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PURIFICATION AND PROPERTIES OF AN ESTERASE FROM *CUCURBITA MAXIMA* FRUIT TISSUE

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; esterase.

Abstract—An esterase from the fruit of *Cucurbita maxima* was purified to apparent homogeneity. The enzyme displays an M_r of 36 000, a pI value of 4.9, a Stokes radius of 2.70 nm, diffusion coefficient of 9.25×10^{-7} cm^2/sec, possesses a homogeneous dimeric quaternary structure with a subunit M_r of 18 000. High esterolytic activity was observed with indophenyl acetate (1510 μmol/min/mg protein and *p*-nitrophenyl acetate (648 μmol/min/mg protein) while the enzyme displayed no carboxypeptidase, amidase, proteinase or aminopeptidase activities. Based on indophenyl acetate as substrate, the esterase has an optimum pH of 7.5 to 8.9 and a K_m value of 0.14 mM at pH 8.0. The esterolytic activity is strongly inhibited by mercaptide-forming and alkylating thiol reagents and by diisopropyl fluorophosphate and phenylmethylsulphonyl fluoride. Product inhibition by indophenyl was competitive (apparent $K_i = 90$ μM) relative to indophenol acetate and parabolic. Acetate was without effect.

INTRODUCTION

Esterases are classified as hydrolases [1], a large and diverse group of enzymes which can hydrolyse peptides, amides and halides as well as esters. Esterases have been purified from a wide variety of sources, animal tissue in particular [2], while those present in higher plants have received relatively little attention [3, 4]. In spite of the wide distribution of esterases, their physiological functions in intermediary metabolism is in many cases unclear [2]. It has been proposed that animal esterases may participate in detoxificating a wide range of substances which include esters of exogenous origin [5, 6], but the function of esterases in plants is more obscure [4]. Classification of esterases can be problematic [4] and involves determining substrate specificities [7, 8] and susceptibilities to inhibitors [9]. During the course of our investigations into the transformation and breakdown of cucurbitacins in *Cucurbita maxima* [10–12], an esterase enzyme has been isolated from the fruit of the Green Hubbard cultivar. In this paper we communicate details regarding the purification of the enzyme as well as some of its molecular and kinetic properties.

RESULTS AND DISCUSSION

Purification and homogeneity

Results of the purification procedure are summarized in Table 1. Both the precipitated protein obtained after 50% saturation with ammonium sulphate and the post 85% ammonium sulphate saturated supernatant were devoid of esterase activity. Two esterase activity peaks were obtained after DEAE chromatography of the 50–85% ammonium sulphate precipitated protein; a minor peak (*ca* 13% of the applied activity) appearing in the non-retained protein peak and the major esterase peak eluting at 0.24 M sodium chloride (Fig. 1). Gel Chromatography of the latter on Sephadex G-75 yielded a single peak of esterase activity (Fig. 2) which was judged to be homogeneous by PAGE (Fig. 3). Esterolytic activity with indophenyl acetate was associated with the single protein band, as indicated by placing unfixed gels, after electrophoresis, in a staining solution whose composition was the same as that used for the enzyme assays (see Experimental).

Table 1. Purification of esterase from *C. maxima* (enzyme activity is expressed as μmol indophenol formed per min).

Step	Purification step	Total protein (mg)	Specific activity (μmol/min/mg protein)	Purification (fold)	Yield (%)
I	Extraction	8200	14	1	100
II	$(NH_4)_2SO_4$ fractionation	1880	52	3.7	81
III	DEAE-*Sephacel*	48	1307	93	53
IV	Sephadex G-75	7.5	5165	369	32

Lyophilization of the purified esterase resulted in an 80% loss in activity, whereas, when stored at 4° in a high ionic strength buffer (50 mM Tris–acetate, pH 8.0, containing 0.1 M NaCl) the loss in activity was *ca* 0.5% per day.

Molecular properties

The M_r, Stokes radius and diffusion coefficient of the purified esterase from *C. maxima* were determined by means of analytical gel chromatography. An apparent M_r value of 36 000, a Stokes radius of 2.7 nm and a diffusion coefficient of 9.52×10^{-7} cm^2/sec were obtained. The

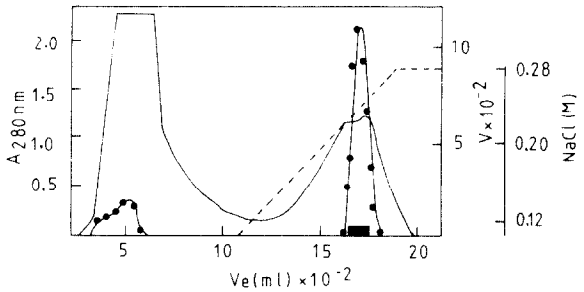

Fig. 1. DEAE-Sephacel ion excahnge chromatography. Enzyme preparation (150 ml containing 1880 mg protein) obtained from the ammonium sulphate precipitation step was applied to the column (5 × 27 cm) at flow rate of 25 ml/hr. The column was developed with 735 ml of 50 mM Tris–acetate buffer, pH 8.0, containing 100 mM NaCl followed by a linear gradient of 100 to 270 mM NaCl in the starting buffer, effective over 800 ml, before the final elution with 270 mM NaCl in the same buffer. 4.2 ml Fractions were collected. —, Protein concentration at A_{280}; ●——●, enzyme activity (v) expressed in μmol indophenol formed/min/ml. Fractions containing enzyme activity were combined as indicated.

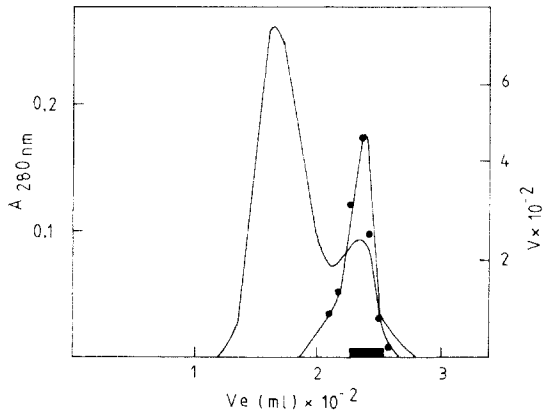

Fig. 2. Sephadex G-75 medium gel exclusion chromatography. Seventeen ml of the enzyme solution (48 mg protein) obtained after DEAE chromatography was applied to the column (2.6 × 95 cm) at a flow rate of 12 ml/hr. The column was developed with 100 mM Tris–acetate buffer pH 8.0, containing 50 mM NaCl. 4.2 ml Fractions were collected. —, Protein concentration at A_{280}; ●—●, enzyme activity (v) expressed in μmol indophenol formed/min/ml.

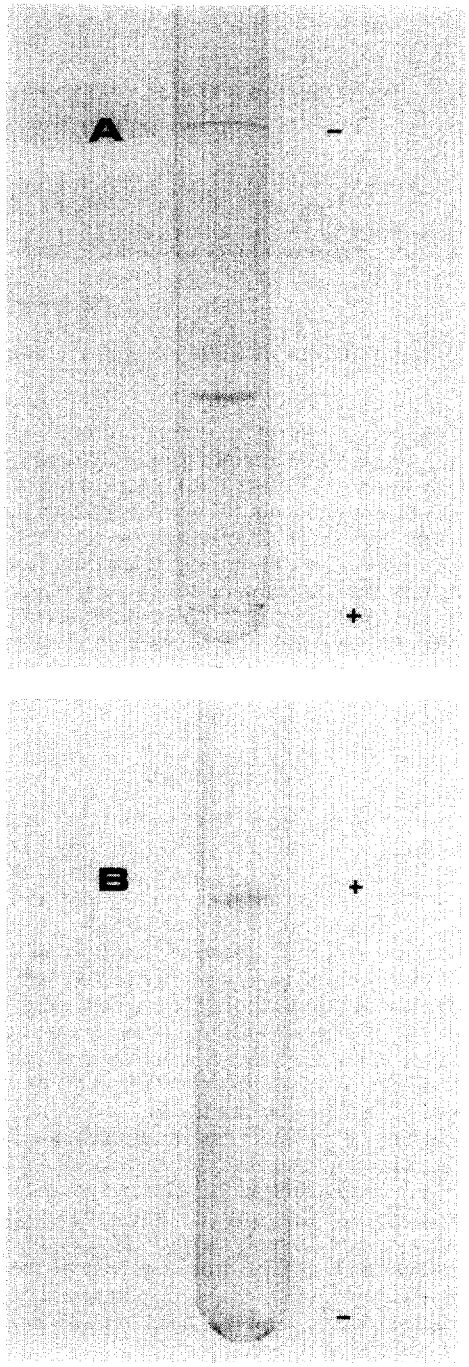

Fig. 3. PAGE of the purified esterase enzyme isolated from *C. maxima* to demonstrate homogeneity. Staining was with Coomassie Brilliant Blue G-250. (A): 10% Gel, pH 8.3/8.9, Tris–glycine buffer. (B): 10% Gel, pH 4.3 β-alanine–acetate buffer.

calculated frictional ration (f/f_0) value of 1.24 is an indication of the globular character of the enzyme.

SDS-PAGE yielded a single protein band (data not shown) corresponding to subunit a M_r value of 18 000. By comparing this value with that obtained after gel chromatography, it appears that the native esterase possesses a homogenous dimeric quaternary structure.

The anionic nature of the esterase is indicated by the pI value of 4.9 obtained after isoelectric focusing (results not shown). This is in accordance with the high acidic amino acid content of the enzyme together with the absence of lysine residues (Table 2). Furthermore, isoelectric focusing also indicated the presence of a single enzyme form isolated from *C. maxima*. Microheterogeneity has been demonstrated for esterases isolated from other sources [5, 13].

The UV spectrum of the esterase exhibited an absorption maximum at 280 nm, with a shoulder at 290–295 nm, indicative of the presence of tryptophan. This is consistent with the results of amino acid analysis, which

Table 2. Amino acid composition of an esterase from the fruit tissue of *C. maxima*

Amino acid	Composition (residues/mol)*
Asp	34
Thr	12
Ser	17
Glu	47
Pro	13
Gly	27
Ala	29
Cys	6
Val	23
Met	4
Ile	19
Leu	25
Tyr	10
Phe	12
Lys	—
His	6
Arg	9
Trp†	4

*Based on M_r of 36000

†Determined spectrophotometrically [27]

revealed the presence of 4 mol of tryptophan/mol of esterase (Table 2). An absorption coefficient, $A_{280}^{1\%} = 18$, for the esterase was calculated from the absorption spectrum and the protein concentration.

Kinetic properties

Substrate specificity studies on the esterase purified from the fruit of *C. maxima* demonstrated that the enzyme does not display carboxypeptidase, amidase, proteinase or aminopeptidase activities but has high esterolytic activities with indophenyl acetate (1510 μmol/min/mg) and *p*-nitrophenyl acetate (648 μmol/min/mg). Furthermore, no enzymatic activity could be observed with cucurbitacins A and B and elaterinide, secondary metabolites produced by *C. maxima* [14], suggesting that this enzyme does not participate in the metabolism of these acetate esters *in vivo*. In a study by Schwartz *et al.* [15], esterase activity with indophenyl acetate, cucurbitacins and acetylcholine was detected in a crude extract of *C. maxima*. At present the specific physiological function of the purified esterase described herein is unknown.

The pH-dependent variation of the initial velocity of the esterase demonstrates a broad pH optimum (pH 7.5–8.0) for the enzymatic reaction with indophenyl acetate (Fig. 4A). No significant change in this pH dependence was observed when concentrations of indophenyl acetate ranging from 0.025 to 0.8 mM were employed. The K_m for this substrate varied with pH (Fig. 4B) reaching minimum values between pH 7.5 and 8.0. At pH 8.0 the K_m value was determined to be 0.14 mM. No substrate inhibition with indophenyl acetate was observed over the concentration range used (i.e. 0.02–1 mM) demonstrating that abortive complex formation with the substrate did not occur [16]. Since the values of both the reaction rate and K_m are pH dependent, it would appear that hydrogen ions act as kinetically mixed non-com-

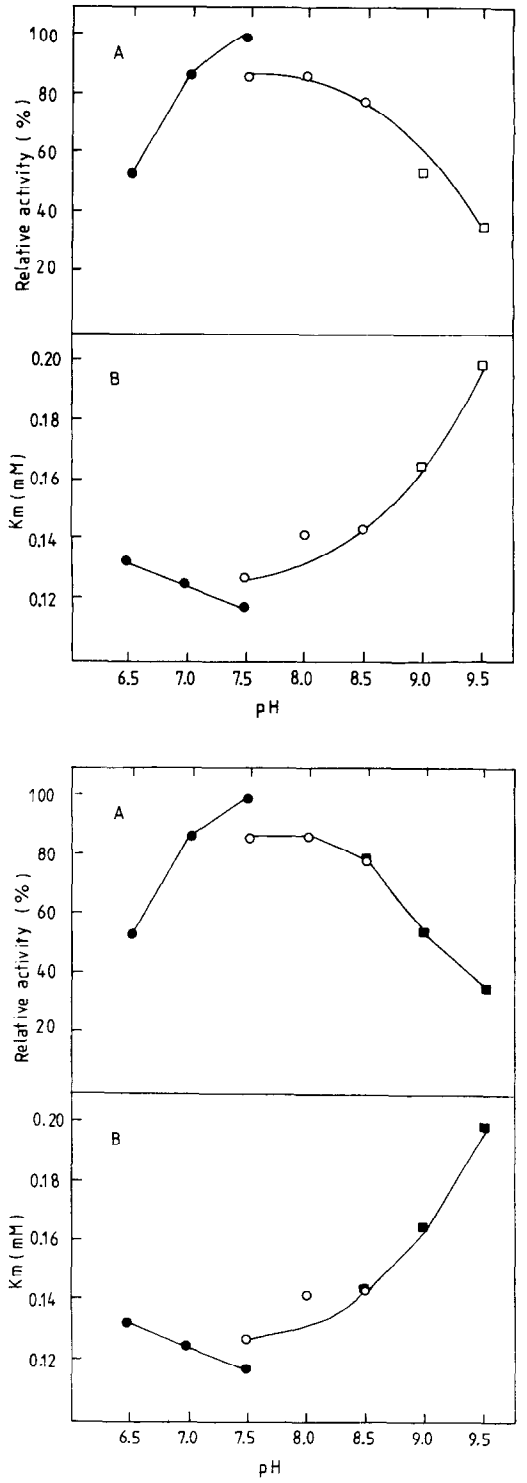

Fig. 4. pH dependence of the esterolytic activity (A) and K_m (B) of the esterase from *C. maxima*. Indophenyl acetate was used as substrate in the following buffer systems (25°): (●) 100 mM sodium phosphate, pH 6.5–7.5; (○) 100 mM Tris–HCl, pH 7.5–8.5 and (■) 100 mM borate, pH 8.5–9.5.

petitive effectors of the enzyme [17]. Apparent ionization constants for the free enzyme and enzyme-substrate complex, determined from plots of log V and log V/K_m against pH (data not shown), were $pK_A^E = 7.2$, $pK_B^E = 8.0$, $pK_A^{EA} = 7.1$ and $pK_B^{EA} = 8.3$. Perturbations in the pK values upon addition of indophenyl acetate may indicate that substrate binding changes the ionization of the relevant amino acid side chains [17]. Because pK values obtained from pH experiments represent complex molecular constants rather than simple group constants, no attempt was made to identify specific groups involved in the catalytic mechanism of the esterase.

Inhibition studies

The effects of various compounds on the esterase activity have been investigated and the results are shown Table 3. EDTA was without effect, suggesting that the enzyme is metallo-independent. Extensive inactivation by diisopropyl fluorophosphate and phenylmethylsulphonyl fluoride indicates a role for serine in the catalytic process. Furthermore, the participation of thiol groups in catalysis is shown by the inhibition of the enzyme by mercaptide-forming and alkylating thiol reagents. Both eserine sulphate (10 μM) and *S*-acetylthiocholine iodide (0.1 mM, 1 mM) did not affect the activity, indicating that the esterase is not an acetylcholinesterase. This is substantiated by the absence of activity with acetylcholine as substrate. According to the resistance and susceptibility of the esterolytic activity toward the inhibitors employed, the esterase purified from *C. maxima* can be classified as Esdp [9].

Product inhibition studies

Inhibition studies with indophenol and acetate as the products were performed in an attempt to obtain some information of the kinetic mechanism of the esterase from *C. maxima*. Increasing concentrations of acetate (0.02–100 mM) did not significantly affect the activity of the enzyme. Indophenol, on the other hand, was inhibitory, and the double-reciprocal plots obtained at different indophenol concentrations (0.02–0.2 mM) intersected at a common point on the $1/v$ axis, indicating that inhibition was competitive. A non-linear replot (not shown) of the slopes from the double-reciprocal plot suggests parabolic inhibition by indophenol. This was confirmed by the concave upward behaviour of the Dixon plots obtained at various fixed indophenyl acetate concentrations (Fig. 5). An approximate K_i value of 90 μM was estimated for indophenol from the Dixon plot. Parabolic inhibition may be the result of the combination of at least two molecules of inhibitor (indophenol) to the enzyme or to abortive complex formation with the product (indophenol) [18].

Table 3. Effects of various esterase and proteinase inhibitors on the hydrolysis of indophenyl acetate by the esterase enzyme from *C. maxima*

Compound	% Inhibition	
	0.1 mM Inhibitor	1 mM Inhibitor
None	0	0
EDTA	0	0
S-Acetylthiocholine iodide	0	0
Iodoacetamide	16	91
O-Iodobenzoate	30	70
Hg^{2+}	75	97
4-Chloromercuribenzoic acid	83	90
Phenylmethylsulphonyl fluoride	75	97
Diisopropyl fluorophosphate	100	100

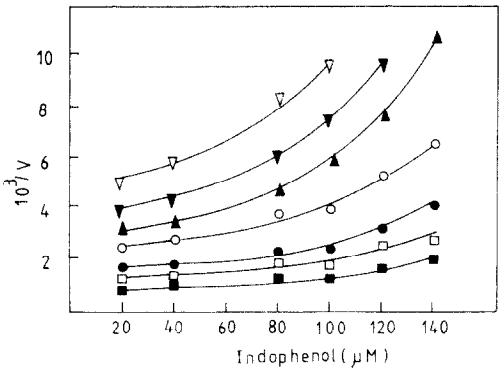

Fig. 5. Dixon plots demonstrating parabolic inhibition of the esterase by indophenol. The concentrations of indophenyl acetate were: (▽) 20 μM, (▼) 25 μM, (▲) 33 μM, (○) 50 μM, (●) 100 μM, (□) 200 μM, (■) 400 μM. The concentration of indophenyl acetate was varied between 33 and 880 μM in 100 mM Tris/HCl buffer, pH 8.0, containing 50 mM NaCl.

Esterases exhibit Uni Bi reaction mechanisms which can include the following: (a) ordered Uni Bi, which involves an acyl-enzyme transition complex with the release of the carboxylic acid product last [19] and (b) rapid equilibrium Random Uni Bi [20]. The interpretation of the obtained results is complicated by the absence of inhibition by acetate and possible dead end complex formation by indophenol and does not allow differentiation between the two kinetic mechanisms.

EXPERIMENTAL

Materials. DEAE *Sephacel*, Sephadex G-75, M_r and pH calibration kits were from Pharmacia. Indophenol sodium salt was from Eastman Kodak. Cucurbitacin A and B and elaterinide were obtained as described elsewhere [10]. All other chemicals were of analytical reagent grade.

Methods. Purification of esterase from *Cucurbita maxima*. All purification steps were performed at 6°. Eight kg of pulped *C. maxima* fruit tissue was diluted with 450 ml of 50 mM NaPi buffer, pH 6.6. The suspension was homogenized in a precooled Atomix MSE homogenizer; passed through cheesecloth and centrifuged at 7000 *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to a final saturation of 50% followed by centrifugation at 10000 g for 20 min. The supernatant was adjusted to 85% saturated with $(NH_4)_2SO_4$ and the precipitated protein, collected by centrifugation at 10000 *g* for 30 min, was redissolved in 50 ml of 50 mM Tris–acetate buffer, pH 8.0, containing 0.1 M NaCl (Buffer A). After dialysis against 2 l of buffer A, the enzyme soln was applied to a column (5 × 27 cm) of DEAE Sephacel, pre-equilibrated with buffer A. After eluting the column with 735 ml of the same buffer, the column was devel-

oped with a linear gradient of NaCl (0.1 to 0.27 M) over 800 ml followed by 800 ml of 50 mM Tris/acetate, pH 8.0, containing 0.27 M NaCl. The major esterase activity peak (Fig. 1) was transferred to a dialysis bag and concentrated against powdered sucrose; applied to a Sephadex G-75 column (2.6 × 95 cm) and eluted with 100 mM Tris–acetate buffer, pH 8.0, containing 50 mM NaCl.

Enzyme assay and protein determination. Indophenyl acetate was synthesized and purified according to the method of ref. [21]. The esterase activity assay mixture at 25° comprised 100 μmol Tris–HCl, pH 8.0, 50 μmol NaCl, 0.5 μmol EtOH and 0.6 μmol indophenyl acetate in a total volume of 1 ml. The reaction was monitored at 640 nm due to the absorbance of the hydrolysed product indophenol. The absorption coefficient of indophenol under the above conditions, excluding enzyme, was determined as 9200 $M^{-1} cm^{-1}$. Protein concentration was determined according to the method of ref. [22] with bovine serum albumin as standard.

Electrophoresis methods. PAGE was performed in 10% gels at pH 8.9 and pH 4.3 by the methods of Gabriel [23]. SDS–PAGE was performed in 10 and 15% gels as described in ref. [24]. Flat bed isoelectric focusing in polyacrylamide gels performed in the pH interval of 3–10 [25]. Protein bands were detected by staining with Coomassie Brilliant Blue G250 or R250.

Analytical gel chromatography. Analytical gel chromatography was carried out on a column (2.6 × 92 cm) of Sephadex G-75, equilibrated with 100 mM Tris–acetate, pH 7.8, containing 50 mM NaCl, as described in ref. [26].

Amino acid analysis. Amino acid analysis was performed on the enzyme after hydrolysis in 6 M HCl for 24 hr at 110°. Threonine and serine values were corrected by extrapolation of the data to zero time. Tryptophan was determined spectrophotometrically by the method of ref. [27].

Kinetic studies. The substrate specificity of the purified esterase was determined at 25° in 100 mM Tris–HCl, pH 8.0, according to published procedures (substrates used are given in parenthesis): carboxypeptidase activity (carbobenzoxy-glycyl-L-phenylalanine [28]; amidase activity (α-*N*-benzoyl-DL-arginine amide [29]); aminopeptidase activity (lysine-*p*-nitro-analide [30]); phenylalanine-β-naphthylamide, proline-β-naphthylamide and lysine-β-naphthylamide [31]); protease activity (heat-denatured casein and bovine albumin [32]); esterolytic activity (*p*-nitrophenyl acetate [33], indophenyl acetate [21]; *N*-benzyloxycarbomyl-L-tyrosine-*p*-nitrophenylester [34]); α-*N*-benzoyl-DL-arginine ethylester [34], *O*-acetylcholine chloride [35] and cucurbitacin A and B [15].

The pH dependence of the esterolytic activity of the purified esterase was determined with indophenyl acetate at 25° using the following buffer systems: 100 mM Na-Pi, pH 6.5–7.5; 100 mM Tris–HCl, pH 7.5–8.5; and 100 mM borate, pH 8.5–9.5.

To assist in the classification of the enzyme, the effects of various known esterase inhibitors (see Table 3) were investigated by preincubating the enzyme with inhibitor for 1 hr at 25° before measuring the esterolytic activity as described.

Product inhibition experiments were performed by varying the indophenyl acetate concentrations from 0.02 to 1 mM while maintaining constant levels of either indophenol or acetate. The analyses were performed in triplicate. Initial rate data were evaluated by means of Lineweaver–Burk plots, replots thereof and Dixon plots [18].

REFERENCES

1. Dixon, M. and Webb, E. C. (1979) in *Enzymes*, 3rd Edn. pp. 207–215. Longmans Green, London.
2. Tsujita, T., Okunda, H. and Yamasaki, N. (1982) *Biochim. Biophys. Acta* **715**, 181.
3. Goodenough, P. W. and Entwistle T. G. (1982) *Eur. J. Biochem.* **127**, 145.
4. Walker, C. H. and Mackness, M. I. (1983) *Biochem. Pharm.* **32**, 3265.
5. Casabe, N. and Zerba. E. N. (1981) *Comp. Biochem. Physiol.* **68C**, 255.
6. Clement, J. G. (1984) *Biochem. Pharm.* **33**, 3807.
7. Aldridge, W. N. (1953) *Biochem. J.* **53**, 110.
8. Augustinsson, K. (1961) *Ann. N. Y. Acad. Sci.* **94**, 844.
9. Hart, N. H. and Cook, M. (1976) *Comp. Biochem. Physiol.* **54B**, 357.
10. Schabort, J. C., Potgieter D. J. and *De Villiers, V.* (1968) *Biochem. Biophys. Acta* **151**, 33.
11. Schabort, J. C. (1978) *Phytochemistry* **17**, 1062.
12. Dirr, H. W., Schabort, J. C. and Weitz, C. (1986) *Biochem. J.* **233**, 649.
13. Heyman, E. and Junge, W. (1979) *Eur. J. Biochem.* **95**, 509.
14. Rehm, S. and Wessels, J. H. (1957) *J. Sci. Food Agric.* **8**, 687.
15. Schwartz, H. M. Biedron, S. I., Von Holdt, M. M. and Rehm, S. (1964) *Phytochemistry* **3**, 189.
16. Cleland, W. W. (1979) *Methods Enzymol.* **63**, 500.
17. Tripton, K. F. and Dixon, H. B. F. (1979) *Methods Enzymol.* **63**, 183.
18. Rudolph, F. B. (1979) *Methods Enzymol.* **63**, 411.
19. Stout, J. S., Sutton, L. D. and Quinn, D. M. (1985) *Biochim. Biophys. Acta.* **837**, 6.
20. Wang, C. and Kloer, H. (1983) *Biochim. Biophys. Acta* **754**, 142.
21. Kramer, D. N. and Gamson, R. M. (1958) *Anal. Chem.* **30**, 251.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
23. Gabriel, O. (1971) *Methods Enzymol.* **22**, 565.
24. Weber, K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol.* **26**, 3.
25. Pharmacia (1982) *Isoelectric Focusing: Principles and Methods.* Upsalla, Sweden. 49.
26. Andrews, P. (1965) *Biochem. J.* **96**, 595.
27. Goodwin, T. W. and Morton, R. A. (1946) *Biochem. J.* **40**, 628.
28. Zuber, H. (1976) *Methods Enzymol.* **45**, 561.
29. Murachi, T. (1970) *Methods Enzymol.* **19**, 273.
30. Roncari, G., Stoll, E. and Zuber, H. (1976) *Methods Enzymol.* **45**, 522.
31. Little, G. H., Starnes, W. L. and Bethal, F. J. (1976) *Methods Enzymol.* **45**, 495.
32. Reimerdes, E. H. and Klostermeyer, H. (1976) *Methods Enzymol.* **45**, 26.
33. Bucholtz, M. L. and Light, R. J. (1976) *J. Biol. Chem.* **251**, 431.
34. Walsh, K. A. and Wilcox, P. G. (1970) *Methods Enzymol.* **19**, 31.
35. Piltz, W. (1974) in *Methods Enzymatic Analysis.* (Bergmeyer, H. U., ed.) Vol. 2, pp. 840–842. Springer Zürich.