Journal of Chromatography, 326 (1985) 103–111
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 565

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC INVESTIGATIONS ON SOME ENZYMES OF PAPAYA LATEX

DEREK H. CALAM*, JANICE DAVIDSON and ROY HARRIS
National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB (U.K.)

SUMMARY

Papaya latex and commercial chymopapain have been examined by cation-exchange chromatography on a TSK SP 5PW column. Multiple components are observed and the resolution is superior to that obtained by low-pressure ion exchange. Most components display amidase activity. Fractions obtained from chymopapain by preliminary chromatography on SP-Sephadex have also been examined by the same procedure and by N-terminal and amino acid analysis. The results are consistent with the existence of chymopapain in multiple forms, the proportions of which alter. The chromatographic profile of chymopapain is influenced by the presence of cysteine in the sample.

INTRODUCTION

The dried latex from *Carica papaya* L. is a commercial source of proteolytic enzymes. A crude preparation containing papain as the main component is widely employed in the food, drink and tanning industries. Preparations of another enzyme, chymopapain, are used medically in the treatment of crushed vertebral discs as an alternative to surgery[1]. The enzymes of papaya latex have been isolated by ion-exchange chromatography[2] and, more recently, by affinity chromatography[3,4] on conventional soft-gel columns at low pressure. The identification and nomenclature of the enzymes found in papaya latex by different groups of workers and described in the literature are confusing. The presence of papain as a monothiol enzyme, which was the first obtained in crystalline from the latex, is beyond dispute. Papain is the least basic enzyme and is eluted first in cation-exchange chromatography. There is also general agreement that the most basic enzyme, variously called papaya peptidase A, papaya proteinase A or III or (probably) chymopapain C, is the last to be eluted in cation-exchange chromatography. However, between these two, a series of peaks has been obtained, the nature, identity and interrelationship of which is unclear. Components in this group are referred to as "chymopapain" but up to seven substances with different properties have been claimed to be present.

The development of surface-modified large-pore packings over the past few years has facilitated analysis of complex protein mixtures by high-performance liquid chromatography (HPLC). In particular the further modification of size-exclusion

0021-9673/85/$03.30

packings has resulted in the preparation of ion exchangers such as the strong cation-exchange packing TSK SP 5PW, based on the G5000PW polymeric size-exclusion material. This packing, with its sulphopropyl exchange groups, may be expected to give similar separations to those obtained with a soft gel such as SP-Sephadex but with enhanced resolution, reduced retention times and, of particular interest in the present case, with less degradation of labile substances during chromatography. We have, therefore, examined the components of papaya latex and of preparations of chymopapain using this high-performance packing.

EXPERIMENTAL

Materials

Papaya latex and chymopapain were from Sigma (Poole, U.K.). Chymopapain was also supplied by Travenol Labs, (Thetford, U.K.). Benzoyl arginine *p*-nitroanilide (BAPNA) was from Aldrich (Gillingham, U.K.). All other chemicals were obtained from BDH (Poole, U.K.), and were of analytical grade or the highest purity available.

HPLC

The chromatograph consisted of a Spectra Physics SP8700 solvent delivery system, SP8750 organiser module, SP8500 dynamic mixer, Cecil CE2112 variable-wavelength UV monitor, and Tekman TE200 recorder. Fractions were collected with a Gilson TDC 80 fraction collector.

Cation-exchange chromatography

High-performance separations were carried out on a TSK SP 5PW column (75 × 7.5 mm) from Toyo Soda (Tokyo, Japan). The solvent gradient was formed from (A) 0.02 *M* to (B) 1.0 *M* sodium acetate buffer (pH 5.0) with the amount of B increasing either from 30% to 50% in 30 min then to 80% in a further 30 min, or from 30% to 100% in 75 min, as indicated on the figures. The flow-rate was 1.0 ml/min and detection was at 280 nm.

Separations on a soft-gel packing were performed on a column (350 × 25 mm) packed with SP-Sephadex C-50 from Pharmacia (Sweden) and equilibrated with 0.1 *M* sodium acetate buffer (pH 5.0), supplied by an LKB Perpex pump at 20 ml/h. Detection with a Cecil 212A UV monitor was at 280 nm. The recorder and fraction collector were as for HPLC. The gradient was formed from 0.1 *M* to 1.0 *M* sodium acetate (pH 5.0), as indicated in the figures.

Preparation of the papaya latex extracts

Crude latex (10 g) was stirred with 150 ml of 0.1 *M* sodium acetate (pH 5.0) for 2 h. The solution was filtered and concentrated at reduced pressure to 50 ml.

Cellulose acetate electrophoresis

Cellulose acetate strips (Whatman 40 × 75 mm) were equilibrated in 0.05 *M* sodium barbitone buffer (pH 8.6). After blotting, the samples were applied in 2 μl of buffer 40 mm from the anode, at intervals of 10 mm. Electrophoresis was carried out in the buffer for 45 min at 7.5 mA. The acetate strip was stained with 0.5% (w/v) Ponceau S in 5% (w/v) trichloroacetic acid, and destained with 10% (v/v) acetic acid.

Polyacrylamide gel electrophoresis (PAGE)

Samples were run on a 10% slab gel in β-alanine buffer (pH 4.3). The gels were stained with 0.5% (w/v) Coomassie Blue in 12.5% (w/v) trichloroacetic acid, and destained with 7% (v/v) acetic acid.

Amidase activity was measured at pH 6.4 in 0.05 *M* citrate–phosphate buffer containing cysteine hydrochloride and disodium EDTA, using N-α-benzoyl DL-arginine *p*-nitroanilide as substrate. The solutions (3.5 ml) were incubated at 37°C for 30 min, the reaction was stopped by adding 0.5 ml of 60% acetic acid, and the absorbance was measured at 410 nm against a corresponding blank. Activity is expressed in pkat/mg protein, the protein content being determined by the method of Bradford[5].

N-Terminal analysis

The method described by Tarr[6] was followed, with the use of phenyl isothiocyanate. The derivatised amino acids were identified by reversed-phase HPLC on a Beckman Ultrasphere ODS column, as described by Noyes[7].

Amino acid analysis

Samples containing between 20 and 100 μg of protein, together with norleucine as internal standard, were hydrolysed with 6 *M* hydrochloric acid at 110°C for 24 h in evacuated sealed tubes. The samples were dried *in vacuo*, neutralised with a small volume of triethylamine, dried again, dissolved in 10–20 μl of a solution containing phenyl isothiocyanate–90% ethanol–triethylamine (1:7:2, v/v/v) and allowed to react at 50°C for 10 min under nitrogen. After drying, the resulting phenylthiocarbamyl amino acids were converted into the more stable phenylthiohydantoin derivatives by reaction with 20 μl of 50% TFA at 50°C for 50 min. The samples were dried, then taken up in methanol–ethyl acetate (1:1, v/v) and analysed by reversed-phase HPLC on a Beckman Ultrasphere ODS column as described by Noyes[7]. Standards were prepared in the same way.

RESULTS AND DISCUSSION

A chromatogram of an extract of crude papaya latex on a SP-Sephadex C-50 cation-exchange column is shown in Fig. 1. No further separation was achieved with smaller samples. Fractions were pooled as marked. Examination by cellulose acetate electrophoresis and for enzyme activity (Table I) revealed the complex composition of all five fractions as well as differences in specific activity. Comparison with the behaviour of an authentic sample confirmed that fraction A contained papain as the main component.

Fig. 2 shows the chromatogram obtained with a portion of the same extract as that in Fig. 1, but examined on the high-performance SP 5PW column. The higher resolution, particularly in the areas at 5–12 min, *ca.* 20 min, and between 30 and 40 min can be seen, as well as the considerable reduction in retention time. Papain was eluted at the void volume of the column.

A sample of commercial chymopapain was examined with the SP 5PW column (Fig. 3). A steeper gradient was employed than in Fig. 2 in order to elute material strongly retained on the column. It can be seen that the relative proportions of the

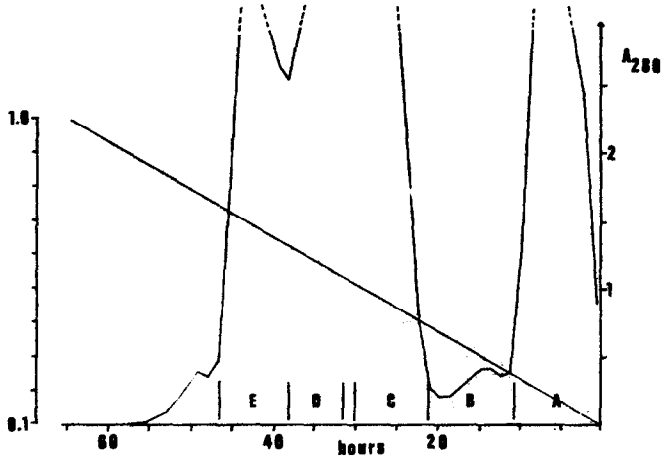

Fig. 1. Fractionation of papaya latex extract by low-pressure cation-exchange chromatography. Column, SP-Sephadex C-50 (350 × 25 mm); mobile phase, gradient 0.1–1.0 *M* sodium acetate buffer (pH 5.0), as indicated by the solid line and at the right; flow-rate, 20 ml/h, UV detection, 280 nm. Fractions were collected every 25 min (*ca.* 8.3 ml) and pooled as indicated.

peaks present differ from those in the latex extract. Many of the fractions collected from this separation were analysed for amidase activity by using the BAPNA assay. The results are given as histograms in the Figure. It is clear that the specific activities of these components vary: those eluted up to *ca.* 14 min are less active relative to the cluster of components eluted on either side of and including the main peak at 19 min. The highest specific activity is, however, displayed by the peak at 44 min. In order to examine these components further, a 2-g sample was chromatographed on the SP-Sephadex column (Fig. 4). The individual fractions from the column were pooled to give seven large fractions as marked. The results of enzyme assay and electrophoresis of these are collected in Table II, and chromatograms of the fractions IV–VII, obtained on the HPLC cation exchange support are reproduced in Fig. 5a–d. These fractions are heterogeneous by both HPLC and electrophoresis, and individual com-

TABLE I

SOME CHARACTERISTICS OF FRACTIONS FROM THE SEPARATION OF PAPAYA LATEX EXTRACT ON SP-SEPHADEX C50

Fraction	*Enzyme activity (BAPNA) (pkat/mg protein)*	*Cellulose acetate electrophoresis*				
		Spots observed (cm from origin)				
		0	*1.4*	*2.6*	*3.9*	*5.1*
A	414.9	×	(×)*	×	×	
B	126.0	×	×	×		
C	341.2	×	×	×	×	
D	278.9	×	×	×		
E	270.6			×	×	×

* Faint spot.

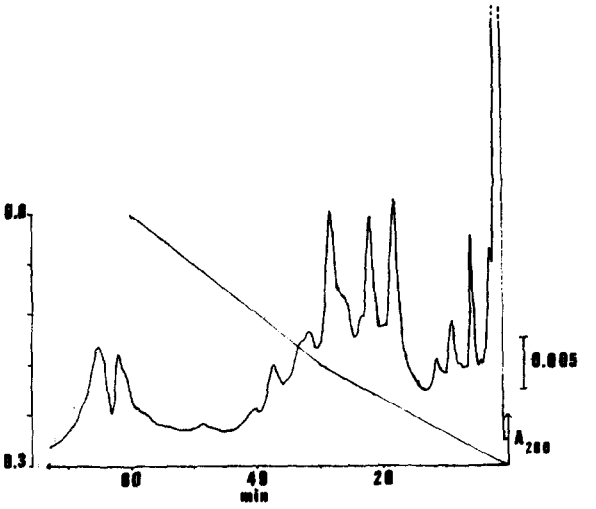

Fig. 2. Separation of papaya latex extract by high-performance cation-exchange chromatography. Column, TSK SP 5PW (75 × 7.5 mm); mobile phase, gradient formed from (A) 0.02 *M* and (B) 1.0 *M* sodium acetate buffer (pH 5.0); the initial composition was 70% A and 30% B, the gradient (indicated by the solid line and at the left) was formed by increasing B from 30 to 50% over 30 min, then from 50 to 80% over a further 30 min; flow-rate, 1.0 ml/min; UV detection, 280 nm.

ponents carry over from one fraction to the next. In Fig. 5d the final peak is probably an artifact, since it is observed in all pooled fractions from the Sephadex column. Comparison of these chromatograms with that of the original chymopapain sample shows some changes in the heights and positions of the peaks, which may result from the time necessary to carry out the separation on the Sephadex column.

Fractions V and VII exhibit the highest specific activity in the BAPNA assay,

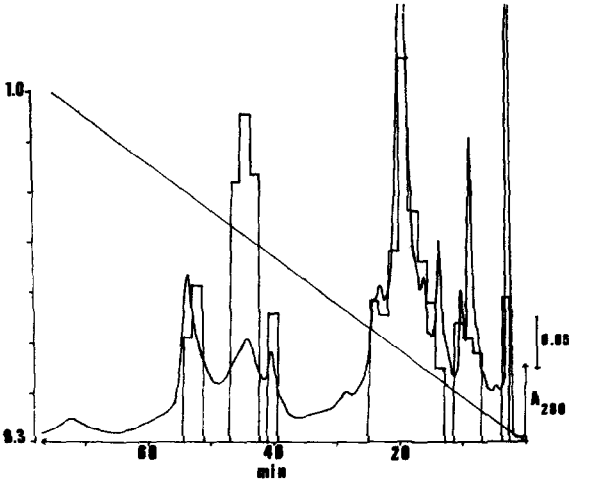

Fig. 3. Separation of chymopapain on SP 5PW. Conditions as in Fig. 2, except that the gradient was formed by increasing the B component from 30% to 100% over 75 min. The activities of the fractions obtained as determined by the BAPNA assay (see text), are shown in the superimposed histograms.

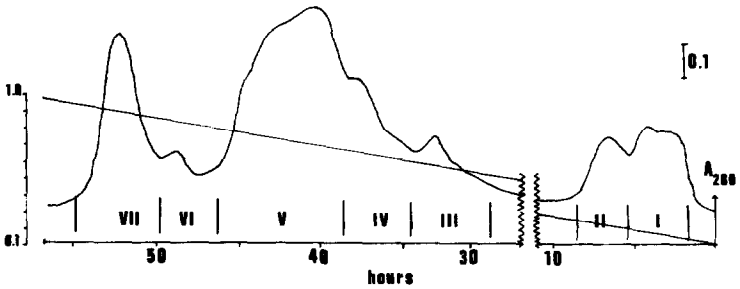

Fig. 4. Fractionation of chymopapain on SP-Sephadex C-50. Conditions as in Fig. 1. Individual fractions were pooled into seven large fractions as indicated.

although all fractions are active. These findings demonstrate the complex composition of chymopapain and the difficulty of obtaining the individual components in a homogeneous state. The fractions were subjected to amino acid analysis with the results shown in Table III. Clear differences are seen, notably between the relative amounts of acidic and basic amino acids. These changes broadly reflect elution position: fraction III contains more acidic amino acid residues relative to basic ones than fraction VII. The high figures for proline in fractions VI and possibly VII should be treated with caution, as they may be artifacts of the analytical procedure.

Further evidence for the nature of the enzymes was sought from N-terminal analysis. Tyrosine was identified as the main amino acid released in fractions III, IV and V. Fraction V also contained leucine. Fractions VI and VII contained predominantly leucine as N-terminal with decreasing amounts of tyrosine.

That chymopapain is obtained as a mixture of components is well recognised (for review see Brocklehurst *et al.*[8]) and has been attributed to the similar physicochemical properties of a number of constituents of the papaya latex. Chymopapain has been reported to contain tyrosine as N-terminal amino acid[2,3,9–11], and this is confirmed by our findings. Isoleucine, which is the N-terminal acid in papain, was not found in the fractions examined, which is consistent with the elution behaviour

TABLE II

SOME CHARACTERISTICS OF FRACTIONS FROM SEPARATION OF CHYMOPAPAIN ON SP-SEPHADEX C-50

Fraction	*Enzyme activity (pkat/mg protein)*	*Electrophoresis*						
		Cellulose acetate, spots (cm from origin)				*Polyacrylamide gel, bands (cm from origin)*		
		1.4	*2.2*	*3.1*	*4.5*	*3.5*	*4.0*	*5.6*
III	26.2			ND*			ND	
IV	16.9	×	×			×		
V	110.5		×			×	×	
VI	38.9		(×)**			×	×	
VII	118.5			×	×		(×)**	×

* ND = Not determined.
** Faint spot.

of papain on these columns. Glutamic acid has been found as the N-terminal amino acid with preparations of chymopapain B[2,10], but was not present in any fractions that we examined and has not been observed in peaks obtained by fractionation of chymopapain on a Mono S column[11].

Identification of leucine as N-terminal amino acid in the late-eluted fractions is consistent with the presence in them of papaya peptidase A, as is the absence of methionine. Lynn[3] identified a Leu-Pro-Glu sequence at the N-terminus of papaya peptidase A although the terminal acid had been assigned previously[10] as isoleucine. Other authors[2] were uncertain about the assignment. The identification of individual amino acids during sequencing has been placed on a firmer basis with the development of HPLC procedures such as that of Noyes[7]. The use of these, together with preliminary high-resolution chromatographic separation of the individual enzymes, should permit resolution of the contradictory reports in the literature. The absence of other N-terminal amino acids in fractions III–V, together with differences in the relative proportions of the chromatographic peaks observed when the SP 5PW col-

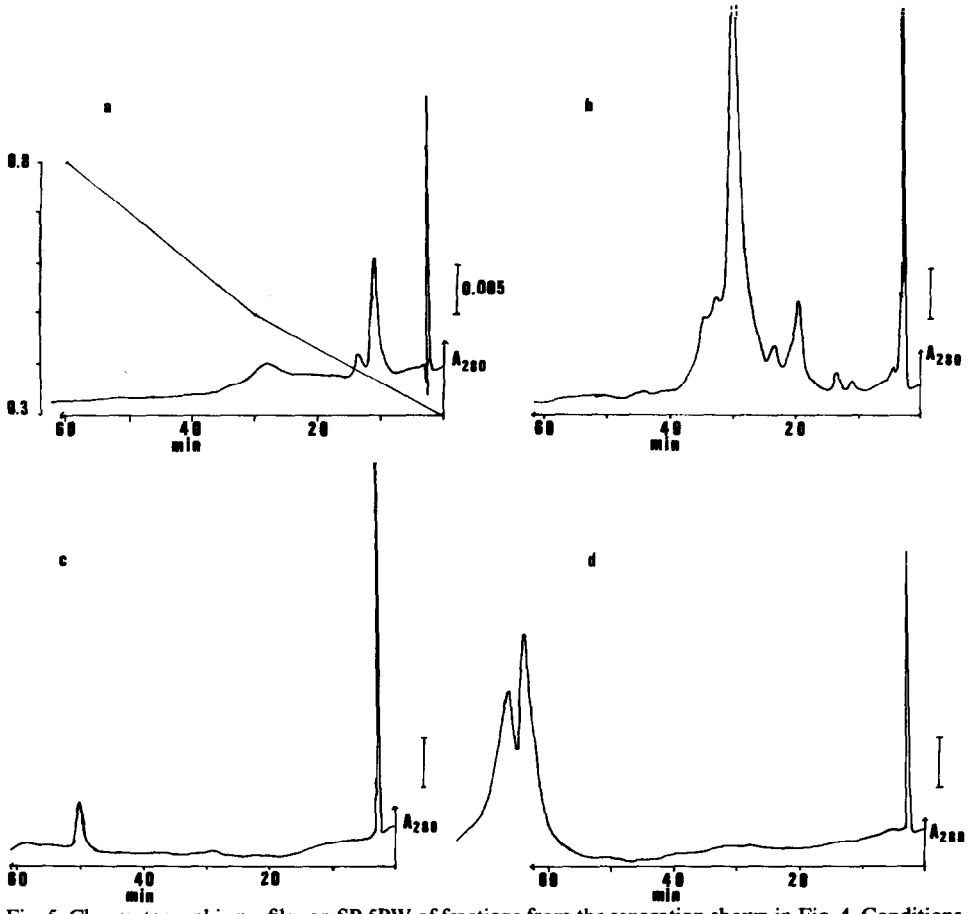

Fig. 5. Chromatographic profiles on SP 5PW of fractions from the separation shown in Fig. 4. Conditions as in Fig. 2. The gradient is shown in (a) and was the same in each case. (a) fraction IV; (b) fraction V; (c) fraction VI; (d) fraction VII.

TABLE III

AMINO ACID ANALYSIS OF FRACTIONS FROM SEPARATION OF CHYMOPAPAIN ON SP-SEPHADEX C-50

Results reported as grams of amino acid per 100 g of protein.

Amino acid	*III*	*IV*	*V*	*VI*	*VII*
Asp	11.92	8.98	9.72	4.55	2.74
Glu	13.33	9.33	10.56	8.45	8.76
Ser	8.98	8.43	9.12	8.31	7.26
Thr	6.38	8.51	7.66	6.32	7.59
Gly	7.48	6.36	6.67	7.43	7.43
His	–	1.58	1.45	–	1.51
Ala	4.53	4.93	4.58	4.97	4.54
Tyr	6.56	8.17	8.01	3.74	8.22
Arg	3.83	4.49	4.49	4.33	6.76
Met	–	–	–	–	–
Val	7.61	7.22	7.65	6.69	8.93
Pro	7.35	10.49	7.63	21.53	13.51
Phe	3.30	3.48	3.23	3.43	1.72
Lys	9.15	8.25	9.24	8.95	10.57
Ile	3.92	3.39	4.38	5.32	5.26
Leu	5.63	5.88	5.60	5.68	5.19

umn is used to examine other samples of chymopapain, suggests that the individual constituents may merely be different forms of a single parent molecule. Buttle and Barrett[11] have recently obtained immunological evidence which provides strong support for this view. They chromatographed chymopapain, from commercial papaya latex, on a Mono S cation-exchange column and showed that the multiple peaks obtained were immunologically identical. In addition, the chymopapain eluted first from the column was the predominant component in fresh latex.

Preparations of chymopapain, both those available commercially and those used therapeutically, contain cysteine. Fig. 6 shows the chromatogram obtained with the SP 5PW column and with such a preparation under the conditions used in other

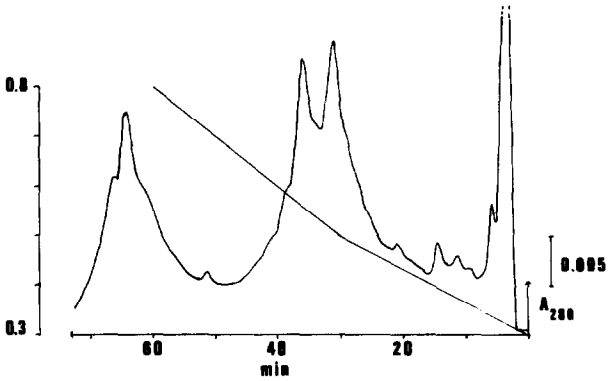

Fig. 6. Chromatographic profile on SP 5PW of chymopapain, containing cysteine. Conditions as in Fig. 2.

experiments. Comparison with Fig. 5 shows that a significant new peak is present at *ca.* 34 min. Cysteine itself is eluted towards the end of the chromatogram. The peak at 34 min could be observed when cysteine–chymopapain mixtures were prepared and analyzed. The size of the peak was found to depend on the relative amount of cysteine and the time between mixing and analysis. It is not known what effect this change may have on the properties of such preparations.

CONCLUSION

High-performance cation-exchange chromatography gives superior resolution of the components of enzymes in papaya latex and preparations of chymopapain than can be achieved with by low-pressure chromatography on soft cation exchangers. This improved resolution and the shorter elution times, together with examination of the individual molecular species by modern protein sequencing methods, including HPLC, should enable the complex interrelationships between the constituents of papaya latex to be unravelled.

ACKNOWLEDGEMENTS

We thank Toyo Soda for the gift of a SP 5PW cation-exchange column, and Ms. J. Lester for skilled technical assistance.

REFERENCES

1 M. C. Javid, E. J. Nordby, L. T. Ford, W. J. Hejna, W. W. Whisler, C. Burton, D. K. Millet, L. L. Wiltse, E. H. Widell, R. J. Boyd, S. E. Newton and R. Thisted, *J. Amer. Med. Assoc.*, 249 (1983) 2489.
2 B. S. Baines and K. Brocklehurst, *J. Protein Chem.*, 1 (1982) 119.
3 K. R. Lynn, *Biochim. Biophys. Acta*, 569 (1979) 193.
4 I. U. Khan and L. Polgar, *Biochim. Biophys. Acta*, 760 (1983) 350.
5 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
6 G. E. Tarr, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 223.
7 C. M. Noyes, *J. Chromatogr.*, 266 (1983) 451.
8 K. Brocklehurst, B. S. Baines and M. P. J. Kierstan, in A. Wiseman (Editor), *Topics in Enzyme and Fermentation Technology 5*, Ellis Horwood, Chichester, 1981, p. 262.
9 D. K. Kunimitsu and K. T. Yasunobu, *Biochim. Biophys. Acta*, 139 (1967) 405.
10 G. W. Robinson, *Biochemistry*, 14 (1975) 3695.
11 D. J. Buttle and A. J. Barrett, *Biochem. J.*, 223 (1984) 81.