# THERMITASE FROM *Thermoactinomyces vulgaris*; AMINO ACID SEQUENCE OF THE LARGE N-TERMINAL CYANOGEN BROMIDE PEPTIDE

Bedřich MELOUN[a], Miroslav BAUDYŠ[a], Manfred PAVLÍK[a], Vladimír KOSTKA[a], Gert HAUSDORF[b] and Wolfgang Ernst HÖHNE[b]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague, Czechoslovakia, and*
[b] *Institute of Physiological and Biological Chemistry, Humboldt-University, Berlin, German Democratic Republic*

Received July 7th, 1984

The large cyanogen bromide fragment (CB1) represents the N-terminal part of the molecule of thermitase and contains 226 amino acid residues. Its molecular weight calculated from sequential data is 22 932 (the C-terminal residue is regarded as a methionine residue in the calculations). The amino acid sequence of fragment CB1 was determined by analysis of peptides obtained by tryptic hydrolysis of the fragment; these data were complemented by sequence analysis of the chymotryptic digest of fragment Mf (residues 75 through 226) and of chymotryptic fragment ET3 (residues 103 through 226) isolated from the limited tryptic digest of fragment CB1. The peptides were purified by high performance liquid chromatography and by thin layer techniques. The sequence analysis of the large peptides was effected in the sequenator, small peptides were sequenced manually by the DABITC/PITC double coupling technique. The results obtained in this study together with those of previous work[5] permitted the complete amino acid sequence of fragment CB1 to be determined.

Thermitase (EC 3.4.21.14) is a thermostable serine proteinase[1] (mol. wt. 31 000) isolated from the culture medium of *Thermoactinomyces vulgaris*. It consists of one polypeptide chain containing one cysteine and one methionine residue[2]; the properties of these residues were utilized to advantage for the preparation of fragments suitable for sequence work. The cleavage of thermitase at the cysteine residue[3] by 2-nitro-5-thiocyanobenzoate gives rise to the N-terminal fragment (CY1) and to an additional fragment (CY2) containing the methionine residue of thermitase. Likewise, cyanogen bromide cleavage of thermitase at the methionine residue[2] yields one large fragment (CB1). The latter represents approximately 80% of the molecule and contains its single cysteine residue. The C-terminal fragment of thermitase (CB2) consists of 53 amino acid residues; its sequence has already been determined[4]. All peptides resulting from chymotryptic digestion of fragment CY1 have been sequenced in the preceding study[5] and the predominant part of the soluble portion of the tryptic digest of fragment CB1 has also been analyzed. The results of these experiments permitted the 75-residue N-terminal sequence of thermitase to be determined. These results and the data derived from the analysis of the peptide containing the only cysteine residue of thermitase[6] enabled us to elucidate completely the N-terminal part of the molecule of thermitase up to lysine 95.

This paper reports on the analysis of the main part of fragment CB1, representing the polypeptide chain of thermitase between the single cysteine residue and the single methionine residue. This middle fragment (Mf) of thermitase composed of more than 150 residues was obtained by additional cleavage of fragment CY2 by cyanogen bromide. The digest obtained contained relatively large, hydrophobic and sparingly soluble peptides; their resolution and analysis permitted the complete amino acid sequence of fragment CB1 to be derived.

## EXPERIMENTAL

### Material

Thermitase, its carboxymethyl derivative and cyanogen bromide fragment CB1 were prepared by procedures described before[1,2]. The tryptic digest of fragment CB1 and the resolution of its insoluble part have been reported in the preceding paper[5] where the fractionation of the soluble part on a Servacel DEAE-50 column has also been described (Fig. 2). The middle fragment (Mf) of thermitase was prepared as follows: thermitase (200 mg) was treated first with 2-nitro--5-thiocyanobenzoate as described elsewhere[3] and the resulting fragment CY2 was subsequently cleaved with cyanogen bromide[2]. The two fragments formed were separated by gel filtration on a column of Sephadex G-75 (1·2 × 120 cm) in 10% formic acid. The larger of the two fragments (Mf) was obtained in a quantity of 25 mg.

The origin of the remaining materials was the following: bovine alpha-chymotrypsin, bovine TPCK-trypsin (trypsin treated with (N-p-tosyl-L-phenylalanine chlormethyl ketone[7]) and hog pepsin were purchased from Worthington (Freehold, NJ, USA). The chemicals for automatic sequential degradation were from Beckman (Berkeley, CA, USA). Polybrene and the Sequenal-grade reagents for manual sequencing (including phenylisothiocyanate) were from Pierce (Rotterdam, Holland), 4-dimethylaminoazobenzene-4′-isothiocyanate (DABITC) was purchased from Fluka (Buchs, Switzerland). Sephadex G-75, G-50 superfine and G-25 fine were from Pharmacia (Uppsala, Sweden). The TLC micropolyamide layer sheets F 1700 were purchased from Schleicher and Schuell (Dassel, FRG). The remaining chemicals were obtained from the sources described in the preceding report[5].

### Methods

The enzymatic digestions of the peptides with trypsin were carried out with 0·1% solutions of the polypeptides in 0·1 mol $l^{-1}$ $NH_4HCO_3$, pH 8·5; the cleavage was allowed to proceed 4 h at 37°C at an enzyme to substrate weight ratio of 1 : 50. Peptic cleavage was carried out in 5% formic acid at an enzyme to substrate weight ratio of 1 : 100, 2 h at 25°C. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed in 15% polyacrylamide gel according to Laemli[8]. Purification of peptides: the technique of fingerprints on Whatman No 3MM paper (electrophoresis at pH 5·6 or 1·9 in the first direction, chromatography in the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12) in the second direction), used for the separation of the chymotryptic digest of fragment Mf, has been described[9], together with the purification of peptides in TLC systems. The latter were used for the isolation of all small peptides from secondary enzymatic digests. Large peptides were purified by HPLC in Beckman-Altex Model 420 Chromatograph equipped with Shimadzu Model SP2-2A Spectrophotometric Detec-

tor. A Lichrosorb RP-2 column (7 μm, 4·6 mm × 25 cm) and two systems[10,11], slightly modified as follows were used. System I — solvent A: 0·1% trifluoroacetic acid in bidistilled water, solvent B: methanol containing 0·1% of trifluoroacetic acid; system II-solvent A: 0·05 mol $l^{-1}$ ammonium acetate, pH 6, solvent B: methanol. Linear gradients were used and 90% concentration of solvent B was achieved in 60 min. The flow rate was 2 ml/min. The peptides were detected by absorbance measurement at 230 nm and the fractions were collected manually. Amino acid analysis: Samples of peptides were hydrolyzed in 6 mol $l^{-1}$ HCl, 20 h at 110°C *in vacuo* and analyzed in Durrum D-500 amino acid analyzer. The hydrolysates of some peptides were incubated in 0·2 mol $l^{-1}$ pyridine acetate buffer at pH 6·5, 1 h at 105°C to convert homoserine lactone to homoserine[12]. Tryptophan was determined according to ref.[13].

*Sequence analysis*: The peptides were sequenced manually using the DABITC/PITC double coupling technique[14]. DABTH-Leucine and DABTH-isoleucine were distinguished in another system[15]. The automated version of the Edman degradation was carried out in Beckman 890C Protein Sequentor using 0·1 mol $l^{-1}$ Quadrol and program No 122 974 modified for the degradation of small peptides[16]. Purified Polybrene[17] (2 mg) and diglycine (300 nmol) were placed in the spinning cup and three complete degradation cycles were carried out before the sequencing of the sample. The polypeptide (40 nmol) to be analyzed was placed in the cup and sequenced according to the program described above. The PTC-derivatives of amino acids were converted into the PTH-derivatives by heating in 0·2 ml of 20% trifluoroacetic acid, 20 min at 80°C, and identified by HPLC on a column of Ultrasphere ODS, 5 μm (4·6 mm × 25 cm), using a gradient of acetonitrile in 0·03 mol $l^{-1}$ acetate buffer at pH 5.

*Insoluble part of tryptic digest of fragment* CB1. The material (48 mg) was dissolved in 3·5 ml of 60% formic acid and separated on a column of Sephadex G-50 fine (3·2 × 60 cm) in 10% formic acid. The fractions (16·3 ml) were collected at intervals of 10 min. The course of the fractionation was monitored by absorbance measurement of the effluent at 280 nm.

*Limited tryptic digest of fragment* CB1: The modification of the lysine residues of fragment CB1 before the tryptic cleavage by treatment with exo-*cis*-3,6-endoxo-$\Delta^4$-tetrahydrophthalic anhydride was carried out by the slightly modified method of Ryley and Perham[18]. Fragment CB1 (100 mg) was dissolved in 1 ml of trifluoroacetic acid and the solution diluted with 5 ml of 0·05 mol $l^{-1}$ HCl; a fine suspension was formed. The following operations were carried out at +2°C. The suspension was treated with 5 ml of 0·2 mol $l^{-1}$ sodium tetraborate, pH 9·45 and the peptides were dissolved by adjusting the pH of the suspension to pH 11. Immediately after the peptides had dissolved the pH of the solution was adjusted to pH 9·45 and the reagent (80 mg) was added within 60 min in portions. The pH of the solution was adjusted 30 min after the addition of the reagent had started to pH 9 and was maintained at this value. The modified fragment was isolated 2 h after the beginning of the reaction by gel filtration on a Sephadex G-25 fine column (1·6 × × 50 cm) in 0·02 mol $l^{-1}$ $(NH_4)_2CO_3$, pH 8·7, at room temperature. The separation was monitored by absorbance measurement of the effluent at 280 nm. Trypsin (1 mg), dissolved in water (2 ml) was added to the solution (41 ml) of modified fragment CB1. The enzymatic digestion was allowed to proceed 60 min at 37°C. Another 1 mg portion of trypsin dissolved in 2 ml of water was added after this period and the digestion was continued for additional 60 min. The tryptic digestion and deacylation were effected by the addition of 5 ml of concentrated acetic acid. The resulting sediment dissolved after the suspension had been heated 4 min at 50°C. The reaction mixture was set aside for 16 h at 23°C. The newly formed insoluble material was separated by centrifugation for 30 min at 3 000 rev/min. The supernatant was decanted off and was not treated further. The sediment was suspended in 3 ml of water and the solution was recentrifuged under identical conditions. This procedure was repeated and yielded the final insoluble fraction (34·9 mg) containing fragment ET3.

*Chymotryptic digest of fragment* Mf: The material (1·8 mg) was dissolved in 200 μl of 0·2 mol . $l^{-1}$ N-methylmorpholine acetate, pH 8·1 and digested with chymotrypsin 90 min at 37°C and at an enzyme to substrate weight ratio of 1 : 60. The same quantity of chymotrypsin was added once again after this period and the digestion was discontinued 60 min later by acidification of the solution by acetic acid and lyophilization. The digest was treated by the fingerprint technique.

*Chymotryptic digest of fragment* ET3: The material (34·7 mg) was dissolved in 2 ml of 0·02 mol . $l^{-1}$ $NH_4OH$, to which 0·35 ml of 1 mol $l^{-1}$ $NH_4HCO_3$ adjusted by ammonia to pH 9·5 had been added, and digested with chymotrypsin (enzyme to substrate ratio 1 : 50, w/v) 2 h at 37°C. The digest was resolved on a column of Sephadex G-50 superfine.

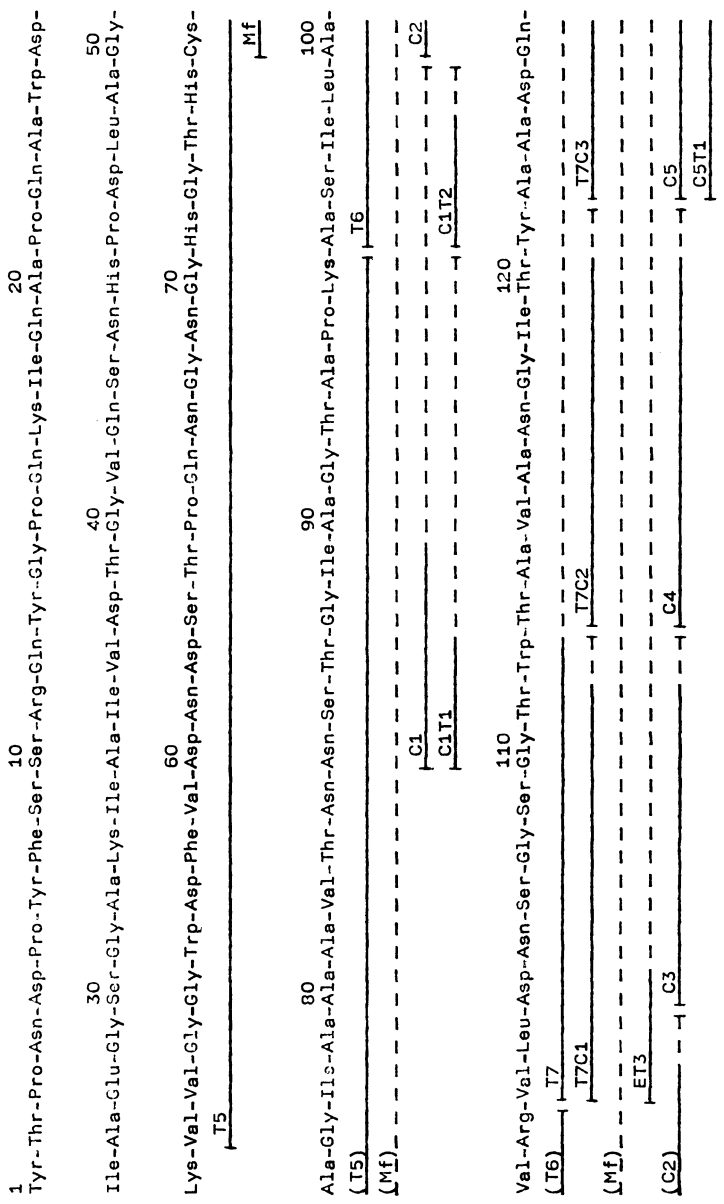
## RESULTS AND DISCUSSION

The position of Cys-75 is of key importance for the strategy of sequence analysis of cyanogen bromide fragment CB1 of thermitase since it permits selective cleavage of the fragment by 2-nitro-5-thiocyanobenzoate thus yielding a peptide accounting for the first third of fragment CB1. The complete chymotryptic digest of the latter has been analyzed in preceding work[5] and the majority of the soluble peptides derived from the tryptic digest of the fragment have also been treated. The results of these experiments enabled us to completely elucidate the N-terminal part of fragment CB1 including tryptic peptide T5 terminated with lysine-95.

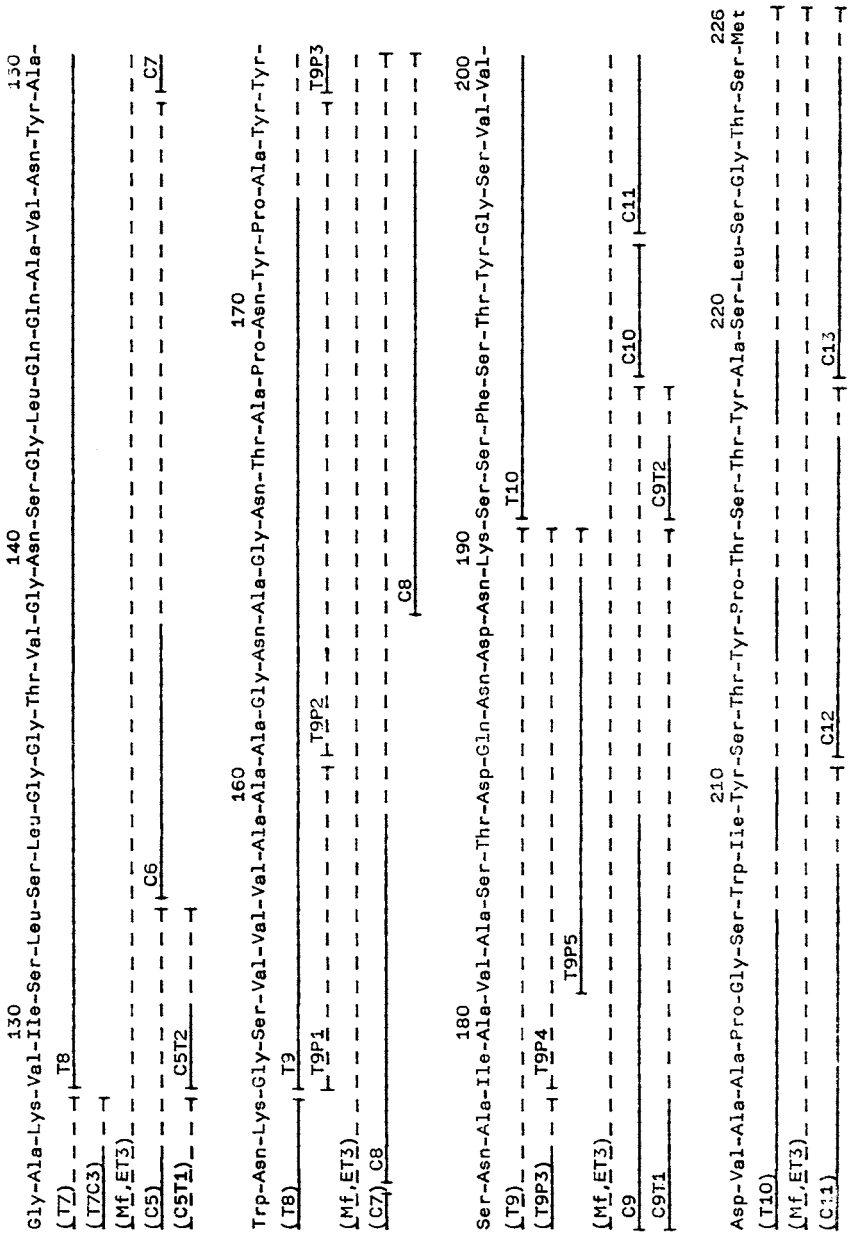
The complete amino acid sequence of cyanogen bromide fragment CB1, which is shown in Fig. 1, was established in this study and the sequences of peptides isolated from the individual digests were determined. Peptides T6–T10 derived from tryptic digestion of fragment CB1 were purified and sequenced; these peptides, with the exception of peptide T8, have been isolated in the material described in the preceding report[5]. Tryptic peptide T6, containing the second of the two arginine residues present in fragment CB1, was isolated from fractions C2–6 (ref.[5], Fig. 2) by TLC techniques. Peptide T7 was isolated from fractions B30–33 (ref.[5]) together with peptide T10 by additional fractionation using HPLC on a reversed phase column. The conditions and the course of the isolation are shown in Fig. 2. A number of tryptic peptides of thermitase are relatively hydrophobic and very little soluble in alkaline solutions. These peptides, however, can be dissolved in strongly acidic media. The insoluble part of the digest was therefore subjected to gel filtration in 10% formic acid and the fraction retained most in the Sephadex G-50 column was resolved on the reversed phase column (Fig. 3); as a result peptide T8 was isolated. The peptide material was so insoluble that it had to be dissolved first in concentrated trifluoroacetic acid, the solution diluted to 10% concentration of trifluoroacetic acid and then applied to the LiChrosorb RP-2 column. The part of the tryptic digest which was insoluble in weakly alkaline solutions mainly contained peptide T8, also a part of peptide T9 and a small quantity of peptide T5. Peptide T9 was isolated to advantage from fractions A23–30 (ref.[5]); it was obtained without difficulties by HPLC

of these fractions (Fig. 4). Peptide T10, the last of the tryptic peptides derived from fragment CB1, was isolated simultaneously with peptide T7 (Fig. 2).

Large, predominantly hydrophobic peptides were isolated from the tryptic digest of fragment CB1; the amino acid composition of these peptides and of the peptides



prepared by their secondary cleavage by other enzymes, is shown in Table I. Peptides T8, T9 and T10 were sequenced in the sequenator, all the remaining peptides were sequenced manually by the DABITC/PITC method.



The data necessary for the arrangement of the tryptic peptides afforded the analyses of the chymotryptic digests of fragments Mf and ET3. The amino acid compositions of these fragments and of peptides isolated from their chymotryptic digests are given in Table II. Fragment Mf (residues 75–226) was prepared by cleavage of thermitase with 2-nitro-5-thiocyanobenzoate and by additional cleavage of the resulting

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 with chymotrypsin (C) or pepsin (P)

| Designation of peptide | Residues | Amino acid analysis |
|---|---|---|
| T6 | 96–102 | Ser 1·0, Ala 1·7, Val 0·9, Ile 0·9, Leu 1·0, Arg 1·0 |
| T7 | 103–128 | Asp 3·9, Thr 3·0, Ser 1·8, Glu 1·2, Gly 4·1, Ala 5·0, Val 2·1, Ile 1·1, Leu 1·0, Tyr 1·2, Lys 1·0, Trp[a] |
| T7C1 | 103–112 | Asp 2·3, Thr 1·3, Ser 2·2, Gly 2·2, Val 1·1, Leu 1·0, Trp[a] |
| T7C2 | 113–121 | Asp 1·2, Thr 1·7, Gly 1·2, Ala 2·0, Val 1·0, Ile 0·9, Tyr 0·7 |
| T7C3 | 122–128 | Asp 1·0, Glu 1·1, Gly 1·2, Ala 2·7, Lys 1·0 |
| T8 | 129–153 | Asp 3·2, Thr 1·2, Ser 2·7, Glu 2·0, Gly 3·9, Ala 2·3, Val 2·7, Ile 0·6, Leu 2·6, Tyr 0·9, Lys 0·8, Trp[a] |
| T9 | 154–190 | Asp 8·0, Thr 1·8, Ser 2·8, Glu 1·2, Pro 2·1, Gly 3·2, Ala 9·0, Val 3·3, Ile 1·0, Tyr 2·9, Lys 1·1 |
| T9P1 | 154–160 | Ser 1·0, Gly 1·0, Ala 1·8, Val 2·7 |
| T9P2 | 161–174 | Asp 2·9, Thr 1·0, Pro 2·0, Gly 2·0, Ala 4·2, Tyr 1·8 |
| T9P3 | 175–178 | Asp 1·0, Ser 0·7, Ala 1·3, Tyr 0·7 |
| T9P4 | 179–190 | Asp 3·8, Thr 1·1, Ser 1·2, Glu 1·1, Ala 2·2, Val 1·0, Ile 0·9, Lys 1·0 |
| T9P5 | 181–190 | Asp 3·5, Thr 1·0, Ser 1·2, Glu 1·1, Ala 1·1, Val 0·7, Lys 0·9 |
| T10 | 191–226 | Asp 1·2, Thr 4·9, Ser 10·0, Hse 0·6, Pro 2·1, Gly 3·4, Ala 3·3, Val 2·3, Ile 1·1, Leu 1·1, Tyr 3·8, Phe 1·0, Trp[a] |

[a] Qualitative determination.

FIG. 1

Sequence analysis of large N-terminal cyanogen bromide fragment CB1 of thermitase. Mf, middle fragment of thermitase (residues 75–226), obtained by cleavage of CB1 before the cysteine residue; ET3, fragment (residues 103–226) isolated from limited tryptic digest of CB1. T, C, P, peptides resulting from cleavage with trypsin, chymotrypsin or pepsin. The peptides are designated by horizontal bars; sequenced regions are marked by full line, unsequenced regions by a dashed line. The sequence of amino acid residues 1–96 including peptide T5 (ref.[6]) was determined in a previous study[5]

TABLE II

Amino acid composition of fragment Mf (residues 75—226), fragment ET3 (residues 103—226) of thermitase and of peptides isolated from their chymotryptic digests (C), including the peptides obtained after additional tryptic cleavage (T). The values are not corrected

| Designation of peptides | Residues | Amino acid analysis |
|---|---|---|
| Mf | 79—226 | Asp 18·0, Thr 12·7, Ser 19·4, Hse 0·9, Glu 4·4, Pro 4·8, Gly 17·1, Ala 27·0, Val 13·4, Ile 6·9, Leu 6·2, Tyr 8·9, Phe 1·1, Lys 4·1, Arg 0·9, Trp 2·6, Cys N.D. |
| ET3 | 103—226 | Asp 15·6, Thr 9·6, Ser 15·3, Hse 0·7, Glu 4·6, Pro 3·5, Gly 13·8, Ala 19·1, Val 9·1, Ile 3·5, Leu 4·7, Tyr 8·6, Phe 0·9, Lys 3·0, Trp[a] |
| C1 | 85— 99 | Asp 1·2, Thr 1·7, Ser 2·0, Pro 0·9, Gly 2·2, Ala 2·7, Ile 1·7, Leu 0·9, Lys 1·0 |
| C1T1 | 85— 95 | Asp 1·1, Thr 1·7, Ser 1·1, Pro 1·1, Gly 2·1, Ala 2·0, Ile 0·9, Lys 1·0 |
| C1T2 | 96— 99 | Ser 0·9, Ala 1·0, Ile 0·9, Leu 0·9 |
| C2 | 100—104 | Ala 1·9, Val 2·0, Leu 1·0, Arg 1·0 |
| C3 | 105—112 | Asp 2·1, Thr 1·0, Ser 2·0, Gly 2·2, Trp[a] |
| C4 | 113—121 | Asp 1·0, Thr 2·2, Gly 1·2, Ala 1·8, Val 0·9, Ile 0·8, Tyr 0·9 |
| C5 | 122—132 | Asp 1·1, Ser 1·2, Glu 1·0, Gly 1·0, Ala 2·7, Val 0·9, Ile 0·7, Leu 0·7, Lys 0·9 |
| C5T1 | 122—128 | Asp 1·0, Glu 1·0, Gly 1·0, Ala 2·9, Lys 1·0 |
| C5T2 | 129—132 | Ser 1·0, Val 1·0, Ile 0·9, Leu 0·9 |
| C6 | 133—149 | Asp 2·0, Thr 0·9, Ser 1·9, Glu 1·8, Gly 3·9, Ala 1·2, Val 1·7, Leu 1·7, Tyr 0·9 |
| C7 | 150—151 | Ala 1·0, Trp 1·1 |
| C8 | 152—175 | Asp 3·8, Thr 1·1, Ser 1·3, Pro 1·9, Gly 3·2, Ala 6·0, Val 2·7, Tyr 2·7, Lys 1·0 |
| C8′ | 164—175 | Asp 2·0, Thr 1·1, Pro 1·9, Gly 1·1, Ala 2·9, Tyr 2·7 |
| C9 | 176—193 | Asp 5·0, Thr 1·0, Ser 3·7, Glu 1·0, Ala 3·1, Val 1·0, Ile 0·9, Phe 0·9, Lys 1·1 |
| C9T1 | 176—190 | Asp 5·0, Thr 1·1, Ser 1·9, Glu 0·9, Ala 2·9, Val 0·9, Ile 0·9, Lys 1·0 |
| C9T2 | 191—193 | Ser 1·8, Phe 1·0 |
| C10 | 194—196 | Thr 0·9, Ser 0·9, Tyr 1·0 |
| C11 | 197—210 | Asp 1·1, Ser 2·2, Pro 0·8, Gly 2·0, Ala 2·1, Val 2·6, Ile 0·9, Tyr 0·9, Trp[a] |
| C12 | 211—218 | Thr 3·0, Ser 2·0, Pro 0·7, Tyr 1·6 |
| C13 | 219—226 | Thr 1·0, Ser 2·9, Hse 0·9, Gly 1·1, Ala 1·0, Leu 0·9 |

[a] Qualitative determination, N.D. not determined.

fragment CY2 by cyanogen bromide. The chymotryptic digest of fragment Mf was resolved by the fingerprint technique. Of the number of peptides isolated the most important one was peptide C1, containing lysine-95, and peptide C2, containing arginine-102 (Fig. 1). These peptides provided evidence necessary for the arrangement of peptides T5, T6, and T7. The other peptides isolated from this digest were C3 and C4, incomplete chymotryptic peptide C8′, and peptides C9, C10, C11 and C12 whose amino acid composition is given in Table II. It follows from the data presented that the second arginine residue of fragment CB1 occupies position 102. In view of this fact the sequence analysis of the major part of fragment CB1 was carried out to advantage with the material prepared by limited tryptic cleavage at the arginine residue using a derivative of the fragment with blocked epsilon-amino groups of lysine residues as starting material. Limited tryptic cleavage gave rise to two portions of high molecular weight material, as shown by polyacrylamide gel electrophoresis (Fig. 5). An insoluble fragment (ET3) had separated during the deacylation; it was homogeneous and its N-terminal sequence was Val-Leu-Asp (103–105). Fragment ET3 (residues 103–226) was digested with chymotrypsin

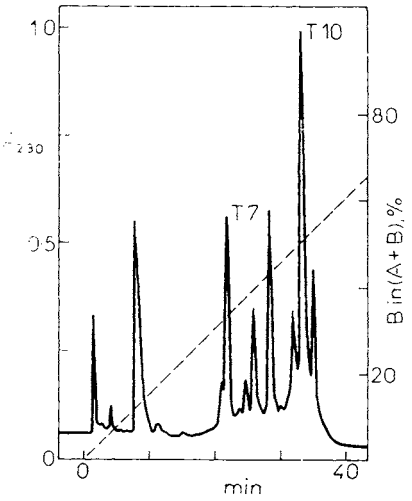



FIG. 2

Resolution of fractions B30-33 (ref.[5]) by HPLC. LiChrosorb RP2 column, 7 μm (4·6 × 25 cm); system II, gradient 0–90% B in (A + B)/60 min, flow rate 2 ml/min. T7 and T10 peptides used for elucidation of amino acid sequence of fragment CB1

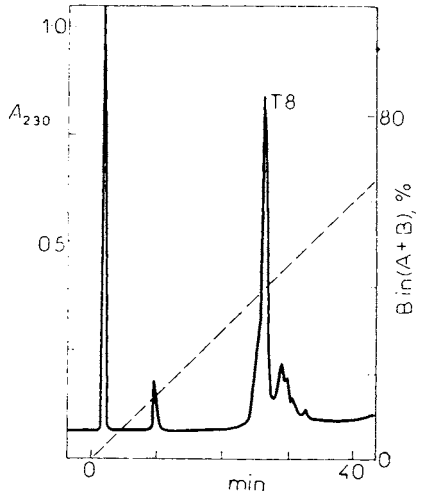



FIG. 3

Final HPLC purification of tryptic peptides from fragment CB1 which were insoluble in weakly alkaline solutions. System I, the remaining conditions are the same as those described in the legend to Fig. 2. Tryptic peptide T8 was isolated

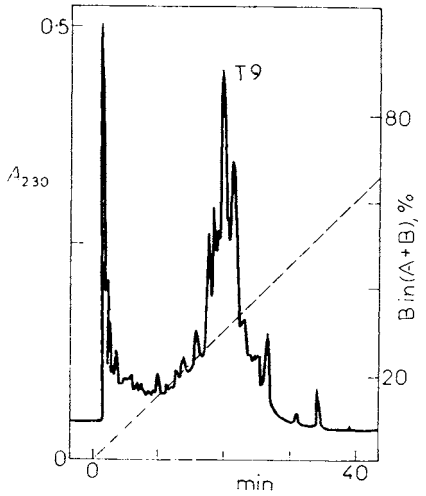



FIG. 4

Resolution of fractions A23—30 (ref.[5]) by HPLC. System II, the remaining conditions are the same as those described in the legend to Fig. 2. Tryptic peptide T9 was subjected to sequence analysis


FIG. 5

Polyacrylamide gel electrophoresis in presence of SDS of peptides from limited tryptic digest of CB1 (see Experimental). *a* insoluble part consisting of fragment ET3 (residues 103—226); *b* unresolved material contained in the limited tryptic digest; *c* soluble part of the digest containing the fragment composed of residues 12—102 and an undetected peptide (residues 1—11)

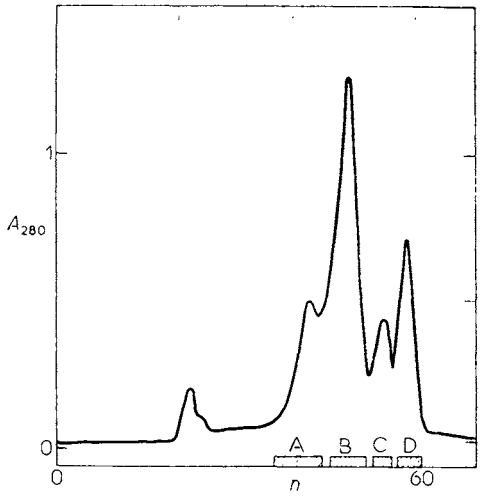



FIG. 6

Gel filtration of chymotryptic digest of fragment ET3 (residues 103—226) on column of Sephadex G-50 fine. The column (1·6 × × 137 cm) was equilibrated in 0·02 mol $l^{-1}$ $NH_4OH$. Fractions 5 ml/15 min; *n* number of fraction. Fractions A—D, marked by hatching, were lyophilized

and the product was resolved on a column of Sephadex G-50 fine (Fig. 6). The peptides contained in fractions A and B were purified by HPLC on a reversed phase column. Peptides C5, C6, C8 and C9 were isolated from fraction A, peptides C4, C11, C12 and C13 from fraction B. Fractions C and D were resolved by TLC techniques. Fractions C contained mostly peptide C10, in fraction D the only tryptophan-containing peptide C7 was present. The total number of peptides isolated from the chymotryptic digests of fragments Mf and ET3 is 13; their sequence characteristics are given in Fig. 1. The chymotryptic digests could be treated without difficulties and afforded data permitting an unambiguous arrangement of tryptic peptides T5 through T10. The amino acid compositions of fragment Mf (residues 75–226) and ET3 (residues 102–226), also shown in Table II, are in good agreement with the numbers of amino acid residues determined by sequence analysis of the corresponding regions, as shown in Fig. 1.

All the data obtained by sequence analysis of cyanogen bromide fragment CB1 of thermitase show that the fragment contains the following 226 amino residues: Asp 12, Asn 18, Thr 18, Ser 25, Glu 1, Gln 10, Pro 11, Gly 27, Ala 33, Cys 1, Val 19, Met 1, Ile 11, Leu 7, Tyr 12, Phe 3, His 3, Lys 7, Arg 2, Trp 5. The molecular weight calculated from these data is 22 932. This value is in accordance with the molecular weight of 23 700 (ref.[2]), calculated from the amino acid composition of fragment CB1, a value based on the molecular weight of 25 000 determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (ref.[2]).

*We wish to thank Mrs A. Kulhánková for her skillful technical assistance during the experimental part of this study. We are indebted to Mrs E. Dršková, Mrs V. Himrová and Mr J. Zbrožek for the amino acid analyses.*

*We are indebted to Dr I. Kluh for the synthesis of* exo-cis-3,6-*endo*-$\Delta^4$-*tetrahydrophthalic anhydride and to Dr J. Čoupek, Laboratorní přístroje, Prague, for his help with the solution of several problems involved in* HPLC. *We are also indebted to Mrs J. Koštovalová, Institute of Molecular Genetics, Czechoslovak Academy of Science, Prague, for the polyacrylamide gel electrophoreses.*

REFERENCES

1. Frömmel G., Hausdorf G., Höhne W. E., Behnke U., Ruttloff H.: Acta Biol. Med. Ger. *37*, 1193 (1978).
2. Hausdorf G., Krüger K., Höhne W. E.: Int. J. Peptide Protein Res. *15*, 420 (1980).
3. Hausdorf G., Krüger K.: Biomed. Biochim. Acta *43*, 3 (1984).
4. Baudyš M., Kostka V., Grüner K., Hausdorf G., Höhne W. E.: Int. J. Peptide Protein Res. *19*, 31 (1982).
5. Meloun B., Baudyš M., Kostka V., Hausdorf G., Höhne W. E.: This Journal *49*, 1835 (1984).
6. Baudyš M., Kostka V. Hausdorf G., Fittkau S., Höhne W. E.: Int. J. Peptide Protein Res. *22*, 66 (1983).
7. Kostka V., Carpenter F. H.: J. Biol. Chem. *357*, 401 (1964).
8. Laemli U. K.: Nature (London) *227*, 680 (1970).
9. Meloun B., Jonáková V., Čechová D.: This Journal *48*, 2558 (1983).

10. Nedkov P., Oberthür W., Braunitzer G.: Hoppe Seyler's Z. Physiol. Chem. *364*, 1537 (1983).
11. Kratzin H., Yang C., Krusche I. U., Hilschmann N.: Hoppe Seyler's Z. Physiol. Chem. *361*, 1591 (1980).
12. Ambler R. P.: Biochem. J. *96*, 32 P (1965).
13. Simpson R. J., Neuberger M. R., Liu T. Y.: J. Biol. Chem. *251*, 1936 (1976).
14. Chang J. Y., Brauer D., Wittmann-Liebold B.: FEBS (Fed. Eur. Biochem. Soc). Lett. *93*, 205 (1978).
15. Yang C. Y.: Hoppe Seyler's Z. Physiol. Chem. *360*, 1673 (1979).
16. Brauer A. W., Margoliesh M. N., Haber E.: Biochemistry *14*, 3029 (1975).
17. Wittmann-Liebold B. in the book: *Synthesis and Sequencing of Peptides and Proteins* (Liu, Schechter, Heinrikson, Eds), p. 75. Elsevier, North Holland, Biochemical Press, Amsterdam (1981).
18. Riley M., Perham R. N.: Biochem. J. *118*, 733 (1970).

Translated by the author (V. K.).