Phytochemistry, 1980, Vol. 19, pp. 43–47. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0101-0043 $02.00/0

ISOLATION AND PROPERTIES OF CARBOXYPEPTIDASE FROM LEAVES OF WOUNDED TOMATO PLANTS*

M. WALKER-SIMMONS and C. A. RYAN†

Department of Agricultural Chemistry and Program in Biochemistry and Biophysics, Washington State University, Pullman, WA 99164, U.S.A.

(*Revised received* 9 *May* 1979)

Key Word Index—*Lycopersicum esculentum;* Solanaceae; tomato; wound-induced; leaves; carboxypeptidase; exopeptidase.

Abstract—A carboxypeptidase was purified to homogeneity from upper, unwounded leaves of tomato plants in which carboxypeptidase activity had been induced to increase over three-fold by severely wounding the lower leaves. The carboxypeptidase was purified by ammonium sulfate precipitation, affinity chromatography, and finally by gel permeation chromatography. Electrophoresis at pH 4.3 and isoelectric focusing showed only a single band. The isoelectric point was 5.2 and the MW 105 000. Tomato carboxypeptidase possessed both peptidase and esterase activities and it sequentially hydrolysed amino acids from the carboxyl-terminal end of insulin chain B. It was optimally active at pH 6–7 on peptidase substrates, and at pH 8 on esterase substrates. The enzyme was inhibited by diisopropylfluorophosphate and incorporated 1 mol of DFP-[^{3}H]. per mol of enzyme. Both peptidase and esterase activities were strongly inhibited by $HgCl_2$, but not by *p*-hydroxymercuribenzoate or iodoacetamide. Carboxypeptidase inhibitor from potatoes did not inhibit the enzyme.

INTRODUCTION

Severe wounding of just one or two lower leaves of a young tomato plant causes a several-fold increase in tomato leaf carboxypeptidase in leaves throughout the plant [1]. The increase in carboxypeptidase is independent of endopeptidase, aminopeptidase, naphthylamidase, and BAPAase activities which remain unchanged ([1] and unpublished results). The increase of carboxypeptidase in leaves of wounded tomato plants parallels the wound-induced synthesis and accumulation of two well characterized proteinase inhibitors in the leaves [2–4].

Carboxypeptidases have been identified in many plant species and several have been purified including enzymes from germinated cotton seed [5, 6], French bean leaves [7], orange leaves and exocarp [8, 9], malted barley [10], and germinated wheat [11, 12]. In tomato, a carboxypeptidase, which has been partially purified from the fruit, reaches maximum activity during the early stage of ripening [13].

In this communication we report the purification and characterization of the carboxypeptidase from leaves of severely wounded plants in which the carboxypeptidase activity had increased about three-fold. The purification of this enzyme is the first step toward our understanding of the regulation of a plant leaf carboxypeptidase activity, its role in tomato leaves following wounding, and its relationship to the accumulation of proteinase inhibitors.

RESULTS AND DISCUSSION

Purification of tomato leaf carboxypeptidase

Extraction and ammonium sulfate preparation. The following procedures were conducted at 0–4°. Unwounded upper leaves (460 g) from plants whose lower leaves had been wounded 3 days previously by crushing between a flat file and a wooden dowel were detached and immediately homogenized for 3 min in 1 l. of 0.01 M NaOAc buffer, pH 5.3, containing 0.1 M sucrose, 1.5 mM EDTA, and 0.1% β-mercaptoethanol and centrifuged for 15 min at 5000 g. Powdered $(NH_4)_2SO_4$ was slowly added to the supernatant to give a 40% satuarated solution. The precipitated proteins were removed by centrifugation and solid $(NH_4)_2SO_4$ was added to the supernatant to give an 80% saturated solution and stirred for 1 hr. The precipitate was recovered by centrifugation, dissolved in a minimal volume of the acetate buffer and dialysed against the same buffer, without β-mercaptoethanol, for 15–20 hr with several changes of buffer.

Affinity chromatography. Carboxypeptidase was further purified by affinity chromatography by the modified procedure of Johansen *et al.* [14] using

* Supported in part by National Science Foundation Grant PCM 75-23629. Scientific paper No. 5244, project 1791, College of Agriculture Research Center, Washington State University, Pullman, WA 99164.

† To whom inquiries should be sent.

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; ATME, *N*-acetyl-L-tryptophan methyl ester; BAPA, *N*-benzoyl-L-arginine-*p*-nitroanilide; BTPA, *N*-benzoyl-L-tyrosine-*p*-nitroanilide; DFP, diisopropyl fluorophosphate; DIP, diisopropyl phosphoryl; EDTA, ethylenediaminetetraacetic acid; IAAm, idodoacetamide; *p*HMB, *p*-hydroxymercuribenzoate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; TLCK, *N*-tosyl-lysine chloromethyl ketone; TPCK, *N*-tosyl-phenylalanine chloromethyl ketone; PIIF, proteinase inhibitor-inducing factor Z-, carbobenzoxy.

aminobenzyl succinate aminocaproyl Sepharose 4B gel [15]. The gel was packed in a 2.5 cm wide ×4.3 cm long column equilibrated with 0.01 M Mes buffer, pH 5. The 40–80% $(NH_4)_2SO_4$ fraction from the previous step was added to the column (30–40 ml per run) and the column was washed with 0.01 M Mes, pH 5, until the *A* 280 nm of the effluent was less than 0.1. The column was washed with 0.01 M NaOAc (pH 4.3) containing M NaCl, until the *A* was less than 0.005. Tomato carboxypeptidase was eluted from the affinity column with a pH gradient of pH 5–7 consisting of 40 ml each of 0.01 M NaPi buffer, pH 5, and the same buffer at pH 7. A small, broad inactive peak followed by a peak of active carboxypeptidase was released by the pH gradient. Several volumes (30–40 ml) of the 40–80% $(NH_4)_2SO_4$ fraction preparation were run through the column per day. The samples containing the carboxypeptidase activity were pooled and concentrated to a volume of about 2 ml with a UM-20 filter in an Amicon ultrafiltration cell.

Gel filtration. The concentrated carboxypeptidase solution was applied to a Sephacryl S-200 Superfine gel column (2.3×72.5 cm) equilibrated with 0.05 M NaOAc buffer, pH 5.25, containing 0.25 M NaCl. The column was eluted with the NaOAc buffer at a flow rate of 6 ml/hr and the fractions containing carboxypeptidase activity were pooled.

A summary of the recovery of tomato leaf carboxypeptidase is presented in Table 1. The purification precedure produced a 3.4% recovery of carboxypeptidase and a 350-fold purification from the crude leaf extract. The recovery of pure tomato leaf carboxypeptidase was low and was thought to be a result of proteolysis by leaf proteolytic enzymes or by autolysis. In order to minimize proteolysis or partial proteolysis of the enzyme during the last stages of purification, DFP was added to the affinity-purified enzyme and the pure inactive, DFP-enzyme was recovered by gel filtration. The affinity-purified, DFP-modified, gel-filtered carboxypeptidase was recovered from Sephacryl S-200 at the same v/v_0 as the native enzyme. The enzyme contained from 0.98 to 1.11 mol of DIP per mol of enzyme as determined by incorporation of DFP-[^{3}H]. The DFP-enzyme was utilized for physical and chemical studies whereas non-inhibited enzyme was utilized for all studies requiring active enzyme.

Properties of tomato carboxypeptidase

Electrophoretic mobility. Disc gel electrophoresis of the purified active enzyme on 5% polyacrylamide, pH 4.3, yielded one enzymatically active band, but two protein bands. Only the slowest moving protein band coincided with enzyme activity. Electrophoresis of the DFP-modified enzyme, purified in the presence of DFP, IAAm, and *p*HMB, yielded a single band that coincided exactly with the enzymatically active band.

Electrophoresis of the DFP-inhibited enzyme on 5% polyacrylamide gel at pH 9.5 yielded one major protein band with a second minor band. The active enzyme yielded several bands upon electrophoresis at pH 9.5, indicating that considerable proteolytic degradation had ensued in the absence of the proteolytic inhibitors.

Isoelectric focusing. The purified carboxypeptidase focused to one band with a pI value of 5.2 in gels containing ampholytes from pH 3 to 10. Gel filtration on Sephacryl S-200 showed that the MW of the purified DFP-inhibited carboxypeptidase was 105 000±2500. This value was confirmed with a preparation of purified active enzyme by monitoring carboxypeptidase activity. The MW of the DFP-inhibited enzyme was examined with 12.5% SDS–urea polyacrylamide slab gel electrophoresis and compared with protein standards. A major protein band was observed, with a MW of 37 000 and a lesser band with a MW of 18 000.

The 105 000 MW native enzyme is not a single polypeptide chain. Either the enzyme is composed of subunits, which has been proposed for the cotton carboxypeptidase [5, 6], or the enzyme has been proteolytically cleaved after synthesis. It may be that the molecule consists of three monomers of 37 000 MW and the band at 18 000 MW represents a proteolytic fragment of the monomer unit. A second possibility is that the enzyme consists of a combination of 37 000 and 18 000 MW subunits. The 18 000 band may also result from an impurity although no such impurities were shown in electrophoretic or isoelectric focusing experiments in the absence of chaotropic agents.

Amino acid analysis. The amino acid composition of the enzyme to the nearest integer value is shown in Table 2. The enzyme contains relatively high amounts of aspartic acid and glycine and low amounts of methionine, histidine and arginine. High aspartic acid and low methionine and histidine residues are also found in carboxypeptidases from barley [10] and cotton seed [5].

pH Optimum and stability. Peptidase activity, determined with BTPA as substrate, exhibited a pH op-

Table 1. Purification of tomato carboxypeptidase

Procedure	Activity (units)*	Protein (mg)	Specific activity (units/mg)	Recovery (%)
1. Induced leaves (460 g)	—		—	—
2. Crude leaf extract	4380	8510	0.51	100
3. 40–80% $(NH_4)_2SO_4$ (dialysed)	1480	704	2.1	34
4. Affinity chromatography	240	4.9	49	5.5
5. Sephacryl chromatography	150	0.86	176	3.4

*Activity was determined with BTPA as described in Experimental.

Table 2. Amino acid composition of tomato leaf carboxypeptidase

Amino acid	Tomato leaf residues for MW = 105 000
Asp	100
Thr	35
Ser	64
Glu	77
Pro	35
Gly	93
Ala	71
½ Cys	12
Val	79
Met	14
Ile	58
Leu	69
Tyr	44
Phe	41
Lys	46
His	19
Arg	19
Trp	53
Total	879

timum of 6–7. The optimal activity with the esterase substrate ATME was pH 8. The peptidase pH optimum is a little higher than the normal range of other plant carboxypeptidases which is 5.0–5.5 [5–13] but the esterase optimum is typical. Tomato leaf carboxypeptidase was stable between pH 5 and 7 when incubated in buffers at the appropriate pH in the presence of 0.1% (v/v) β-mercaptoethanol at 4°.

Enzyme specificity. Tomato carboxypeptidase hydrolysed the synthetic peptidase substrate, BTPA, and also hydrolysed carbobenzoxy dipeptides (Table 3). The enzyme preferred peptides with phenylalanine in the R_2 position (of $R_1R_2R_3$ where $R_1 = Z$) and exhibited a much lower enzyme activity when glycine or proline was in the R_2 position. The enzyme also exhibited esterase activity with acetyltryptophan-*p*-nitrophenyl ester, ATME and ATEE.

Table 3. Hydrolysis of carbobenzoxy dipeptide substrates by tomato carboxypeptidase

Substrate	Enzyme activity (ΔA 570/min-unit)
Z-phe-leu	0.590
Z-phe-ala	0.520
Z-phe-phe	0.400
Z-gly-phe	0.004
Z-pro-phe	0.0002

Enzyme assays were conducted with 25–50 μl of affinity purified carboxypeptidase and 0.5 ml of 3.3 mM substrate in 0.05 M phosphate, pH 6.2. Enzyme was incubated with the substrate for 0–20 min at 30° and the sample was measured with the ninhydrin assay as described in Experimental. Activity is expressed as change in *A* at 570 nm per unit of BTPAase activity.

Carboxypeptidase activity of the enzyme toward a polypeptide substrate was demonstrated by analysis of the free amino acids released from oxidized bovine insulin chain B. The enzyme hydrolysed the polypeptide chain with a sequential release of alanine and lysine and a very small amount of proline. The order of amino acid release is identical to the sequence of the first three amino acid residues of the COOH-terminus of the polypeptide.

The ability of tomato carboxypeptidase to hydrolyse potato and tomato proteinase inhibitors was also examined. Tomato carboxypeptidase showed no proteolytic activity on potato or tomato proteinase inhibitors I and II. No release of amino acids was detected when measured with the ninhydrin reagent. In addition, no decrease in the immunological reactivity of the proteinase inhibitors was detectable after incubation with the tomato enzyme. However, the proteinase inhibitors, when reduced and carboxymethylated, became substrates for the carboxypeptidase. Quantitation of the specific amino acids released was not determined but the rate of release of ninhydrin-positive material from the action of the enzyme on reduced, denatured inhibitor II was about four times that released from reduced, carboxymethylated inhibitor I.

Enzyme inhibitors. As shown in Table 4, DFP is a potent inhibitor of the enzyme. The sulfhydryl reagent $HgCl_2$ also inhibits, but the enzyme is not affected by *p*HMB or IAAm. In general $HgCl_2$ is inhibitory toward plant carboxypeptidases and *p*HMB only slowly reacts with these enzymes or does not react at all [9, 10, 13]. The activity of purified tomato carboxypeptidase was not affected when preincubated with purified inhibitors I and II, or carboxypeptidase inhibitor from potato [16] or with crude extracts of potato tubers or tomato leaves.

Immunological characterization. Mouse anti-DFP-inhibited carboxypeptidase antibodies were employed to compare the immunological characteristics of the enzyme from control and wounded plants and during enzyme purification. In Ouchterlony double diffusion assays, the DFP-inhibited enzyme was compared with affinity-purified enzyme and the crude enzyme preparation, precipitated with $(NH_4)_2SO_4$ to concentrate the enzyme. The precipitin lines that formed with each preparation were fully cross-reacting with the others, with no indication of the presence of spurs among the three preparations. $(NH_4)_2SO_4$ fractions from control leaves were also fully cross-reacting.

Immunoelectrophoresis comparing the pure enzyme with the $(NH_4)_2SO_4$ fraction prepared from wounded leaves indicated that the major antigen in the $(NH_4)_2SO_4$ precipitate was not identical to the pure carboxypeptidase. A minor antigen, however, migrated identically with the pure enzyme. Since Ouchterlony assays showed full cross-reactivity between the pure enzyme and all antigens in the $(NH_4)_2SO_4$ precipitate, the major cross-reacting species must be very similar to the pure enzyme. The major antigen did not bind to the affinity columns and could be identified immunologically in the unbound proteins eluting in the breakthrough peak, which contained no carboxypeptidase activity. Thus, the major cross-reacting

Table 4. Effects of enzyme inhibitors on tomato carboxypeptidase activity

Inhibitor	Concentration (mM)	% Activity remaining: Peptidase activity	% Activity remaining: Esterase activity
Control		100	100
DFP	1.0	0	0
$HgCl_2$	0.2	26	37
	2.0	0	7
*p*HMB	1.0	102	106
IAAm	12.5	—	111
	20.0	102	—
	100.0	98	100
TLCK	0.5	102	—
	1.0	80	80
TPCK	0.05	101	—
	0.25	96	99
EDTA	10.0	99	92
β-Mercaptoethanol	0.1% (v/v)	97	104

Inhibitors were pre-incubated with the enzyme for 90 min at room temperature before assaying. Substrates for peptidase and esterase assays were BTPA and acetyl-trp-*p*-nitrophenyl ester, respectively. Conditions for these assays are in the text.

species may be an inhibited enzyme or a zymogen. Further experiments to isolate and identify this protein species are in progress.

This report of the purification of the wound-enhanced tomato leaf carboxypeptidase is the first step towards understanding how the increase in carboxypeptidase activity is regulated and the possible relationships of this process to the accumulation of inhibitors and to the wound hormone PIIF [2, 3].

EXPERIMENTAL

Aminobenzyl-succinate-amino caproyl-Sepharose 4B gel was a gift of Dr. Bert Vallee, Biophysics Laboratory, Harvard Medical School. The following reagents were purchased from the sources indicated: ATEE, BAPA, BTPA, DFP, *p*HMB, leucine-*p*-nitroanilide, phenylalanyl-β-naphthylamide, and iodoacetamide were from Sigma; *N*-acetyl-L-tryptophan methyl ester from Aldrich; *N*-acetyl-L-tryptophan-*p*-nitrophenyl ester from Bachem; DFP-[^{3}H] (3.4 Ci/mmol) from Amersham; oxidized bovine insulin chain B from Boehringer; and Sephacryl S-200 Superfine was from Pharmacia.

Potato proteinase inhibitor I was prepared as previously described [17] and potato proteinase inhibitor II by the method of ref. [18]. Tomato proteinase inhibitors I and II were prepared according to ref. [4]. Carboxymethylated proteinase inhibitors were prepared by G. Plunkett of this laboratory by the modified method of ref. [19].

Tomato plants (*Lycopersicum esculentum* var. Bonny Best) were grown in a growth chamber uner 10 000 lx with a 17 hr day, a daytime temp. of 31° and a night-time temp. of 21°. The plants were utilized *ca* 3 weeks after planting when they had two large expanding leaves plus smaller apical leaves. The lowest leaf was wounded by crushing between a flat file and a wooden dowel. Three days later the adjacent upper unwounded leaf was detached for carboxypeptidase isolation.

Protein concns were determined according to ref. [20] with BSA as the standard. Proteinase inhibitors I and II were determined by the immunological method of radial diffusion in agar gels containing specific antibodies [21].

Enzyme assays. Peptidase activity was routinely assayed with the substrate BTPA [22]. Aliquots of enzyme (50–100 μl) were incubated with 1.35 ml 0.1 M NaPi buffer, pH 6.2, and 0.2 ml 3 mM BTPA, dissolved in *N,N*-dimethylformamide. Samples were incubated for 0–2 hr at 35°. The assay was terminated by adding 1 ml 30% HOAc. A unit of activity is defined as the change in *A* of 0.01 per min at 410 nm. Peptidase activity on protein or polypeptide substrates was monitored by measurement of release of amino acids using ninhydrin [23, 24].

Endopeptidase activity was measured by radial diffusion in agar plates containing 1.5% gelatin [25]. BAPAase activity was measured at 410 nm at pH 8 with the substrate BAPA [26]. Aminopeptidase activity was determined at 410 nm at pH 6.7 with leucine-*p*-nitroanilide as the substrate [27]. Naphthylamidase activity was measured at 525 nm at pH 7.3 with the substrate phenylalamine-β-naphthylamide [28]. Esterase was measured with the substrates ATEE and ATME, at pH 8 [29], and acetyl-tryptophan-*p*-nitrophenyl ester at pH 6.2 [30]. ATEE hydrolysis was measured at 230 nm, ATME at 300 nm and acetyl-tryptophan-*p*-nitrophenyl ester hydrolysis at 410 nm.

Disc gel electrophoresis was performed with 5% acrylamide gels (0.6×9 cm) at pH 4.3 [31] and pH 9.5 [32] usually for 3 to 5 hr at 2.5 ma/tube. Protein was stained with Coomassie blue.

SDS-gel electrophoresis was conducted in slabs (100×160 cm) of 12.5% acrylamide containing 7 M urea according to the modified method of ref. [33]. Samples were prepared to a final concn of 0.16 M Tris, 0.07 M SDS, 2.5 M urea, and 0.65 M dithioerythritol, and then incubated at 100° for 1.5 min. Gel buffer was 0.2 M Tris, 0.66 M glycine, 0.2% (w/v) SDS, pH 8.9, and the reservoir buffer was 0.05 M Tris, 0.4 M glycine, 0.1% (w/v) SDS, pH 7.8. Standards for MW estimation were BSA, 68 000; chymotrypsinogen, 24 000; ribonuclease, 13 500; cytochrome, 12 500 and insulin chain B, 3400. DFP-[^{3}H] distribution in protein in the SDS gel was measured by slicing the gel into 3 mm sections. The sections were

dissolved in 0.5 ml 30% H_2O_2 for 15 hr at 70°, and radioactivity was counted in 15 ml of ScintiVerse counting fluid.

Isoelectric focusing was conducted in 8% polyacrylamide gels containing 0.27% bisacrylamide, 0.03% ammonium-persulfate, 0.06% TEMED (tetramethylethylenediamine), and 2% ampholytes, pH 3–10. Gels were run for 6 hr at 33 V/gel. The upper and lower reservoirs contained H_3PO_4 and 0.15 M ethanolamine, respectively. The gels were soaked in 16% TCA containing 0.2 M sulfosalicylic acid for 2 hr at 50° and stained with 0.04% Brilliant blue G in 0.9% $HClO_4$. The pH gradient in the gels was determined in 3 mm segments of unstained gels which were incubated separately in 1 ml H_2O for 6 hr, before pH measurement.

Amino acid analysis of tomato carboxypeptidase was carried out according to the method of ref. [34]. Samples (1 mg) were hydrolysed under N_2 in sealed ampoules at 110° for 24 and 48 hr. A standard amount of norleucine was added to each sample before hydrolysis. The hydrolysed samples were dried *in vacuo* and analyses were performed with an amino acid analyser. A separate 1 mg sample was performic acid-oxidized, hydrolysed, and analysed separately for determination of half-cystine.

MW was estimated from the elution vol. on Sephacryl S-200 gel. The column (2.3×72.5 cm) was calibrated with the following standard proteins: γ-globulin (155 000), almond β-glucosidase (117 000), calf mucosa alkaline phosphatase (100 000), human transferrin (80 000), BSA (68 000), ovalbumin (45 000), and chymotrypsinogen (24 000).

Immunological studies. Antibodies specific for DFP-inhibited carboxypeptidase were prepared in mice ascites serum fluid. The ascites fluid was induced to accumulate in the abdomen of mice by an initial intraperitoneal injection of 0.2 ml complete Freund's adjuvant (diluted 1:1 with H_2O) followed by weekly injections of 0.2 ml incomplete Freund's adjuvant (diluted 1:1 with H_2O). When the ascites fluid was present in sufficient quantities, the mice were inoculated every 2 weeks with 0.1 mg of purified DFP-inhibited tomato carboxypeptidase in 0.2 ml of incomplete Freund's adjuvant (diluted 1:1 with H_2O). Ascites fluid (0.5–3 ml) was removed from the intraperitoneal cavity once a week.

Ouchterlony double-diffusion assays [35] were performed in 2% Noble agar in 0.1 M Na barbital, pH 8.2, containing 0.9% NaCl and 0.1% thimerosol. Immunoelectrophoresis was carried out with 1.5% Noble agar in 0.10 M Na barbital, pH 8.6, on glass slides (2.5×7.5 cm) as described in ref. [17]. Electrophoresis was conducted for 3 hr at 3 mA/slide. A longitudinal well was filled with mouse ascites fluid and the slide was developed overnight at 4°.

Acknowledgements—We thank Dr. Bert Vallee for the generous gift of aminobenzylsuccinate-amino-caproyl-Sepharose 4B gel, Mr. Gregory Plunkett for preparing the carboxymethylated proteinase inhibitors, and Mr. Richard Hamlin for growing the tomato plants. The help of Dr. William Davis in preparing antibodies is gratefully acknowledged.

REFERENCES

1. Walker-Simmons, M. and Ryan, C. A. (1976) *Biochem. Biophys. Res. Commun.* **74,** 411.
2. Green, T. R. and Ryan, C. A. (1972) *Science* **175,** 776.
3. Green, T. R. and Ryan, C. A. (1973) *Plant Physiol.* **51,** 19,
4. Gustafson, G. and Ryan, C. A. (1976) *J. Biol. Chem.* **251,** 7004.
5. Ihle, J. N. and Dure, L. S. (1972a) *J. Biol. Chem.* **247,** 5034.
6. Ihle, J. N. and Dure, L. S. (1972b) *J. Biol. Chem.* **247,** 5041.
7. Carey, W. F. and Wells, J. R. E. (1972) *J. Biol. Chem.* **247,** 5573.
8. Zuber, H. (1976) in *Methods in Enzymology* (Lorand, L., ed.) Vol. 45B, pp. 561–568. Academic Press, New York.
9. Kubota, Y., Shoji, S., Funakoshi, T. and Ueki, H. (1973) *J. Biochem.* **74,** 757.
10. Ray, L. E. (1976) *Carlsberg Res. Commun.* **41,** 169.
11. Preston, K. R. and Kruger, J. E. (1976) *Plant Physiol.* **58,** 516.
12. Preston, K. R. and Kruger, J. E. (1977) *Phytochemistry* **16,** 525.
13. Matoba, T. and Doi, E. (1974) *Agric. Biol. Chem.* **38,** 1901.
14. Johansen, J. T., Breddam, K. and Ottesen, M. (1976) *Carlsberg Res. Commun.* **41,** 1.
15. Peterson, L. M., Sokolovsky, M. and Vallee, B. L. (1976) *Biochemistry* **25,** 2501.
16. Ryan, C. A., Hass, G. M. and Kuhn, R. W. (1974) *J. Biol. Chem.* **249,** 5495.
17. Melville, J. C. and Ryan, C. A. (1972) *J. Biol. Chem.* **247,** 3445.
18. Bryant, J., Green, T. R., Gurusaddaiah, T. and Ryan, C. A. (1976) *Biochemistry* **15,** 3418.
19. Crestfield, A. M., Moore, S. and Stein, W. H. (1963) *J. Biol. Chem.* **238,** 622.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
21. Ryan, C. A. (1967) *Analyt. Biochem.* **19,** 434.
22. Aibara, S., Hayashi, R. and Hata, T. (1971) *Agric. Biol. Chem.* **35,** 658.
23. Moore, S. and Stein, W. H. (1954) *J. Biol. Chem.* **211,** 907.
24. Moore, S. (1968) *J. Biol. Chem.* **243,** 6281.
25. Santarius, K. and Ryan, C. A. (1977) *Analyt. Biochem.* **77,** 1.
26. Preiser, H., Schmitz, J., Maestracci, D. and Crane, R. K. (1975) *Clin. Chim. Acta* **59,** 169.
27. Chrispeels, M. J. and Boulter, D. (1975) *Plant Physiol.* **55,** 1031.
28. Kolehmainen, L. and Mikola, J. (1971) *Arch. Biochem. Biophys.* **145,** 633.
29. Matern, H., Betz, H. and Holzer, H. (1974) *Biochem. Biophys. Res. Commun.* **60,** 1051.
30. Bai, Y., Hayashi, R. and Hata, T. (1975) *J. Biochem.* **78,** 617.
31. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404.
32. Reisfield, R. A., Lewis, U. J. and Williams, D. E. (1962) *Nature* **195,** 281.
33. Swank, R. T. and Munkres, K. D. (1971) *Analyt. Biochem.* **39,** 462.
34. Moore, S. and Stein, W. (1963) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N., eds.) Vol. VI, pp. 819–831. Academic Press, New York.
35. Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26,** 507.