ISOLATION AND SEQUENTIAL STUDIES OF PEPTIDES FROM TRYPTIC DIGEST OF TRICHLOROACETIC ACID PRECIPITATE OF PARTLY AUTOLYZED ALKALINE PROTEASE FROM Aspergillus flavus*

Otakar MIKEŠ, Nguyen bao TOAN** and Jaroslava TURKOVÁ

Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, 166 10 Prague 6

A partly autolyzed preparation of the alkaline protease from *As. flavus*, denatured by trichloro-acetic acid, was resolved into the high and low molecular weight part; the amino acid composition of both parts was similar to that of the native protease. The high molecular weight part was subjected to tryptic digestion; the digest was fractionated by chromatography on a sulfonated ion exchange resin and by paper electrophoresis and paper chromatography techniques. The peptides isolated were characterized by amino acid analysis and terminal end group analysis. Peptides obtained in larger quantities were sequenced. The data on peptides obtained in this study are compared with the results of earlier work on nonradioactive peptides from a partial hydrolysate of the [^{32}P]DIP-labeled enzyme.

In the preceding study[2] we reported on experimental details of the isolation and characterization of nonradioactive peptides from a partial hydrolysate of the [^{32}P]-diisopropylphosphoryl derivative of oxidized alkaline protease from *Aspergillus flavus*. We also presented a summary of all our papers dealing with this subtilisin--related protease of the serine protease type, isolated and characterized by us earlier[3,4]. The aim of this study was the characterization and sequential analysis of peptides from the tryptic digest of the TCA precipitate of the alkaline protease, intended to contribute to the elucidation of the complete amino acid sequence of the enzyme. The results of sequential analysis of the main peptides from this digest have been reported before[5]. Another aim of this study was to cast light on certain problems of the early stages of autolysis of this enzyme resulting in its inhomogeneity demonstrated by its N-terminal end group analysis.

EXPERIMENTAL

Material and Methods

The technical preparation of the *As. flavus* protease was produced in the Central Research Institute of Food Industry, Prague, Czechoslovakia, with the perspective of industrial application[6]. Silica gel layer sheets for thin-layer chromatography (Silufol) were from Kavalier, Votice, Czechoslovakia. All the remaining materials for chromatography were of analytical purity and were supplied by Lachema, Brno, Czechoslovakia. The organic solvents were distilled before use.

Methods

Preparation of pure alkaline protease: A standard preparation of the alkaline protease from *A. flavus* is not commercially available. We had to work[3] with individual batches of the crude enzyme, obtained from another laboratory in low quantities and at long intervals. The individual preparations of the enzyme isolated (which were homogeneous when characterized as described before[3,7] and showed the presence of N-terminal glycine only) were lyophilized and stored in the freezer at −20°C. The enzyme did not loose its proteolytic activity during this treatment, it was, however, partly autolyzed as evidenced by N-terminal end group analysis of preparations stored for longer periods.

Fractionation of partly autolyzed protease by trichloroacetic acid. The protease (1·5 g) was dissolved in 50 ml of ice-cold distilled water and the solution was immediately treated with cold 15% solution of trichloroacetic acid until the formation of the precipitate had ceased (to pH c. 2). The suspension of the precipitate was set aside for 1 h at room temperature. The protein precipitate was centrifuged off and washed repeatedly with water until the pH of the supernatant was 5. The supernatants were pooled. The precipitate was washed with acetone and ether. The denatured dry protein (weight 1·04 g) was used in subsequent work. The pooled aqueous supernatants were freed of trichloroacetic acid by repeated extraction with ether; the latter was removed by passing a stream of air through the water phase. The solution of small peptides, arising from autolysis of the protease and not precipitated by trichloroacetic acid, was then lyophilized. (Weight of hygroscopic product 0·45 g.) This preparation was not examined in this study.
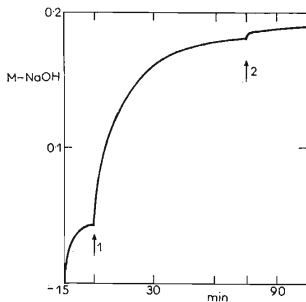
Fig. 1

Course of the Tryptic Digestion of the Suspension of the Denatured Alkaline Protease from *Aspergillus flavus* as Recorded in the Autotitrator

Ordinate: uptake of 0·2M-NaOH. Abscissa: time of hydrolysis. The suspension was first adjusted to constant pH (8·5) in the autotitrator. At zero time 1 mol% of trypsin (1 = first addition) was added. A second addition of 0·5 mol% of trypsin was made after 75 min (2 = second addition). The digestion was discontinued after 105 min by acidification.

Tryptic digestion of denatured alkaline protease. The insoluble denatured enzyme (1·04 g) was suspended in water to a 2% suspension (in terms of dry weight), heated at 37°C and made alkaline by 0·2M-NaOH to pH 8·5 in the autotitrator. The suspension was treated with 1 mol% of trypsin (Fig. 1). The tryptic digestion was essentially complete after 70 min since an addition of 0·5 mol% of trypsin did not lead to further hydrolysis. The digest was acidified with formic acid to pH 3, freed of a small amount of sediment by centrifugation, and subjected to ion exchange chromatography without concentration.

Ion exchange chromatography of tryptic digest. The solution of the digest of 1·04 g of denatured alkaline protease was placed on a column of Dowex 50 equilibrated with buffer A (Table I). The digest was chromatographed by a system of buffers (Fig. 2, Table I). A 0·3 ml aliquot taken from every odd fraction was tested according to Lowry[8] and a 0·5 ml aliquot taken from every even fraction was dried in a desiccator *in vacuo* and tested by paper chromatography in system S_1
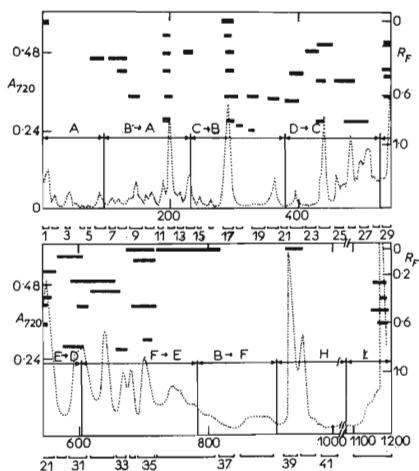
Fig. 2

Ion Exchange Chromatography of Tryptic Digest of Alkaline Protease from *A. flavus*

Column (66 × 4·5 cm) of Dowex 50X2, 200—400 mesh, equilibrated with buffer A. Flow rate 20 ml/20 min (1 fraction), at room temperature. A—I, buffers shown in Table I. The symbols B → A to G → F designate regions where linear gradients were applied. Left ordinate: absorbance of aromatic peptides with the Lowry reagent[8], marked by a dashed line. Right ordinate: R_F-values of aliquots of fractions chromatographed in system S_1 (Table II) and stained with ninhydrin; the results are designated by horizontal bars set in boldface type. Abscissa: *n*, number of fractions, ⌊___⌋ with numerals mark pooled fractions.

(Table II). According to the results of these tests the corresponding effluent fractions were pooled and rotary evaporated. The total number of pooled fractions was 42.

Treatment of pooled fractions obtained by ion exchange chromatography. The further separation of material contained in the pooled fractions was effected by paper chromatography in system S_1 (Table II), high voltage electrophoresis[9] in system E_1 (3000 V, 100—120 min), descending electrophoresis[10] in system E_2 (1 500 V, 90 min), or by a combination of these methods. During semimicropreparative operations on paper the margins of the peptide zones were detected by chlorination[18] or stained with ninhydrin with subsequent stabilization[19]. The peptides were eluted by 25—50% aqueous pyridine, exceptionally by water or 5% acetic acid. The methods of separation were chosen with respect to the result of orienting experiments. Fractions unlikely to be succesfully separated were not treated further. A complete survey of fractionation procedures including their order in which they were applied to the pooled fractions is given in Table III. The peptides isolated are designated with respect to their occurrence in the individual zones obtained by the treatment of the pooled fractions as follows: First number — number of fraction from ion exchange chromatography (Fig. 2), capital letter — zone obtained by paper chromatography (in order from the origin), high voltage electrophoresis E_1, (in order from the anode), or descending electrophoresis E_2 (in order from the cathode). The numbers and letters were affixed during subsequent fractionations according to the same principle. Peptides obtained in small quantities are not recorded in Table III.

Characterization of peptides isolated. Aliquots (c. 0·025 μmol) of all peptides isolated were tested for homogeneity by paper chromatography in systems S_1–S_3 or by paper electrophoresis in systems E_1 and E_2 (Table II). Peptides which appeared homogeneous according to these tests were subjected to orienting chromatography of their total acid hydrolysate in system S_2

TABLE I

Buffers used for Ion Exchange Chromatography of Tryptic Digest of Alkaline Protease from *As. flavus* on Dowex 50—X2 and Elution Scheme

Designation[a]	Pyridine M	pH-Adjustment by	pH of buffer	Total effluent volume l
A	0·10	HCOOH	3·0	1·9
B	0·15	HCOOH	3·5	4·5
C	0·20	CH_3COOH	4·0	7·5
D	0·30	CH_3COOH	4·5	10·5
E	0·40	CH_3COOH	5·0	12·0
F	0·60	CH_3COOH	6·0	15·5
G	0·80	CH_3COOH	7·0	18·0
H	2·00	H_2O	9·3	21·4
I	2·00	NH_4OH	12·0	23·2

[a] The designation of buffers in the Table is identical with their designation in Fig. 2.

TABLE II

Solvent Systems Used for Electrophoresis or Chromatography of Amino Acids, Their Derivatives, and Peptides on Paper or Silica Gel Thin Layers

Systems E_1 and E_2 were used for paper electrophoresis, systems S_1-S_5 for paper chromatography, and systems S_6-S_{16} for thin-layer chromatography on silica gel.

Designation	Composition	Reference
E_1	Acetic acid–formic acid–water (pH 1·9) (3 : 1 : 16)	9
E_2	Pyridine–acetic acid–water (pH 5·6) (4 : 1 : 995)	10
S_1	1-Butanol–pyridine–acetic acid–water (30 : 20 : 6 : 24)	11
S_2	1-Butanol–acetic acid–water (144 : 13 : 43)	10
S_3	Phenol–collidine–water (8 : 2 : 1)	
S_4	Toluene–pyridine–ethylenechlorohydrine–0·8M ammonia (10 : 3 : 6 : 6)	12
S_5	1·5M Phosphate buffer (pH 6)	12
S_6	Chloroform–ethyl acetate–methanol–acetic acid (45 : 75 : 22·4 : 1)	13
S_7	Ethyl acetate–isopropanol–25% ammonia (30 : 50 : 15)	13
S_8	1-Butanol–toluene–25% ammonia (8 : 1 : 1)	14
S_9	Chloroform–toluene–1-butanol–acetic acid (7 : 1 : 1 : 1)	14
S_{10}	Ethanol–toluene–25% ammonia (7 : 2 : 1)	14
S_{11}	Benzene–pyridine–acetic acid (80 : 20 : 5)	15
S_{12}	Chloroform–benzyl alcohol–acetic acid (70 : 30 : 3)	15
S_{13}	o-Xylene (layers impregnated with formamide before application of samples)	16
S_{14}	Butyl acetate–water–propionic acid–formamide (60 : 1 : 1·8 : 8)	16
S_{15}	Ethylene chloride–acetic acid (30 : 4)	16
S_{16}	Xylene–acetone (5 : 1)	17

TABLE III

Separation of Pooled Fractions Obtained by Ion-exchange Chromatography of the Tryptic Digest of the TCA-precipitate of the Alkaline Protease from *A. flavus*

F pooled fractions obtained by ion-exchange chromatography (Fig. 2); S_1, E_1, E_2 order of separation methods (Table II); P running designation of peptide isolated.

F	S_1	E_1	E_2	P	F	S_1	E_1	E_2	P	F	S_1	E_1	E_2	P
9	—	1	—	9E	27	1	2	—	27G3	36	1	—	2	36F2
10	2	1	—	10A1	28	2	1	—	28A3	36	1	—	2	36F3
10	2	1	—	10B1	28	2	1	—	28C1	36	1	—	—	36F4
11	1	2	—	11A2	28	2	1	—	28C3	37	1	—	—	37E
12	2	—	1	12B1	29	—	1	—	29A	37	1	—	—	37F
14	1	—	—	14A	29	—	1	—	29B	37	1	—	—	37G
16	—	—	1	16C	29	—	1	—	29C	39	1	—	—	39A
17	1	—	—	17A	30	—	—	1	30A	42	3	1,4	2	42A1C1
17	1	2	—	17C3	31	—	1,3	2	31A3E	42	3	1,4	2	42A1C2
17	1	—	2	17D2	31	—	1	—	31B	42	3	1,4	2	42A1D1
17	1	—	2	17D3	31	—	1	—	31C	42	3	1,4	2	42A1D2
18	1	2	—	18D1	31	—	1	—	31D	42	3	1,4	2	42A1D3
19	1	—	—	19E	31	—	1	—	31E	42	3	1,4	2	42A1E1
20	1	2,3	—	20A2	32	—	1	2	32A1	42	3	1,4	2	42A1E2
20	1	—	—	20B	32	—	1	2	32A3	42	3	1	2	42B1A
21	2	1	—	21A4	32	—	1	—	32B	42	3	1,4	2	42B1C1
21	2	1	—	21B3	32	—	1	—	32C	42	3	1,4	2	42B1C2
21	—	1	—	21C	33	1	2	—	33D4	42	3	1,4	2	42B1C3
21	2	1	—	21D2	33	1	—	—	33G	42	3	1,4	2	42B1C4
22	—	1	—	22A	33	1	—	—	33H	42	3	1,4	2	42B1C6
22	—	1	—	22B	33	1	—	—	33I	42	3	1,4	2	42B2C1
22	—	1	—	22C	34	1	—	—	34B	42	3	1,4	2	42B2D1
23	—	—	—	23	34	1	—	—	34G	42	3	1	2	42C2D
24	—	2	1	24B1	35	1	—	—	35C	42	3	1,4	2	42C2E1
24	—	2	1	24B2	35	1	—	—	35D	42	3	1,4	2	42C2E2
24	—	2	1	24B3	35	1	2	—	35D2	42	3	1,4	2	42C2E3
24	—	—	1	24C	35	1	2	—	35D3	42	3	1,4	2	42C2F2
26	—	—	—	26	35	1	2	—	35D4	42	3	1	2	42D1A
27	1	—	—	27C	35	1,2	—	—	35E3	42	3	1	2	42D1D
27	1	2	—	27F1	35	1,2	—	—	35E4	42	3	1	2	42D2F
27	1	2	—	27G1	35	1,2	—	—	35E5	42	—	1	—	42E
27	1	2,3	—	27G2A	36	1	2	—	36A1					
27	1	2,3	—	27G2B	36	1	—	2	36E1					

and then to amino acid analysis[20] (Table VI). The hydrolysis was carried out by 6M-HCl in evacuated tubes, 20 h at 110°C. The presence of tryptophan was assayed with independent aliquots using a modified method[21]. The net charge of the peptides was determined by paper electro-

phoresis[10] in system E_2. The N-terminal amino acids were qualitatively determined by the dansyl technique[22] and the derivatives were examined by thin-layer chromatography on Silufol in systems $S_6 - S_{15}$ (Table II). The quantitative determination of the N-terminal amino acid was carried out with peptides isolated in larger quantities by the dinitrophenylation technique[23]; the DNP-derivatives were chromatographed[12] in systems S_4 and S_5 (Table II). The yellow spots were cut out, the absorbance of their eluates determined at 360 nm and evaluated according to recorded data[24]. The C-terminal end groups of peptides were determined by hydrazinolysis[25] modified as described elsewhere[26].

Sequential analysis of peptides characterized. The Edman degradation of peptides isolated in larger quantities was carried out according to method[27] using a modified[17] technique; the PTH amino acids were chromatographed in the systems given in Table II. Peptides isolated in a small quantity only were sequenced according to[22]. The new N-terminal amino acid was determined in each step by dansylation. The subfragmentation of peptides was performed by combined acid hydrolysis[28] and enzymatically. Tryptic digestion of peptides was carried out with a 5% solution of the peptide in 0·1M ammonium bicarbonate, pH 8·5 (ref.[29,30]) at a molar enzyme to substrate ratio of 1 : 100, 5 h at 37°C. The solution was cooled down, acidified to pH 2—3 by formic acid, and taken to dryness *in vacuo* over sodium hydroxide. Chymotryptic digestion was carried out in the same manner yet for 8 h. For peptic digestion the peptide was dissolved in water to a 1—5% solution and pepsin was added (molar ratio 1 : 50). The pH was adjusted to 2 by 0·1M-HCl. The incubation was allowed to proceed 10 h at 37°C and the pH was checked occasionally. The digest was dried *in vacuo*, dissolved in water, and again dried. The partial hydrolysates were fractionated, with respect to the results of orienting experiments, by the paper techniques described above.

Determination of proteolytic activity and pH-*activity curve.* The proteolytic activity of the alkaline protease from *As. flavus* was determined by a modification[3] of Anson's method[31] using a haemoglobin solution in Britton–Robinson buffer at pH 7 or 3—12 (for determination of the pH-optimum curve).

RESULTS AND DISCUSSION

Analysis of Protease, Its Molecular Weight, and Precipitation with Trichloroacetic Acid

The pH-optimum of activity of the alkaline protease lies in the range of pH 7—9. A more detailed investigation of the autolysis of the protease showed, however, that in 0·05M phosphate buffer at pH 7·5 (*i.e.* at a value which is close to its maximal activity) and 37°C the protease lost after 28 h only 6% of its activity[32]. However, even during the initial stages of autolysis N-terminal alanine appeared in addition to N-terminal glycine; the quantity of both N-terminal amino acids became almost equimolar during a short period. Such preparations give two bands on electrophoresis in 8M urea[33] whereas in the absence of urea the protease moves as a single band, similarly to the unautolyzed enzyme. Other N-terminal amino acids appear later in the course of autolysis and their number rapidly increases. If a salt-free preparation of the protease is autolyzed the presence of all amino acids present in the protein can be determined by dinitrophenylation. Only entirely fresh preparations of the

protease were perfectly homogeneous. The presence of N-terminal alanine was observed, to a small degree, even with preparations stored for a few days at −20°C. Salt-containing preparations were considerably more homogeneous. The examples of two lyophilized preparations stored for various periods are given in Table IV.

Since the pH-optimum of the alkaline protease from *A. flavus* lies in the range of the pH-optimum of trypsin, the protease cannot be digested in active state with this enzyme. Therefore trichloroacetic acid was chosen as a denaturing agent; this treatment permitted us also to separate the low molecular weight products of autolysis from its high molecular weight part. The starting material before the addition of trichloroacetic acid, the protease precipitate, and the peptides present in the supernatant were analyzed both by paper chromatography in system S_1 (Table II) and by amino acid analysis of the total acid hydrolysate (Table V). It was shown by paper chromatography that the starting preparation of the protease (A) contained, in addition to a considerable amount of high molecular weight material remaining on the origin also a mixture of peptides of different R_F-values. The precipitated protein (B) contained only the high molecular weight material remaining on the origin and was entirely free of migrating peptides. The supernatant (C) contained exclusively migrating peptides and was free of high molecular weight material on the origin. Even though paper chromatography indicated the completeness of the precipitation procedure, the amino acid analysis (Table V) revealed no essential differences between fractions (A), (B), and (C) which all corresponded in their amino acid composition to that of the intact protease (D) and its assumed molar ratio of amino acid residues (E) (ref.[34]).

TABLE IV

Effect of Length of Storage Period on Degree of Limited Autolysis of Alkaline Protease from *A. flavus*

The preparations were stored in lyophilized state at −20°C for the period given. The values of N-terminal amino acids are calculated from the data given before with respect to N-terminal DNP-glycine ($= 1$ mol). The values are in mol.

Amino acid	Preparation	
	stored one month	stored six months
DNP-glycine	1·00	1·00
DNP-alanine	0·62	0·95
DNP-threonine	0·42	0·57
DNP-serine	0·27	0·36
DNP-aspartic acid	0·23	0·22

The finding that fractions (B) and (C) of the partly autolyzed protease (A) retain a very similar amino acid composition (Table V) deserves special interest. Such an agreement would be impossible if the protease were cleaved by autolysis at random and gradually. The results obtained, however, indicate a rapid decomposition of the entire molecule to small fragments. We explain this phenomenon by a hypothesis assuming a big difference in the susceptibility to attack the native and denatured protein. Whereas the active protease (even though partly cleaved at specific sites

TABLE V

Amino Acid Composition of Partly Autolyzed Protease from *A. flavus* and of Fractions after its Precipitation with Trichloroacetic Acid

Starting preparation of partly autolyzed protease before the precipitation, B trichloroacetic acid precipitate, C mixture of small peptides arising from autolysis, D homogeneous protease with single N-terminal glycine, E derived number of amino acid residues.

Amino acid	A^a	B^a	C^a	D^b	E^c
Lys	16·99	17·59	16·52	17·30	17
His	5·50	5·78	5·87	5·82	6
Arg	3·45	4·23	3·32	3·82	4
Asp	32·91	34·53	37·50	32·93	33
Thr	17·40	16·85	17·10	18·15	18
Ser	31·76	31·06	31·89	34·27	34
Glu	20·44	19·66	20·65	20·30	20
Pro	6·16	6·61	7·14	7·60	8
Gly	31·27	33·04	30·57	31·60	31—32
Ala	38·00	38·00	38·00	38·00	38
Val	18·88	18·17	19·91	24·75	25
Met	1·97	1·85	1·52	1·95	2
Ile	13·87	14·26	15·07	16·15	16
Leu	15·02	14·59	15·86	15·02	15
Tyr	8·45	8·53	9·69	8·50	8—9
Phe	8·13	8·21	8·67	8·05	8
Trp^d				3·45	3—4
-NH$_2$				28·00	28

^{a} The values were obtained after 20-h hydrolysis in 6M-HCl at 110°C and are not corrected for zero time of hydrolysis or completeness of hydrolysis. All values are calculated assuming the presence of 38 alanine residues in the protease; ^{b} mean corrected values obtained by analyses of freshly prepared, unautolyzed protease samples are given for the sake of comparison; ^{c} final amino acid composition of the alkaline protease from *A. flavus* obtained by graphical evaluation[34]; ^{d} tryptophan was not determined in A, B, and C.

which are not essential for its activity) is relatively resistant to the effect of active molecules of the same enzyme, after loss of its activity and denaturation it becomes readily accessible to cleavage and is readily degraded (*cf.* also Discussion in paper[32]). The fact that the protease retains a high activity also after partial autolysis[35] (even though lacking disulfide bonds) can also be explained by assuming that limited proteolysis does not interfere with the productive complementation of the peptide chains of the enzyme[36,37].

Another problem awaiting elucidation is the actual value of the molecular weight of the protease and the total number of its amino acid residues. Preliminary reports[5] on the sequential data were in disagreement with the number of amino acid residues corresponding to the originally considered molecular weight of the protease (18 000, ref.[3]) which was therefore subjected to a revision[7]. In another study[1] the molecular weight was reexamined with the following results: Two experiments carried out by gel chromatography gave values of 28 000 and 29 000 and the values of 27 000 to 36 000 were obtained by the method of sedimentation analysis (Yphantis). The mean values of molar amino acid ratios, obtained by a great number of analyses of various preparations, were treated by the graphical method of determination of integral number of amino acid residues[34]; the amino acid composition thus obtained is given in Table V (columns D and E). This composition corresponds to a molecular weight of 29 400.

Sequential Analysis of Tryptic Peptides

By analyzing the nonradioactive peptides from the partial hydrolysate of the [32P]DIP-labeled alkaline protease[2] we utilized the material left over after the determination of the amino acid sequence around the serine active center[4]. These peptides represented a suitable starting material because they were obtained from the proteolytically inactive enzyme which in the form of its DIP-derivative had been chromatographed once more on Amberlite IRC-50 (ref.[3]) and thus the products of autolysis removed which might have been formed during the labeling with diisopropyl fluorophosphate. This protein contained one single N-terminal amino acid, glycine, and therefore the composition of its tryptic digest was less complex. The quantity of this material, however, was very small and therefore only a few of the peptides obtained could be sequentially characterized[2]. Because of the limited accessibility of the alkaline protease from *A. flavus* we had to use in our sequential studies also the material which was stored for different periods and thus partly autolyzed. We assumed that the trichloroacetic acid precipitate would contain, besides the intact polypeptide chain, also large polypeptide fragments which productively complemented each other in the active protease. We had to expect though that after tryptic hydrolysis we would obtain, in addition to specific fragments, also smaller fragments arising from chains cleaved by autolysis and that homogeneous peptides would be obtained after a more complicated fragmentation procedure.

The second tryptic digest, described here, contained a larger quantity of material and we were therefore able to determine the amino acid sequence of the main peptides. The peptides isolated from the tryptic digest (Fig. 1) of the precipitated protein (B, Table V) by ion exchange chromatography (Fig. 2) and other separation techniques (Table III) were characterized by analytical techniques given in Table VI. Peptides isolated in a sufficient quantity were sequenced. Smaller peptides were degraded by the dansyl-Edman or the classical Edman method. The amide content of the peptides was judged by their net charge and presence of amino acids with ionogenic side chains. The following smaller peptides were obtained:

Tetrapeptide 14A:	Asn.Gly.Asn.Ala
Tripeptide 21C:	Ser.Ala.Phe
Tetrapeptide 22B:	Asp.Val.Val.Lys
Tripeptide 23:	Asp.Val.Lys
Octapeptide 27Gl:	Leu.Leu.Ala(Asn,Asn,Gly,Ala,Tyr)
Tripeptide 29B:	Thr.Ser.Lys
Tripeptide 29C:	Ala.Ser.Lys
Tetrapeptide 31E:	Gly.Ile.Ala.Lys
Tetrapeptides 34B, 35B:	Ser.Asn.Asn.Arg

Peptide 27Gl was obtained in a small quantity not permitting its complete sequencing by the dansyl-Edman technique. When neutral peptides 22B and 23 were degraded, they became basic after the first step; this indicates the presence of N-terminal aspartic acid. Because we were not certain (*cf.* peptide 014, ref.[2]) whether the molar ratio of Ser : Asn : Arg is 1 : 2 : 1 or 1 : 3 : 1 we repeated the amino acid analysis of peptides 34B and 35B several times and were able to show that the peptides contain two aspartic acid residues. This finding was also confirmed by sequential analysis. Certain peptides were sequenced by the dansyl-Edman technique even though their quantity was small; the amino acids in the first or second step were detected in negligible amounts only and the sequence is therefore not certain. This is the case of the following peptides:

36F4:	Ala.Asp(Arg,Asp$_5$,Thr,Ser$_4$,Glu$_5$,Gly$_4$,Ala$_{4-5}$,Val$_5$,Met,Ile$_{1-2}$,Leu, Tyr$_{1-2}$,Phe$_2$,Lys)
42B2D1:	Ala.Ser(Asp,Ser,Gly,Phe$_2$,Lys)
42C2F2:	Thr.Ala.Gly(Ile,Tyr,Lys)

TABLE VI

Characterization of Fractions Isolated from the Tryptic Digest of the TCA-precipitate of the Alkaline Protease from *A. flavus*. The net charge of the peptides was determined by descending electrophoresis in system E_2 (Table II).

Designation of fraction	Quantitative amino acid analysis																	Number of residues	Net charge	N-Terminal amino acid
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp			
9E	—	—	—	0·95	0·76	1·20	—	—	0·90	0·90	—	—	—	—	—	—	—	3	0	Gly
10A1	—	—	—	1·13	0·29	1·26	—	—	—	—	—	—	—	—	—	—	—	4	0	
10B2	—	—	—	—	—	—	—	—	0·41	1·85	—	—	—	—	—	—	—	4	0	
11A2	—	—	—	1·38	—	0·94	0·29	1·96	0·95	—	—	—	1·01	—	—	—	—	5	0	Gly
12B1	0·38	—	—	2·15	0·85	1·77	1·54	1·08	1·15	3·69	1·23	—	0·93	—	—	—	—	14—15	—	
14A	0·02	—	—	2·15	0·02	0·04	0·06	—	0·94	0·94	1·03	—	—	—	—	—	—		0	Asp
16C	—	—	—	1·10	2·03	1·19	1·26	—	1·94	1·03	—	—	0·19	0·19	0·65	—	—	9	—	Asp
17A	0·99	—	—	7·68	1·98	3·96	6·14	2·48	3·17	10·2	4·06	1·03	1·88	0·99	—	1·98	—	45	—	Ile
17C3	0·56	—	—	1·19	4·15	5·03	—	1·67	2·17	2·00	—	—	1·76	—	—	—	—	20	—	
17D2	1·00	—	—	8·50	2·00	4·33	5·84	3·00	3·62	11·5	4·30	—	2·00	1·86	—	2·00	—	50—53	—	
17D3	0·94	—	—	6·85	1·97	3·63	5·56	2·00	3·02	9·90	4·35	—	1·94	0·97	—	1·80	—	42—44	—	
18D1	0·25	—	—	3·66	1·00	2·17	2·50	—	2·16	4·33	1·83	—	1·00	1·00	—	0·83	—	19—21	—	Asp
19E	—	—	—	0·16	—	0·22	—	—	0·22	0·27	1·09	—	1·09	1·91	—	—	—	3		
20A2	—	—	—	1·95	—	—	0·08	—	1·06	1·57	—	—	—	0·22	0·22	—	—	4	0	Ala
20A4	—	—	—	—	—	—	—	—	—	—	—	—	—	1·00	—	—	—	1		
20B	—	—	—	1·21	—	0·95	0·27	—	1·21	0·95	—	—	1·10	0·63	—	—	—	5	0	Gly
21A4	—	—	—	1·51	1·10	1·90	0·80	—	2·60	1·90	—	—	—	1·20	—	—	—	11—13		
21B3	0·73	—	—	—	—	0·90	—	1·10	1·10	2·20	—	—	—	0·93	—	—	—	5		
21C	—	—	—	—	—	—	—	—	—	0·90	—	—	—	—	—	1·20	—	3	0	
21D2	0·86	—	—	0·86	—	1·29	—	0·93	—	—	—	—	—	3·20	—	—	—	3	—	Leu
22A	0·93	—	—	1·95	0·90	—	0·97	—	1·05	4·00	1·02	—	—	—	—	—	—	15	0	Ala
22B	0·96	—	—	1·03	—	—	—	—	—	—	—	—	—	—	—	—	—	4	0	Asp
22C	1·00	—	—	3·00	0·14	1·19	0·14	—	0·33	2·80	1·14	—	0·91	0·07	0·03	—	—	10	0	
23	0·90	—	—	1·12	0·11	0·16	0·15	—	0·17	0·24	0·98	—	0·07	3·31	0·03	0·05	—	3	0	Ala
24B1	0·09	—	—	1·97	0·19	1·73	0·44	1·83	1·01	2·14	0·24	—	0·19	3·31	1·05	0·12	—	13	0	Asp

The table below is printed sideways on the page. Rows are the residue lines (amino acid, marker, residue number); columns are the peptide fractions (24B2 … 34G). A dash (—) indicates no value in a cell.

Amino acid		No.	24B2	24B3	24C	26	27C	27F1	27G1	27G2A	27G2B	27G3	28A3	28C1	28C3	29A	29B	29C	30A	31A3E	31B	31C	31D	31E	32A1	32A3	32B	32C	33D4	33G	33H	33I	34B	34G
Gly	0	8	0·03	—	—	—	—	—	—	—	—	—	—	1·08	0·10	1·00	—	—	1·03	—	—	0·83	1·05	—	—	0·08	2·01	0·83	—	—	—	—	—	—
Ser	0	4	—	—	0·80	—	—	—	—	—	—	—	—	1·20	2·00	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gly	¡	17–18	0·79	—	—	—	—	—	—	—	—	—	1·82	2·62	1·73	2·76	—	2·51	1·02	—	2·13	—	0·98	1·00	—	—	—	—	—	—	—	—	—	—
Glu	0	5	0·95	0·09	—	0·22	—	—	—	—	—	—	0·23	1·00	0·26	1·01	—	0·24	1·21	—	0·11	0·27	—	1·00	—	0·13	0·45	0·15	—	—	—	—	—	—
	0	9	1·01	—	—	—	—	—	—	—	—	—	1·40	1·09	1·68	0·95	—	2·08	1·26	—	0·49	0·27	2·01	0·94	—	1·26	5·29	2·77	—	—	—	—	—	—
Leu	0	35	—	—	—	—	—	—	—	—	—	—	5·04	3·02	4·28	3·02	—	4·54	5·29	0·76	2·01	2·01	2·03	—	—	—	—	—	—	—	—	—	—	—
Thr	0	8	—	—	—	—	—	—	—	—	—	—	1·85	0·19	0·26	0·10	—	1·14	—	—	0·10	1·94	0·13	—	—	—	—	—	—	—	—	—	—	—
Ala	0	2	—	—	—	—	0·04	—	—	—	—	—	—	0·94	—	—	—	—	0·93	—	1·03	—	0·93	—	—	—	—	—	—	—	—	—	—	—
Ala	0	2	—	—	—	—	1·07	—	—	—	—	—	0·92	0·33	0·08	0·92	—	0·11	1·12	—	1·07	0·88	—	—	—	—	—	—	—	—	—	—	—	—
	0	2	—	—	—	—	—	—	—	—	—	—	0·86	0·04	1·07	—	—	1·23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gly		4	1·00	—	—	—	1·00	—	—	—	—	—	1·00	0·75	1·25	1·00	—	0·96	0·09	—	1·10	—	0·75	—	—	—	—	—	—	—	—	—	—	—
Thr		3	1·03	—	—	—	1·00	—	—	—	—	—	—	0·89	1·08	1·04	—	0·03	0·09	—	—	—	0·98	—	—	—	—	—	—	—	—	—	—	—
Ala		6	1·10	—	—	—	1·10	—	—	—	—	—	—	0·15	1·04	—	—	—	0·98	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
G·y		6	1·12	—	—	—	1·12	—	—	—	—	—	—	—	—	—	0·87	2·00	1·07	—	0·14	0·79	—	—	—	—	—	—	—	—	—	—	—	—
		3	0·86	—	—	—	0·81	—	—	—	—	—	1·07	1·85	1·00	1·00	—	1·00	1·24	—	0·85	2·52	1·09	—	—	—	—	—	—	—	—	—	—	—
Ala	+	10	0·81	—	—	—	1·00	—	—	—	—	—	1·00	—	2·28	0·57	—	1·04	0·99	0·92	1·03	1·05	—	—	—	—	—	—	—	—	—	—	—	—
Ala	+	13	1·00	—	—	—	1·00	—	—	—	—	—	—	1·73	—	—	—	0·04	0·91	0·15	1·10	—	—	—	—	—	—	—	—	—	—	—	—	—
Gly	+	7	1·07	—	—	—	1·07	0·09	—	—	—	—	0·09	0·26	1·04	0·91	—	0·36	0·91	—	1·04	1·02	—	—	—	—	—	—	—	—	—	—	—	—
Ala	+	4	0·96	—	—	—	0·96	—	—	—	—	—	—	0·88	—	—	—	0·92	1·09	1·11	1·04	—	—	—	—	—	—	—	—	—	—	—	—	—
		4	0·19	1·00	0·97	—	0·19	—	—	—	—	—	2·55	0·84	2·20	1·00	—	1·78	1·26	2·77	0·06	0·19	0·71	0·81	—	—	—	—	—	—	—	—	—	—
Ala	+	15–17	1·00	0·90	0·95	—	1·00	—	—	—	—	—	2·72	0·90	2·98	0·57	—	1·94	3·91	—	2·52	0·91	1·04	—	—	—	—	—	—	—	—	—	—	—
Ser	+	18	0·90	—	0·11	—	0·90	—	—	—	—	—	3·07	1·04	1·51	0·15	—	0·22	0·93	0·13	0·94	1·02	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	1·08	3·14	—	—	—	—	0·13	—	—	—	1·04	0·09	1·11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		3	1·08	—	—	—	1·11	0·96	—	—	—	—	—	—	1·73	—	—	0·88	1·06	2·71	0·65	0·91	0·81	1·0	—	—	—	—	—	—	—	—	—	—
His?	+	16–18	0·23	—	0·65	—	—	—	—	—	—	—	2·52	2·80	—	2·48	—	3·20	1·06	1·68	2·30	2·17	1·04	0·86	—	—	—	—	—	—	—	—	—	—
	+	25–26	1·11	—	—	—	—	—	—	—	—	—	2·72	5·10	1·38	—	—	3·28	3·20	—	—	2·00	0·91	—	—	—	—	—	—	—	—	—	—	—
	+	18–19	—	—	—	—	1·00	—	—	—	—	—	1·04	0·23	1·51	0·20	—	2·67	2·06	1·13	2·06	2·00	0·11	—	—	—	—	—	—	—	—	—	—	—
	0	29	0·23	—	—	—	—	—	—	—	—	—	2·16	0·82	3·56	0·42	—	5·20	4·03	1·79	3·81	3·85	0·23	—	—	—	—	—	—	—	—	—	—	—
Ser		4	—	—	—	—	—	—	—	—	—	—	0·72	2·38	—	0·85	—	—	—	—	0·30	0·30	—	—	—	—	—	—	—	—	—	—	—	—
	+	3	—	—	—	—	—	—	—	—	—	—	0·29	—	—	—	1·35	—	1·00	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE VI
(*Continued*)

Designation of fraction	Quantitative amino acid analysis																	Number of residues	Net charge	N-Terminal amino acid
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp			
35B	—	—	1·00	2·00	—	0·81	—	—	—	—	—	—	—	—	—	—	—	4	+	Ser
35C	1·87	—	—	2·03	—	2·81	—	—	5·05	3·18	—	1·12	3·17	1·68	—	—	—	21	+	Ala?
35D2	0·84	—	—	1·12	—	1·12	—	—	2·50	1·40	—	0·56	1·12	1·12	1·12	—	—	10—12		Ile
35D4	0·96	—	—	—	—	—	—	—	—	—	—	—	1·04	—	—	—	—	2	0	
35E3	—	—	—	0·68	—	2·14	1·12	1·91	1·01	3·14	—	—	—	—	—	—	—	10	0	
35E5	—	—	—	—	—	—	—	1·00	—	—	—	—	—	—	—	—	—	2	0	
36A1	0·86	0·71	0·68	6·70	3·20	5·15	5·96	0·71	5·83	4·70	4·65	0·12	2·12	1·12	2·84	2·12	—	49	+	Ala
36E1	1·00	—	—	0·91	0·36	1·82	—	—	2·87	1·92	—	0·82	1·10	0·96	0·62	—	—	13	+	Ala
36F2	0·96	—	—	1·07	0·28	0·22	—	—	2·88	1·92	0·96	0·96	1·13	1·07	0·69	—	—	11	0	Ala
36F3	2·00	—	0·90	4·25	1·80	5·38	2·24	—	7·18	5·60	1·90	1·34	3·13	2·70	—	0·90	—	38—40	+	Ala
36F4	0·98	—	0·87	6·20	1·30	3·90	5·20	—	4·35	5·65	4·78	0·33	1·63	1·20	1·30	1·96	—	37—41	+	Ala
37E	1·00	—	—	3·10	0·70	2·40	2·30	—	3·60	3·70	2·10	0·30	1·30	1·10	0·50	0·80	—	24	0	Ala
37F	1·10	—	—	4·95	2·05	4·73	3·00	—	4·33	5·20	1·73	—	2·13	1·97	—	1·81	+	33	0	
37G	1·08	0·24	0·29	2·03	1·26	3·06	0·97	0·72	2·70	3·05	1·38	—	2·05	2·28	—	0·90	+	22	0	
39A	0·92	0·25	—	4·68	1·75	5·75	2·66	0·35	4·20	3·72	2·85	0·15	2·24	1·95	0·37	2·18	+	33—35	+	Lys
42A1C1	1·10	—	1·10	1·10	—	2·20	—	—	4·20	2·00	—	0·80	—	0·90	—	0·50	—	13—14	+	
42A1C2	0·84	—	—	—	0·08	1·95	0·19	—	3·15	—	—	0·94	—	1·02	0·74	—	—	9		
42A1D1	0·67	—	—	1·16	—	2·00	—	—	3·16	1·50	—	0·83	1·00	—	1·00	—	—	12—13		
42A1D2	1·00	—	—	—	—	2·20	—	—	4·00	—	—	—	1·00	1·20	0·60	—	—	9		
42A1D3	1·00	—	—	—	—	1·11	—	—	3·11	—	—	—	—	0·80	0·55	—	—	7		
42A1E1	—	—	—	1·12	0·10	0·10	—	—	0·02	1·78	—	—	1·06	—	—	—	—	4		Ala
42A1E2	—	—	—	0·98	0·10	0·10	—	—	0·10	—	—	—	1·02	—	—	—	—	2		Ile
42B1A	0·85	0·10	—	0·24	0·08	0·15	—	—	1·16	0·15	0·15	0·04	—	—	—	0·05	—	2	0	Gly
42B1C1	0·21	—	—	1·21	—	1·95	—	—	0·74	0·95	0·15	—	—	—	—	1·05	—	6	0	Ala?
42B1C2	0·10	—	—	1·02	—	0·98	—	—	0·29	0·12	—	—	—	—	—	0·93	—	3	0	Ser?
42B1C3	1·23	—	—	1·23	0·16	2·79	—	—	2·80	0·82	—	0·25	—	0·74	0·57	1·80	—	13	+	

Peptide	Ala	His?	(—)	Gly	Ile	Thr?	His	Gly	(—)	(—)	(—)	(—)	Lys
(symbol)			0		+				+				
(total)	8	3	8	8	11–12	6	4	5	6	2	5	3	4
42B1C4	0·75	—	—	1·00	1·25	—	—	—	—	—	3·00	1·00	1·00
42B1C6	1·11	—	—	0·09	—	—	—	—	0·85	—	1·07	0·17	0·11
42B2C1	0·77	—	1·22	1·77	—	—	—	—	1·77	—	1·33	1·00	—
42B2D1	0·57	0·79	1·26	1·98	—	—	—	—	2·11	—	1·26	0·97	—
42C2D	0·92	—	0·25	1·16	—	—	—	0·17	—	0·08	1·96	1·12	0·71
42C2E1	0·72	—	1·35	1·24	—	—	—	—	0·83	0·83	0·93	0·93	—
42C2E2	0·93	—	—	—	—	—	—	—	—	—	1·20	0·93	0·93
42C2E3	1·20	—	—	0·16	—	—	—	—	0·81	0·81	1·20	0·89	0·89
42C2F2	1·05	—	—	—	—	—	—	—	0·72	0·72	1·00	0·94	0·97
42D1A	0·94	—	—	1·77	0·07	—	—	1·10	—	—	0·07	—	—
42D1D	0·15	—	—	—	0·37	—	—	—	—	—	2·13	—	0·37
42D2F	0·87	—	—	0·87	—	—	—	—	—	—	1·30	—	—
42E	2·16	1·08	—	—	—	—	—	—	—	—	0·76	—	—

TABLE VII

Characterization of Subfragments Isolated from Partial Hydrolysates of Peptides Prepared by Tryptic Digestion of Alkaline Protease from *A. flavus*

Designation of subfragment	Quantitative analysis													Number of residues	Net charge	N-Terminal amino acid
	Lys	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr			
22Ap2	—	—	1·03	—	—	—	0·96	1·03	1·98	—	—	—	—	5	—	Asp
22Ap3A	—	—	—	0·99	—	—	—	—	—	0·99	—	—	—	3	+	Val
22Ap5	—	—	1·15	—	—	1·00	—	—	—	—	—	0·86	—	3	—	
22Ap6A	—	—	1·12	—	—	1·05	—	—	—	—	—	1·83	—	4		
22Ap7	—	—	—	—	—	—	—	—	2·20	—	—	0·83	—	3		
22Ap8	—	—	—	—	—	—	—	—	1·06	—	—	0·94	—	2		
24Cc2	—	—	1·06	0·97	0·97	1·94	—	1·00	—	—	—	—	0·70	7	—	Gly
24Cc3	—	—	1·12	1·93	0·99	1·25	—	2·02	0·94	—	0·72	—	1·03	11		
24Cp1	—	—	1·00	1·00	1·00	2·00	—	1·00	—	—	—	—	—	6	—	
24Cp3	—	—	0·90	1·30	1·10	1·10	—	1·70	0·90	—	—	—	0·40	9	0	
24Cp4A	—	—	—	0·75	1·00	1·00	—	2·00	trace	—	—	—	—	5	0	
24Cp4B	—	—	—	1·58	1·10	0·98	—	1·95	0·98	—	—	—	0·37	7—8		
24Cp4C	—	—	—	1·00	—	1·00	—	2·00	1·00	—	—	—	—	4	—	
24Cp5A	—	—	1·00	1·72	1·14	1·14	—	2·00	1·00	—	0·86	—	0·86	10	—	
24Cp5B	—	—	1·75	1·75	2·24	1·00	—	1·50	1·25	—	1·00	—	0·25	11—12	—	Thr

26a1B	1·03	—	—	0·97	—	—	—	—	—	—	—	—	—	2		Thr
26a4A	—	—	—	—	—	0·98	—	—	1·11	—	—	0·92	—	3	—	Glu
26a4B	—	—	—	—	—	1·00	—	—	—	—	—	1·00	—	2	—	
29Aa3	0·95	—	—	1·90	1·18	—	—	—	—	—	—	—	—	4	+	Thr
29Aa4	—	—	—	—	—	—	—	1·17	—	—	—	0·82	—	2	0	Gly
29Aa5	—	—	—	1·79	1·21	—	—	—	—	—	—	—	—	2	—	Thr
31Ca2B	0·82	—	—	—	0·89	—	—	—	—	1·29	—	—	—	3		
31Ca2C	1·09	—	—	—	0·91	—	—	—	—	1·09	—	—	—	3	+	Ser
31Ca6A	—	—	—	—	0·67	—	—	—	—	—	0·97	1·03	—	3		
31Ca6B	—	—	—	—	0·99	—	—	—	0·82	—	1·06	1·13	—	4		Ala
31Ca6C	—	—	—	—	0·94	—	—	—	—	—	0·98	1·10	—	3		(I)le
32A3c5	—	1·00	—	—	—	1·00	—	1·10	—	—	—	—	—	3		Glu

Some peptides, given in Table VI, were obtained in large amounts; they were subjected to enzymic or partial acid hydrolysis. The subfragments were isolated and their analytical characteristics are presented in Table VII. The subfragments bear the symbol of the original peptide and a small letter expressing the method of additional cleavage namely *a* for combined partial hydrolysis, *c* for chymotryptic cleavage, and *p* for peptic cleavage. Next follows a numerical and alphabetical designation illustrating the method of separation of the partial hydrolysate. The system described for the original peptides under "Experimental" is again used. The structure of the following peptides was solved by means of subfragmentation:

Pentadecapeptide 22A. Lysine was C-terminal according to hydrazinolysis. Peptic hydrolysis and chromatography in system S_1 afforded subfragments 22A-pl

Table VIII

Primary Structure of Pentadecapeptide 22A Derived from Subfragments Obtained by its Peptic Digestion

Designation	Sequential data	Note
22A:	Leu.Ala.Ala.Leu.Glu.(Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val,Thr)Lys	*a*
— pl	Asp.Gly.Pro.Ala.Ala	
— p3A	Val(Thr,Lys)	
— p5	Glu.Asn.Leu	*b*
— p6A	Leu(Glx,Asx,Leu)	
— p7	(Leu,Ala,Ala)	*c*
— p8	(Leu,Ala,Ala,Leu)	*d*
22A:	Leu.Ala.Ala.Leu.Glu.Asn.Leu.Asp.Gly.Pro.Ala.Ala.Val.Thr.Lys	

a The sequential degradation of the original peptide was carried out by a modification[17] of the Edman method; chromatography showed the presence of glutamic acid in the fifth step. The amino acid sequence of the subfragments was determined by the dansyl-Edman method[22]; *b* hydrazinolysis of subfragment — p5 gave leucine. After the first step of Edman degradation the originally acidic peptide became neutral; after the second step the peptide remained neutral. Its first amino acid must therefore be glutamic acid and its second residue asparagine; *c* the N-terminal amino acid could not be identified; the second residue was alanine; *d* the N-terminal amino acid could not be identified, the second and third residue was alanine.

to 22A-p8 (marked -p1 etc. in what follows). Subfragment -p3 was purified by descending electrophoresis in system E_2 and afforded fraction -p3A. Similarly, -p6 gave -p6A when subjected to high voltage electrophoresis in system E_1. All subfragments and the derived primary structure of the peptide are given in Table VIII.

Heptadecapeptide 24C. Hydrazinolysis of the original peptide revealed C-terminal tyrosine. Chymotryptic digestion and descending electrophoresis (E_2) afforded fragments 24C-c2 and -c3. Peptic hydrolysis and chromatography in system S_1 and purification by high voltage electrophoresis (E_1) gave subfragments -p1 to -p5B which after being aligned gave the primary structure of the peptide (Table IX).

Pentapeptide 26. The first three steps of the neutral peptide were determined by the classical Edman method. Combined partial acid hydrolysis, chromatography in system S_1, and purification by high voltage electrophoresis (E_1) yielded subfragments 26-a1B to -a4B. Subfragment -a4A was acidic. The results can be arranged as follows:

TABLE IX

Primary Structure of Heptadecapeptide 24C Derived from Subfragments Obtained by its Chymotryptic and Peptic Digestion

Designation	Sequential data	Note
24C	Gly.Gln.Gln.Ser.Thr(Asx,Tyr,Ile,Tyr,Thr,Asx,Ala,Ser,Glx,Gly,Gly,Thr)Tyr	a
—c2	Gly(Gln,Gln,Ser,Thr,Asp,Tyr)	b
—c3	(Ile,Tyr,Thr,Asx,Ala,Ser,Glx,Gly,Gly,Thr,Tyr)	
—p1	(Gly,Gln,Gln,Ser,Thr,Asp)	b
—p3	(Thr,Asn,Ala,Ser,Gln,Gly,Gly,Thr,Tyr)	c
—p4A	(? ,Ser,Gln,Gly,Gly,Thr)	c
—p4B	(Ala,Ser,Glu,Gly,Gly,Thr,Tyr)	
—p4C	(Glu,Gly,Gly,Thr)	
—p5A	(Ile,Tyr,Thr,Asx,Ala,Ser,Glx,Gly,Gly,Thr,Tyr)	
—p5B	(Ser,Thr,Asx,Tyr,Ile,Tyr,Thr,Asx,Ala,Ser,Glx,Gly,Gly)	
24C:	Gly.Gln.Gln.Ser.Thr.Asp.Tyr(Ile,Tyr)(Thr,Asn)(Ala,Ser)(Gln,Gly,Gly)Thr.Tyr	

[a] Glutamine was detected in the 2nd and 3rd step by chromatography of the PTH derivative
[b] Since the acidic fragment is derived from the enzymic digest of the peptide where an amide was found to occupy positions 2 and 3, we assume the presence of aspartic acid in position 6;
[c] The neutrality of the peptides indicates the amide form of acidic amino acids.

26:	Glu.Leu.Ala(Thr,Lys)
26-a1B	Thr.Lys
26-a4A	Glu(Leu,Ala)
26-a4B	(Glu,Leu)
26:	Glu.Leu.Ala.Thr.Lys

Hexapeptide 29A. The first three steps were determined by the dansyl-Edman technique. Combined partial acid hydrolysis and descending electrophoresis (E_2) afforded subfragments 29A-a3 to -a5 which can be summarized as follows:

29A:	Gly.Leu.Thr(Thr,Gln,Lys)
29A-a3	Thr(Thr,Gln,Lys)
29A-a4	Gly.Leu
29A-a5	Thr(Thr,Gln)
29A:	Gly.Leu.Thr.Thr.Gln.Lys

In the subsequent experiment the complete amino acid sequence was determined by the classical manual Edman method.

Hexapeptide 31C. Combined partial acid hydrolysis, chromatography in system S_1, and final purification by electrophoresis (E_1) afforded subfragments 31C-a2B to -a6C. The sequential data obtained by analysis of these fragments are summarized in Table X.

Octapeptide 32A3. The peptide was sequenced by the classical manual Edman method and the products in the first five steps were identified. Only the arginine-containing tripeptide (-c5) was isolated in a sufficient quantity from the chymotryptic digest by high voltage electrophoresis (E_1). Dansylation of the tripeptide revealed its N-terminal glutamic acid. The results can be summarized in the following partial structure:

Ala.Tyr.Val.Val.Asp(His,Asx$_2$,Ser,Glx$_2$,Gly,Val$_2$,Phe)Glx.Gly.Arg .
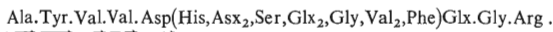
A survey of all peptides isolated from the tryptic hydrolysate of the trichloro-acetic precipitate of the alkaline protease together with their sequential characterization is given in Table XI. Some of the peptides isolated are obviously identical, *e.g.* 27G2B = 27G3, 32A1 = 32A3 = 33D4, 34B = 35B. The amino acid composition of peptides 42B2C1 and 42B2D1 is entirely identical, yet since there are certain doubts as to their N-terminal amino acid we do not regard them as identi-

cal. Polypeptides 17D2 and 17D3 are very similar and most likely derived from the same site of the molecule; since there are, however, minor differences in their amino acid composition, we cannot decide whether they are identical or not. It follows from the survey given in Table XI that many specific tryptic peptides were obtained yet also a great number of nonspecific fragments. It is therefore worthwhile to compare the results obtained with this digest and those which gave the preceding[2] digest. The identity of several peptides is obvious at a first glance.

I (ref.[2])	II (present study)	Composition
03	29A	Gly.Leu.Thr.Thr.Gln.Lys
06	23	Asp.Val.Lys
07	22B	Asp.Val.Val.Lys
09	29C	Ala.Ser.Lys
012	31C	Ala.Ser.Ile.Leu.Ser.Val.Lys
014	34B; 35B	Ser.Asn.Asn.Arg

These peptides were obviously not cleaved by autolysis and they can be regarded as specific because they are C-terminated with basic amino acids. Other peptides, even though differring in amino acid composition, are most likely derived from the same site of the chain and complement one another:

TABLE X
Sequential Analysis of Subfragments from Partial Acid Hydrolysate of Heptapeptide 31C

Designation	Sequential data	Note[a]
31C:	Ala.Ser(Ile,Leu,Ser,Val,Lys) $\longrightarrow \longrightarrow$	
−a2B	(Ser,Val,Lys)	
−a2C	Ser.Val.Lys $\longrightarrow \longrightarrow$	[a]
−a6A	(Ser,Ile,Leu)	
−a6B	Ala.Ser.Ile.Leu $\longrightarrow \longrightarrow \longrightarrow \longrightarrow$	[b]
−a6C	(Ile,Leu,Ser)	
31C:	Ala.Ser.Ile.Leu.Ser.Val.Lys $\longrightarrow \longrightarrow \longrightarrow$	[c]

[a] Determined by the dansyl-Edman method; [b] a negligible amount of DNS-Ile was detected after the 3rd degradation step; [c] three steps were determined by the classical manual Edman degradation.

Designation of peptides	Quantity isolated according to amino acid analysis μmol	Number of amino acid residues	Composition of fragments
9E	0·08	3	Gly(Ser,Ala)
10A1	0·05	4	(Asn,Thr,Ser,Ala)
10B1	0·05	4	(Asn,Ala$_2$,Ile)
11A2	0·075	5	Gly(Asx,Ser,Pro$_2$)
12B1	0·15	14—15	(Asx$_2$,Thr,Ser$_2$,Glx$_{1-2}$,Pro,Gly,Ala$_4$,Val,Ile)
14A	7·5	4	Asn.Gly.Asn.Ala
16C	0·65	9	Asx(Thr$_2$,Ser,Glx,Gly$_2$,Ala)Tyr
17A	0·28	45	Ala(Asx$_8$,Thr$_2$,Ser$_4$,Glx$_6$,Pro$_2$,Gly$_3$,Ala$_9$,Val$_4$,Phe$_2$, Ile$_2$,Leu)Lys
17C3	0·08	20	Ile(Asx,Thr$_4$,Ser$_5$,Pro$_2$,Gly$_2$,Ala$_2$,Met,Ile,Lys)
17D2	0·05	50—53	(Asx$_{8-9}$,Thr$_2$,Ser$_{4-5}$,Glx$_6$,Pro$_3$,Gly$_4$,Ala$_{12}$,Val$_{4-5}$, Ile$_2$,Leu$_2$,Phe$_2$,Lys)
17D3	0·28	42—44	(Asx$_7$,Thr$_2$,Ser$_{3-4}$,Glx$_{5-6}$,Pro$_2$,Gly$_3$,Ala$_{10}$,Val$_4$,Ile$_2$, Leu,Phe$_2$)Lys
18D1	0·1	19—21	Asx(Asx$_{2-3}$,Thr,Ser$_2$,Glx$_{2-3}$,Gly$_2$,Ala$_{4-5}$,Val$_2$,Ileu, Leu,Phe,Lys$_{C-1}$)
19E	0·1	3	(Ile,Leu$_2$)
20A2	0·08	4	Ala(Asn$_2$,Gly)
20B	0·08	5	Gly(Asn,Ser,Ala,Leu)
21A4	0·05	11—13	(Asx$_{1-2}$,Thr,Ser$_2$,Glx,Gly$_{2-3}$,Ala$_2$,Ile,Leu)
21B3	0·05	5	(Ala$_2$,Gly,Leu,Lys)
21C	0·55	3	Ser.Ala.Phe
21D2	0·05	3	(Asx,Ser,Lys)
22A	13·8	15	Leu.Ala.Ala.Leu.Glu.Asn.Leu.Asp.Gly.Pro.Ala.Ala .Val.Thr.Lys
22B	10·0	4	Asp.Val.Val.Lys
22C	0·25	10	Ala(Asp,Asn$_2$,Ser,Ala$_2$,Val,Ile)Lys
23	37·50	3	Asp.Val.Lys
24B1	0·35	13	Asn(Asn,Ser$_2$,Pro$_2$,Gly,Ala$_2$,Leu$_3$)Tyr
24B2	0·18	8	Gly(Asn,Ser,Pro,Ala,Leu$_2$)Tyr
24B3	0·08	4	Ser(Arg,Asp,Ser)
24C	6·3	18	Gly.Gln.Gln.Ser.Thr.Asp.Tyr(Ile,Tyr)(Thr,Asn)(Ala, Ser)(Gln,Gly,Gly)Thr.Tyr
26	25·0	5	Glu.Leu.Ala.Thr.Lys
27C	0·1	9	(Asx,Thr.Ser$_2$,Glx,Gly$_2$,Ala,Lys)

TABLE XI
(Continued)

Designation of peptides	Quantity isolated according to amino acid analysis μmol	Number of amino acid residues	Composition of fragments
27F1	0·05	35	$(Asx_5,Thr_3,Ser_4,Glx_3,Gly_5,Ala_5,Val_3,Met,Ile_2,Leu_2, Phe,Lys)$
27G1	0·35	8	$Leu.Leu.Ala(Asn,Asn,Gly,Ala,Tyr)$
27G2A	0·08	2	$Thr.Tyr$
27G2B	0·08	2	$Ala.Tyr$
27G3	0·08	2	$Ala.Tyr$
28A3	0·05	4	(Asn,Ser,Gln,Gly)
28C1	0·05	3	(Asx,Ser,Lys)
28C3	0·05	6	$(Asx,Thr,Ser,Glx,Gly,Ala)$
29A	20·0	6	$Gly.Leu.Thr.Thr.Gln.Lys$
29B	13·0	3	$Thr.Ser.Lys$
29C	13·0	3	$Ala.Ser.Lys$
30A	0·55	2	$Gly.Lys$
31A3E	0·18	10	$(Asx,Thr_2,Ser,Glx,Gly_2,Ala,Tyr,Lys)$
31C	5·0	7	$Ala.Ser.Ile.Leu.Ser.Val.Lys$
31D	0·15	4	$Ala(Gln,Ile)Lys$
31E	3·5	4	$Gly.Ile.Ala.Lys$
32A1	0·3	15—17	$Ala(His,Asx_{2-3},Ser,Glx_{2-3},Gly_2,Val_3,Tyr,Phe)Arg$
32A3	1·8	18	$Ala.Tyr.Val.Val.Asp(His,Asx_2,Ser,Glx_2,Gly,Val_2, Phe)Glx.Gly.Arg$
32B	0·35	5	$Ala(Ser,Ile,Leu,Lys)$
32C	1·8	3	$Ser.Val.Lys$
33D4	0·05	16—18	$Ala(His,Asx_{2-3},Ser,Glx_{2-3},Gly_3,Val_3,Tyr,Phe,Arg)$
33G	0·18	25—26	$(Asn_3,Thr_3,Ser_{5-6},Gln,Gly_3,Ala_3,Val_2,Ile_2,Leu_2,Phe)$
33H	0·18	18—20	$(His,His_2,Asx,Thr_{0-1},Ser_{1-2},Pro,Gly_3,Ala_2,Val,Ile_2, Leu_2,Lys)Tyr_{0-1}$
33I	0·18	29	$(His,Asp_2,Thr,Ser_4,Pro_2,Gly_5,Ala_4,Val_2,Ile_4,Leu_4)$
34B, 35B	7·5	4	$Ser.Asn.Asn.Arg.$
34G	0·05	3	(Pro,Ala,Trp)
35C	0·13	21	$(Asn,Asx,Ser_3,Gly_5,Ala_3,Met,Ile_3,Leu_2,Lys_2)$
35D2	0·1	10—12	$(Asx,Ser,Gly_{2-3},Ala_{1-2},Met,Ile,Leu,Tyr,Lys)$
35D4	1·13	2	$Ile.Lys$
35E3	0·1	10	$(Asn,Ser_2,Gln,Gly,Ala_3,Pro_2)$
35E5	0·55	2	(Pro,Trp)
36A1	0·25	49	$(Lys,His,Arg,Asx_7,Thr_3,Ser_5,Gly_6,Phe_2,Pro,Glx_6,Ala_5 Val_5,Ile_2,Leu,Tyr_3)$
36E1	0·08	13	$Ala(Asn,Ser_2,Gly_3,Ala,Met,Ile,Leu,Tyr,Lys)$

TABLE XI
(*Continued*)

Designation of peptides	Quantity isolated according to amino acid analysis μmol	Number of amino acid residues	Composition of fragments
36F2	0·08	11	Ala(Asn,Gly$_3$,Ala,Met,Ile,Leu,Tyr,Lys)
36F3	0·08	38—40	Ala(Arg,Asx$_4$,Thr$_2$,Ser$_{5-6}$,Glx$_2$,Gly$_7$,Ala$_{4-5}$,Val$_2$, Met,Ile$_3$,Leu$_3$,Phe,Lys$_2$)
36F4	0·13	37—41	Ala.Asx(Arg,Asx$_5$,Thr,Ser$_4$,Glx$_5$,Gly$_4$,Ala$_{4-5}$,Val$_5$, Met$_{0-1}$,Ile$_{1-2}$,Leu,Tyr$_{1-2}$,Phe$_2$,Lys)
37E	0·13	23—24	Ala(Asn$_3$,Thr,Ser$_{2-3}$,Gln$_2$,Gly$_4$,Ala$_3$,Val$_2$,Ile,Leu, Tyr,Phe,Lys)
37F	0·08	33	(Asx$_5$,Thr$_2$,Ser$_5$,Glx$_3$,Gly$_4$,Ala$_5$,Val$_2$,Ile$_2$,Leu$_2$,Phe$_2$, Lys)
37G	0·08	22	Lys(Asx$_2$,Thr,Ser$_3$,Glx,Gly$_3$,Pro,Ala$_3$,Val,Ile$_2$,Leu$_2$, Phe,Trp)
39A	2·50	33—35	(Asx$_5$,Thr$_2$,Ser$_6$,Glx$_{2-3}$,Gly$_3$,Ala$_{3-4}$,Val$_3$,Ile$_2$,Leu$_2$, Tyr$_{0-1}$,Phe$_2$,Trp,Lys)
42A1C1	0·05	13—14	(Asn,Ser$_2$,Gly$_2$,Ala$_2$,Met,Ile,Leu,Phe$_{0-1}$,Lys)
42A1C2	0·05	9	(Ser$_2$,Gly$_3$,Met,Leu,Tyr,Lys)
42A1D1	0·05	12—13	(Asx,Ser$_2$,Gly$_3$,Ala$_{1-2}$,Met,Ile,Leu,Tyr,Lys)
42A1D2	0·05	9	(Ser$_2$,Gly$_4$,Leu,Tyr,Lys)
42A1D3	0·05	7	(Ser,Gly$_3$,Leu,Tyr,Lys)
42A1E1	0·10	4	Ala(Asp,Ala,Ile)
42A1E2	0·10	2	Ile.Asn
42B1A	0·18	2	Gly.Lys
42B1C1	0·18	6	(Ala,Asn,Ser$_2$,Gly,Phe)
42B1C2	0·18	3	Ser.Asn.Phe
42B1C3	0·23	13	(Asn,Ser$_3$,Gly$_3$,Ala,Leu,Tyr,Phe$_2$,Lys)
42B1C4	0·05	8	(Ser,Thr,Gly$_3$,Ala,Ile,Lys)
42B1C6	0·23	3	(Gly,Phe,Lys)
42B2C1	0·05	8	(Phe,Asp,Ser$_2$,Gly,Ala,Phe,Lys)
42B2D1	0·15	8	Ala.Ser(Asx,Ser,Gly,Phe$_2$,Lys)
42C2D	0·05	11—12	(His,Thr$_2$,Ser,Gly$_2$,Ala,Val,Ile$_2$Tyr$_{0-1}$,Lys)
42C2E1	0·05	6	(Asx,Ser,Gly,Ala,Phe,Lys)
42C2E2	0·08	4	Gly(Ala,Ile,Lys)
42C2E3	0·08	5	Ile(Gly,Ala,Phe,Lys)
42C2F2	0·20	6	Thr.Ala.Gly(Ile,Tyr,Lys)
42D1A	0·20	2	His.Lys
42D1D	0·23	5	Gly(Ser$_2$,Gly,Leu)
42D2F	0·05	3	(Ser,Gly,Lys)
42E	0·05	4	Lys(Arg,Gly,Lys)

I (ref.[2])	II (present study)	Composition
04	18D1	Asx(Asx$_{2-3}$,Thr,Ser$_2$,Glx$_{2-3}$,Pro$_{0-1}$,Gly$_2$,Ala$_5$,Val$_2$, Ile,Leu,Phe,Lys)
		(peptide 04 is longer by proline and specifies the number of lysine and alanine residues in 18D1)
011	42C2F2	Thr.Ala.Gly(Ile,Tyr,Lys)
		(specifies threonine and tyrosine in 011)
016b	36E1	Ala(Asn,Ser$_2$,Gly$_3$,Ala,Met,Ile,Leu,Tyr,Lys)
		(specifies tyrosine in peptide 016b)

The histidine-abundant peptide 021 from the Ist hydrolysate is obviously derived from the same region of primary structure as peptide 33H from the IInd hydrolysate, also rich in histidine. These peptides, however, cannot be identical and were split off due to a different specificity.

Certain fragments, which are completely different in amino acid composition can be derived (with regard to the content of minority amino acids) from the same region of primary structure. This is the case of the following peptides:

I (ref.[2])	II (present study)	Composition
018		Phe(Ser,Ala)Ser.Asn.Phe.Gly.Lys
	42B1C1	(Ser,Ala,Ser,Asn,Phe,Gly)
	42B1C2	Ser.Asn.Phe
	42B1C6	(Phe,Gly,Lys)
	42C2E1	(Ala,Ser,Asx,Phe,Gly,Lys)

The final alignment of peptides in this region, however, is prevented by the existence of a peptide similar in amino acid composition, namely

42B2D1   Ala.Ser(Phe,Ser,Asx,Phe,Gly,Lys)

A fact deserving interest is that peptide 018 isolated from the Ist hydrolysate in a relatively large quantity was not isolated from the IInd hydrolysate at all or in a negligible quantity provided that unsequenced peptide 42B2C1 is identical with peptide 018 and not with peptide 42B2D1. This could be ascribed to autolysis since we found the subfragments mentioned above in the IInd hydrolysate.

On the other hand, we found large quantities of certain other peptides in the second hydrolysate, e.g.: peptide 22A, Leu.Ala.Ala.Leu.Glu.Asn.Leu.Asp.Gly.Pro.Ala.Ala. .Val.Thr.Lys which were not detected in the first hydrolysate. It is possible that these peptides are linked to long regions which in the first hydrolysate were contained in the insoluble core or in large radioactive peptides B, C (ref.[4]) and which were

present in autolyzed form in the second hydrolysate. On similar assumptions we cant also explain the fact that the following peptides from the first hydrolysate[2] were not found in the second hydrolysate: Dodecapeptides 02 and 08, dipeptide 013b, 25−28-residue peptides 016a and 017a, nonapeptide 017b, dipeptide 017c, 25-residue peptide 021, and tripeptide 022. Free basic amino acids liberated by specific tryptic hydrolysis and found in the first hydrolysate (05-Lys and 013a-Arg) were not included, together with some other free amino acids, into the population of peptides selected for detailed characterization. The differences in the specificity of cleavage observed with the two hydrolysates, whose causes have been discussed before, represent a valuable tool for arranging peptides into the primary structure of the alkaline protease since they permit us to link several peptides one to another.

Evaluation of Results Obtained

The evaluation of the tryptic digests is usually accompanied by balancing the numbers of amino acid residues. Such a balancing requires a complete population of peptides derived either from the intact protein or from defined fragments. In this study we started with a partially autolyzed protein from which some short fragments had been separated. We cannot therefore set together a complete balance from the present data. We can try, however, to evaluate the situation with respect to the first four amino acids (in order of minority), *i.e.* methionine, tryptophan, arginine, and histidine.

The methinone peptides can be divided into two groups which belong to nonoverlapping regions of the primary structure. The first group is related to the known sequence[4] around the active center, Gly.Thr.Ser.Met.Ala contained in all these peptides. These are the 20-residue peptide 17C3 overlapping in the region of the active center peptides 27F1, 36F3, and 36F4. Radioactive polypeptides B and C isolated elsewhere[4] also belong to this group; their content of basic amino acids was not analytically determined. All these peptides cannot be aligned with the second group of methionine peptides not containing threonine, *i.e.* peptides 35D2, 35C, 36E1, 36F2, 42A1C1, 42A1C2, and 42A1D1. The existence of these two groups of peptides which cannot be aligned with one another indicated the presence of two methionine residues in the molecule of the alkaline protease and stimulated the revision of the molecular weight[7] and amino acid composition[34] of the protease. These two groups are also important because they characterize the links between three cyanogen bromide fragments[38] of the alkaline protease and will be discussed later when being aligned.

The protease contains 3−4 tryptophan residues according to amino acid analysis[34]. Whereas no tryptophan-containing peptide was isolated from the first digest (since the oxidized protease was used to start with), the second tryptic digest contains two short tryptophan peptides 34G (Ala,Pro,Trp) and 35E (Pro,Trp), which probably

are derived from the same region of primary structure, and two other peptides, 37G and 39A, which according to their amino acid composition may contain the same tryptophan residue. Other tryptophan peptides were not found and should therefore occur in the low molecular weight portion of the autolysate.

From the amino acid composition of the protease[34] we can expect four arginine peptides in its digests. Tetrapeptides 24B3 and 34B = 35B cannot overlap and involve therefore two different arginine residues. These peptides cannot overlap octapeptide 32A1 = 32A3 = 33D4 which represents the third residue. Short arginine peptides 24B3 and 34B = 35B, however, can be reconciled with large arginine peptides 36A1, 36F3, and 36F4. Similarly, peptide 32A1 = 32A3 = 33D4 can be contained in peptide 36A1 and can overlap peptides 36F3 and 36F4. We isolated, however, a small quantity of a strongly basic tetrapeptide 42E, Lys(Gly,Lys,Arg), which has not been sequenced and which probably contains the fourth arginine residue.

The protease contains six histidine residues. Octapeptide 32A1 = 32A3 = 33D4 containing one histidine is derived from an autonomous region of the primary structure and cannot overlap dipeptide 42D1A. This small peptide, however, can be contained in undecapeptide 42C2D and together with the latter in the 49-residue peptide 36A1 containing one histidine. This peptide can partly overlap, however, the octa- or nonadecapeptide 33H containing three histidines and account for one of them similarly to octadecapeptide 32A1 = 32A3 = 33D4. This comparison shows that as yet we have found only three unambiguously nonoverlapping histidine peptides; as regards the remaining peptides we cannot decide whether they are overlapping or nonoverlapping and whether the peptides comprising all the histidine residues of the enzyme have been isolated or not. A discussion of other amino acids in order of their minority (Phe,Pro,Tyr) does not seem worthwhile in view of the incomplete sequential data.

The peptides isolated in this study can be complemented by a number of smaller peptides isolated and characterized during the treatment of the supernatant remainig after precipitation of the autolyzed protease by trichloroacetic acid. These sequential relations will be reported later. The peptides from the tryptic digest of the trichloroacetic acid precipitate of the partly autolyzed alkaline protease from *As. flavus* yielded a great deal of valuable information necessary for the elucidation of the primary structure of the enzyme. The contribution of these peptides will be discussed from this viewpoint in forthcoming papers.

REFERENCES

1. Nguyen bao Toan: *Thesis.* Czechoslovak Academy of Sciences, Prague 1970.
2. Nguyen bao Toan, Mikeš O., Turková J.: This Journal *43*, 1175 (1978).
3. Turková J., Mikeš O., Gančev K., Boublík M.: Biochem. Biophys. Acta *178*, 100 (1969).
4. Mikeš O., Turková J., Nguyen bao Toan, Šorm F.: Biochim. Biophys. Acta *178*, 112 (1969).
5. Mikeš O., Turková J.: *8th International Symposium on the Chemistry of Natural Products.* (*IUPAC*), *New Delhi 1972.* Abstract No D — 8, p. 216.
6. Šorm F., Turková J., Mikeš O., Hanus J.: Czech. 133 247, 1969.
7. Turková J., Mikeš O.: This Journal *37*, 1408 (1972).
8. Lowry O. H., Lopez J. A.: J. Biol. Chem. *162*, 421 (1946).
9. Prusík Z., Keil B.: This Journal *25*, 2049 (1960).
10. Mikeš O.: This Journal *22*, 831 (1957).
11. Walley S. G., Watson J.: Biochem. J. *55*, 328 (1953).
12. Levy A. L.: Nature (London) *174*, 126 (1954).
13. Nedkov P., Genov N.: Biochim. Biophys. Acta *127*, 544 (1966).
14. Novotný J., Franěk F.: Chem. Listy *62*, 995 (1968).
15. Deyl Z., Rosmus J.: J. Chromatogr. *20*, 514 (1965).
16. Edman P., Sjöquist J. S.: Acta Chem. Scand. *10*, 1507 (1956).
17. Grüner K.: Chem. Listy *64*, 1160 (1970).
18. Reindel F.: Hoppe H.: Ber. *87*, 1103 (1954).
19. Wieland T., Kawerau E.: Nature (London) *168*, 77 (1951).
20. Spackman D., Stein W. H., Moore S.: Anal. Chem. *30*, 1185 (1958).
21. Meloun B., Morávek L., Šorm F.: This Journal *32*, 1947 (1967).
22. Gray H. R., Hartley B. S.: Biochem. J. *89*, 379 (1963).
23. Sanger F.: Proc. Chem. Soc., London *1963*, 76.
24. Porter R. R., Sanger F.:: Biochem. J. *42*, 287 (1948).
25. Akabori S., Ohno K., Narita K.: Bull. Chem. Soc. Jap. *25*, 214 (1952).
26. Meloun B., Vaněček J., Šorm F.: This Journal *23*, 1788 (1958).
27. Gray W. R.: Methods Enzymol. *11*, 469 (1969).
28. Mikeš O., Holeyšovský V., Tomášek V., Šorm F.: This Journal *26*, 1048 (1961).
29. Anfinsen C. B., Aquist S. E. G., Cook J. P., Bjornson B.: J. Biol. Chem. *234*, 1119 (1959).
30. Kostka V., Meloun B., Vaněček J., Šorm F.: This Journal *27*, 882 (1962).
31. Anson M. L.: J. Gen. Physiol. *22*, 78 (1938).
32. Turková J., Mikeš O.: This Journal *36*, 2739 (1970).
33. Mikeš O., Turková J., Toan N. B., Šorm F.: 5th FEBS Meeting, Prague 1968, Abstr. No 905.
34. Mikeš O.: Int. J. Peptide Protein Res. *11*, 282 (1978).
35. Turková J., Mikeš O., Šorm F.: 5th FEBS Meeting, Prague 1968, Abstr. No 904.
36. Anfinsen Ch. B.: Developmental Biology Supplement *2*, 1 (1968).
37. Anfinsen Ch. B.: Science *181*, 223 (1973).
38. Nišanjan P. G., Mikeš O.: C. R. Acad. Bulg. Sci. *28*, 71 (1975).

Translated by V. Kostka.