culture supernatant by using bacitracin-silica affinity chromatography as described previously (van den Burg *et al.*, 1989). Fractions showing proteolytic activity were pooled and desalted by gel filtration on Sephadex G-25 (medium grade) (Pharmacia-LKB, Uppsala, Sweden). Desalted protein solutions were freeze-dried and stored at -20 °C.

Determination of conditions for limited autodigestion. To obtain limited autodigestion, neutral proteinase was dissolved in 50 mM-Tris/HCl buffer, pH 7.0, containing 5 mM-CaCl₂ (0.5 mg/ml) and incubated at the selected temperature. Samples containing the specific neutral-proteinase inhibitor phosphoramidon, at a final concentration of 2 mM, were incubated under the same conditions. [Inhibition studies indicated that at a concentration of 0.25 mM-phosphoramidon some residual proteolytic activity (approx. 10%) was still present. Therefore we used the high concentration of 2 mM of this inhibitor, to be completely sure that autolytic activity was prevented.] After incubation, phosphoramidon was also added to the experimental samples to prevent further breakdown.

Protein samples to be used in gel-filtration experiments were incubated in the presence and in the absence of 20% (v/v) propan-2-ol, replacing phosphoramidon, since the latter inhibitor absorbs at 280 nm, which impedes the detection of small breakdown products.

Slab gel electrophoresis and Western blotting. PAGE was performed in the presence of SDS essentially according to the procedure of Laemmli (1970) with the Minitan gel-electrophoresis system (Bio-Rad Laboratories). Stacking gels were 3% polyacrylamide and separating gels were 12.5%. After electrophoresis proteins were blotted on Immobilon [poly(vinylidene difluoride)] membranes (Millipore, Bedford, MA, U.S.A.) in 25 mM-Tris/glycine buffer, pH 8.5, containing 20% (v/v) methanol at 100 V for 60 min. Blots were either stained with Coomassie Brilliant Blue R-250, as described by Matsudaira (1987), or treated with polyclonal antibodies against the *B. subtilis* neutral proteinase raised in rabbits. Bound antibodies were detected with alkaline-phosphatase-labelled anti-(rabbit IgG) antibodies, essentially as described by the supplier. Molecular-mass markers (Pharmacia-LKB) ranged from 14 400 to 94 000 Da.

Coomassie Brilliant Blue-stained gels and blots were scanned with an LKB Ultrascan XL densitometer (Pharmacia-LKB).

Gel-filtration chromatography. Analytical gel filtration was performed with an f.p.l.c. system equipped with a Superose 12 column (1 cm × 30 cm) (Pharmacia-LKB) in 20 mM-sodium acetate buffer, pH 5.0, containing 5 mM-CaCl₂, 50 mM-NaCl and 20% propan-2-ol. Protein samples (25 µl) were loaded on to the column. The flow rate was 38 ml/cm² per h. The absorbance of the fractions was monitored directly at 280 nm with a single-path UV-1 monitor (Pharmacia-LKB).

N-Terminal sequence determination. Peptides were separated on SDS/PAGE and electroblotted on Immobilon membranes. After staining with Coomassie Brilliant Blue R-250, peptide bands of interest were cut out and used for N-terminal sequence

determination in an automated gas-phase protein sequenator (Applied Biosystems, Foster City, CA, U.S.A.).

Activity measurements. Neutral proteinase activity was determined with Azocoll as a substrate by the method of Chavira *et al.* (1984). In those cases in which further autodigestion of the neutral proteinase was stopped by the addition of propan-2-ol to a final concentration of 20% (v/v), the preparations were diluted to a non-inhibitory concentration of less than 1% propan-2-ol in the activity assays.

RESULTS AND DISCUSSION

Determination of conditions for limited autodigestion

One of the major problems in the determination of autodigestion target sites in *B. subtilis* neutral proteinase, in particular at elevated temperatures, is the rapid degradation of the peptides formed. After incubation at the optimum temperature of the enzyme (52 °C), almost no breakdown products were detectable, although the amount of mature proteinase decreased rapidly upon incubation. Fig. 1 illustrates this observation. After incubation of the purified neutral proteinase for 60 min at 52 °C, approx. 30% of the mature enzyme had been degraded, whereas after 120 min 75% had disappeared. Under these conditions only minor amounts of breakdown products were detectable in 12.5% polyacrylamide gels. Incubation of the neutral proteinase at room temperature for 16 h resulted in an even higher decrease of the amount of mature proteinase (Fig. 1, lane 4), but still no significant accumulation of large breakdown products was observed. Apparently the first fissions gave rise to unfolded peptides that were highly susceptible to further degradation. Similar observations have been made by Pace & Barrett (1984), who showed that the rate of hydrolysis of the unfolded form of ribonuclease T1 was increased 1700-fold compared with that of the folded form of the enzyme.

Fig. 2 documents the successful accumulation of specific peptides by incubating the purified neutral proteinase on ice for 16 h. Four major peptides were produced, the largest of

Fig. 1. Autodigestion of *B. subtilis* neutral proteinase

SDS/12.5%-PAGE was performed on purified *B. subtilis* neutral proteinase (7.5 µg) that had been incubated on ice for 120 min in the presence of 2 mM-phosphoramidon (lane 1), incubated at 52 °C for 60 min (lane 2), incubated at 52 °C for 120 min (lane 3), or incubated at 20 °C for 16 h (lane 4). Gels were stained with Coomassie Brilliant Blue R-250. The positions of the molecular-mass markers (M) are also indicated.

Fig. 2. Production of specific fragments of *B. subtilis* neutral proteinase

B. subtilis neutral proteinase was incubated for 16 h on ice and subsequently subjected to electrophoresis. (a) Western blot of SDS/12.5%-PAGE gel of incubated neutral proteinase. Lane 1, 30 µg of purified enzyme was incubated on ice for 16 h; after electrophoresis proteins were transferred on to Immobilon membranes and stained with Coomassie Brilliant Blue R-250. The positions of the native neutral proteinase (N) and the major peptides formed upon incubation are indicated. Molecular-mass markers (M) are also shown. (b) Immunoblot of incubated neutral proteinase. Lane 2, 5 µg of purified enzyme was incubated on ice for 16 h; lane 3, as lane 2, but in the presence of 2 mM-phosphoramidon. Proteins from the SDS/12.5%-PAGE gel were transferred on to Immobilon membranes and detected with polyclonal antibodies raised against purified *B. subtilis* neutral proteinase as described in the Materials and methods section.

which had an estimated molecular mass of 30 kDa and was immunologically reactive. The second major peptide, of molecular mass approx. 22 kDa, was immunologically barely reactive. The 20 kDa peptide was immunologically reactive, whereas the 15 kDa peptide was not. The observed difference in immunological reactivity might be caused by a differing degree of folding of the peptides after electro-blotting. Conceivably the immunologically reactive peptides adopted a native-like folding after blotting, whereas the non-reactive peptides remained unfolded. Similar differences in immunological reactivity were also observed by Fontana *et al.* (1983) for fragments of thermolysin, considerably differing in their degree of folding, as determined by c.d. measurements. In addition, variation of the number of epitopes present on the peptides may underlie the variation in immunological reactivity. In the range between 22 and 30 kDa immunologically reactive peptides were present, but apparently in amounts too small to be detected in the Coomassie Brilliant Blue-stained membranes.

To ascertain that formation of the peptides was due to autodigestion, *B. subtilis* neutral proteinase was incubated in the presence of the specific inhibitor phosphoramidon. The results are presented in Fig. 2, lane 3, and show that almost no breakdown products were formed. Under the conditions used neutral proteinase activity was completely absent (results not shown). Most probably the peptides present were formed before the addition of inhibitor to the dissolved enzyme. They represented only a minor fraction of the total amount of protein, because they were absent from a Coomassie Brilliant Blue-stained blot (Fig. 1, lane 1, and Fig. 2, lane 1).

B. subtilis neutral proteinase incubated in the presence and in the absence of 20% propan-2-ol 16 h on ice was subjected to gel-filtration chromatography on Superose 12 (results not shown). The amount of protein eluted at the position of native neutral proteinase in both cases was determined. The data showed that

in the absence of propan-2-ol the amount of protein eluted at that position had decreased by 30%. Scanning of the Coomassie Brilliant Blue-stained blot (Fig. 2, lane 1) showed that approx. 50% of the total protein had a higher mobility than the native proteinase on SDS/PAGE. Therefore autodigestion at 0 °C led to the nicking of at least 50% of the proteinase present, but approx. 40% of this fraction retained the molecular mass of the native enzyme under non-denaturing conditions. Apparently autodigestion at 0 °C led to fission of peptide bonds, and specific fragments were released from the enzyme molecule, after which the remaining nicked molecules dissociated upon being subjected to SDS/PAGE.

Determination of the *N*-termini of the autodigestion products

To determine the fission sites in the enzyme, the four peptide bands depicted in Fig. 2, lane 1, were cut out, and subjected to sequencing.

The results of the *N*-terminal sequencing of these peptides are listed in Table 1. Although some heterogeneity was present in all four bands, comparison with the known amino acid sequence of the *B. subtilis* neutral proteinase (Yang *et al.*, 1984; Fig. 3) allowed the accurate localization of the fission sites.

The 30 kDa band contained two peptides, present in equimolar amounts. One of these had the *N*-terminal sequence of the mature neutral proteinase. The other peptide appeared to start at Ala-114 of the primary sequence of the neutral proteinase. On the basis of the estimated molecular mass (30 kDa) of this peptide it might well contain the *C*-terminus of the mature neutral proteinase.

The second protein band also contained two peptides, again one having the *N*-terminus of the mature protein, whereas the other started at Ile-157.

For the 20 kDa protein band three *N*-terminal sequences could be deduced. In addition to peptides starting at amino acid-1 and Ile-157, the majority of peptides present in this band started at Leu-156. The 15 kDa protein band also contained three peptides. The major peptide started at Leu-198. A peptide starting at Met-121 could also be identified. As in the bands described above, a peptide starting at amino acid-1 was present in low concentration.

Fig. 3 indicates the positions of the fissions in the amino acid sequence of the neutral proteinase.

Table 1. *N*-Terminal amino acid sequences of the major autodigestion products of *B. subtilis* neutral proteinase formed upon incubation for 16 h on ice

Four major peptide bands, indicated in Fig. 2, were cut out and subjected to *N*-terminal sequencing. The relative amounts of the peptides were estimated after scanning of the gel presented in Fig. 2, lane 1.

Peptide	<i>N</i> -Terminus	Relative amount (%)
Mature enzyme	Ala-Ala-Ala-Thr-Gly-Ser-Gly	52
30.0 kDa	Ala-Ala-Ala-Thr-Gly-Ser-Gly; Ala-Ala-Trp-Thr-Gly-Asp-Gln	11.5
22.0 kDa	Ala-Ala-Ala-Thr-Gly-Ser-Gly; Ile-Tyr-Glu-Asn-Gln-Pro-Gly	3.5
20.0 kDa	Leu-Ile-Tyr-Glu-Asn-Gln-Pro; Ala-Ala-Ala-Thr-Gly-Ser-Gly; Ile-Tyr-Glu-Asn-Gln-Pro-Gly	9
15.0 kDa	Leu-Ser-Asn-Pro-Thr-Lys-Tyr; Met-Ile-Tyr-Gly-Asp-Gly-Asp; Ala-Ala-Ala-Thr-Gly-Ser-Gly	21

Fig. 3. Positions of identified fission sites in the primary amino acid sequence of mature *B. subtilis* neutral proteinase

The *B. subtilis* neutral proteinase primary sequence is according to Yang *et al.* (1984). Arrows indicate the positions of the fission sites, as determined by *N*-terminal sequencing of the peptides shown in Fig. 2.

Fission sites in *B. subtilis* neutral proteinase in relation to substrate-specificity and autodigestion target sites in thermolysin

Substrate-specificities of thermolysin and *B. subtilis* neutral proteinase have been studied in detail with synthetic peptides (Mori-hara & Tsuzuki, 1970) and have indicated that these enzymes preferentially cleave the chain between a large hydrophobic C-terminal residue (P_1' residue, according to the nomenclature of Schechter & Berger, 1967) and a smaller N-terminal residue (P_1 residue). The autodigestion target sites identified in *B. subtilis* neutral proteinase are in agreement with this cleavage specificity. In most cases large hydrophobic residues, such as leucine, methionine and isoleucine, were found at the P_1' position, and smaller residues were present at the P_1 position.

Fontana *et al.* (1986) subjected thermolysin to limited proteolysis and identified several sites highly susceptible to autodigestion. These sites are indicated in the three-dimensional model of thermolysin (Fig. 4). This Figure, in which the positions of the fission sites determined in *B. subtilis* neutral proteinase, after alignment of the two primary structures, are also indicated, shows that the location of three of the five sites identified in the *B. subtilis* neutral proteinase are identical with those in thermolysin, notwithstanding the fact that the two enzymes have only 47.2 % sequence identity. The two peptide bonds (Asn-155-Leu-156 and Leu-156-Ile-157 in *B. subtilis* neutral proteinase and Gly-154-Leu-155 and Leu-155-Ile-156 in thermolysin) near the α -helix connecting the two domains appeared to be favoured autodigestion target sites in both enzymes. In thermolysin the peptide bonds in this region are highly accessible and therefore sensitive to proteolysis (Fontana *et al.*, 1986).

A third fission site common to the two neutral proteinases is located between Ser-197 and Leu-198 (Ser-204 and Met-205 in thermolysin). These residues are located in an exposed loop

Fig. 4. Three-dimensional model of thermolysin

The positions of the autodigestion target sites as determined by Fontana *et al.* (1986) are indicated by white arrows. The positions of autodigestion target sites determined in *B. subtilis* neutral proteinase (black arrows) were modelled in the thermolysin three-dimensional model after alignment of the primary sequences of both enzymes. Amino acid numbering is that of thermolysin. The open circle indicates the Zn²⁺ ion, and the numbered circles indicate Ca²⁺ ions present in thermolysin.

characterized by high flexibility in thermolysin, probably allowing its fitting into the active site of the enzyme and subsequent chain cleavage.

Two additional fission sites specific for *B. subtilis* neutral proteinase are both located in exposed areas of the molecule, as was shown by comparison of the thermolysin three-dimensional structure and a three-dimensional model of the *B. subtilis* neutral proteinase, constructed on the basis of the program What If (Vriend, 1990; V. G. H. Eijssink, G. Vriend, B. van den Burg, G. Vemema & B. K. Stulp, unpublished work). The fission between Asn-113 and Ala-114 is especially interesting in view of the fact that the five residues at the *N*-terminus of the peptide produced were highly similar to the *N*-terminus of the mature enzyme (four out of five residues were identical). Consequently the maturation site of the pre-pro-precursor of the enzyme, from which the pro sequence is autocatalytically removed during enzyme maturation (Toma *et al.*, 1989), is very similar to this autocatalytic fission site. Possibly this site becomes available for fission after partial unfolding in this region, thus rendering it accessible to the proteinase. Although at present no crystallographic data on *B. subtilis* neutral proteinase are available, the results presented here suggest that the tertiary structure of the *B. subtilis* neutral proteinase is grossly similar to that of thermolysin, since both enzymes share identical autodigestion target sites. Thus it would seem that using the three-dimensional structure of thermolysin in modelling studies *B. subtilis* neutral proteinase is helpful with respect to the identification of sequences involved in stability of the latter enzyme.

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