PRIMARY STRUCTURE OF THE N-TERMINAL REGION (1—95) OF THERMITASE, A THERMOSTABLE PROTEINASE FROM *Thermoactinomyces vulgaris*

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The 95-residue N-terminal amino acid sequence of thermitase, a thermostable serine proteinase from *T. vulgaris*, is the following: Tyr-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-Gln-Tyr-Gly--Pro-Gln-Lys-Ile-Gln-Ala-Pro-Gln-Ala-Trp-Asp-Ile-Ala-Glu-Gly-Ser-Gly-Ala-Lys-Ile-Ala-Ile--Val-Asp-Thr-Gly-Val-Gln-Ser-Asn-His-Pro-Asp-Leu-Ala-Gly-Lys-Val-Val-Gly-Gly-Trp-Asp--Phe-Val-Asp-Asn-Asp-Ser-Thr-Pro-Gln-Asn-Gly-Asn-Gly-His-Gly-Thr-His-Cys-Ala-Gly-Ile--Ala-Ala-Ala-Val-Thr-Asn-Asn-Ser-Thr-Gly-Ile-Ala-Gly-Thr-Ala-Pro-Lys. The sequential data were obtained by analyses of peptides isolated from the soluble part of the tryptic digest of the N-terminal cyanogen bromide fragment of the enzyme and from the chymotryptic digest of a thermitase fragment obtained by 2-nitro-5-thiocyanobenzoate cleavage of the enzyme at its single cysteine residue.

Thermitase, a thermostable serine proteinase from *Thermoactinomyces vulgaris*[1], consists of a single polypeptide chain which shows a considerable homology with the subtilisins as regards its amino acid composition[2] and some partial sequences[3,4]. Unlike the subtilisins thermitase contains one methionine residue and a single cysteine residue; the modification of the latter with mercury compounds leads to complete inactivation of the enzyme[5].

Cyanogen bromide cleavage of the enzyme[2] gives rise to a large fragment (CB1) of molecular weight 25 000 and a small fragment (CB2) of 6 000. The amino acid sequence of all 53 residues of the latter fragment has been determined[3]. In another study a peptide containing the single cysteine residue of thermitase was isolated from the tryptic digest of the enzyme by thiol disulfide interchange using a TP-Sepharose column[4].

In a recent paper[6] the chemical cleavage at the single cysteine residue of thermitase was described. This cleavage results in the formation of two peptide fragments of molecular weights 10 000 (CY1) and 20 000 (CY2). The present study reports on the determination of the amino acid sequence of N-terminal peptide CY1 and

on the final location of the cysteine residue in the polypeptide chain of the enzyme. The amino acid sequence reported here has been derived from analyses of peptides contained in the soluble part of the tryptic digest of the large cyanogen bromide fragment (CB1). Chymotryptic digestion of the N-terminal fragment (CY1) of thermitase was used to obtain the overlaps of the tryptic peptides.

EXPERIMENTAL

Material

Thermitase was isolated from a crude preparation, which had been obtained by sodium sulfate precipitation of the *Thermoactinomyces vulgaris* culture medium and purified to homogeneity by single-step isoelectric focusing[1]. The preparation of the S-carboxymethyl derivative of thermitase, its cleavage by cyanogen bromide, and the isolation of the N-terminal cyanogen bromide fragment (CB1) were carried out as described earlier[2]. Fragment CY1 of thermitase was prepared by S-cyanylation using 2-nitro-5-thiocyanobenzoate[7] as described elsewhere[6]. From 160 mg of the enzyme 77 mg of fragment CY1 was obtained.

Bovine TPCK-trypsin (trypsin treated with chloro-(N-*p*-tosyl-L-phenylalanyl) methane[8]) and bovine chymotrypsin were purchased from Worthington (Freehold, NJ, USA), thermolysin from Calbiochem (Los Angeles, CA, USA). 4-dimethylaminoazobenzene-4′-isothiocyanate (DABITC) was from Fluka (Buchs, Switherland), and phenylisothiocyanate (PITC) from Pierce (Rotterdam, Holland). Sephadex G-75, G-50 superfine, and G-25 fine were purchased from Pharmacia (Uppsala, Sweden), cellulose MN 300 from Macherey-Nagel (Düren, FRG). All the remaining chemicals were products of Serva (Heidelberg, FRG).

Methods

The conditions of the separation of peptide mixtures by paper electrophoresis have been described before[9]. Individual peptides (0·1% solutions) were subjected to enzymatic cleavage in 0·1 mol l^{-1} N-methylmorpholine acetate buffer, pH 8·1, usually 3 h at 37°C. The final enzyme/substrate weight ratio was 1 : 50. The digests were fractionated by techniques of thin-layer chromatography and electrophoresis[10] on cellulose plates. The peptides were eluted by 0·1 mol . l^{-1} NH_4OH. The amino acid analyses were carried out in a Durrum D-500 amino acid analyzer with samples hydrolyzed in 6 mol l^{-1} HCl, 20 h at 110° *in vacuo*. The presence of tryptophan on the peptide maps was determined by the Ehrlich reagent and verified by sequence analysis. The latter was carried out manually with 8—20 nmol of the peptides using the DABITC/PITC double coupling method[11]. The same technique was used with the large fragments CB1 and CY1, yet the quantity of the reagents was doubled.

Tryptic digest of cyanogen bromide fragment CB1 *of thermitase*: Fragment CB1 (80 mg) was dissolved in 10 ml of 0·1 mol l^{-1} NH_4OH; 0·8 mg of trypsin dissolved in 1 ml of 0·005 mol l^{-1} HCl was added and the pH of the solution was adjusted to 8·6 by 1 mol l^{-1} NH_4HCO_3. The cleavage was allowed to proceed at 37°C. The same quantity of trypsin was added again after 90 min and the digestion was discontinued after a total period of 3 h. The solution was centrifuged 30 min at 3 000 rev./min to separate the insoluble part of the digest. The latter was frozen and kept at —20°C. The soluble part of the digest (about 80%) was applied to a column of Sephadex G-50, equilibrated with 0·1 mol l^{-1} NH_4HCO_3 (pH 8·5, adjusted by ammonia). Four fractions A—D, were collected (Fig. 1). The total quantity of material treated was 3 × 80 mg.

Each fraction was subjected to ion-exchange chromatography on a column of Servacel DEAE-52. The course of the fractionation is shown in Fig. 2.

Chymotryptic digest of fragment CY1 *of thermitase*: The fragment (9 mg) was dissolved in 9 ml of 0·02 mol l^{-1} NH_4HCO_3, and digested with chymotrypsin (enzyme/substrate weight ratio 1 : 50) 4 h at 37°C. The digest was lyophilized and then separated on a column of Sephadex G-25. The elution profile is shown in Fig. 3. Three fractions, designated I, II and III, were collected and subjected to additional fractionation by paper electrophoresis on Whatman 3MM paper in a buffer at pH 5·6 (fraction I and II) and at pH 1·9 (fraction III). The details of these separation procedures have been described elsewhere[9].

RESULTS AND DISCUSSION

The N-terminal sequence analysis of cyanogen bromide fragment CB1 of S-carboxymethylated thermitase (Fig. 4) permitted unambiguous interpretation of the sequence of the first 9 residues; serine, not estimated unambiguously, was the predominant residue in the 10th step. This result is in perfect agreement with the sequenator data reported by Stepanov and coworkers[12], and shows that fragment CB1 represents the N-terminal part of thermitase, as expected from earlier experiments[2]. The fragment is soluble in neutral solutions with difficulties only, it is, however, readily dissolved in trifluoroacetic acid or in alkaline media (ammonia). It was therefore dissolved in a relatively weak solution of ammonium hydroxide. Trypsin was added and the pH of the solution was adjusted to 8·6 by ammonium bicarbonate. The fine suspension thus formed dissolved gradually during the cleavage. The remaining insoluble part of the digest was centrifuged off. The fractionation of the soluble part on a column of Sephadex G-50 was perfectly reproducible and very

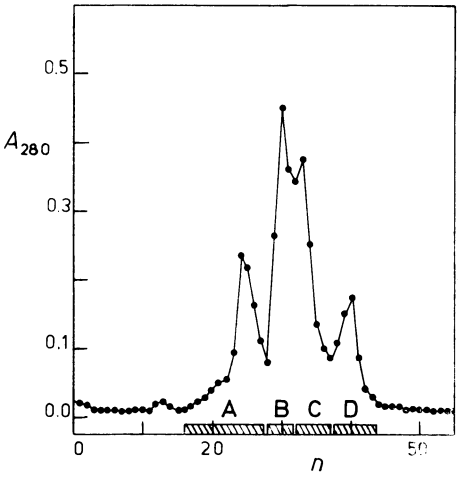


FIG. 1

Gel filtration of tryptic digest of large cyanogen bromide fragment CB1 (80 mg) of thermitase on column of Sephadex G-50 superfine. The column (3·8 × 83 cm) was equilibrated with 0·1 mol l^{-1} NH_4HCO_3. Fractions 21 ml/30 min, *n* fraction number. The fractions were pooled and lyophiziled as showed by hatching

effective. The effluent was pooled (Fig. 1) to appropriate fractions (A, B, C, D). The total quantity of material processed in repeated runs was 240 mg of fragment CB1. Each of the fractions was subjected to additional separation on a Servacel DEAE-52 column. An elution gradient developed with 0·02 and 1·00 mol l^{-1} NH_4HCO_3 was used yet all the components were eluted at concentrations of NH_4HCO_3 lower than 0·5 mol l^{-1}. The majority of peptides contained in the peak fractions, which

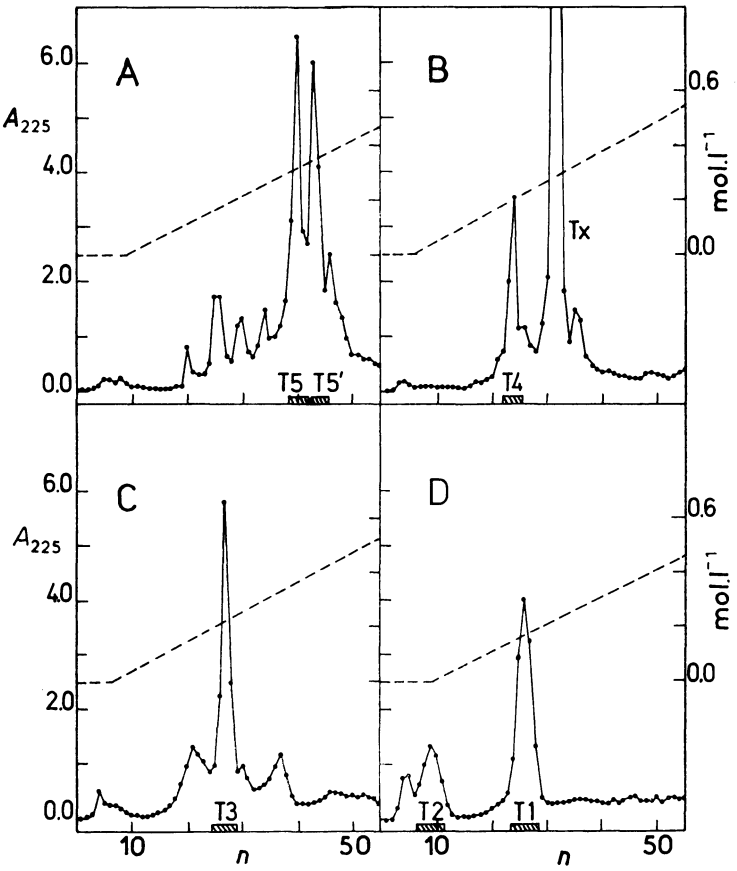


FIG. 2

Ion exchange elution profiles of tryptic peptides contained in fractions A—D (Fig. 1). The lyophilized material of the fractions was always dissolved in 13 ml of 0·01 mol l^{-1} NH_4HCO_3 and placed on a column (2 × 18 cm) of Servacel DEAE-52 equilibrated with 0·02 mol l^{-1} NH_4HCO_3. A linear gradient (0·02 to 1 mol l^{-1}, total volume 1 210 ml) was applied. Fractions 12·7 7 ml/10 min; *n* fraction number. T1—T5 stands for tryptic peptides, numbered in accordance with their order in the parent enzyme. T5′ represents the deamidated form of peptide T5 and T_x an unresolved mixture of peptides

had been identified by absorbance measurement at 225 nm, were pure enough for the determination of amino acid composition (Table I) and sequence studies. The peptide maps of fractions A—D were prepared to advantage by thin layer techniques on microcellulose layers.

In parallel experiments the chymotryptic digest of the small N-terminal fragment of S-cyanylated thermitase (CY1) was studied. The fragment, characterized by amino acid analysis and SDS polyacrylamide gel electrophoresis was perfectly homogeneous: it gave one electrophoretic zone only and the sequence of its first two amino acids was Tyr-Thr-. Both fragment CY1 and the products of its chymotryptic digestion were readily soluble in 0·1 mol l^{-1} NH_4HCO_3. The fractionation of the chymotryptic digest of fragment CY1 on Sephadex G-25 (Fig. 3) afforded a simple resolution pattern (fractions I, II, and III). From these fractions the chymotryptic peptides (Table II) were isolated by electrophoretic techniques on paper.

The amino acid sequence of the N-terminal part of the polypeptide chain of thermitase, shown in Fig. 4, was derived from sequence data obtained by the analysis of the tryptic and chymotryptic digest.

Five main peptides were obtained both from the tryptic and from the chymotryptic digest. The N-terminal part of thermitase is represented by peptide T1; the latter was unambiguously sequenced up to the eighth residue, the subsequent two serines were determined beyond doubt only after the analysis of the chymotryptic digest of peptide T1 (peptide T1C2, Phe-Ser-Ser-Arg). The overlap of tryptic peptides T1 and T2 afforded chymotryptic peptides C2 and C2a. The 16-residue tryptic peptide T3 was sequenced up to tryptophan, the first one in the chain of thermitase. Additional data on the amino acid sequence of peptide T3 were obtained by se-

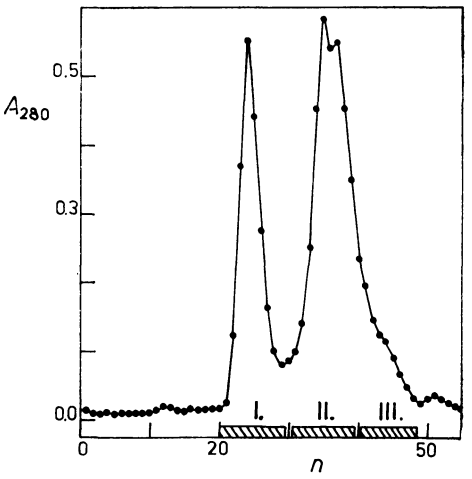
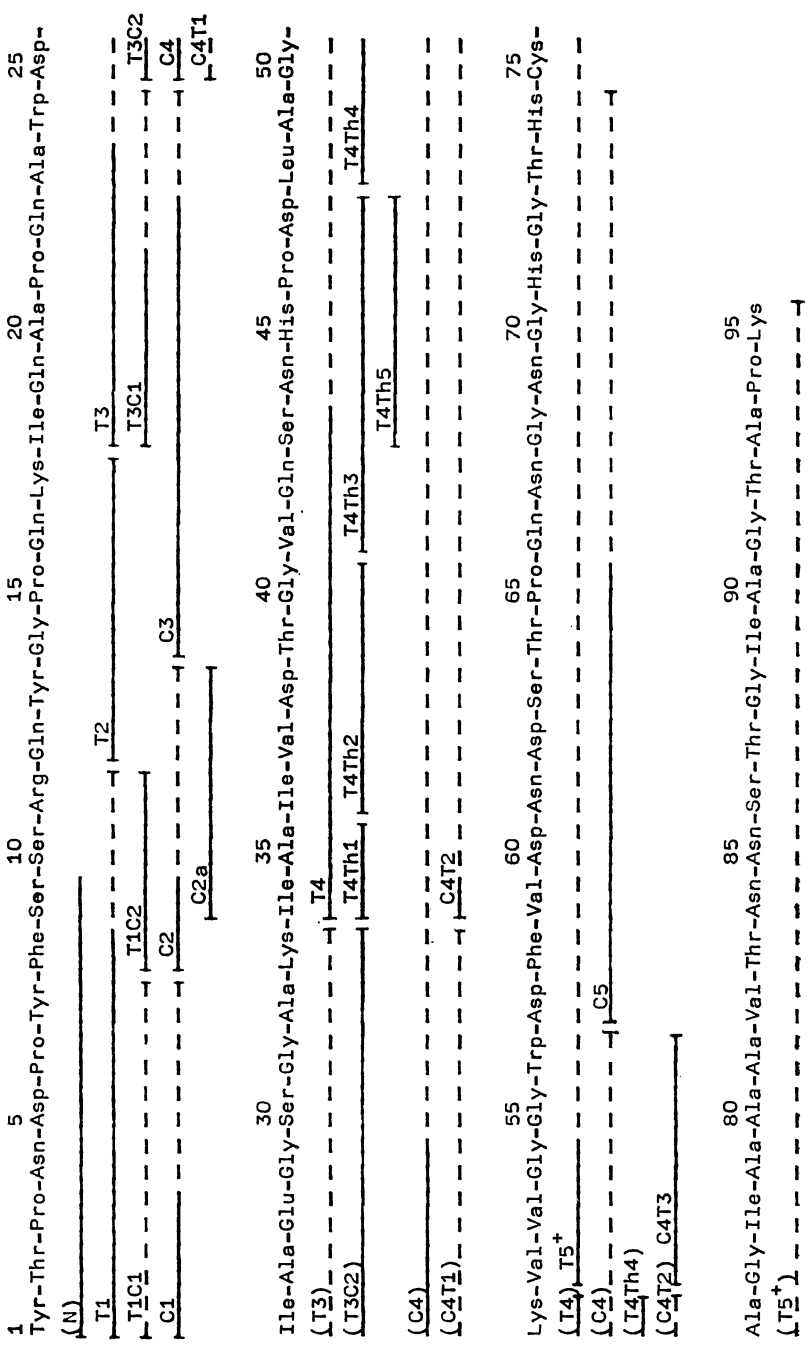


FIG. 3

Gel filtration of chymotryptic digest of small fragment CY1 (9 mg) of S-cyanylated thermitase on column of Sephadex G-25 fine. The column (1·5 × 60 cm) was equilibrated with 0·05 mol l^{-1} NH_4HCO_3. Fractions 2·2 ml/10 min; *n* fraction number. The fractions were pooled and lyophilized as shown by hatching


1 5 10 15 20 25
Tyr-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-Gln-Tyr-Gly-Pro-Gln-Lys-Ile-Gln-Ala-Pro-Gln-Ala-Trp-Asp-
(N)
T1
T1C1
C1
T1C2
C2
C2a
T2
C3
T3
T3C1
T3C2
C4
C4T1
30 35 40 45 50
Ile-Ala-Glu-Gly-Ser-Gly-Ala-Lys-Ile-Ala-Ile-Val-Asp-Thr-Gly-Val-Gln-Ser-Asn-His-Pro-Asp-Leu-Ala-Gly-
(T3)
(T3C2)
T4
T4Th1
T4Th2
T4Th3
T4Th5
T4Th4
(C4)
(C4T1)
C4T2
55 60 65 70 75
Lys-Val-Val-Gly-Gly-Trp-Asp-Phe-Val-Asp-Asn-Asp-Ser-Thr-Pro-Gln-Asn-Gly-Asn-Gly-His-Gly-Thr-His-Cys-
(T4) T5+
(C4)
C5
(T4Th4)
(C4T2) C4T3
80 85 90 95
Ala-Gly-Ile-Ala-Ala-Ala-Val-Thr-Asn-Asn-Ser-Thr-Gly-Ile-Ala-Gly-Thr-Ala-Pro-Lys
(T5+)

quence analysis of its chymotryptic fragment T3C2. The overlap of peptides T2 and T3 afforded chymotryptic fragment C3. Peptide T4 containing 18 residues was sequenced in 10 steps by the DABITC/PITC double coupling technique. Additional

TABLE I

Amino acid composition of peptides isolated from tryptic (T) digest of cyanogen bromide fragment CB1 of S-carboxymethylated thermitase and of peptides obtained by additional cleavage of the tryptic fragments by chymotrypsin (C) or thermolysin (Th)

Designation of peptide	Residues	Amino acid analysis
T1	1—11	Asp 1·9, Thr 1·0, Ser 1·8, Pro 2·0, Tyr 2·0, Phe 1·0, Arg 1·0
T1C1	1—7	Asp 2·0, Thr 1·1, Pro 1·9, Tyr 1·7
T1C2	8—11	Ser 2·0, Phe 1·0, Arg 1·0
T2	12—17	Glu 2·0, Pro 1·0, Gly 1·1, Tyr 1·0, Lys 1·0
T3	18—33	Asp 1·4, Ser 1·2, Glu 2·7, Pro 0·9, Gly 2·0, Ala 3·8, Ile 1·7, Lys 1·0, Trp[a]
T3C1	18—24	Glu 2·0, Pro 0·9, Ala 2·0, Ile 0·7, Trp[a]
T3C2	25—33	Asp 1·0, Ser 1·0, Glu 1·1, Gly 2·1, Ala 2·0, Ile 0·9, Lys 1·0
T4	34—51	Asp 3·1, Thr 1·1, Ser 1·2, Glu 1·1, Pro 1·0, Gly 2·1, Ala 2·3, Val 1·7, Ile 1·6, Leu 1·0, His 0·9, Lys 1·0
T4Th1	34—35	Ala 1·0, Ile 09
T4Th2	36—40	Asp 1·1, Thr 1·0, Gly 1·1, Val 0·8, Ile 0·8
T4Th3	41—47	Asp 2·0, Ser 1·0, Glu 1·1, Pro 0·9, Val 0·8, His 0·9
T4Th4	48—51	Gly 1·2, Ala 1·1, Leu 0·8, Lys 1·0
T4Th5	43—47	Asp 1·9, Ser 0·7, Pro 1·2, His 1·0
$T5^+$	52—95	Cys 1·0, Asp 8·0, Thr 4·8, Ser 2·3, Glu 1·4, Pro 2·1, Gly 8·0, Ala 6·3, Val 3·6, Ile 2·1, Phe 1·1, Trp[a], His 1·9, Lys 1·2

[a] Qualitative determination.

FIG. 4

Sequence analysis of the N-terminal part of the polypeptide chain of thermitase. N, sequence analysis of intact cyanogen bromide fragment CB1 of thermitase; T, C, Th, peptides obtained by cleavage with trypsin, chymotrypsin and thermolysin, respectively. The peptides are designated by horizontal bars; sequences determined completely are marked by full lines, undetermined sequences by dashed lines. The amino acid sequence of fragment $T5^+$ has been reported elsewhere[4]

information provided the sequence analysis of peptides from the thermolytic digest of fragment T4 (T4Th1-T4Th5); the majority of these peptides were completely sequenced without difficulties with the exception of peptide T4Th5, Ser-Asn-His-Pro--Asp(43–47). As regards the latter, a weak histidine was observed in addition to the strong spot of asparagine No 44. A weak spot of histidine and a spot of proline were found in position No 45 whereas the assignment of proline to position No 46 was unambiguous. These anomalies were not observed when peptide T4Th3 was sequenced. The discussed part of fragment T4 is overlapped by chymotryptic fragment C4 containing 32 amino acid residues including lysines No 33 and 51. Peptide C4 overlaps a part of fragment T3, the whole fragment T4 (including the -His-Pro- bond), and the N-terminal part of fragment $T5^+$. A small part of peptide C4 only was sequenced, the rest was digested with trypsin to peptides C4T1-C4T3. The sequence of the tryptophan-containing peptide C4T3 is identical with the N-terminal sequence of peptide $T5^+$. The 15-residue peptide C5 was sequenced to the 9th step. The sequence is identi-

TABLE II

Amino acid composition of fragment CY1 of S-cyanylated thermitase and of peptides isolated from its chymotryptic digest (C), including peptides obtained after additional tryptic digestion of the chymotryptic peptides. The values are not corrected

Designation of peptide	Residues	Amino acid analysis
CY1	1—74	Asp 11·7, Thr 3·8, Ser 4·8, Glu 6·6, Pro 4·5, Gly 9·8, Ala 6·0, Val 4,5, Ile 3·3, Leu 1·2, Tyr 2·8, Phe 1·7, His 2·4, Lys 2·9, Arg 1·2, Trp 2·0
C1	1—7	Asp 2·0, Thr 1·0, Pro 2·0, Tyr 2·1
C2	8—13	Ser 2·0, Glu 1·0, Tyr 1·0, Phe 1·0, Arg 1·1
C2a	9—13	Ser 1·7, Glu 1·1, Tyr 0·8, Arg 1·0
C3	14—24	Glu 3·0, Pro 1·9, Gly 1·1, Ala 2·3, Ile 1·0, Trp[a], Lys 1·2
C4	25—56	Asp 4·3, Thr 1·2, Ser 1·9, Glu 1·9, Pro 0·7, Gly 5·6, Ala 3·5, Val 2·9, Ile 2·1, Leu 0·8, Trp[a], His 1·2, Lys 1·7
C4T1	25—33	Asp 1·3, Ser 0·9, Glu 1·1, Gly 2·4, Ala 1·8, Ile 0·9, Lys 0·8
C4T2	34—51	Asp 2·7, Thr 1·0, Ser 1·1, Glu 1·1, Pro 1·0, Gly 2·1, Ala 2·0, Val 1·4, Ile 1·4, Leu 0·8, His 0·9, Lys 1·0
C4T3	52—56	Gly 2·0, Val 1·7, Trp[a]
C5	57—74	Asp 5·9, Thr 2·0, Ser 1·1, Glu 1·2, Pro 0·9, Gly 3·3, Val 1·0, Phe 0·9, His 2·0

[a] Qualitative determination.

cal with the N-terminal region of the tryptic peptide containing the cysteine residue essential for the activity of thermitase and determined in earlier work[4]. This permits

TABLE III

Homology of the N-terminal sequence of thermitase (residues 1—95) with the sequences of the corresponding regions of the subtilisin molecules (residues 1—87) considering either identical residues only or both identical residues and most conservative amino acid replacements[15]

Sequences compared	Number of total positions	Identical residues %	Identical residues and most conservative replacements %
Thermitase/subtilisin BPN′	97	39	48
Thermitase/subtilisin Amylosaccharitícus	97	40	50
Thermitase/subtilisin Carlsberg	96	38	46
Subtilisin BPN′/subtilisin Amylosaccharitícus	87	90	92
Subtilisin BPN′/subtilisin Carlsberg	87	64	70
Subtilisin Carlsberg/subtilisin Amylosaccharitícus	87	67	72

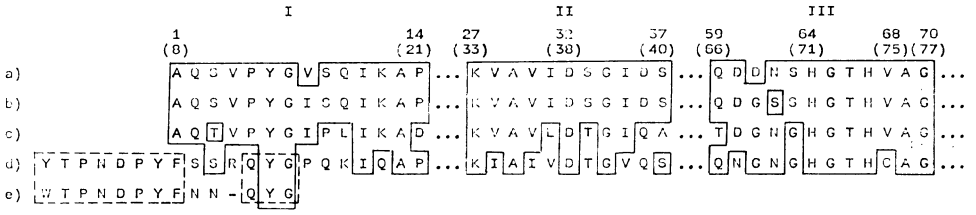


FIG. 5

Sequence homology of thermitase, *B. thuringiensis* alkaline proteinase and the subtilisins. The N-terminal parts of the enzyme molecule (I) and regions around the active site residues Asp 32 (II) and His 64 (III) only are shown. The sequences are numbered according to the subtilisins, thermitase numbering is given in brackets. The single cysteine 75 of thermitase corresponds to Val 68 of the subtilisins. Amino acid residues identical in all enzymes are marked by boxes drawn in solid lines. Broken lines are used for residues identical in thermitase and *B. thuringiensis* proteinase. *a*) subtilisin BPN′[13], *Bacillus amyloliquefaciens*; *b*) subtilisin amylosaccharitícus[14], *Bacillus amylosaccharitícus*; *c*) subtilisin Carlsberg[13] *Bacillus licheniformis*; *d*) thermitase, (this paper and N-terminal 14 residues also (ref.[12]), *Thermoactinomyces vulgaris*; *e*) Thuringiensis alkaline proteinase[12], *Bacillus thuringiensis*.

us to define the complete 95-residue N-terminal sequence of thermitase shown in Fig4; the cysteine residue occupies position No 75.

The amino acid composition of fragment CY1 (Table II) used as starting material is in good agreement with the sequence data, with the exception of the prolines (5 residues found by amino acid analysis and 6 residues found in peptides).

The amino acid sequence of the 95 N-terminal residues of thermitase represents roughly a half of the large cyanogen bromide fragment of thermitase. A comparison of this partial sequence with the corresponding region of the subtilisins shown in Fig. 5, reveals a high degree of homology, especially around the active site residues Asp 32 and His 64 (according to the subtilisin numbering). A comparison of the 74--residue N-terminal part of thermitase with the corresponding 68-residue region of the subtilisins shows that 40% of amino acid residues are identical. The homology is almost 50% if "the most conservative amino acid replacements" (ref.[15]) are regarded as identical residues (Table III). The degree of homology near the N-terminus is low. Thermitase moreover is longer by 7 residues at the N-terminus. This explains the lack of homology in the 14-residue N-terminal region of thermitase and the subtilisins, originally reported by Stepanov[12] and coworkers. Interest deserves, however, the striking sequence homology between thermitase and another serine proteinase with a free SH-group, *i.e.* with the proteinase from *B. thuringiensis*, observed by these authors.

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