PURIFICATION AND SOME PROPERTIES OF TWO PROTEINASE INHIBITORS FROM *ERYTHRINA ACANTHOCARPA* SEED

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ABSTRACT.—Two proteinase inhibitors (DE-1 and DE-2) were purified from *Erythrina acanthocarpa* seed by gel filtration followed by ion exchange chromatography on DEAE-cellulose and DEAE-sepharose. They contain 163–164 amino acids (molecular weight 18000) including four half-cystine residues and resemble the Kunitz-type proteinase inhibitors. The N-terminal amino acid sequence of DE-1 also shows homology with those of the Kunitz-type inhibitors. For DE-2 no free N-terminal amino acid was found. DE-1 contains a potent inhibitor for both porcine trypsin and bovine α-chymotrypsin. Inhibitor DE-2 inhibits α-chymotrypsin strongly and it has practically no action on trypsin.

Proteinase inhibitors are of widespread occurrence in the animal and plant kingdoms and they have been isolated from various Leguminosae seeds (1). Members of the Leguminosae are usually divided into three subfamilies, namely, Mimosoideae, Caesalpinioideae and Lotoideae (Papilionoideae) (2). A number of proteinase inhibitors from the Lotoideae, which includes most of the common food and fodder legumes of agricultural importance, and several from the Caesalpinioideae have been isolated and characterized (3). Recently, Odani *et al.* (4) and Kortt and Jermyn (5) reported on proteinase inhibitors from legume seeds which belong to the subfamily Mimosoideae.

The genus *Erythrina*, a legume of subfamily Papilionoideae (6), consists of approximately 108 species of trees and shrubs distributed throughout tropical to warm temperature regions of the world (7, 8). It has been established that seeds from Southern African species of *Erythrina*, *viz.*, *E. acanthocarpa*, *E. caffra*, *E. humeana*, *E. latissima*, and *E. lysistemon* contain large concentrations of proteinase inhibitors. The purification and characterization of two proteinase inhibitors (DE-1 and DE-3) from the seed of *E. latissima*, have recently been described (9). The present communication describes the purification and some of the properties of two proteinase inhibitors from the seeds of *E. acanthocarpa*.

EXPERIMENTAL

MATERIAL.—*Erythrina ancanthocarpa* seeds were supplied by the Forest Research Institute, Ketjen Street, Pretoria West. Procine trypsin (3 times crystallized) was supplied by Miles Laboratories (Pty) Ltd., Cape Town. Bovine α-chymotrypsin was obtained from Worthington. *N*-α-benzoyl-L-arginine ester hydrochloride (BzArgOEt) and *N*-acetyl-L-tyrosine ethyl ester (AcTyrOEt) was obtained from BDH Chemicals and Merck, respectively. Diethylaminoethyl cellulose (DEAE-cellulose) was a microgranular preparation (DE-52) from Whatman. Sephadex G-50 (fine) and DEAE-sepharose CL-6B were obtained from Pharmacia.

PHYSICOCHEMICAL METHODS.—Sephadex G-50, DEAE-cellulose and DEAE-sepharose columns were prepared as recommended by the manufacturers and the eluates were monitored at 280 nm with a Beckman spectrophotometer. Estimation of molecular weights by gel filtration was carried out as described by Andrews (10) on a Sephadex G-50 column (0.9 x 150 cm). Markers used were soybean trypsin inhibitor (20 100), myoglobin (17 800), ribonuclease (13 700) and *Naja nivea* toxin α (7 900).

Disc electrophoreis at pH 8.9 was performed with a 15% gel according to the method of Ornstein and Davis (11). Sodium dodecyl sulfate gel electrophoresis at pH 7.2 carried out with a 10% gel as described by Weber and Osborne (12).

PROTEINASE INHIBITOR ASSAYS.—Assays used were based on the methods developed by Schwert and Takenaka (13). The rates of hydrolysis at 30° of *N*-α-benzoyl-L-arginine ethyl ester by porcine trypsin and of *N*-acetyl-L-tyrosine ethyl ester by bovine chymotrypsin, were recorded as a change in absorption at 253 nm and 247 nm, respectively.

Both enzymes were kept as stock solution of 3 mg/ml in 0.001 M hydrochloric acid. The substrates were used at concentrations of 0.001 M for both *N*-α-benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester in 0.05 M Tris/HCl, 0.01 M calcium chloride, pH 8 and 0.05 M potassium phosphate pH 7 containing 10% methanol, respectively.

To assess the inhibition of trypsin and chymotrypsin by increasing levels of the inhibitors, the enzymes were incubated with suitable quantities of the inhibitors in 0.1 M Tris/HCl pH 8, for 5 min at room temperature and thereafter assayed for enzyme activity remaining. The concentration of the enzymes was corrected for inactive materials as determined by active-site titrations (14).

One unit of enzyme activity was defined as that amount of enzyme causing a change in the amount of substrate of 1 μmol/min at 30°. One unit of inhibitor activity was defined as that amount of inhibitor which inhibited one unit of enzyme activity. Specific inhibitor activity was expressed as inhibitor units per mg inhibitor.

CHEMICAL ANALYSIS METHODS.—Amino acid analyzes were performed with an automatic Beckman amino acid analyzer. Samples were hydrolyzed with 6 N hydrochloric acid for 24 h in sealed evacuated tubes; phenol was added to prevent destruction of tyrosine (15). Half-cystine was determined as cysteic by the method of Hirs (16). For the determination of tryptophan the samples were hydrolyzed with 3 M *p*-toluene sulphonic acid as described by Liu and Chang (17). Free sulphydryl groups were assayed in intact proteinase inhibitor samples in 6 M guanidinium chloride according to Ellman (18).

N-TERMINAL AMINO ACID SEQUENCE.—The N-terminal sequence of reduced and S-carboxymethylated proteinase inhibitor samples was determined with a Beckman sequencer as described (19).

PREPARATION OF THE CRUDE PROTEINASE INHIBITOR.—Ground defatted *E. acanthocarpa* seeds (100 g) were extracted with 0.5 M sodium chloride solution (1 liter) overnight at 10°. The suspension was then macerated for 5 min in a Waring blender. The extract was clarified by centrifugation at 10 000 rpm and brought to 70% saturation with ammonium sulfate, and the precipitate was recovered by centrifugation. The precipitate was redissolved in 0.05 M sodium chloride solution, dialyzed against distilled water and lyophilized. The yield of the extract was 10.7 g.

RESULTS

The elution profile obtained for the crude extract on Sephadex G–50 in 0.2 M ammonium hydrogencarbonate solution is shown in fig. 1. Various peaks were

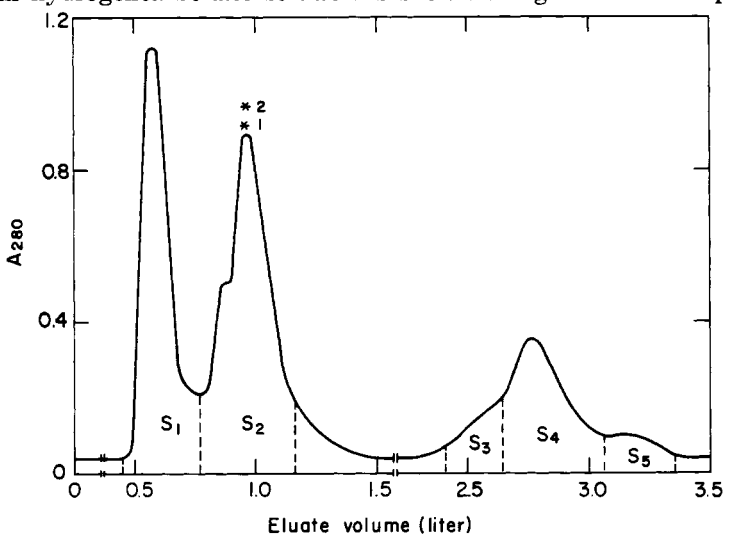

FIG. 1. Gel filtration of the crude extract of the seeds *E. acanthocarpa*. Crude extract (2 g) was loaded on Sephadex G–50 column (3.8 x 150 cm) and elution effected with 0.2 M ammonium hydrogencarbonate solution at a flow rate of 50 ml/h. The column temperature was 20° and the elute was monitored at 280 nm. *1 indicates trypsin inhibitor activity and *2 chymotrypsin inhibitor activity.

evident with only peak S_2 exhibiting trypsin as well as chymotrypsin inhibitor activities. Peak S_2 was lyophilized and further fractionated on DEAE-cellulose on a linear sodium chloride gradient (0.2 M over 2 *l*) in 0.05 M Tris/HCl at pH 8. This revealed four major proteinase inhibitor peaks (fig. 2). Peaks C_1, C_2, C_3 and C_4 were each rechromatographed on DEAE-sepharose columns (0.9 x 15 cm) on a linear sodium chloride gradient (0–0.2 M over 1 *l*) at a flow rate of 12 ml/h

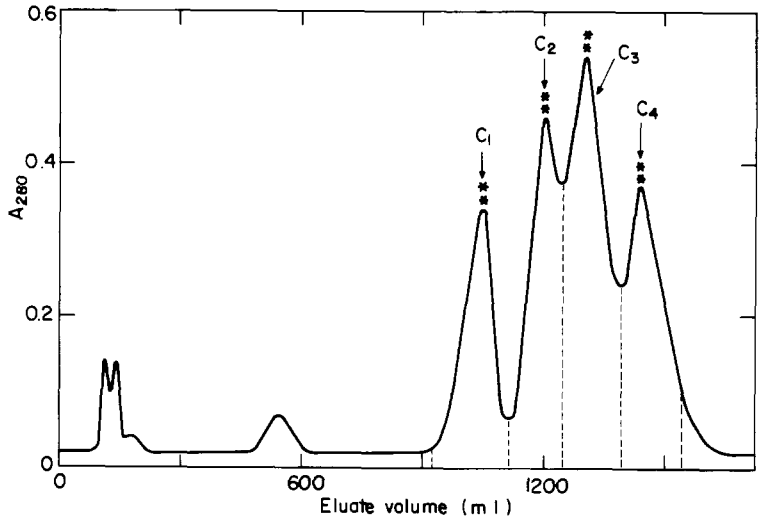

FIG. 2. Chromatography of peak S_2 on DEAE-cellulose. Peak S_2 (0.25 g) was loaded on DEAE-cellulose (0.9 x 15 cm) column and elution effected by a linear sodium chloride gradient (0–0.2 M over 2 liter) in 0.05 M Tris/HCl at pH 8 at a flow rate of 50 ml/h. The column temperature was 20° and the elute was monitored at 280 nm. *1 indicates trypsin inhibitor activity and *2 chymotrypsin inhibitor activity.

in 0.05 M Tris/HCl pH 8. The chromatograms revealed for C_1, C_2 and C_3, respectively, major peaks DE–1, DE–2 and DE–3 and for C_4 two major peaks DE–4a and DE–4b. The purification of the proteinase inhibitors is summarized in table 1.

Disc electrophoresis both in the absence and presence of dodecyl sulfate showed that the proteinase inhibitors DE–1 and DE–2 were probably homogenous, but disc electrophoresis revealed two major bonds for DE–3, DE4a, and DE–4b (fig. 3). Further purification of DE–3, DE–4 and DE–4b was attempted but it was not successful. Some of the properties of inhibitors DE–1 and DE–2 are summarized

TABLE 1. Summary of the purification of proteinase inhibitors DE–1, DE–2, DE–3, DE–4a and DE–4b.

Step	Protein [mg]	Total inhibitor activity [units x 10^3]	Specific inhibitor activity [units/mg protein]	Yield [%]
Crude preparation	2 000	T 2 380	1 190	100
		C 2 240	1 120	100
Sephadex G–50	542	T 1 520	2 810	63.8
		C 1 590	2 940	71.0
DEAE-cellulose and DEAE-sepharose DE–1	32	T 319	9 980	13.4
		C 150	4 680	6.7
DE–2	41	T 7.8	190	0.3
		C 405	9 870	18.1
DE–3	55	T 303	5 510	12.7
		C 221	4 020	9.9
DE–4a	28	T 136	4 890	5.7
		C 73	2 620	3.3
DE–4b	22	T 144	6 590	6.1
		C 87	4 030	3.9

T = trypsin inhibitor and C = α-chymotrypsin.

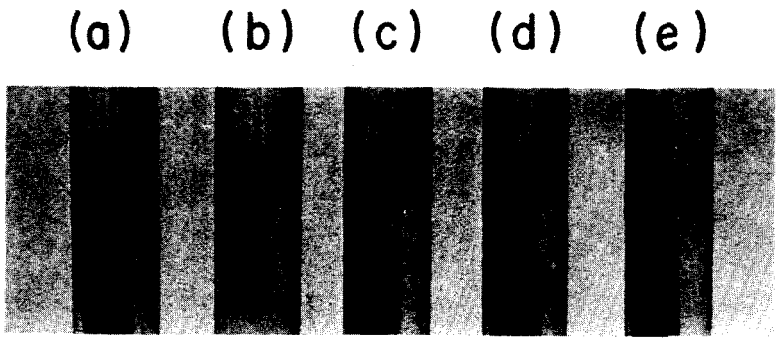

FIG. 3. Polyacrglamide gel electrophoresis of proteinase inhibitor fractions of *E. acanthocarpa* seed. (a) DE-1; (b) DE-2; (c) DE-3; (d) DE-4a and (e) DE-4b.

TABLE 2. Some of the properties of proteinase inhibitors DE-1 and DE-2.

Property	DE-1	DE-2
Disc electrophoresis	one band	one band
SDS[a]-gel electrophoresis	one band	one band
Molecular weight		
(i) gel filtration[b]	18,300	18,100
(ii) SDS-gel	17,400	18,100
Inhibitor activities	Trypsin Chymotrypsin	— Chymotrypsin
Free SH	none	none
N-terminal amino acids	Valine	none found

[a]Sodium dodecyl sulfate.
[b]In 0.05 M Tris/HCl pH 8+0.2 M NaCl.

TABLE 3. Amino acid composition of proteinase inhibitors DE-1 and DE-2. Values are given as mol of residue per mol inhibitor on the basis of a molecular weight of 18,000.

Amino acid	DE-1	DE-2	Kunitz soybean trypsin inhibitor (20-22)
Aspartic acid	17.4(17)	18.3(18)	26
Threonine	8.9(9)	11.1(11)	7
Serine	13.1(13)	11.7(12)	11
Glutamic acid	22.2(22)	21.1(21)	18
Proline	10.4(10)	11.1(11)	10
Clycine	13.8(14)	12.3(12)	16
Alanine	6.2(6)	11.9(12)	8
Half-cystine[a]	4.0(4)	3.7(4)	4
Valine	11.1(11)	10.6(11)	14
Methionine	0.04(0)	0.02(0)	2
Isoleucine	5.9(6)	6.7(7)	14
Leucine	15.8(16)	15.8(16)	15
Tyrosine	6.8(7)	5.7(6)	4
Phenylalanine	5.3(5)	5.0(5)	9
Lysine	11.8(12)	6.8(7)	10
Histidine	1.8(2)	2.4(2)	2
Arginine	6.7(7)	7.3(7)	9
Tryptophan	1.9(2)	1.8(2)	2
Total	163	164	181

[a]Determined as cysteic acid by the method of Hirs (16).

in Table 2 and their amino acid composition together with that of the Kunitz soybean trypsin inhibitor (20–22) is given in table 3.

The N-terminal primary structure of reduced and S-carboxymethylated proteinase inhibitor DE–1, determined on the Beckman sequencer, is given in table 4. However, Edman degradation with the sequencer failed to yield any N-terminal amino acid for intact proteinase inhibitor DE–2. The N-terminal amino acid could be blocked with an acetyl group or a pyroglutamyl residue (23).

TABLE 4. N-terminal sequence of reduced and S-carboxymethylated proteinase inhibitor DE–1[a].

Step	High-performance liquid chromatography
1	Val 137
2	Leu 157
3	Leu 108
4	Asp 110
5	Gly 65
6	Asn 101
7	Gly 79
8	Glu 87
9	Val 114
10	Val 114
11	Glu 55
12	Asn 48
13	Gly 41
14	Gly 50
15	Thr 20
16	Tyr 42
17	Tyr 48
18	Leu 40
19	Leu 45
20	Pro 31
21	Gln 24

[a]250 nmol was loaded. The quadrol programme (Beckman No. 12294 mod.) was used. The numbers signify yields of phenylthiodantoin-amino acids in nmol.

Inhibition of porcine trypsin and bovine α-chymotrypsin at pH 8 by increasing levels of inhibitor DE–1 and DE–2 is shown in fig. 4.

DISCUSSION

The proteinase inhibitors from leguminosae may be divided into two general groups depending on their molecular weight and cystine content, namely, the Bowman-Birk-type inhibitors and the Kunitz-type inhibitors. The Bowman-Birk-type inhibitors have molecular weights of 8 000–10 000 and a high cystine content (usually 7 disulphides) (24–31). The Kunitz-type inhibitors have molecular weights of approximately 20 000 and a low cystine content (usually two disulphides). The Kunitz soybean trypsin inhibitor (20–22) is a typical example of this group. The inhibitors from winged bean (*Psophocarpus tetragonolobus*) (32, 33), the seeds of *Albizzia julibrissin* (4), *Erythrina latissima* (9) and *Acacia elata* (5) also belong to the Kunitz-type.

The molecular weights of proteinase inhibitors DE–1 and DE–2 from *E. acanthocarpa* by gel filtration and dodecyl sulfate gel electrophoresis were of the order of 18 000, and hence the inhibitors comprise 163–164 amino acid residues including four half-cystine residues (tables 2 and 3). Since no sulphydryl groups could be detected in the intact inhibitors, they are cross-linked by two intramolecular disulphide bonds. The molecular weights and low disulphide content of the *E. acanthocarpa* inhibitors resemble the Kunitz-type proteinase inhibitors. Furthermore, in fig. 5 the N-terminal primary structure of inhibitor DE–1 is compared

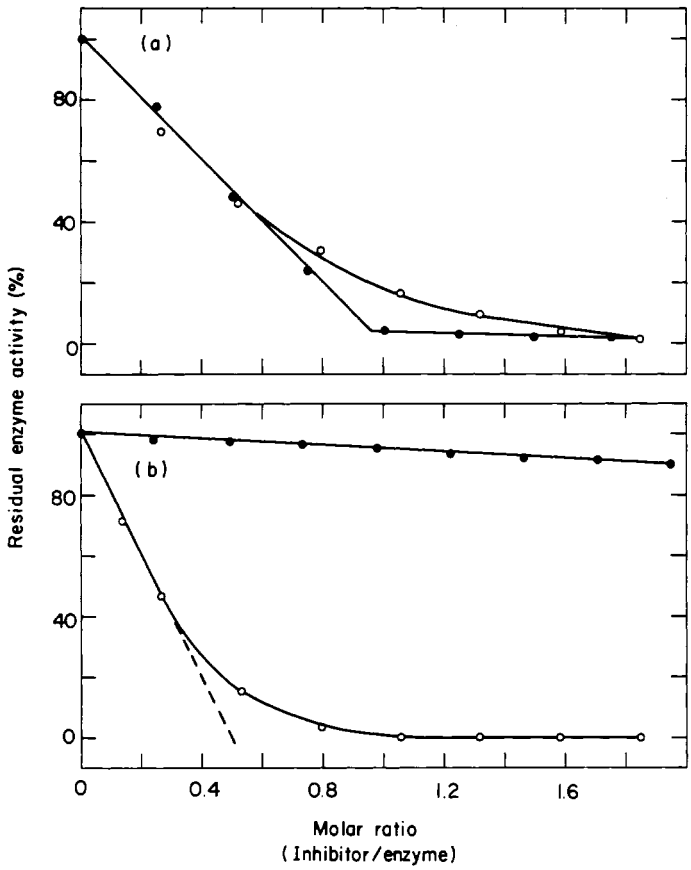

FIG. 4. Inhibition of porcine trypsin and bovine α-chymotrypsin by increasing amount of Kunitz-type proteinase inhibitors from *E. acanthocarpa* seed (a) DE–1 and (b) DE–2.
Inhibition of trypsin ●——●
Inhibition of α-chymotrypsin ○——○
A molecular weight of 18 000 for the two inhibitors was used.

with those of Kunitz soybean trypsin inhibitor (20–22) and Kunitz-type inhibitors from the seeds of *Albizzia julibrissin* (A–II and B–II) (4) and *E. latissima* (9). The high degree of homology is quite obvious.

The inhibitory activity characteristics of the two Kunitz-type proteinase in-

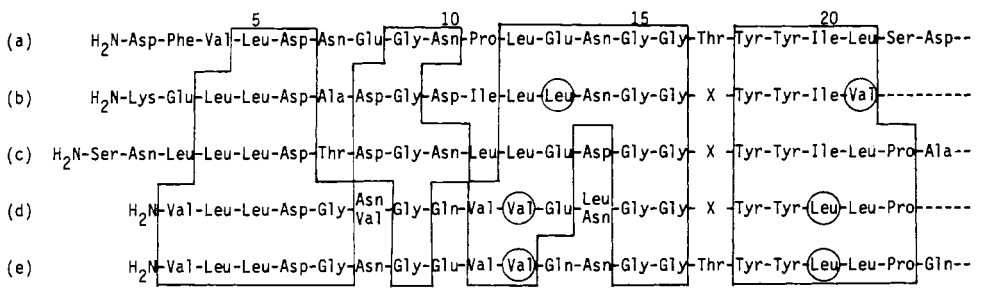

FIG. 5. Comparison of the N-terminal primary structures of Kunitz-type proteinase inhibitors from various sources.
(a) Kunitz soybean trypsin inhibitor (20–22);
(b) *Albizzia julibrissin* A–II (4);
(c) *Albizzia julibrissen* B–II (4);
(d) *Erythrina latissima* DE–3 (9) and
(e) *Erythrina acanthocarpa* DE–1 (this paper). The circles in a boxed region indicate variant amino acid. X indicates unidentified amino acid residues.

hibitors from *E. acanthocarpa* are different. Inhibitor DE–1 contains a very potent inhibitor for both porcine trypsin and bovine α-chymotrypsin (fig. 4a). DE–1 stoichiometrically inhibited trypsin in a molar ratio of 1:1. For α-chymotrypsin the titration curve was at first linear, and extrapolation of the data indicates that one mol of DE–1 reacts with approximately one mol of α-chymotrypsin. This was also the case of some of the Kunitz-type inhibitors of the *Psophocarpus tetragonolobus* bean (32, 33). At high molar ratios, DE–1 inhibited trypsin and α-chymotrypsin almost completely.

Inhibitor DE–2 comprises a very strong inhibitor for α-chymotrypsin (fig. 4b). The titration curve was at first linear and extrapolation of the data indicates that one mol of the inhibitor binds approximately two mols of α-chymotrypsin. At high molar ratios DE–2 inhibited α-chymotrypsin completely, but trypsin was only very weakly inhibited.

The inhibitory characteristics of the Kunitz-type proteinase inhibitors are varied. A few inhibitors are reported to be specific for chymotrypsin and do not inhibit trypsin (4, 9, 33). Some Kunitz-type inhibitors are potent inhibitors for trypsin but also inhibit α-chymotrypsin to varying degrees (4, 5, 32). The inhibitory activity properties of the proteinase inhibitors from *E. acanthocarpa* resemble those of the Kunitz-type inhibitors from other legume seeds and beans.

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LITERATURE CITED

1. H. Tschesche, *Angew. Chem. Internat. Edit.* **13**, 10 (1970).
2. V. H. Heywood, "Chemotaxonomy of the Leguminosae," Academic Press, London, 1971, p. 1.
3. B. Kassel and M. J. Williams, "Handbook of Biochemistry," 3rd Edn. CRC Press, Cleveland, 1976, Vol. 2, p. 583.
4. S. Odani, S. Odani, T. Ono and T. Ikenaka, *J. Biochem. (Tokyo)*, **86**, 1795 (1979).
5. A. A. Kortt and M. A. Jermyn, *Eur. J. Biochem.*, **115**, 551 (1981).
6. K. C. Palgrave, "Trees of South Africa," C. Struik Publishers, Cape Town, 1977, p. 214.
7. P. H. Raven, *Lloydia*, **37**, 543 (1974).
8. B. A. Krukoff and R. C. Barneby, *Lloydia*, **37**, 332 (1974).
9. F. J. Joubert, F. H. H. Carlsson and T. Haylett, *Hoppe-Seyler's Z. Physiol. Chem.*, **362**, 531 (1981).
10. P. Andrews, *Methods Biochem. Anal.*, **18**, 1 (1970).
11. L. Ornstein and B. J. Davis, "Disc electrophoresis," reprinted by Distillation Products Industries, Rochester, New York, 1962.
12. K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
13. G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).
14. F. J. Kezdy and E. T. Kaiser, *Methods Enzymol.*, **19**, 3 (1970).
15. F. Sanger and E. O. P. Thompson, *Biochim. Biophys. Acta*, **71**, 468 (1963).
16. C. H. W. Hirs, *Methods Enzym.*, **11**, 59 (1967).
17. T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
18. G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
19. F. J. Joubert, *Hoppe-Seyler's Z. Physiol. Chem.*, **356**, 53 (1975).
20. T. Koide and T. Ikenaka, *Eur. J. Biochem.*, **32**, 401 (1973).
21. T. Koide, S. Tsunasawa and T. Ikenaka, *Eur. J. Biochem.*, **32**, 408 (1973).
22. T. Koide and T. Ikenaka, *Eur. J. Biochem.*, **32**, 417 (1973).
23. K. Narita, H. Matsuo, and T. Nakajima, "End group determination. In Protein sequence determination, (Edited by S. B. Needleman), Springer-Verlag, Berlin, p. 30 (1975).
24. S. Odani and T. Ikenaka, *J. Biochem. (Tokyo)*, **71**, 839 (1972).
25. S. Odani and T. Ikenaka, *J. Biochem. (Tokyo)*, **82**, 1523 (1977).
26. S. Odani and T. Ikenaka, *J. Biochem. (Tokyo)*, **83**, 737 (1978).
27. C. G. L. Tan and F. C. Stevens, *Eur. J. Biochem.*, **18**, 503 (1971).
28. F. C. Stevens, S. Wuerz and J. Krahn, *Bayer-Symp.*, **5**, 344 (1974).
29. K. A. Wilson and M. Laskowski, Sr., *J. Biol. Chem.*, **250**, 4261 (1975).
30. C. Ishikawa, S. Nakamura, K. Watenabe and K. Takahashi, *Febs Lett.*, **99**, 97 (1979).
31. F. J. Joubert, H. Kruger, G. S. Townshend and D. P. Botes, *Eur. J. Biochem.*, **97**, 85 (1979).
32. A. A. Kortt, *Biochim. Biophys. Acta*, **577**, 371 (1979).
33. A. A. Kortt, *Biochim. Biophys. Acta*, **624**, 237 (1980).