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ISOLATION AND ACTIVITIES OF THE TRYPSIN-MODIFIED *VICIA ANGUSTIFOLIA* PROTEINASE INHIBITOR LACKING CARBOXYL-TERMINAL HEXAPEPTIDE

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Summary

The *Vicia angustifolia* proteinase inhibitor was incubated with *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone-trypsin (EC 3.4.21.4) and a main product was isolated. The purified product was different to the first trypsin-modified *V. angustifolia* inhibitor. The C-terminal residues of the new derivative were arginine, which was also the C-terminal of the cleaved antitryptic site; lysine was a newly exposed C-terminal. These results suggest that the new derivative lacks the C-terminal portion of the native inhibitor, which has asparagine at its C-terminus.

The liberated C-terminal peptide had the following amino acid sequence: H-Glu-Glu-Val-Ile-Lys-Asn-OH.

The derivative lacking the C-terminal hexapeptide still possesses inhibitory activities against trypsin and α-chymotrypsin (EC 3.4.21.1), however, its antichymotryptic activity was inactivated by incubation with chymotrypsin at pH 8.0.

Introduction

Among the proteinase inhibitors of natural origin, legume seeds contain double-headed type inhibitors such as those from soybean (Bowman-Birk) (inhibitor B) [1,2], lima bean (inhibitor L) [3], garden bean [4] and several others [5—8].

Abbreviations: inhibitor B, Bowman-Birk soybean inhibitor; inhibitor L, lima bean inhibitor; inhibitor V, *V. angustifolia* inhibitor; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

We have reported previously [9,10] the isolation of a proteinase inhibitor (inhibitor V) from the seeds of *Vicia angustifolia* L. var. *segetalis* Koch which inhibits trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1). We determined the homology of its partial amino acid sequence around the antitryptic site to inhibitor B.

Most proteinase inhibitors undergo limited hydrolysis at the reactive site peptide bonds on exposure to catalytic amounts of the proteinases at acidic pH [11]. We also reported the isolation and properties of the first trypsin-modified (the antitryptic site Arg-Ser bond specifically cleaved) inhibitor V [10], which was prepared at pH 3 for 4 h with a catalytic amount of *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin.

Under similar conditions, when the incubation period was prolonged, a new derivative was obtained and this still retained inhibitory activities against both trypsin and α-chymotrypsin. In this paper, we report the isolation and characterization of the second trypsin-modified form and also compare its inhibitory activities with those of the first trypsin-modified inhibitor V.

Materials and Methods

Materials. V. angustifolia proteinase inhibitor was prepared as described previously [9]. Bovine trypsin and bovine α-chymotrypsin were obtained from Boehringer. TPCK-trypsin was prepared by the method of Wang and Carpenter [12] and passed through a column of soybean trypsin inhibitor-Sepharose 4B prepared according to the method of Knights and Light [13]. Diisopropylphosphorofluoridate-treated carboxypeptidases A and B were obtained from Worthington. Casein and 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride were purchased from Merck and Tokyo Kasei, respectively. Sephadex G-type resins and DEAE-Sephadex A-25 were from Pharmacia. Polyamide thin-layer sheets were from Cheng Chin Corp. All other chemicals were of reagent grade.

Amino acid analysis. Amino acid analyses were performed by the method of Spackman et al. [14] with a JEOL model JLC-6AH amino acid analyzer after 24 h hydrolysis in sealed, evacuated tubes.

Determination of N- and C-terminal residues. The N-terminal residue was determined by either dansylation [15] or Edman degradation [16]. The resulting phenyltiohydantoin derivaties were identified on polyamide sheets [17] and, in some cases, by amino acid analyses after acid hydrolyses of phenylthiohydantoin [18] or thiazolinone derivatives [19].

Activity measurements. Proteolytic activities of trypsin and α-chymotrypsin in the presence or absence of inhibitor were estimated by casein digestion [20] at pH 8.0 and 37°C with 2 min preincubation.

Results

Preparation and identification of the second trypsin-modified inhibitor V

A new product was isolated from the reaction mixture of the inhibitor V and a catalytic amount of TPCK-trypsin by Sephadex G-50 (Fig. 1) followed by the first DEAE-Sephadex A-25 (Fig. 2). And after the second DEAE-

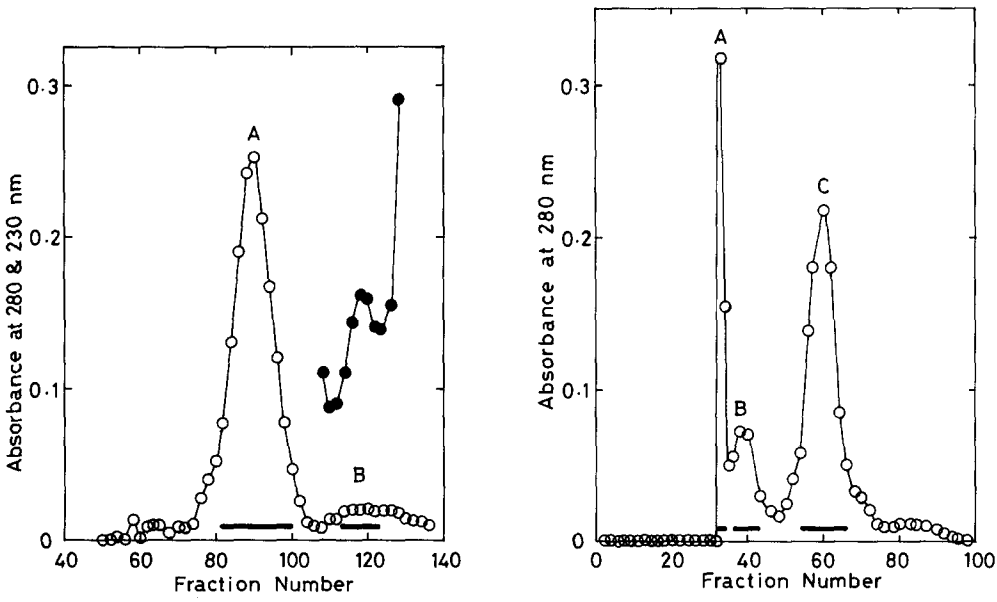

Fig. 1. Sephadex G-50 chromatography of the reaction mixture of *V. angustifolia* inhibitor with a catalytic amount of trypsin. 51.3 mg native inhibitor V (6.3 μmol) and 1.9 mg TPCK-trypsin (0.08 μmol) in 2 ml 0.05 M citrate buffer (pH 3), 0.01 M $CaCl_2$, were incubated at 37°C for 78 h. The trypsin was then precipitated by the addition of 0.5 ml 25% trichloroacetic acid. After 30 min at room temperature, the precipitate was removed by centrifugation at 4000 × *g* for 10 min, and the supernatant was applied to a column (2.4 × 150 cm) of Sephadex G-50 Fine equilibrated with 0.01 M ammonium acetate (pH 7). Elution was performed with the same buffer (flow rate, 30 ml/h; 5-ml fractions). ○, absorbance at 280 nm; ●, absorbance at 230 nm.

Fig. 2. First DEAE-Sephadex A-25 chromatography of the second trypsin-modified *V. angustifolia* inhibitor. Peak A (Fig. 1) was applied directly to a column (1.2 × 30 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient 250 ml each 0.01 and 0.2 M ammonium acetate (pH 7; 5-ml fractions).

Sephadex chromatography of the main peak C (Fig. 2), the derivative with a relative electrophoretic mobility 0.47 was obtained with enough purity (Fig. 3) for the further characterization.

Dansylation revealed that the purified new derivative possessed two N-terminal residues, a Gly and a Ser. The same results had been obtained in the case of the first modified form [10]. The C-terminal residues of the new derivative were determined as an Arg and a Lys by carboxypeptidase B treatment, while the first modified form, as an Arg and an Asn [10]. These results revealed that the new derivative had the same cleaved site as that of the first modified form, however, lacked the C-terminal portion of the native inhibitor.

Isolation and characterization of small and large fragments of the second trypsin-modified form

Table I summarizes the results of amino acid analyses of the two fragments obtained by Sephadex G-50 gel filtration of the reduced and *S*-carboxymethylated second modified form (Fig. 4). The small fragment consisted of 16 amino acid residues and was found to have the same composition as that of the

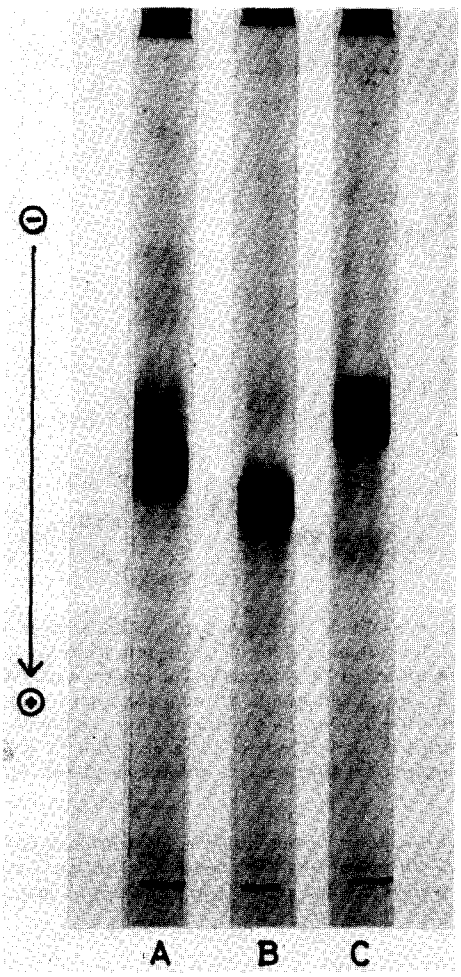

Fig. 3. Polyacrylamide gel electrophoresis of the purified inhibitor derivatives. Electrophoreses were carried out essentially by the method of Davis [21] at 3 mA/tube for 1 h using 5% acrylamide gels. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 7% acetic acid and destained in the same acid solution. (A) Native inhibitor V. (B) First trypsin-modified form. (C) Second trypsin-modified form.

first trypsin-modified form which was recalculated from the reported value [10] on the basis of the partial sequence determined (Table II). The large fragment consisted of about 50 residues and the sum of both the fragments accounted, within experimental error, for that of the intact second modified form (65 residues). However, compared to the native inhibitor, about six amino acid residues were missing in the second modified form. Absence of an Ile residue in the large fragment suggests that it lacks the C-terminal oligopeptide present in the native inhibitor, because an Ile present in the inhibitor V located at the second position from the C-terminus [10].

Dansylation of both the fragments revealed that a Gly and a Ser were the N-terminal of the small and the large fragments, respectively, and the

TABLE I

AMINO ACID COMPOSITIONS OF THE TRYPSIN-MODIFIED *V. ANGUSTIFOLIA* INHIBITOR LACKING C-TERMINAL HEXAPEPTIDE, ITS FRAGMENTS OBTAINED BY THE REDUCTION AND S-CARBOXYMETHYLATION, AND THE C-TERMINAL HEXAPEPTIDE

Native are recalculated values from the data given in Ref. 9 on the basis of Phe = 2. All other data are analysis of 24-h hydrolysates. n.d. not determined

Amino acid	Native		Second modified		Fragments							
					Small		Large		Hexapeptide		Sum of them	
Aspartic acid	8.61	9	7.68	8	3.12	3	5.16	5	1.23	1	9	
Threonine	4.03	4	3.81	4	1.86	2	1.97	2	0.18	0	4	
Serine	5.84	6	5.21	5	1.09	1	4.51	5	0.35	0	6	
Glutamic acid	7.18	7	5.24	5	0.12	0	5.38	5	2.33	2	7	
Proline	4.66	5	5.34	5	0.05	0	5.08	5	0.11	0	5	
Glycine	2.45	2	2.26	2	1.08	1	1.12	1	0.25	0	2	
Alanine	3.28	3	3.18	3	1.06	1	2.07	2	0.23	0	3	
Half-cystine	14.10 *	14	12.62	13	3.81 *	4	9.29 *	9	0	0	13	
Valine	4.67	5	4.01	4	0.80	1	3.30	3	1.00	1	5	
Methionine	0.24 **		0.30 **		0.24 **		0		0.02	0		
Isoleucine	0.72	1	0.17	0	0	0	0	0	0.84	1	1	
Leucine	1.31	1	1.16	1	1.00	1	0	0	0.15	0	1	
Tyrosine	1.77	2	1.74	2	0	0	1.78	2	0	0	0	
Phenylalanine	2.00	2	2.22	2	0	0	2.17	2	0	0	2	
Lysine	5.25	5	4.10	4	1.09	1	3.23	3	1.29	1	5	
Histidine	3.97	4	4.00	4	0.05	0	4.08	4	0.02	0	4	
Arginine	3.20	3	2.94	3	0.92	1	2.00	2	0.07	0	3	
Tryptophan	0	0	n.d.		n.d.		n.d.		n.d.			
Total		73		65		16		50		6	72	

* Determined as CM-cysteine.

** See the footnote to Table II.

C-terminal, as an Arg and a Lys. Only difference between the large fragments is the C-termini, that is, a Lys in the case of the second, and an Asn (which is also the C-terminus of the native inhibitor) in the first modified form. These determinations also confirmed the lack of oligopeptide from the C-terminal portion of the second trypsin-modified form.

Isolation and determination of the amino acid sequences of the C-terminal hexapeptide

The C-terminal oligopeptide was isolated from the reaction mixture described above. The peak B (Fig. 1) was pooled, lyophilized, and further purified through a column (2.5 × 56 cm) of Sephadex G-25 (not shown) and a single peak was obtained. Purity of the pooled and lyophilized material was estimated by dansylation. A main dansyl-Glu spot was obtained as well as a few minor amino acid derivatives. From this result and from amino acid analysis (Table I), the material was judged to be pure enough for the determination of its amino acid sequence.

As shown in Table I, this material is composed of six amino acid residues and well makes up for the lost part of the second modified form. Amino acid sequence of the hexapeptide was determined by Edman degradation as H-Glu-Glu-Val-Ile-Lys-Asn-OH (Table II).

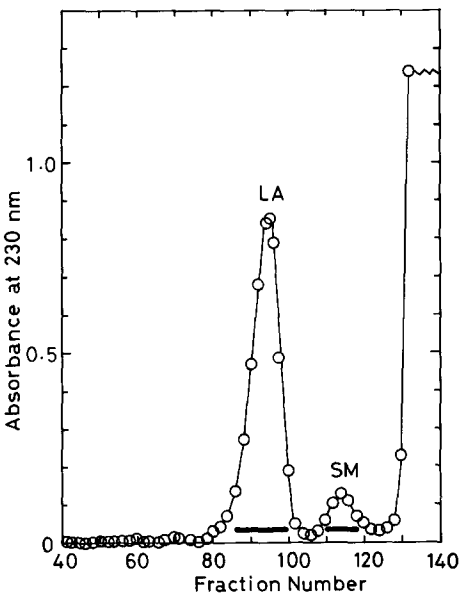

Fig. 4. Sephadex G-50 gel filtration of the reduced and S-carboxymethylated second trypsin-modified *V. angustifolia* inhibitor. Reduction and S-carboxymethylation of the inhibitor derivative were performed as described by Hirs [22]. The reaction mixture, after acidification with acetic acid to pH 4, was passed through a column (2.4 × 151 cm) of Sephadex G-50 equilibrated with 0.2 M acetic acid. Elution was performed with the same acid (5-ml fractions). Peaks LA and SM were pooled and lyophilized.

TABLE II

PARTIAL SEQUENCES OF *V. ANGUSTIFOLIA* INHIBITOR, SECOND TRYPSIN-MODIFIED FORM, ITS FRAGMENTS OBTAINED BY REDUCTION AND S-CARBOXYMETHYLATION, AND C-TERMINAL HEXAPEPTIDE

Arrows indicate the results of Edman degradation (→), dansylation (⇒), and digestion with carboxypeptidase A and B (←).

Sample	Partial sequence determined
	1 5 10 15
Native	H-Gly-Asp-Asp-Val-Lys-Ser-Ala-Cys-Cys-Asp-Thr-Cys-Leu-Cys-Thr-Arg-
	(Met) *
	1 16
Second modified	H-Gly— —Arg-OH
	1 15 16
Small fragment	H-Gly— —Thr-Arg-OH
	17 19 73
Native	Ser-Gln-Pro— — — — — — — — — — — — — — — — — — —Ile-Lys-Asn-OH
	17 67
Second modified	H-Ser— — — — — — — — — — — — — — — —Lys-OH
	17 20 67
Large fragment	H-Ser-Gln-Pro-Pro-Thr— — — — — — — —Lys-OH
	68 70 73
C-Terminal peptide	H-Glu-Glu-Val-Ile-Lys-Asn-OH

* The ratio of Val to Met, estimated by amino acid analysis after acid hydrolysis of the thiazolinone derivatives, was about 2 : 1.

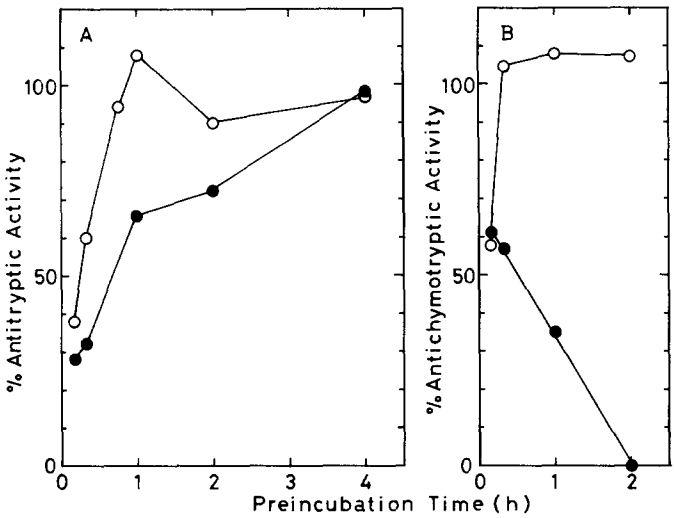

Fig. 5. Time course of the inhibitory activities of the first and the second trypsin-modified *V. angustifolia* inhibitors. A 1 : 1 (w/w) mixture of the inhibitor V and trypsin (or α-chymotrypsin) in 0.1 M Tris-HCl buffer (pH 8), 5 mM $CaCl_2$, was incubated at 37° C. At appropriate time periods, aliquots were withdrawn for the activity measurements by the casein method [20]. Native inhibitor was incubated with either enzyme under the same conditions and activities were expressed by the per cent of inhibition of each derivative on the basis of that of the native inhibitor at each time period as 100%. Enzyme control was also included in each case. (A) Antitryptic activities. (B) Antichymotryptic activities. ○, first trypsin-modified form; ●, second trypsin-modified form.

Inhibitory activities of the second trypsin-modified form

Time course of the inhibitory activities of the second trypsin-modified form was compared with those of the first modified form on the basis of the activities of the native inhibitor at each time period taken as 100% (Fig. 5). As shown in Fig. 5A, the second modified form takes longer incubation time (about 4 h) than the first modified form to attain the same level of the antitryptic activity to the native inhibitor. However, more drastic difference was observed on the antichymotryptic activity (Fig. 5B). That is, the antichymotryptic activity of the first modified inhibitor increased with incubation time and it took about 20 min to attain the same level to the native form, while that of the second modified form declined with incubation time and completely lost after 2 h incubation with chymotrypsin at pH 8.

Discussion

It is well established that the reactive site peptide bonds of the proteinase inhibitors undergo limited cleavage at acidic pH with catalytic amounts of proteinases [11]. However, the research on the additional cleavage during modification by enzyme has been limited except in the cases of the temporary inhibitors of the animal origin [23—25]. One of the legume inhibitors which undergo additional cleavage on prolonged incubation at acidic pH has been shown in the report of Sakura and Timasheff [26] on inhibitor L. In this case, however, the product of the additional cleavage has neither been isolated nor

characterized. The double-headed type legume inhibitor V, which we have isolated and characterized [9], is considerably different from the other double-headed inhibitors reported so far [1—8] on the point that this inhibitor undergoes more rapid sequential cleavage at multiple sites of the molecule under acidic condition or even at neutral pH with a catalytic amount of trypsin or α-chymotrypsin (Abe, O., unpublished data).

The first incubation product formed at acidic pH with a catalytic amount of trypsin has been isolated and characterized as the first trypsin-modified inhibitor V in which the antitryptic site Arg-Ser bond has been specifically cleaved [10]. The second product, isolated and characterized in this report, is also a trypsin-modified form, although its C-terminal hexapeptide, H-Glu-Glu-Val-Ile-Lys-Asn-OH, has been missing. The peptide contains in its sequence, the characteristic C-terminal portion of the native inhibitor, that is, -Ile-Lys-Asn-OH [10]. Hence, the whole process for the production of the second derivative seems to follow the scheme shown in Fig. 6.

The second trypsin-modified form lacking the C-terminal peptide still possesses inhibitor activities against trypsin and α-chymotrypsin, however, they are somewhat different from those of the first modified form, especially in the antichymotryptic activity (Fig. 5B). Antitryptic activity also has a slight difference but on incubation with trypsin at pH 8 the activity increases depending on the incubation time to approach, anyhow, to that of the native inhibitor. These facts might depend on the location of the reactive sites on the inhibitor molecule. That is, the antitryptic site Arg(16)-Ser(17) locates on the N-terminal half region of the inhibitor, while the antichymotryptic site Tyr(43)-Ser(44), on the other half (Abe, O., unpublished data). Therefore, lack of the C-terminal peptide considerably affected on the antichymotryptic activity, and not on the antitryptic activity. In addition, further proteolysis of the modified inhibitor might also be responsible for the progressive loss of the antichymotryptic activity.

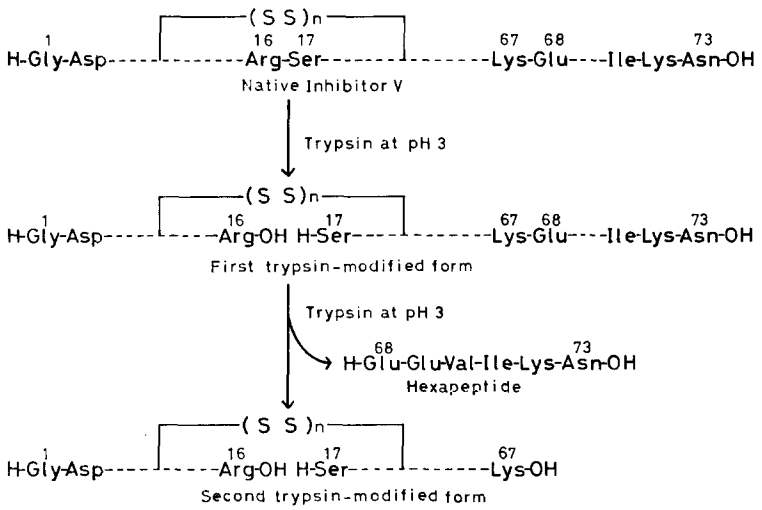

Fig. 6. Schematic presentation of the sequential tryptic cleavage of the *V. angustifolia* inhibitor at pH 3.

On liberation of the C-terminal peptide, the inhibitor derivative evidently loses a negative charge of a Glu residue and becomes more basic. This agrees with the mobility on polyacrylamide gel electrophoresis (Fig. 3), that is, the second modified form is less mobile than the native or the first modified form.

Purification and characterization of the minor product of the incubation mixture are now under investigation in our laboratory.

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References

1 Bowman, D.E. (1946) Proc. Soc. Exp. Biol. Med. 63, 547—550
2 Birk, Y. (1961) Biochim. Biophys. Acta 54, 378—381
3 Jones, G., Moore, S. and Stein, W.H. (1963) Biochemistry 2, 66—71
4 Wilson, K.A. and Laskowski, M., Sr. (1973) J. Biol. Chem. 248, 756—762
5 Belew, M., Porath, J. and Sundberg, L. (1975) Eur. J. Biochem. 60, 247—258
6 Yoshida, C. and Yoshikawa, M. (1975) J. Biochem. (Tokyo) 78, 935—945
7 Gennis, L.S. and Cantor, C.R. (1976) J. Biol. Chem. 251, 734—740
8 Odani, S. and Ikenaka, T. (1977) J. Biochem. (Tokyo) 82, 1513—1522
9 Abe, O., Ohata, J., Utsumi, Y. and Kuromizu, K. (1978) J. Biochem. (Tokyo) 83, 1737—1748
10 Abe, O., Shimokawa, Y., Araki, T. and Kuromizu, K. (1978) J. Biochem. (Tokyo) 83, 1749—1756
11 Laskowski, M., Jr., and Sealock, R.W. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 3, pp. 375—473, Academic Press, New York
12 Wang, S.-S. and Carpenter, F.H. (1965) J. Biol. Chem. 240, 1619—1625
13 Knights, R.J. and Light, A. (1974) Arch. Biochem. Biophys. 160, 377—386
14 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
15 Woods, K.R. and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369—370
16 Edman, P. and Henschen, A. (1975) in Protein Sequence Determination (Needleman, S.B., ed.), 2nd edn., pp. 232—279, Springer Verlag, Berlin
17 Kulbe, K.D. (1974) Anal. Biochem. 59, 564—573
18 Van Orden, H.O. and Carpenter, F.H. (1964) Biochem. Biophys. Res. Commun. 14, 399—403
19 Mendez, E. and Lai, C.Y. (1975) Anal. Biochem. 68, 47—53
20 Kunitz, M. (1947) J. Gen. Physiol. 30, 291—310
21 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
22 Hirs, C.H.W. (1967) Methods Enzymol. 11, 199—203
23 Tschesche, H. and Klein, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1645—1656
24 Schneider, S.L., Stasiuk, L. and Laskowski, M., Sr. (1973) J. Biol. Chem. 248, 7207—7214
25 Schneider, S.L. and Laskowski, M., Sr. (1974) J. Biol. Chem. 249, 2009—2015
26 Sakura, J.D. and Timasheff, S.N. (1974) Biochim. Biophys. Acta 336, 37—45