ISOLATION AND STRUCTURE DETERMINATION OF THE PEPTIDES FROM THE CHYMOTRYPTIC HYDROLYSATE OF THE ALKALINE PROTEASE FROM *Aspergillus flavus**

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From the chymotryptic hydrolysate of 1·08 g of the precipitate obtained on treatment of alkaline protease from *Aspergillus flavus* with trichloroacetic acid 134 peptides were isolated by means of ion exchange chromatography, paper electrophoresis and paper chromatography. Among these 38 peptides containing 311 amino acids were isolated in amounts exceeding 0·50 μmol. The peptides were characterized by amino acid analysis, electric charge and also mostly by the terminal groups determination. In the case of peptides isolated in larger amounts the complete or at least the partial sequence of amino acids has been determined. In all peptides the total isolated amount in μmol was determined. The peptides containing basic amino acids were subfractionated with trypsin.

Alkaline protease from *Aspergillus flavus* belongs among mould serine enzymes the primary structures of which have not been resolved so far although these enzymes do not contain disulfide bridges. This fact facilitates the sequence analysis. In preceding papers we described preliminarily the isolation of peptides from cyanogen bromide fragmentation[2] of this enzyme, non-radioactive peptides[3] from partial hydrolysate of [^{32}P]-diisopropylphosphoryl derivative of the enzyme, left after the isolation of the readioactively labelled active centre[4], and the peptides from tryptic hydrolysate[5] of the enzyme denatured with trichloroacetic acid.

In this paper we describe the isolation, characterisation and sequence analysis of the peptides from the chymotryptic hydrolysate of the trichloroacetic acid precipitate of the enzyme. The main results of the sequence analysis of the peptides from all three hydrolysates, including the chymotryptic one (ref.[3,5] and this paper) have been preliminarily announced earlier[6].

* This study contains part of the thesis presented by Nguyen bao Toan[1] (in fulfilment of his CSc degree).

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EXPERIMENTAL

Material

The starting alkaline protease from *As. flavus* was of the same origin as indicated in the preceding paper[5] and it was also worked up and stored at −20°C in an analogous manner. All the chemicals were supplied by Lachema (Brno, Czechoslovakia) in an analytically pure state. The same firm also supplied all chromatographic materials, except the silica gel plates Silufol, which were supplied by Kavalier (Votice, Cezchoslovakia).

Methods

Trichloroacetic acid precipitation: The protease which was partly damaged by autolysis during storage (*cf.* a detailed discussion in ref.[5]) was precipitated with trichloroacetic acid in the manner described[5]. From 2·08 g of the starting freeze-dried protease about 1·09 g of the denatured precipitate was obtained after extraction of the trichloroacetic acid and drying.

Chymotryptic hydrolysis: This operation was carried out in an autotitrator at pH 8·5, using a technique described earlier[5]. 1·08 g of the precipitate was hydrolysed with 15 mg of chymotrypsin. Further chymotrypsin (7·5 mg) was added after one hour's treatment. The last addition (7·5 mg) was made after 30 min, but it did not produce any further hydrolysis. Thus the total molar ratio of enzyme to substrate increased up to 1 : 30. The digestion was stopped by acidification with formic acid to pH 2·9, the solution was freed of a small amount of turbidity by centrifuging and pumped without concentration directly onto the ion exchanger column.

Ion exchange chromatography on Dowex 50 (Fig. 1): The combined groups of coinciding fractions C 1 to C 49 were concentrated on a rotatory evaporator. The volatile buffer was eliminated by repeated evaporation after the addition of water. The fractions were dried in a vacuum desiccator over solid sodium hydroxide and concentrated sulfuric acid. They were stored under vacuum in the presence of sodium hydroxide. Three of the combined fractions (C2, C3 and C44) did not contain a workable amount of peptides and they were discarded. The remaining combined fractions were submitted to further separation on paper.

Preparative paper electrophoresis and chromatography: Further fractionation of the combined fractions C1—C49 was carried out using known[5] methods of high-voltage paper electrophoresis E_1 at pH 1·9, descending electrophoresis E_2 at pH 5·6, and paper chromatography in 1-butanol–pyridine–acetic acid–water 30 : 20 : 6 : 24 (system S_1), see Table II in ref.[5]. The method of labelling fractions is also based on the method used in this reference. A survey of the fractionations carried out is given in Table I in which the subfractions obtained in excessively low amounts are not indicated. They were labelled, however, in the same manner during the working up procedure.

The control analyses and the final testing of homogeneity of the isolated peptides as well as their characterization were carried out as in ref.[5]. The amino acid analyses, N-terminal amino acids, and the electric charges of the peptides isolated are surveyed in Table II. The sequence analysis of the isolated peptides, their further subfragmentation (by enzymic and partial acid hydrolyses), separation, and the characterization of the subfragments were carried out according to ref.[5]. When larger amounts were available the structure was determined by manual Edman's method, while with small amounts the DANSYL-Edman method was used. The analyses and the characterizations of the subfragments are summarized in Table III.

TABLE I

Survey of the Separation of Combined Fractions of Chymotryptic Hydrolysate of the TCA-Precipitate of Alkaline Protease from *As. flavus* after Ion Exchange Chromatography

C-Labelling of the combined fractions after ion exchange chromatography (Fig. 1); S_1 (paper chromatography), E_1 (high voltage electrophoresis), E_2 (descending electrophoresis) are the separation methods described in the text[5] and Table II, ref.[5]; the numbers in the columns under them give the order of the methods used; P working label of the isolated peptides.

C	S_1	E_1	E_2	P	C	S_1	E_1	E_2	P	C	S_1	E_1	E_2	P
C1	2		1	C1B1	C8	1	2		C8B1	C15			1	C15E
C1			1	C1C	C8	1	2		C8B2	C16	2		1	C16B6
C4	2		1	C4B1	C8	1		2	C8E2	C16	2		1	C16C2
C4	2		1	C4C1	C9	1			C9A	C16		2	1	C16D1
C5	1		2	C5A2	C9	1			C9B	C16		2	1	C16D2
C5	1		2	C5A3	C9	1	2		C9D1	C17	1			C17B
C5	1	2		C5D1	C10	1			C10A	C18		1	2	C18A2
C5	1		2	C5E2	C10	1	2		C10B1	C18	2	1		C18B1
C6	1		2	C6A1	C10	1	2		C10B2	C18	2	1		C18B2
C6	1		2	C6A2	C10	1	2		C10B3	C18	2	1		C18B6
C6	1		2	C6E1	C11	1			C11A	C18	2	1		C18B7
C6	1		2	C6E2	C11	1	2		C11B1	C18	2	1		C18B8
C6	1		2	C6E3	C11	1			C11C	C18	2	1		C18C1
C7	1	2		C7A1	C12			1	C12B	C18	2	1		C18C3
C7	1	2		C7A2	C14	1,2			C14D4	C18	2	1		C18C5
C7	1	2		C7A3	C15	2		1	C15B2	C18	2	1		C18C6
C7	1			C7B	C15			1	C15C	C18		1		C18D
C19	1			C19D	C29	1	2		C29C2	C37	1			C37A
C21	1		2	C21A1	C29	1		2	C29D2	C37	1		2	C37B
C21	1		2	C21C2	C30	1		2	C30A1	C37	1		2	C37C1
C22		2	1	C22A1	C30	1			C30C	C37	1		2	C37C2
C22			1	C22B	C31			1	C31B	C37	1			C37E
C23	1			C23A	C32	2		1	C32B4	C37	1	3	2	C37G1A
C23	1			C23C	C32	2		1	C32C6	C38	1,2			C38A1
C23	1			C23E	C33	2		1	C33A1	C38	1,2			C38A2
C24	1			C24A	C33	2		1	C33A2	C38	1	2		C38B1
C24	1	2		C24C1	C33	2			C33B3	C38	1	2		C38B2
C24	1	2		C24C2	C33	2		1	C33B4	C39	1			C39A
C24	1	2		C24D1	C33	2		1	C33D1	C39	1	2		C39C1
C25	2		1	C25A8	C33	2		1	C33D3	C40	2		1	C40A2
C25	2		1	C25A9	C34	2		1	C34A3	C40	2		1	C40A3
C25			1	C25C	C34	2		1	C34B5	C41	1			C41C
C27	1	2		C27C1	C35			1	C35A	C42	1	2		C42A1
C28		2	1	C28A1	C35			1	C35B	C42	1	1,2		C42C1A
C28		2	1	C28A2	C35	2		1	C35C6	C43	1			C43A
C28		2	1	C28A3	C35			1	C35E	C43	1			C43C
C28		2	1	C28A4	C36	2		1	C36A2	C45	1		2	C45A1

TABLE I
(Continued)

C	S_1	E_1	E_2	P	C	S_1	E_1	E_2	P	C	S_1	E_1	E_2	P
C28		2	1	C28A5	C36	2		1	C36B1	C45	1			C45D
C28		2	1	C28A6	C36	2		1	C36B6	C46	1,2			C46B2
C28		2	1	C28B1	C36	2		1	C36C1	C47	1	2		C47B1
C28			1	C28C	C36			1	C36E	C49	1	2		C49A1
C49	1	2	3	C49A2A	C49	1		2	C49C2	C49	1	2,3	4	C49E1B2
C49	1	2	3	C49B1A	C49	1	3	2	C49C3A	C49	1	3	2	C49E2B
C49	1,2		3	C49B1C	C49	1	3	2	C49C3B	C49	1	3	2	C49E2C
C49	1,2		3	C49B1D	C49	1	3	2	C49D1B	C49	1	3	2	C49E2D
C49	1,2			C49B4	C49	1	3	2	C49D1C	C49	1	3	2	C49E3A
C49	1,2		3	C49B5A	C49	1	3	2	C49D1D	C49	1	2		C49E4
C49	1,2		3	C49B5B	C49	1	3	2	C49D1E	C49	1		2	C49F1
C49	1		2	C49C1	C49	1	2,3	4	C49E1A3					

RESULTS

Sequence Analysis of Individual Peptide Fractions

Individual peptides were isolated from the chymotryptic hydrolysate in very different amounts, determining the possible depth of their sequence analysis. Some of them were obtained in such small amounts that they could be characterized merely by their amino acid analysis or, in some cases, maximally by the terminal amino group determination (Table II). These peptides are given in the table of results (Table IV) without further comment. The isolated amounts were calculated from amino acid analyses. The structures of some peptides obtained in larger amounts were in some cases not resolved owing to their evident identity with the peptides already sequenced in other fractions, given in Table IV.

If the amount of some of the isolated peptides was higher the sequence of all or at least part of the amino acids could be done by gradual degradation as the sole approach to their sequence analysis. The results are again presented directly in Table IV. In this manner the structures of the following fractions were studied:

C5A3	C11C	C18D	C29C2	C37G1A
C6E3	C15B2	C23A	C30A1	C38B2
C7A2	C15C	C24A	C31B	C39A
C7B	C16B6	C24C1	C33D3	C45A1
C9A	C16D1	C25C	C36B6	C49B1D
C10A	C18B8	C28C	C36E	C49E3A

In the case of the decapeptide C25C the manual Edman degradation of 1 μmol afforded the first four steps normal and pure, the 5th step was almost void, with

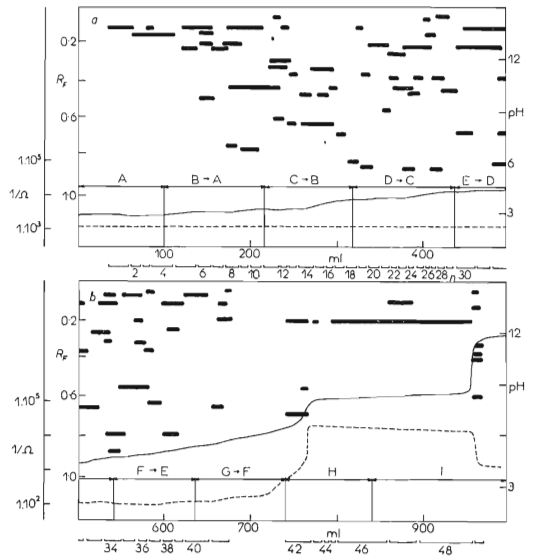

FIG. 1

Chromatography of Chymotrypsin Hydrolysate of Denatured Alkaline Protease on a Column of Dowex 50X2, 200–400 mesh (66 × 4·5 cm)

The solution of hydrolysate was pumped without concentration onto a column equilibrated in buffer A. For the volatile pyridine–formate and pyridine–acetate buffers used see ref.[5] (Table I). The elution of the first part (buffer A) and the last two parts (buffers H, I) of the chromatogram was carried out isocratically. In the central parts a system of linear gradients was employed; mixed buffers are indicated by arrows. The fractions (15 ml, 20 min) were collected right from the beginning of the sample application. 0·3 ml aliquots of each second fraction were evaporated *in vacuo* and tested by paper chromatography using ninhydrin for detection and 1-butanol–pyridine–acetic acid–water (S_1, Table II, ref.[5]) for development. The positions of the spots are indicated by thick horizontal lines in the upper part of the figure, the conductivity of the fractions (mho = $1/\Omega$) is indicated with a broken line, pH is indicated with a thin full line, *n* is the number of the fraction. The numbered segments under the horizontal line indicate the combination of the fractions. These materials, 1–49, are indicated in further documentation as C1–C49.

a small amount of glycin, the 6th and the 7th steps displayed a considerable heterogeneity, while the 8th and the 9th steps were again normal. The degradation can be expressed by the following survey:

Step	1.	2.	3.	4.	5.	6.	7.	8.	9.
Amino acid	Val.	Val.	Asp.	Ser.	X	Gly.	Asn.	Val.	Asp.....
					(Gly)	(Val)	(Val)		

The resulting sequence in Table IV.

Val.Val.Asp.Ser.Gly.Val.Asn.Val.Asp.(His,Glx,Glx)Phe was formulated with respect to the amino acid analysis of C25C and also in relation to the sequence analysis of other peptides from the same section of the primary structure. The sole Gly in the 6th step was considered as the unreacted residue from the 5th step, the amino acid in the 6th place is valine which also reacted only partly and passed into the 7th step, where the amino acid proper is asparagine. The reasons for a faulty course of the Edman degradation in some steps – also observed in some other cases – are not known.

The peptides obtained in still higher amount than in the above-mentioned group could be submitted to sequence analysis by combination of several methods, enabling thus the comparison of partial results. The methods of solving their structures and the results obtained are described below.

Do- to Tridecapeptides C1B1 *and* C4B1

The manual sequencing with Edman's method afforded a similar result in both fractions. In individual steps the following amino acids were found:

Step	1.	2.	3.	4.	5.	6.	7.	8.
C1B1	Ser.	Ser.	Val.	Ala.	Ala.	Ala.	Gly.	Asn.....
		(Val)	(Ala)			(Gly)	(Asn)	(Glu)
C4B1	Ser.	Ser.	Val.	Ala.	Ala.	Ala.	Gly.	.....
		(Val)				(Gly)	(Asn)	

It seems that we were concerned with a mixture of two components of which one was by one N-terminal serine shorter. The amount of isoleucine in fraction C4B1 did not increase even after prolonged hydrolysis; it probably belongs to one of the mixture components. This fraction afforded a spot at the level of proline by hydrazinolysis and paper chromatography, which was not observed in the case of fractions C1B1 and C8E2 which have a similar composition. 3·13 μmol of fraction C4B1 were submitted to combined partial hydrolysis and fractionation by descending

TABLE II

Characterization of the Peptides Isolated from Chymotryptic Hydrolysate of the TCA-precipitate of Alkaline Protease from *As. flavus*

The hydrolyses for amino acid analysis were carried out in 6M-HCl at 110°C for 20 h. When more valine residues were present the hydrolysis took up to 72 h. The charge of the peptide was determined electrophoretically in the system E_2 (Table II, ref.[5]). Tryptophan was determined qualitatively only[5]. The peptides do not contain Cys.

Symbol of peptide	Quantitative analysis (in number of residues)																Number of residues	Charge	Terminal amino acid	
	Lys	His	Arg	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp			N	C
C1B1				1·87	0·90	1·76	1·19	1·23	0·94	3·41	1·19		0·79				—	13—14	—	Ser	
C1C				3·75	2·18	5·21	3·26	1·00	2·97	4·60	1·93		1·04	0·74			—	27	—	Asx	Ala
C4B1				2·11	1·03	1·94	1·03	1·00	1·03	3·05	1·20		0·50				—	12—13	—	Ser	Pro
C4C1	0·03			1·90	0·06	0·21	0·10	tr.	1·00	1·15	0·06	tr.	tr.	tr.			—	4	—		Ala
C5A2	0·87			0·17	1·12	2·78	1·96		1·12								—	8	—		
C5A3				2·00					1·04	1·04							—	4	—		
C5D1				0·27	2·06	2·00	0·27		1·13	0·68		0·94	1·26				—	7—8	0	Ser	
C5E2				2·71	1·17	0·63	1·17	1·04	1·17	3·70	0·95	tr.	0·17	1·67			—	14—15	—	Ala	
C6A1	0·90			0·20	1·15	3·00	2·15		1·10	0·15							—	8	—		
C6A2				3·01			0·97		0·10	1·09	1·00						—	6	—	Asx	
C6E1				0·28	1·00	1·00	0·28		1·00	1·00	0·14	0·14	0·50	0·07			—	4—5	0		
C6E2				3·17	1·74	0·96	0·01	1·74	0·96	5·00	1·82		0·91	2·26			—	20	—		
C6E3				2·20	0·50	tr.	0·98	1·00	0·95	3·79	1·02			2·24			—	13—15	—	Ala	
C7A1				1·24	1·07	2·52	0·20		0·97	0·97			0·86	0·07			—	8	0		
C7A2				0·10	1·98		1·00										—	3	0	Thr	
C7B				1·04	1·04	2·82	tr.		0·96	1·07			0·96				—	8	0	Ile	
C8E2				1·49	0·93	0·93	1·24	0·67	1·02	2·85	0·91		0·02	2·44			—	12—14	—	Leu	
C9A	0·03			1·88	0·04	0·10	0·06		1·06	1·09							—	4	0	Asp	Ala
C9B				1·18	0·20	0·30	0·30		0·76	1·09	0·09		0·09				—	3	0	Gly	
C10A	0·02			1·99	0·04	0·10	0·06		1·04	0·97							—	4	0	Asp	Ala
C10B1				1·12	3·24	2·00	3·24		2·12	2·00			1·12	2·12			—	16			
C10B2	0·08			2·00	1·83	2·20	2·00	1·00	1·96	1·96		0·08	1·54	2·24			—	17			

C11A	1·09		6·04	2·81	5·38	3·09	2·95	3·05	8·67	2·18		1·43							—	36	—								
C11B1	0·10		0·79	0·89	0·93	0·75		1·25	1·14	0·20									—	6	0								
C11C			0·42	0·10	0·31	0·96		0·20	2·02	0·04		1·13	0·10						—	4	0	Ala							
C12B			1·03	1·94	0·98	1·04		1·98	1·05	0·04	tr.	0·02	0·07	0·85					—	9		Asx	Tyr						
C14D4			0·05		0·06	0·05		1·24		0·84		0·74	1·19						—	4	0	?	Leu						
C15B2			1·04	0·02	0·04	0·96	1·00	1·04	1·05			0·91	1·00						—	7	—	Ala	Leu						
C15C	0·04		0·04	0·16	0·32	1·94		1·18	0·36	0·88		0·18	1·12		0·06				—	5	—	Glx	Leu						
C15E			1·01	0·03	0·09	0·20		1·07	0·12	0·12		0·03	0·11		0·93				—	3	—	Asx	Phe						
C16B6			1·02	0·01	0·02	1·04	0·93	1·04	1·02	0·03		0·98	0·97						—	7	—	Ala							
C16C2	0·05		0·39	0·05	0·11	1·96		1·16	0·29	0·93		0·20	1·11		0·06				—	5	—								
C16D1			2·89			3·05		1·00	2·09	2·00	tr.		1·09		1·08				—	13	—	Asx	Leu						
C16D2			3·00		0·03	2·92		1·02	2·00	2·02			1·04		1·00				—	13	—	Asx	Leu						
C18A2			2·09	0·53		1·20	1·18	1·12	4·92	0·97		0·11	2·92	0·88					—	16	—								
C18B1	0·37	0·16	1·72	0·95	2·00	1·14	1·00	1·00	2·62	0·86		0·37		0·08	0·08				—	11—12									
C18B2	0·25		2·00	0·56	0·56	1·62	0·93	1·25	2·50	1·06		0·37		0·25	0·19				—	10—12									
C18B6	0·24		2·80	1·13	2·20	1·75	tr.	2·12	2·87	0·88		0·88	1·25	0·24	1·13				—	17									
C18B7			2·00	0·60	0·40	1·70	tr.	2·00	2·50	1·00		1·10	2·90		0·60				—	14—17									
C18B8			0·98	0·20	0·25	0·98	1·00	1·10	1·05	0·05		1·00	2·97						—	9									
C18C1	0·80		2·30	0·50	0·60	1·20	tr.	1·30	3·10	0·90	tr.	0·80							—	12									
C18C5	0·71		5·70	1·72	2·15	2·15	0·86	2·72	8·00	1·86	1·00	1·43	3·57		1·14				—	33—35									
C18C6			2·15	1·00	1·72	1·29	tr.	3·15	3·29			2·29	3·15						—	17									
C18D			0·07	tr.	0·89			0·09	1·10		tr.	0·99	1·07						—	4	0	Ala	Leu						
C19D			0·05		0·13	0·05		1·75	0·10	1·14		1·08	2·08						—	6	0	Ile	Leu						
C21A1		0·95		2·81	2·05		1·14	1·24	1·05		0·91	1·05							—	11	+	Thr							
C21C2	0·83		1·92	1·08	0·33	1·08	1·17	1·25	3·32	1·00		2·00							—	13	—	Ala							
C22A1	0·11		1·12	0·11	1·00	0·19		0·19	0·50		tr.	0·23	0·30		0·85				—	3	0								
C22B	0·96		2·08	1·08	0·46	1·04	0·92	1·17	3·96	0·92	0·08	0·12	1·96	0·12	0·08				—	14	—	Ala	Lys						
C23A	0·92		2·20		1·04	0·06		0·14	2·00	0·75		0·76	0·06		0·09				—	8	0	Ala	Lys						
C23C			1·10		0·83	0·02		0·06	0·06			0·01	0·02		1·10				—	3	0	Ser	Phe						
C23E								0·65		0·22		0·17	3·00						—	2—3	0	Leu	Leu						
C24A	1·08		2·30		1·08				2·21	0·63		0·70							—	8	0	Ala	Lys						
C24C1	1·15		2·16	1·00	0·15	1·00	0·96	1·08	3·78	1·00			3·00		0·10				—	15	0	Leu	Lys						
C24C2	0·25		2·80	0·25	1·95	0·30		0·40	1·05	0·25			0·80		2·15				—	9	0								

TABLE II
(*Continued*)

Symbol of peptide	Quantitative analysis (in numbers of residues)																Number of residues	Charge	Terminal amino acid	
	Lys	His	Arg	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp		N	C
C24D1													0·003*	0·049*			—	2—3	0	Leu	
C25A8				0·80	1·00	1·20	1·40		2·00	3·60			0·80	1·00		1·20	—	13	0		
C25A9	0·09			0·13	0·09	0·26	0·26		2·08	0·22	1·04		0·78	4·09			—	8	0		
C25C		0·98		2·98		0·96	2·00		1·10		4·00				0·07	1·07	—	13	—		
C27Cl							2·04		1·08	1·00	1·04			1·00		1·00	—	7	—		
C28A2	0·22	0·22			0·89	2·30	0·56		4·67	2·00	0·44	1·00	1·00	1·89	0·32		—	13—17	0		
C28A4	2·00			4·22		2·11	0·22		2·00	3·78	1·11	0·22	1·11	1·67			—	18	0	Ala	
C28A5	1·55			2·82		1·09	tr.	1·27	1·18		2·09						—	10—11	0		
C28B1				0·33	0·18	0·29	2·19		1·28	1·19	0·95			0·81		0·76	—	7	—	Ala	
C28C				1·17	1·94	1·06	1·08		2·02	1·09			0·96	0·04	1·79	0·06	—	11	—	Ile	Tyr
C29C2	0·05			0·10	2·04	0·27	1·04		2·78	0·15			0·10	1·02	0·41		—	8	0	Gly	Tyr
C29D2				1·00	1·85	1·13	0·96		2·06	1·17			1·02		1·96		—	11	—		Tyr
C30A1		0·89			0·99	0·05		1·02	0·08	1·18							—	4	+	Ala	
C30C				0·07	0·07	0·07	0·05		0·10	1·02			0·04	0·05	1·00		—	2		Ala	Tyr
C31B	0·04	0·72		0·08	0·10	2·18	0·06		1·10	0·08			1·17		0·04		—	5	+		Tyr
C32B4	1·24			tr.	1·09	2·52	0·19		1·00	0·86			0·86				—	7—8	+	Ala	
C32C6				0·11			0·11		0·28	4·06			0·17	1·16		0·67	—	6	0	Ala	
C33A1	1·04			0·04	0·96	1·02			0·11	0·13							—	3	+		
C33A2	1·89	0·22		0·11	2·17	0·89	0·17		0·45	1·22							—	6—7	+		
C33B3	2·90			0·60	1·00	1·70	1·90		1·10	4·10	0·30		2·00				—	15—16	+	Ala	
C33B4	1·12			0·37	1·37	0·88	0·96		1·12	1·25	0·25		0·79	0·54			—	7—8	+		
C33D1	0·77		2·00	1·15	0·62	1·23	2·69		3·15	0·92	0·46		0·38	0·15	0·15			11—14	0		
C33D3						1·30			5·15					1·94	0·19		—	8	0	Ser	Tyr

* Data given in micromoles.

C34A3	1·82	0·23		0·32	3·00	2·23	0·41			2·36	0·91			2·00	0·32			–	12	+					
C34B5						0·93				0·03					1·22		0·85	–	3	0	Ser	Tyr			
C35A	1·00			0·17	0·10	1·03	0·10			0·26	0·13							–	2	+	Ser				
C35B	2·00			2·94	0·40	1·73	0·50			0·87	1·00	0·33		0·94				–	10	+					
C35C6		1·06				1·94				1·97				1·06	0·97			–	7	+	Gly	His			
C35E	1·10			2·88	0·12	1·02	2·88			1·14	3·03	1·93		0·08	1·05	0·10	1·98	–	17	–	Ser				
C36A2	0·10			0·02	0·01	0·05	0·04			0·89	1·08			1·10	0·01			–	3–4	+	Gly	Lys			
C36B1	2·03			2·26	0·10	1·08				0·10	2·23	0·69	0·10	0·79				–	9	+	Ala	Lys			
C36B6	0·10	1·00				1·97				1·97	0·10			1·13	1·10			–	7	+	Gly	His			
C36C1	2·40			3·20	1·08	1·28	0·40	0·88		1·24	1·16	2·12						–	12–14	+					
C36E				1·84		0·02	0·03			1·04	0·04					0·97	+	–	5	–	Asx	Trp			
C37A	2·45			3·10	1·10	1·49	0·33	0·84		1·40	1·21	1·90	tr.	0·25				–	12–14	+	Ala				
C37B	0·85	1·38		2·74	0·50	1·41	1·32			2·60	2·06	1·03		0·91				–	14–16	+					
C37C1	2·04			2·15		0·98	0·10			0·37	2·10	0·75		0·63				–	9	+	Ala	Lys			
C37C2	0·33	1·57		2·10	tr.	0·97	0·90			2·77	2·12	1·03		1·06				–	13	+		His			
C37E	3·00		0·20	2·68	1·53	2·13	0·80			2·40	4·80	0·87	1·07	3·14	0·60		0·27	–	23–24	0	Gly				
C37G1A	1·08			0·93	0·03	0·06	0·08			0·90	0·06	2·23	0·02	0·04	0·05		0·90	–	6	0	Gly	Phe			
C38A1	1·01			1·01	0·97	0·95	2·00			1·01						0·88		–	8		Lys	Tyr			
C38A2	0·77	0·34		1·16	0·96	1·00	2·00			1·16	0·02	0·01				0·55		–	8–9						
C38B1		1·07		0·10	1·80	0·15	tr.	1·00		2·06	0·87	1·13		0·87	1·67			–	11	+	Ala				
C38B2		1·44			1·85			1·74		2·04	1·85	1·04		1·04	1·93			–	14	+	Ala				
C39A	1·05			1·12	1·02	1·00	2·02			1·00						0·83		–	8	+	Lys				
C39C1	1·10			1·30		0·80	0·30			0·60	2·20			1·00				–	6–7	0					
C40A2	2·85			3·08	0·77	0·93		1·08		1·15	1·08	2·93						–	14	+	Ala	Pro			
C40A3	2·60	0·28		3·08	1·04	1·20	0·28	1·00		1·40	0·92	2·32		0·20	0·08			–	13–14	+					
C41C	3·25		0·25	4·05	1·10	1·95	0·80	1·05		2·40	2·30	2·45	0·10	0·60	0·95	0·15		–	19–22	0					
C42A1	2·16	0·48		3·24	1·12	1·52	0·40	1·12		1·88	1·36	2·60		0·28	1·96	0·08	0·16		16–19						
C42C1A				0·07		1·07					0·93							+	3	0	Ser	Trp			
C43A	0·86	0·29	0·86	1·43	1·00	1·86	2·58			2·28	1·14							–	11–13	0					
C43C	0·20			2·00						1·80	1·20	1·60		1·00	1·00			+	9–10	0					
C45A1		3·02		2·03	0·99	0·99	0·97			3·70	2·18	1·00		1·01				–	16	+	Asx	His			
C45D	1·00		0·50	2·00	1·17	2·17	1·50			3·00	2·00	1·17		1·17	1·17		0·84	+	17–19	0					
C46B2	2·83		0·10	3·14	1·21	1·52	0·52	1·07		1·97	1·58	2·45		0·27	1·79	0·10	0·17	–	17–18	+					

TABLE II
(*Continued*)

Symbol of peptide	Quantitative analysis (in numbers of residues)																Number of residues	Charge	Terminal amino acid	
	Lys	His	Arg	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp			N	C
C47B1	0·09	2·55		1·19	1·02	1·03	1·05		4·00	2·02	0·97		1·00				—	15	+	Ala	
C49A1	3·70		1·00	4·30	1·67	4·30	2·00		4·00	3·70							—	25—26	0		
C49A2A	1·13		1·00	0·26	0·09	1·04	0·83		1·04	1·00							—	6	+		
C49B1A	1·28		1·08	0·33	0·10	1·11	0·89		1·17	2·17			0·16		0·75	0·09	—	8	+		
C49B1C	3·74	0·67	2·40	2·94	1·00	3·87	1·93		3·20	5·40	0·73		1·33	0·33	1·07	0·87	—	29—30	+		
C49B1D	0·18		0·89	0·20	0·08	0·97	0·89		1·11	1·16			0·11				—	5	0		
C49B4	1·70		0·97	1·18	1·34	2·04	1·04		3·10	3·24	0·73		1·23	0·27	0·27	0·27	—	16	0		
C49B5A	0·93					0·12	0·08		0·12	1·17					0·87		—	3	+	Lys	
C49B5B	0·86			0·21	tr.	0·43	0·29		0·93	2·14					1·14		—	5	0		
C49C1	2·84	0·83	1·00	0·67	1·67	2·17	1·33		3·00	2·50			2·17	1·16			—	19—20	+		
C49C2	1·93	0·24	0·81	1·11	1·44	2·03	1·10	0·75	1·10	2·30	0·30	0·06	0·67	0·37	0·45	0·46	+	14—17	+		
C49C3A	2·13		2·05	4·13	1·32	4·36	1·05	0·73	1·55	3·86	0·91		0·95	0·95	0·50	1·73	—	25—27	0		
C49D1B	1·00			0·40		1·20			0·80	1·20						0·80	+	6	+		
C49D1C	1·08		0·07	0·06	0·06	0·91	0·06	0·25	0·12	1·21			0·07	0·06	0·25	0·81	+	5	+	Phe	
C49D1D	1·00		0·08			0·84			0·11	1·18					0·08	1·00	—	4	+	Ser	
C49D1E	1·95					0·95			0·24	2·81					1·14	1·28	—	8	+	Lys	
C49E1A3				2·11	1·00	0·10	1·00	1·11	1·19	3·81	1·08			3·12			—	14	—		
C49E1B2	0·87				2·00	0·96	0·06		2·04	1·05	1·04		1·04		0·80		—	10	+		
C49E2B	2·00		2·14		0·53	3·20	0·87		0·87	3·94			1·33	1·07		2·33	—	17—18	+	Lys	
C49E2C	1·12		1·41			0·83	0·96		0·17	1·08			1·00	1·08		0·92	—	8	+		Leu
C49E2D	0·95		0·80			0·20	1·10		0·20	0·25			1·05	1·25		0·20	—	5	+		
C49E3A	0·86				1·86	1·07			2·10	1·21	1·03		1·10		0·75		—	10	+		
C49E4	1·16		0·89			0·16	1·05		0·10	0·16			0·89	1·16			—	5	+	Arg	Leu
C49F1	2·96					2·52	0·17		0·22	1·04	4·04		0·87	1·04		2·08	—	15	+	Lys	

electrophoresis E_2, affording 8 subfractions of which the last two, C4B1a7 and C4B1a8, were free glutamic and aspartic acids, respectively. The characterization of acid subfractions C4B1a2 to −a6 is given in Table III and the results of the sequence analysis of fraction C4B1 are summarized in Table V. A small amount of serine in subfractions −a4 and −a5 could probably be explained by the presence of a smaller amount of this amino acid in the blank from paper, but the content of serine in −6a suggests an alternative interpretation, *i.e.* that serine might be present in the section of the three alanine residues, where it could be hardly detected owing to the cumulation of this amino acid during the Edman degradation of the mixture of peptides of similar composition.

Hexapeptide C19D

The sequencing by the manual Edman's method, started with 1·5 μmol, afforded isoleucine in the 1st step, but did not afford any amino acid in the 2nd and the 3rd steps. Therefore 1 μmol was taken for a combined partial hydrolysis, which after paper electrophoresis E_1 afforded 3 zones, C19Da1 to −a3 (Table III). The subfraction C19Aa1 was evidently the unchanged starting peptide. It was sequenced by the DANSYL–Edman method to the 2nd step. On hydrazinolysis the subfragment −a2 afforded glycine and the subfragment −a3 set free leucine only on hydrolysis. The combination of these results into the final structure is given in Table V.

Peptides C23E *and* C24D1

The neutral fraction C23E was not completely homogeneous and it did not separate on high-voltage electrophoresis E_1. During the preliminary chromatography of a small amount of it in S_1 it gave a double spot in an about 1/4 : 1 ratio of the ninhydrin coloration, which, however, merged into one spot in preparative separation. The N-terminal group of the preparation – leucine – was single. Since in hydrazinolysis the preparation gave in addition to a large amount of leucine also a smaller amount of glycine, we consider the fraction to be a mixture of a smaller amount of an undefined glycine peptide with a main amount of a leucine oligomer which differs in its R_F value substantially from leucine (it is much more hydrophobic). According to the analysis (Table II) the neutral fraction C24D1 is substantially more homogeneous; in high-voltage electrophoresis it is above all free amino acids and therefore it cannot be identical with leucine from which it also differs substantially chromatographically. We failed to identify whether it is dileucine or trileucine.

Lysine and Arginine Peptides

In the chymotrypsin hydrolysate of alkaline protease a number of peptides were found which contained one lysine or arginine residue not at the C-terminus, or several

TABLE III

Characterization of Subfragments Isolated from Partial Hydrolysates of Peptides Prepared from Chymotryptic Hydrolysate of Alkaline Protease from *As. flavus*

Subfragmentation by partial acid hydrolysis is indicated by letter a, hydrolysis with chymotrypsin by letter c and with trypsin by letter t after the symbol of the starting peptide. The hydrolyses for amino acid analysis were carried out in 6M-HCl in an evacuated sealed tube for 20 h at 110°C. None of the analysed subfragments contained cysteine methionine or tryptophan.

Symbol of sub-fragments	Quantitative analysis (in numbers of residues)														Number of residues	Charge of the peptide	N-terminal amino acid
	Lys	His	Arg	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe			
C4B1a2				2·43	0·79	1·95	0·79	1·42	0·89	3·00	0·95	0·69				13	—	
C4B1a3				2·42	0·67	1·59	1·41	0·92	1·34	2·77	0·92	0·25				12	—	
C4B1a4				1·00	0·26	0·63	0·84		0·69	0·95	0·37	0·21	0·26			5	—	
C4B1a5				0·61	0·09	0·37	0·73		0·84	0·49	0·23					3	—	
C4B1a6				2·30	0·24	1·27	0·80	0·31	0·67	0·91	0·16					6	—	
C11At1	0·90			4·05	1·94	3·80	2·22	2·00	2·11	0·70	1·16	1·00				20		
C18Da5				0·03	0·01	0·25	0·04			0·24	0·02	0·93	1·08			2		
C19Da1				0·08	0·08	0·26	0·16		1·89	0·13	1·12	1·03	1·94		0·13	6		Ile
C19Da2						0·40			2·20		0·60	1·00				4		
C35Bt1	1·84			2·76		1·45			0·40	0·40		0·26				5—6		
C45B2	1·10					0·30			0·80	1·10		0·80				4		
C35Et1	0·07			2·93		0·14	2·96		1·04	3·13	1·88		1·01		2·03	15	—	Ala
C35Et3	1·03					0·97										2	+	Ser
C37At1				0·94		1·03			1·03							3 (4)	0	Gly
C37At2	1·04			0·96	0·17						2					4	0	Asp
C37At3	1·29			1·10		0·20			0·30		0·90					3	0	Asp
C37At4	1·06				1·06					0·91						3	+	Ala

C37Bt3	0·27	1·81		2·08		1·25	0·97		3·07	2·09	0·84	0·97			13		
C37Bt4	1·00			1·00											2	0	
C37Et1	1·04			2·09					0·78	2·09					6		Ala
C37Et2	1								1·00	1·00		1·00			4		
C40A2tA	0·13			1·03		0·97		1·03	0·97						4	0	Gly
C40A2tB	1·14			1·05							1·81				4		
C40A2tC	0·91			0·98							1·12				3	0	Asp
C40A2tD	0·97				0·97					1·06					3	+	Ala
C49B1AtA				0·01		0·01			0·02	1·00				0·94	2		
C49B1AtB			0·93				0·93		1·13						3		
C49B1AtC	1·06					1·06			0·02	0·89					3		
C49E4tB	0·18		1·00	0·12			0·12					0·12			1		Arg
C49E4tD	1·20		0·90				0·18					1·00			3		Arg
C49E4tE	1·00						0·20					1·00			2	—	Ile
C49E4F							0·69						1·21		2	—	Glu

TABLE IV

Results of Sequential Analysis of Peptides from Chymotryptic Hydrolysate of Alkaline Protease from *As. flavus*, Characterized in Table II and the Amounts Isolated

Symbol of fractions	Amount isolated μmol	Number of amino acids	Achieved degree of the determination of the structure
C1B1	5·13	12 – 13	[Ser].Ser.Val.Ala.Ala.Ala.Gly.Asn.Glu(Asx,Thr,Pro,[Ile?])
C1C	0·68	27	Asx.(Asx_3,Thr_2,Ser_5,Glx_3,Pro,Gly_3,Ala_4,Val_2,Ile,Leu).Ala
C4B1	5·85	12 – 13	[Ser?].Ser.Val.Ala.Ala.[Ser?].Ala.Gly.Asn.Glu.Asx.(Thr, ,Pro,[Ile?])
C4C1	0·46	4	(Asx,Gly,Asx).Ala
C5A3	0·04	4	Asx.Gly.(Asx,Ala)
C5D1	0·08	8	Ser.(Thr,Ile,Gly,Thr,Ser,Met,Ala)
C5E2	0·04	14 – 15	Ala.(Ala,Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val,Thr, ,Ser_{0-1},Asx)
C6A1	0·04	8	(Lys,Thr,Ser_3,Glu_2,Gly)
C6A2	0·13	6	Asx.(Asx,Ala,Val,Glx,Asx)
C6E1	0·03	4 – 5	(Thr,Ser,Gly,Ala,Ile_{0-1})
C6E2	0·03	20	(Asx_3,Thr_2,Ser,Glx,Pro_2,Gly,Ala_5,Val_2,Ile,Leu_2)
C6E3	0·16	12 – 14	Ala.Ala.(Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val, ,Thr_{0-1},Ser_{0-1},Asx_{0-1})
C7A1	0·03	8	(Ile,Gly,Ser,Ser,Ser,Ala,Thr,Asn)
C7A2	0·21	3	Thr.Gln.Thr
C7B	2·55	8	Ile.Gly.Ser.Ser.Ser.Ala.Thr.Asn
C8E2	1·00	12 – 14	Leu.(Leu_{1-2},Ser,Val,Ala,Ala,Ala,Gly,Asx,Glx,Asx_{0-1}, ,Thr,Pro)
C9A	3·38	4	Asn.Gly.Asn.Ala
C9B	0·18	3	Gly.(Asn,Ala)
C10A	7·55	4	Asn.Gly.Asn.Ala
C10B1	0·04	16	(Asx,Thr_3,Ser,Glx_3,Gly_2,Ala_2,Ile,Leu_2)
C1OB2	0·04	17	(Asx_2,Thr_2,Ser_2,Glx_2,Pro,Gly_2,Ala_2,Ile_2,Leu_2)
C11A	0·28	34 – 36	(Asx_4,Thr_2,Ser_4,Glx_2,Pro_2,Gly_2,Ala,[Val],Ile,Lys).(Asx_2, ,Thr,Ser,Glx,Pro,Gly,Ala_8,[Val])
C11B1	0·04	6	(Asn,Thr,Ser,Gln,Gly,Ala)
C11C	4·13	4	Ala.Ala.Ile.Gln
C12B	3·85	9	Asx.(Thr,Ser,Ala,Gly,Glx,Gly,Thr).Tyr
C14D4	0·35	4	(Gly,Val,Ile).Leu
C15B2	1·96	7	Ala.Pro.Gly.(Glx,Asx,Ile,Leu)

TABLE IV
(*Continued*)

Symbol of fractions	Amount isolated μmol	Number of amino acids	Achieved degree of the determination of the structure
C15C	1·48	5	Glu.Gln.Gly.Val.Leu
C15E	1·10	3	$\overrightarrow{\text{Asp.Gly.Phe}}$
C16B6	3·25	7	Ala.Pro.Gly.Gln.Asp.Ile.Leu
C16C2	0·17	5	$\overrightarrow{\text{(Glx,Glx,Gly,Val,Leu)}}$
C16D1	3·93	13	Asn.Asp.Ala.Val.Glu.Asn.Ala.Phe.Glu.Gln.Gly.Val.Leu
C16D2	2·58	13	$\overrightarrow{\text{Asx.(Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,Glx,Gly,Val).Leu}}$
C18A2	0·13	16	(Ala,Tyr,Leu,Ala,Ala,Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala, ,Ala,Val,Thr)
C18B1	0·04	12	(Ser,Ser,Val,Ala,Ala,Gly,Asx,Glx,Asx,Thr,Pro)
C18B2	0·04	11—12	([Ser],Ser,Val,Ala,Ala,Ala,Gly,Asx,Glx,Asx,Thr,Pro)
C18B6	0·04	17	$(Asx_3,Thr,Ser_2,Glx_2,Gly_2,Ala_3,Val,Ile,Leu,Phe)$
C18B7	0·04	14—17	$(Asx_2,Thr,Ser,Glx_2,Gly_2,Ala_{2-3},Val,Ile,Leu_3,Phe_{0-1})$
C18B8	0·13	9	$\overrightarrow{\text{Ala.Pro.(Gly,Glx,Asx,Ile,Leu}_3)}$
C18C1	0·04	12	$\overrightarrow{\text{(Lys,Asx}_2\text{,Thr,Ser,Glx,Gly,Ala}_3\text{,Val,Ile)}}$
C18C5	0·04	33—35	$(Lys,Asx_6,Thr_2,Ser_2,Glx_2,Pro,Gly_3,Ala_8,Val_2,Phe,Met, ,Ile_{1-2},Leu_{3-4})$
C18C6	0·04	17	$(Asx_2,Thr,Ser_2,Glx,Gly_3,Ala_3,Ile_2,Leu_3)$
C18D	6·38	4	$\overrightarrow{\text{Ala.Ser.Ile.Leu}}$
C19D	3·83	6	$\overrightarrow{\text{Ile.Gly.Val.Gly.Leu.Leu}}$
C21A1	0·08	11	(Thr?).(Ser,Ile,Gly,Thr,Ser,Met,Ala,Thr,Pro,His)
C21C2	0·05	13	Ala.(Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val,Thr,Lys)
C22A	0·04	3	(Ser,Asn,Thr)
C22B	0·30	14	Ala.(Ala,Ala,Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Val,Thr). .Lys
C23A	1·48	8	$\overrightarrow{\text{Ala.Ala.Asn.Asp.Ile.Val.Ser.Lys}}$
C23C	8·21	3	$\overrightarrow{\text{Ser.Asn.Phe}}$
C23E	c. 0·4	2—3	Leu.Leu or Leu.Leu.Leu
C24A	2·78	8	$\overrightarrow{\text{Ala.Ala.Asn.Asp.Ile.Val.Ser.Lys}}$
C24C1	1·53	15	$\overrightarrow{\text{Leu.Ala.Ala.Leu.Glu.Asn.Leu.(Asx,Gly,Pro,Ala,Ala,Val, ,Thr).Lys}}$
C24C2	0·25	9	$(Asn_3,Ser_2,Ala,Leu,Phe_2)$
C24D1	c. 0·5	2—3	Leu.Leu or Leu.Leu.Leu
C25A8	0·02	13	$(Asn,Thr,Ser,Gln,Gly_2,Ala_4,Ile,Leu,Phe)$
C25A9	0·05	8	(Ile,Gly,Val,Gly,Leu_4)
C25C	1·48	13	Val.Val.Asp.Ser.Gly.Val.Asn.Val.Asp.(His,Glx,Glx).Phe
C27C1	0·30	7	$\overrightarrow{\text{Ala.(Phe,Glx,Glx,Gly,Val,Leu)}}$

TABLE IV
(*Continued*)

Symbol of fractions	Amount isolated μmol	Number of amino acids	Achieved degree of the determination of the structure
C28A2	0·07	13—17	(Thr,Ser_{2-3},Gln_{0-1},Gly_{4-5},Ala_2,Val_{0-1},Met,Ile,Leu_2)
C28A4	0·15	18	Ala.(Lys_2,Asx_4,Ser_2,Gly_2,Ala_3,Val,Ile,Leu_2)
C28A5	0·08	10—11	(Asx,Val,[Val],Lys,Asx,Val,Lys,Gly,Ser,Pro,Asx)
C28B1	0·16	7	Ala.(Phe,Glx,Glx,Gly,Val,Leu)
C28C	6·43	11	Ile.Tyr.Asp.Thr.Ser.Ala.Gly.Glu.Gly.Thr.Tyr
C29C2	0·30	8	$\overrightarrow{\text{Gly.Gly.(Thr,Thr,Gln,Gly,Leu).Tyr}}$
C29D2	2·55	11	$\overrightarrow{\text{(Ile,Tyr,Asx,Thr,Ser,Ala,Gly,Glx,Gly,Thr).Tyr}}$
C30A1	7·66	4	$\overrightarrow{\text{Ala.Thr.Pro.His}}$
C30C	12·50	2	$\overrightarrow{\text{Ala.Tyr}}$
C31B	3·00	5	$\overrightarrow{\text{Gly.Ser.Ile.Ser.His}}$
C32B4	0·16	7—8	(Ala,Lys,Thr,Ser_{2-3},Gly,Ile)
C32C6	0·18	6	Ala.(Ala_3,Leu,Phe)
C33A1	0·25	3	(Thr,Ser,Lys)
C33A2	0·08	6—7	(Lys_2,Thr_2,Ser,Ala,Gly_{0-1})
C33B3	0·10	15—16	(Ala, Lys_3,Asx_{0-1},Thr,Ser_2,Glx_2,Gly,Ala_3,Ile_2)
C33B4	0·08	7—8	(Lys,Thr,Ser,Gln,Gly,Ala,Ile,Leu_{0-1})
C33D1	0·06	11—14	(Lys,Arg_2,Asx,Thr_{0-1},Ser,Glx_{2-3},Gly_3,Ala,Val_{0-1})
C33D3	2·80	8—9	Ser.Leu.Gly.Gly.Gly.Gly.(Gly,Leu).Tyr(?)
C34A3	0·08	12	$\overrightarrow{\text{($Lys_2$,$Thr_3$,$Ser_2$,$Gly_2$,Ala,$Ile_2$)}}$
C34B5	2·60	3	Ser.Leu.Tyr
C35A	0·05	2	Ser.Lys
C35B	0·18	10	[(Asx_3,Ser_2,Lys),(Gly,Ile,Ala,Lys)]
C35C6	1·98	7	Gly.(Leu,Gly,Ser,Ile,Ser).His
C35E	1·00	17	Ser.Lys.Ala.(Phe,Asx,Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,,Glx,Gly,Val,Leu)
C36A2	0·45	3—4	Gly.(Ile,Ala).[Lys?]
C36B1	1·55	9	Ala.(Ala,Asx,Asx,Ile,Val,Ser).Lys.Lys
C36B6	2·25	7	Gly.Leu.Gly.Ser.Ile.Ser.His
C36C1	0·08	12—14	$\overrightarrow{\text{Ala,Thr,Lys,Asx,Val,Val,Lys,Asx,Val_{0-1},Lys_{0-1},Gly,Ser,}}$,Pro,Asn)
C36E	11·10	5	Asp.Gly.Phe.Asn.Trp
C37A	0·22	14	$\overrightarrow{\text{Ala.Thr.Lys.[(Asp.Val.Val.Lys),(Asp.Val.Lys),Gly.(Ser,Pro,}}$,Asn)]
C37B	0·33	15—16	Asx.Lys.(Asx,Ala,Ala,Gly,Gly,Ser,Val,Gly,Asx,Ile,Glx,His,,[His],[Thr?])
C37C1	1·25	9	Ala.(Ala,Asx,Asx,Ile,Val,Ser).Lys.Lys

TABLE IV
(*Continued*)

Symbol of fractions	Amount isolated μmol	Number of amino acids	Achieved degree of the determination of the structure
C37C2	0·18	13	(Asx,Ala,Ala,Gly,Gly,Ser,Val,Gly,Asx,Ile,Glx,His).His
C37E	0·16	22—24	Gly.(Ile,Ala,Lys).[Ala.(Ala,Asx_2,Gly,Lys),(Lys,Asx,Thr_{1-2},Ser_2,Glx,Ala_3,Val,Met,Ile,Leu_{0-1})]
C37G1A	1·80	6	Gly.Lys.Val.Val.Asp.Phe
C38A1	5·28	8	Lys.(Gly,Glx,Glx,Ser,Thr,Asx).Tyr
C38A2	0·16	8—9	(His_{0-1},Lys,Gly,Glx,Glx,Ser,Thr,Asx,Tyr)
C38B1	0·16	11	Ala.(Thr,Pro,His,Leu,Val,Gly,Ile,Thr,Leu,Gly)
C38B2	1·00	14	Ala.Thr.Pro.His.Leu.Val.Gly.(Ile,Thr,Leu,Gly,Ala,Pro,His)
C39A	6·93	8	Lys.Gly.Gln.Gln.(Ser).Thr.(Asp).Tyr
C39C1	0·03	7	(Lys,Asp,Ser,Gly_{0-1},Ala_2,Ile)
C40A2	2·00	14	Ala.Thr.Lys.Asp.Val.Val.Lys.Asp.(Val,Lys).Gly(Ser,Pro,Asn)
C40A3	0·08	13—14	(Ala,Thr,Lys,Asx,Val,Val_{0-1},Lys,Asx,Val,Lys,Gly,Ser,Pro,Asn)
C41C	0·10	19—22	(Lys_3,Asx_4,Thr,Ser_2,Glx,Pro,Gly_{2-3},Ala_2,Val_{2-3},Ile_{0-1},Leu)
C42A1	0·10	16—19	(Lys_2,His_{0-1},Asx_3,Thr_1,Ser_{1-2},Glx_{0-1},Pro,Gly_2,Ala,Val_3,Leu_2)
C42C1A	0·53	3	Ser.Ala.Trp
C43A	0·02	11—13	(Lys,Arg,Asx_{1-2},Thr,Ser_2,Glx_{2-3},Gly_2,Ala)
C43C	0·04	9—10	(Asp_2,Gly_2,Ala,Val_1-_2,Ile,Leu,Trp)
C45A1	4·10	16	Asn.Ala.Ala.Gly.Gly.Gly.Ser.Val.Gly.Asp.Ile.(Glx,His,His,Thr).His
C45D	0·04	17—19	(Lys,Arg_{0-1},Asx_2,Thr,Ser_2,Glx_{1-2},Gly_3,Ala_2,Val,Ile,Leu,Phe,Trp)
C46B2	0·16	17—19	([Glx],Leu,Ala_{1-2},Thr,Lys,Asx,Val,Val,Lys,Asx,Val,Lys,Gly_2,Ser,Pro,Asx,Leu)
C47B1	0·16	15	Ala.(Ala,Gly,Gly,Gly,Ser,Val,Gly,Asp,Ile,Glx,His,His,Thr,His)
C49A1	0·03	25—26	(Lys_4,Arg,Asx_4,Thr_2,Ser_{4-5},Glx_2,Gly_4,Ala_4)
C49A2A	0·03	6	(Glx,Gly,Arg,Ala,Ser,Lys)
C29B1A	0·35	8	[(Glx,Gly,Arg),(Ala,Ser,Lys)],(Ala,Tyr)
C49B1C	0·08	29—30	(Lys_4,His,Arg_{2-3},Asx_3,Thr,Ser_4,Glx_2,Gly_3,Ala_5,Val,Ile,Tyr,Phe)
C49B1D	0·66	5	Glu.Gly.(Arg,Ala,Ser)
C49B4	0·10	16	(Lys_2,Arg,Asx,Thr,Ser_2,Glx,Gly_3,Ala_3,Val,Ile)
C49B5A	0·25	3	Lys.(Ala,Tyr)

TABLE IV
(*Continued*)

Symbol of fractions	Amount isolated μmol	Number of amino acids	Achieved degree of the determination of the structure
C49B5B	0·03	5	(Lys,Gly,Ala_2,Tyr)
C49C1	0·03	19—20	(Lys_3,His,Arg,Asx,Thr_2,Ser_2,Glx,Gly_3,Ala_{2-3},Ile_2,Leu)
C49C2	0·41	14—17	Lys_2,Arg,Asx,Thr_{1-2},Ser_2,Glx,Pro,Gly,Ala_2,Ile,Tyr_{0-1}, ,Phe_{0-1},Trp)
C49D1B	0·03	6	(Gly,Lys,Phe,Ser,Ala,Trp)
C49D1C	0·28	5	(Lys,Phe,Ser,Ala,Trp)
C49D1D	0·21	4	Ser.(Lys,Ala,Phe)
C49D1E	0·05	8	(Ala,[Ser],Lys,Ala,Tyr,Ser,Lys,Ala,Phe) or (Lys,Ala,Tyr, ,Ala,Ser,Lys,Ala,Phe)
C49E1A3	0·10	14	(Leu,Ala,Ala,Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val, ,Thr)
C49E1B2	0·18	10	(Val,Ser,Gly,Thr,Ala,Gly,Thr,Tyr,Ile,Lys)
C49E2B	0·03	17—18	Lys(?).(Lys,Arg_2,Thr_{0-1},Ser_3,Glx,Gly,Ala_4,Ile,Leu,Phe_2)
C49E2C	0·18	8	(Ser,Ala,Phe,Arg,Ile,Lys,Glx).Leu
C49E2D	0·08	5	(Arg,Ile,Lys,Glx,Leu)
C49E3A	0·63	10	Val.Ser.Gly.(Thr,Ala,Gly,Thr,Tyr,Ile,Lys) $\rightarrow\rightarrow\rightarrow$ Arg.Ile.Lys.Glu.Leu
C49E4	0·68	5	Lys.(Lys_2,Ser_3,Ala,Val_4,Ile,Leu,Phe_2)
C49F1	0·33	15	

lysine residues. Their tryptic hydrolysis could therefore lead to obtaining subfragments, classifiable among the already described peptides from tryptic hydrolysates[3,5], and so enable the combination of partial structures into larger units. Therefore tryptic hydrolysis was tested in a number of cases as the main method of subfragmentation wherever their amount permitted it.
They were the following:

C11A	C36B1	C37C1	C38A1
C35B	C37A	C37E	C40A2
C35E	C37B	C37G1A	C49B1A
			C49E4

Since the work-up of these fractions was identical, the subfragmentations that were carried out can be summarized in a uniform manner in Table VI and completed by a short comment in the text. The trypsin hydrolysate (enzyme : substrate =

TABLE V

The Results of Subfragmentation of Peptide Fractions that Did not Contain Basic Amino Acids

Characterization of the starting fractions is given in Table II, of the subfragments in Table III. Further details are explained in the text.

Symbol of fractions	Starting, partial and resulting sequence information
Step	1. 2. 3. 4. 5. 6. 7. 8. 9.
C4B1	[Ser].Ser.Val.Ala.Ala.[ ].Ala.Gly.Asn.Glu.(Asx,Thr,Pro,[Ile])
C4B1a2	(Ser, Ser,Val,Ala,Ala, Ala,Gly,Asx,Glx, Asx,Thr,Pro,[Ile])
—a3	(Ser, Ser,Val,Ala,Ala, Ala,Gly,Asx,Glx, Asx,Thr,Pro)
—a4	([Ser], Ala,Gly,Asx,Glx)
—a5	(Gly,Asx,Glx)
—a6	(Ser, Ala,Gly,Asx,Glx Asx)
C4B1	[Ser].Ser.Val.Ala.Ala.[Ser?].Ala.Gly.Asn.Glu.Asx(Thr,Pro,[Ile])
C19D	Ile(Gly,Val,Gly,Leu)Leu
C19Da1	Ile.Gly(Val,Gly,Leu,Leu)
—a2	(Ile,Gly,Val)Gly
—a3[a]	Leu.Leu
C19D	Ile.Gly.Val.Gly.Leu.Leu

[a] According to paper chromatography this fraction is free leucine.

TABLE VI

The Results of Subfragmentation of Lysine and Arginine Peptides by Tryptic Hydrolysis
Characterization of the starting peptides is given in Table II, of the subfragments in Table III.

Symbol	Starting, partial, and resulting sequentional information
C11A	(Asx$_4$,Thr$_2$,Ser$_4$,Glx$_2$,Pro$_2$,Gly$_2$,Ala, Val, Ile,Lys, Asx$_2$,Thr,Ser,Glx,Pro,Gly,Ala$_8$, Val)
−t1	(Asx$_4$,Thr$_2$,Ser$_4$,Glx$_2$,Pro$_2$,Gly$_2$,Ala,[Val],Ile,Lys)
C11A	(Asx$_4$,Thr$_2$,Ser$_4$,Glx$_2$,Pro$_2$,Gly$_2$,Ala,[Val],Ile,Lys).(Asx$_2$,Thr,Ser,Glx,Pro,Gly,Ala$_8$,[Val])
C35B	(Asx$_3$,Ser$_2$,Lys,Gly,Ile,Ala,Lys)
−t1	(Asx$_3$,Ser$_2$,Lys)
−t2	(Gly,Ile,Ala,Lys)
C35B	[(Asx$_3$,Ser$_2$,Lys),(Gly,Ile,Ala,Lys)]
C35E	Ser(Lys,Ala,Phe,Asx,Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,Glx,Gly,Val,Leu)
−t1	Ala(Phe,Asx,Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,Glx,Gly,Val,Leu)
−t2	Ser.Lys
C35E	Ser.Lys.Ala(Phe,Asx,Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,Glx,Gly,Val,Leu)
C37A	Ala(Thr,Lys, Asx, Val,[Val],Lys,Asx, Val,Lys,Gly,Ser$_{1-2}$,Pro,Asx)]
−t1	Gly(Ser, [Pro],Asn)
−t2	Asp.(Val, Val, Lys)
−t3	Asp.(Val,Lys)
−t4	Ala.Thr.Lys.
C37A	Ala.Thr.Lys.[Asp.(Val, Val, Lys),Asp.(Val,Lys),Gly.(Ser, Pro, Asn)]
C37B	(Asx,Lys, Asx,Ala,Ala,Gly,Gly,Ser,Val,Gly,Asx,Ile,Glx,Thr$_{0-1}$,His, His$_{0-1}$)
−t3	(Asx,Ala,Ala,Gly,Gly,Ser,Val,Gly,Asx,Ile,Glx — His, His)
−t4	Asx.Lys
C37B	Asx.Lys.(Asx,Ala,Ala,Gly,Gly,Ser,Val,Gly,Asx,Ile,Glx,[Thr?], His,[His])

C37E	Gly.(Ile,Ala,Lys, Ala, Ala,Asx_2,Gly,Lys, Lys,Asx,Thr_{1-2},Ser_2,Glx,Ala_3,Val,Met,Ile,Leu_{0-1}
−t1	Ala(Ala,Asx_2,Gly,Lys)
−t2	(Gly,Ile,Ala,Lys)
C37E	Gly.(Ile,Ala,Lys).[Ala.(Ala,Asx_2,Gly,Lys),(Lys,Asx,Thr_{1-2},Ser_2,Glx,Ala_3,Val,Met,Ile,Leu_{0-1})]
C40A2	Ala(Thr,Lys,Asx,Val,Val,Lys,Asx,Val,Lys,Gly,Ser,Pro,Asx)
−tA	Gly(Ser,Pro,Asn)
−tB	(Asx,Val,Val,Lys)
−tC	Asp(Val,Lys)
−tD	Ala(Thr,Lys)
C40A2	Ala.Thr.Lys.Asp.Val.Val.Lys.Asp.(Val,Lys).Gly(Ser,Pro.Asn) → → → → → → → →
C49B1A	(Glx,Gly,Arg, Ala,Ser,Lys, Ala,Tyr)
−t1	(Ala,Tyr)
−t2	(Glx,Gly,Arg)
−t3	(Ala,Ser,Lys)
C49B1A	[(Glx,Gly,Arg),(Ala,Ser,Lys)],(Ala,Tyr)
C49E4	Arg.(Ile,Lys,Glx).Leu
−tB	Arg
−tD	Arg.(Ile,Lys)
−tE	Ile.Lys
−tF	Glu.Leu
C49E4	Arg. Ile.Lys.Glu.Leu

= 1 : 75; 4 h, 37°C) was in all cases separated by high-voltage electrophoresis E_1, with the exception of the last fraction C49E4, where paper chromatography in system S_1 was used. The labelling of subfragments is ruled by the same principles as in main fractions. The composition and the characterization of the subfragments is given in Table III. In view of the specificity of trypsin it may be assumed that all subfragments contain basic amino acids at the C-terminus. However, only confirmed results are given in the tables. Information on sequences of individual starting peptides after evaluation by tryptic hydrolysis is summarized in Table IV.

In view of the relatively small amount of the peptide the results obtained for fraction C11A (Table VI) should be considered as preliminary only. The subfragment 35Bt1 is probably contaminated by the subfragment C35Bt2, and after its subtraction the composition of C35Bt1 can be interpreted as (Lys, Asx_3, Ser_{1-2}). The C36B1 and C37C1 split off free lysine easily on tryptic hydrolysis, so that the C-terminal sequence -Lys.Lys (Table IV) may be assumed. The peptide C37B can represent a difficultly separable mixture of two related peptides: One terminating with the sequence ...Glx.His.His and the other with ...Glx.His.His.Thr.His. Both correspond to the specificity of the chymotryptic cleavage and explain the problems with one threonine residues. The results characterizing the peptide C37E may be considered as preliminary only, since it was obtained in a relatively small amount. The peptide 37G1A split off a subfragment on orienting tryptic hydrolysis, having in electrophoresis in systems E_1 and E_2 and in chromatography in S_1 the same mobility as a parallelly applied basic dipeptide, Gly.Lys. The second acid subfragment (Val,Val, ,Asp)Phe was ninhydrin-negative and it was observed only on detection with chlorine according to Reindel (for reference see ref.[5]). Later on it was shown that other peptides too, beginning with the Val.Val... sequence, are ninhydrin-negative. In addition to this the sequence of the whole peptide was also determined using the manual Edman procedure (Table IV). Peptide C38A1 was not cleavable with trypsin, so that we assume lysine to be at the N-terminal, which was confirmed by the dinitrophenylation method (Table IV). In addition to tryptic cleavage peptide C40A2 was also analysed by the manual Edman degradation to the 8th step. This peptide behoved in a different way from peptide C40A3 during preparation, although they have coinciding amino acid composition. From the results of the complete determination of the structure of this sequence it follows that the existence of the differing fractions C37A, C40A2 and C40A3 cannot be explained by the splitting off of natural amides. We have no further explanation. The fragments C49B1A-t2 and -t3 (Table VI) have evidently basic amino acids at their C-terminus, and owing to the specificity of trypsin they should precede the neutral fragment -t1, which, in view of the specificity of the chymotryptic cleavage, will probably end with tyrosine. The simultaneous isolation of the subfragments C49E4tB and -tD (Table VI) shows clearly that after the N-terminal arginine with a free α-amino group (similarly as in the case of N-terminal lysine) trypsin also cleaved only slowly under the given conditions.

TABLE VII

Survey of Peptides with the Same Amino Acid Composition. Their Collective Indication and the Total Amount Isolated from Various Fractions of the Chymotryptic Hydrolysate

Individual fractions are characterized in Table IV; they are discussed in the order given there.

Symbol of fractions	Amount isolated μmol	Collective symbol Table VIII	Symbol of fractions	Amount isolated μmol	Collective symbol Table VIII
C1B1	5·13		C22A1	0·04	
C4B1	5·85		C23C	8·21	
C18B1	0·04			8·25	Chy 7
C18B2	0·04		C23A	1·48	
	11·06	Chy 4	C24A	2·78	
C4C1	0·46			4·26	Chy 12
C5A3	0·04		C23E	0·40	
	0·50	Chy 38	C24D1	0·50	
C5A2	0·04			0·90	Chy 32
C6A1	0·04		C28C	6·43	
	0·08	Chy 40	C29D2	2·55	
C5E2	0·04			8·98	Chy 6
C6E3	0·16		C35C6	1·98	
	0·20	Chy 39	C36B6	2·25	
C7A1	0·03			4·23	Chy 14
C7B	2·55		C36B1	1·55	
	2·58	Chy 22	C37C1	1·25	
C9A	3·38			2·80	Chy 20
C10A	7·55		C36C1	0·08	
	10·93	Chy 5	C37A	0·22	
C15B2	1·96		C40A2	2·00	
C16B6	3·25			2·30	Chy 23
	5·21	Chy 11	C38A1	5·28	
C15C	1·48		C39A	6·93	
C16C2	0·17			12·21	Chy 2
	1·65	Chy 25	C49E1B2	0·18	
C16D1	3·93		C49E3A	0·63	
C16D2	2·58			0·81	Chy 33
	6·51	Chy 9	C49E2D	0·08	
			C49E4	0·68	
				0·76	Chy 34

TABLE VIII

The Main Peptides from Chymotryptic Hydrolysate of Alkaline Protease from *As.flavus*, Ordered According to Their Total Amount Isolated

Symbol	Total isolated amount μmol	Main fraction	Number of amino acids	Achieved degree of sequence analysis
Chy 1	12·50	C30C	2	Ala.Tyr
Chy 2	12·21	C39A	8	Lys.Gly.Gln.Gln.Ser.Thr.Asp.Tyr
Chy 3	11·10	C36E	5	Asp.Gly.Phe.Asn.Trp
Chy 4	11·06	C4B1	12—13	[Ser].Ser.Val.Ala.Ala.[Ser?].Ala.Gly.Asn.Glu.Asx.(Thr,Pro,[Ile?])
Chy 5	10·93	C10A	4	Asn.Gly.Asn.Ala
Chy 6	8·98	C28C	11	Ile.Tyr.Asp.Thr.Ser.Ala.Gly.Glu.Gly.Thr.Tyr
Chy 7	8·25	C23C	3	Ser.Asn.Phe
Chy 8	7·66	C30A1	4	Ala.Thr.Pro.His
Chy 9	6·51	C16D1	13	Asn.Asp.Ala.Val.Glu.Asn.Ala.Phe.Glu.Gln.Gly.Val.Leu
Chy 10	6·38	C18D	4	Ala.Ser.Ile.Leu
Chy 11	5·21	C16B6	7	Ala.Pro.Gly.Gln.Asp.Ile.Leu
Chy 12	4·26	C24A	8	Ala.Ala.Asn.Asp.Ile.Val.Ser.Lys
Chy 13	4·10	C45A1	16	Asn.Ala.Ala.Gly.Gly.Gly.Ser.Val.Gly.Asp.Ile.(Glx,His,His,Thr).His
Chy 14	4·23	C36B6	7	Gly.Leu,Gly.Ser.Ile.Ser.His
Chy 15	4·13	C11C	4	Ala.Ala.Ile.Gln
Chy 16	3·85	C12B	9	Asx(Thr,Ser,Ala,Gly,Glx,Gly,Thr)Tyr
Chy 17	3·83	C19D	6	Ile.Gly.Val.Gly.Leu.Leu
Chy 18	3·00	C31B	5	Gly.Ser.Ile.Ser.His
Chy 19	2·80	C33D3	8—9	Ser.Leu.Gly.Gly.Gly.Gly.(Gly,Leu).Tyr[?]
Chy 20	2·80	C36B1	9	Ala.(Ala,Asx,Asx,Ile,Val,Ser).Lys.Lys
Chy 21	2·60	C34B5	3	Ser.Leu.Tyr

Chy 22	2·58	C7B	8	Ile.Gly.Ser.Ser.Ser.Ala.Thr.Asn
Chy 23	2·30	C40A2	14	Ala.Thr.Lys.Asp.Val.Val.Lys.Asp.(Val,Lys).Gly.(Ser,Pro,Asn)
Chy 24	1·80	C37G1A	6	Gly.Lys.Val.Val.Asp.Phe
Chy 25	1·65	C15C	5	Glu.Gln.Gly.Val.Leu
Chy 26	1·53	C24C1	15	Leu.Ala.Ala.Leu.Glu.Asn.Leu.(Asx,Gly,Pro,Ala,Ala,Val,Thr).Lys
Chy 27	1·48	C25C	13	Val.Val.Asp.Ser.Gly.Val.Asn.Val.Asp.(His,Glx,Glx).Phe
Chy 28	1·10	C15E	3	Asp.Gly.Phe
Chy 29	1·00	C8E2	12—14	Leu.(Leu_{1-2},Ser,Val,Ala,Ala,Ala,Gly,Asx,Glx,Asx_{0-1},Thr,Pro)
Chy 30	1·00	C35E	17	Ser.Lys.Ala.(Phe,Asx,Asx,Ala,Val,Glx,Asx,Ala,Phe,Glx,Glx,Gly,Val,Leu)
Chy 31	1·00	C38B2	14	Ala.Thr.Pro.His.Leu.Val.Gly.(Ile,Thr,Leu,Gly,Ala,Pro,His)
Chy 32	0·90	C24D1	2—3	Leu.Leu or Leu.Leu.Leu
Chy 33	0·81	C49E3A	10	Val.Ser.Gly.(Thr,Ala,Gly,Thr,Tyr,Ile,Lys)
Chy 34	0·76	C49E4	5	Arg.Ile.Lys.Glu.Leu
Chy 35	0·68	C1C	27	Asx.(Asx_3,Thr_2,Ser_5,Glx_3,Pro,Gly_3,Ala_4,Val_2,Ile,Leu).Ala
Chy 36	0·66	C49B1D	5	Glu.Gly.(Arg,Ala,Ser)
Chy 37	0·53	C42C1A	3	Ser.Ala.Trp
Chy 38	0·50	C4C1	4	(Asx,Gly,Asx),Ala
Chy 39[a]	0·20	C6E3	12—14	Ala.Ala.(Leu,Glx,Asx,Leu,Asx,Gly,Pro,Ala,Ala,Val,Thr_{0-1},Ser_{0-1},Asx_{0-1})
Chy 40[a]	0·08	C5A2	8	(Lys,Thr,Ser_3,Glu_2,Gly)

[a] The last two peptides, spread over various fractions, are not considered as principal ("main peptides") and they are mentioned here to be systematic and for the sake of completeness of Table VII; further peptides, isolated from the chymotryptic hydrolysate individually and in small amounts (*i.e.* under 0·5 μmol) will be further indicated by symbols from Table IV only.

TABLE IX

Possible Mutual Alignment of Various Peptides from Chymotryptic Hydrolysate of Alkaline Protease from *As. flavus*

Key to one-letter notation of amino acids is in the footnote on p. 2027. Sequence characteristics were taken from Tables VIII and IV. The arrows indicate the assumed specific splitting by chymotrypsin.

Symbol	Amount μmol	Number of amino acids	Alignment of structures
Chy2	12·21	8	K G Q Q S T D Y
C38A2	0·16	9	([H],K,G,Z,Z,S,T,D,Y)
	12·37	9	[H] K G Q Q S T D Y
			↑ ↑
Chy 3	11·10	5	D G F N W
Chy 28	1·10	3	D G F
	12·20	5	D G F N W
			↑ ↑
Chy 4	11·06	12—13	[S]S V A A[S]A G N E B (T P,[I])
Chy 29	1·00	12—14	L(L, [L]S,V,A,A,—A,G, B,Z,[B],T,P)
	12·06	13—16	L L[L?] [S]S V A A A G N E B (T,P,[I])
			↑ ↑
Chy 5	10·93	4	N G N A
C9B	0·18	3	G(N,A)
	11·11	4	N G N A
			↑
Chy 6	8·98	11	I Y D T S A G E G T Y
Chy 16	3·85	9	B(T,S,A,G,Z,G,T)Y
	12·83	11	I Y D T S A G E G T Y
			↑ ↑

Chy8	7·66	4
Chy 31	1·00	14
C21A1	0·08	11
C5D1	0·08	8
C38B1	0·16	11
	8·98	21
Chy 9	6·51	13
Chy 25	1·65	5
C27C1 + C28B1	0·46	7
C6A2	(0·13)	6
Chy 30	1·00	17
C49D1D	(0·21)	4
	9·62	17
Chy 11	5·21	7
C18B2	0·13	9
Chy 32	0·90	2—3
	6·24	
Chy 12	4·26	8
C35A	0·05	2
Chy 20	2·80	9
	7·11	
Chy 13	4·10	16
C47B1	0·16	15
C37C2	0·18	13
C37B	0·33	15—16
	4·77	17—18

```
                    A T P H
                    A T P H L V G(I,T,L,G,  A,P,H)
T(S,  I,G,T,S,M,A,T,P H)
S(T,  I,G,T,S,M,A)
                    A(T,P,H,L,V,G,I,T,L,G)
(S,T).(I,G,T,S,M)A T P H L V G(I,T,L,G).(A,P,H)
                  ↑       ↑                 ↑

          N D A V E N A F E Q G V L
                          E Q G V L
                        A(F,Z,Z,G,V,L)
              B(B,A,V,Z, B)
S K A(F, B, B,A,V,Z, B,A,F,Z,Z,G,V,L)
S (K,A,F)
S K A F N D A V E N A F E Q G V L
      ↑           ↑   ↑         ↑

A P G Q D I L
A P(G Z B I L L L)
                [L]L L
A P G Q D I L L L
                ↑

A A N D I V S K
                S K
A(A B B I V S)K K
A A N D I V S K K

      N A A G G G S V G D I (Z,H,H,  T)H
          A(A,G,G,G,S,V,G, B,I,Z,H,H,  T,H)
      (B,A,A,G,G,-, S,V,G, B,I,Z,H)H
B K(B,A.A,G,G.-, S,V,G, B,I,Z,H[H],[T])
B K N A A G G[G]S V G B I Z H H T H
  ↑                           ↑   ↑
```

TABLE IX
(*Continued*)

Symbol	Amount µmol	Number of amino acids	Alignment of structures
Chy 14	4·23	7	G L G S I S H
Chy 18	3·00	5	G S I S H
	7·23		G L G S I S H
			↑ ↑
Chy 17	3·83	6	I G V G L L
C25A9	0·05	8	(L,L, I,G,V,G,L,L)
	3·88	8	[(L,L), I G V G L L]
			↑ ↑
Chy 23	2·30	14	A T K D V V K D(V,K)G (S,P,N)
C40A3	0·08	14	(A,T,K, B,V_1[V],K,B,V,K,G, S,P, B)
C28A5	0·08	11	(B, V, V, K,B,V,K,G, S,P, B)
C46B2	0·16	17–18	([Z],L,A,T,K,B,V, V, K, B,V,K,G_2,S,P,N,L)
	2·46	17	[([Z],L),A T K D V V K D(V,K)G (S,P,N),L]
			↑ ↑
Chy 26	1·53	15	L A A L E N L(B,G,P,A,A,V, T)K
Chy 39	0·20	12–13	A A(L,Z, B,L,B,G,P,A,A,V)[T]
C18A2	0·13	16	(A,Y,L,A,A,L,Z, B,L,B,G,P,A,A,V, T)
C21C2	0·05	13	A(L,Z, B,L,B,G,P,A,A,V, T,K)
C22B	0·30	14	A(A,L,Z, B,L,B,G,P,A,A,V, T)K
C49E1A3	0·10	14	(L,A,A,L,Z, B,L,B,G,P,A,A,V, T)
	2·31	17	(A,Y)L A A L E N L(B,G,P,A,A,V, T)K
			↑ ↑
Chy 34	0·76	5	R I K E L
C49E2C	0·18	8	(S,A,F, R,I,K,Z)L
	0·94	8	(S,A,F)R I K E L
			↑ ↑

Chy 36	0·66	5	E G(R, A,S)
C49A2A	0·03	6	(Z,G,R, A,S,K)
C49B1A	0·35	8	[(Z,G,R),(A,S,K)](A,Y)
C49B5A	1·25	3	K (A,Y)
	1·29	8	E G R (A,S)K (A,Y)
			↑
Chy 37	0·53	3	S A W
C49D1C	0·28	5	(K,F)S A W
C49D1B	0·03	6	(G,K,F)S A W
	0·84	6	G(K,F)S A W
			↑ ↑

Multiple Isolation of the Same Peptides and the Arrangement of the Main Peptides According to the Amount Obtained

From the results of the characterization of the individual fractions, summarized in Table IV, it follows that some fractions evidently contain the same peptide. The cases which showed the identity of the isolated peptides were summarized in Table VII, where the sum of individual isolated amounts is also given. In this table equally long peptides are arranged together, originating with great probability from the same site, even though we are unable to explain unambiguously in all instances why they were sometimes found in rather distant fractions (for example C1B1 and C18B1).

In chymotryptic hydrolysis we started with 1·08 g of substrate, *i.e.* about 37 μmol. 30 mg of enzyme, *i.e.* 1·2 μmol, were employed. In order to prevent an erroneous exchange of the peptides from the substrate for a peptide of the cleaving (autolysed) enzyme, Table VIII was composed of the so-called main peptides from tryptic hydrolysate. It was composed in the order of the total amount isolated, taking in view the sums in Table VII. When Table VIII was composed the resulting sequence information was marked with the symbol Chy with a number and these peptides will be labelled in this manner in further studies. Those peptides are considered as belonging undoubtedly to the substrate under study, which were obtained in exceeding 1 μmol. However, since the losses during the separation of the peptides on paper very often exceed 50% and more, peptides are registered in Table VIII up to the total amount of 0·5 μmol. However, in view of the very large losses during the isolation of some peptides and in view of the fragments split off unspecifically and in small yield the majority of peptides obtained in amounts less than 0·5 μmol can also be used for the evaluation of the sequence analysis of the substrate if it is in agreement with the sequence information obtained from other sources. In some instances the peptides isolated in small amounts can be the carriers of sequence information obtainable with great difficulty in other ways. Therefore we present them all in Table IV.

Tables VIII and IV represent a summary of experimentally found results obtained in the working up of chymotryptic hydrolysate during this study. Their interpretation and the search for mutual relationships is the subject of the following discussion.

DISCUSSION*

From the data in Table VIII it is evident that some non-identical fragments were very probably cleaved from the same segment of the primary structure in consequence of the merely group specificity of the enzyme used. Some bonds of the substrate protein could have already been damaged by autolysis of the starting enzyme[5] which often splits even after lysine. Of course, this increases the total amount of the isolated overlapping parts, given in micromol from individual peptides. Possible alignment

of this type are summarized in Table IX, in the order of peptides from Table VIII. However, only the larger fragments of those well sequentionally characterized can be connected with a larger measure of probability, which overlap to a larger extent, or also such which contain minor amino acids. (The number of residues[8] in the protein studied is given in brackets: Met (2), Trp (3–4), Arg (4), His (6), Phe (8), Pro (8), Tyr (8–9).

Even the largest amount isolated in the case of combined peptides (12·83 μmol in the case of the group Chy 6, Table IX) does not exceed half the limit calculated on p. 2026, *i.e.* 37/2 = 18·5 μmol. It can hardly be assumed that some of the sequences of Table IX would occur in the protein twice. Later we shall comment on the groups in the order given in Table IX. In the group of the peptides Chy 3, isolated in a relatively large amount (12·20 μmol) we must suppose that it was split off specifically and therefore, evidently, an aromatic acid precedes it in the sequence, most probably tyrosine. The same is true of Chy 5, Chy 6, Chy 11, Chy 12, Chy 14 and Chy 17, unless the original chain preceding them was damaged by autolysis. This evidently took place in the case of peptide Chy 13, since alkaline protease often behaves as a less specific trypsin.

In the case of peptides aligned in the group Chy 8 there is a discrepancy in the determination of the N-terminal group (C21A1 and C5D1) and therefore we formulate the resulting fragment provisionally as (S, T).... The extension of the fragments Chy 17 and Chy 37 by peptides obtained in small amounts did not permit a closer sequentional characterization of longer fragments.

The peptides obtained in this study originate from the hydrolysate of the trichloroacetic acid precipitate of the partly autolysed alkaline protease from *As. flavus*. They are therefore without low-molecular fragments that cannot be precipitated with the reagent used. The results obtained so far can be further corrected and made more precise by the study of the peptides split off from alkaline protease by autolysis[5], which is the subject of a subsequent paper[9].

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* In this discussion internationally accepted[7] one-letter symbols for amino acids will be used for the sake of brevity and clearness. In order to facilitate the understanding of the discussion we present a short list of them

A — Ala	G — Gly	N — Asn	V — Val
B — Asx	H — His	P — Pro	W — Trp
C — Cys	I — Ile	Q — Gln	X — unknown
D — Asp	K — Lys	R — Arg	Y — Tyr
E — Glu	L — Leu	S — Ser	Z — Glx
F — Phe	M — Met	T — Thr	

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