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PURIFICATION AND PROPERTIES OF β-CYANO-L-ALANINE SYNTHASE FROM *LATHYRUS LATIFOLIUS**

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Key Word Index—*Lathyrus latifolius*, Leguminosae, β-cyano-L-alanine synthase, cysteine synthase; enzyme purification; substrate specificity, amino acid composition, L-cysteine; *O*-acetyl-L-serine, β-cyano-L-alanine; heterocyclic β-substituted alanines.

Abstract—β-Cyano-L-alanine synthase was purified *ca* 470-fold to homogeneity from the aerial parts of *Lathyrus latifolius*. The purified enzyme has an apparent M_r of 56 000 and can be dissociated into identical subunits of M_r 28 000, each containing one molecule of pyridoxal 5′-phosphate The K_m value is 1 6 mM for L-cysteine and 0.51 mM for cyanide. The purified enzyme also catalysed the formation of some heterocyclic β-substituted alanines and *S*-substituted L-cysteines as additional catalytic activities. Significant differences were found between this enzyme and β-cyano-L-alanine synthases from other sources. Several properties, including the amino acid composition of the purified enzyme, are also described

INTRODUCTION

In the preceding paper [1], we described the purification and properties of β-cyano-L-alanine (BCA) synthase from *Spinacia oleracea* leaves and we presented evidence that BCA synthase could also catalyse the formation of some heterocyclic β-substituted alanines and *S*-substituted L-cysteines in the presence of L-cysteine or *O*-acetyl-L-serine and suitable precursors We recently also purified cysteine synthases from several plant species [2–6]. These results suggest that naturally occurring heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine and L-quisqualic acid are synthesized by a reaction mechanism comparable to the one that results in the formation of BCA or L-cysteine.

Some leguminous plants belonging to the genera *Lathyrus* and *Vicia* contain toxic amino acids and amines which are the cause of different forms of lathyrism in man and animals [7–9]. In the seeds of the perennial sweet pea (*Lathyrus latifolius*) small amounts of BCA were detected [10] together with the lathyrotoxic compounds α,γ-diaminobutyric acid and β-aminopropionitrile [11–13]. These lathyrotoxins can be regarded as the products of the reduction and the decarboxylation of BCA, respectively, although no unequivocal proof for such metabolic relationships has been given yet. From the work on the biosynthesis *in vitro* of BCA, using either L-cysteine or *O*-acetyl-L-serine as amino-substrates, it was concluded that BCA is the first intermediate in the process of cyanide assimilation in higher plants [14] Since cyanide is formed naturally from the ethylene pathway in higher plants [15, 16], BCA synthase is essential for the detoxification of this endogenous toxin and at the same time may form the potential precursor for the lathyrotoxins α,γ-diaminobutyric acid and β-aminopropionitrile in *L. latifolius*

During our continuing study on the biosynthesis of this group of uncommon amino acids, we have now attempted the purification of BCA synthase from the aerial parts of *L. latifolius*, which contains a highly active enzyme for the formation of BCA, in order to make a detailed comparison with BCA synthases described before [1, 17–22], and also to provide information for the further study of the biosynthesis of the lathyrogens.

In this paper we describe the purification and the properties of BCA synthase from *L. latifolius* and compare these properties with those of BCA synthases and cysteine synthases from other sources

RESULTS

Purification

The methods described in our previous paper [1] were used for the extraction and purification of BCA synthase from 7 5 kg fresh weight of the aerial parts of *L. latifolius*. The enzyme was prepared simultaneously with the cysteine synthase activity by a procedure including acetonized preparation of mitochondria, ammonium sulphate fractionation, gel filtration on Sephadex G-100 or Ultrogel AcA 44, ion-exchange chromatography on DEAE-Sephadex A-50, hydrophobic chromatography on AH-Sepharose 4B and preparative polyacrylamide gel electrophoresis (PAGE) as summarized in Table 1.

The protein demonstrating BCA synthase activity was completely separated from cysteine synthase activity after the first DEAE-Sephadex A-50 column and the AH-Sepharose 4B column were eluted with a concentration gradient of K-Pi buffer, respectively. BCA synthase eluted at 105–130 mM on the DEAE-Sephadex A-50 column and eluted at 110–140 mM on the AH-Sepharose 4B column as shown in Fig. 1.

* Parts of this work were reported at the 31st Annual Meeting of Kanto Branch of the Pharmaceutical Society of Japan at Tokyo, 7 November 1987 (Abstracts p. 90)

Table 1 Summary of the purification of β-cyano-L-alanine synthase from *Lathyrus latifolius*

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Acetonized preparation of mitochondria*	3750	13300	0 282	100	1
2. Ammonium sulphate precipitate†	2220	3420	0 649	59 3	2 3
3. Sephadex G-100 (peak fractions)	1520	1350	1 13	40 6	4 0
4 1st DEAE-Sephadex A-50 (105–130 mM)	1130	243	4 65	30 0	16
5 Ultrogel AcA 44 (peak fractions)	998	48	20 8	26 6	74
6 AH-Sepharose 4B (110–140 mM)	378	4 4	85 9	10 1	305
7 Polyacrylamide-gel electrophoresis	86	0 67	128	2 3	454
8 2nd DEAE-Sephadex A-50 (75–85 mM)	74	0.56	132	2 0	468

*The acetonized preparation of mitochondria was produced from 7 5 kg of the aerial parts of *Lathyrus latifolius* with the specific activity 0 040 units/mg protein and with the total activity 5580 units

†40–60% saturation and desalted on Sephadex G-25

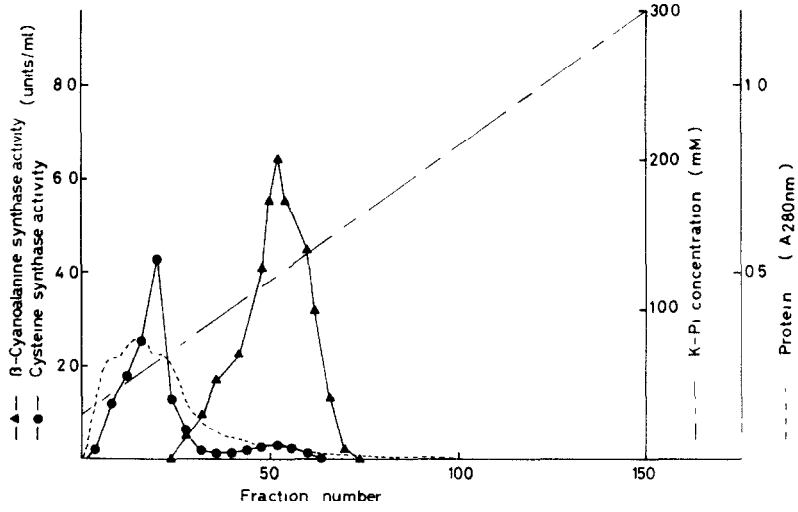

Fig 1 Elution patterns of β-cyano-L-alanine synthase and cysteine synthase after the AH-Sepharose 4B column chromatography β-Cyano-L-alanine synthase activity (▲—▲), cysteine synthase activity (●—●) and protein (A_{280}, ---) were monitored as shown in the Experimental

The complete procedure as shown in Table 1 afforded an apparent purification of *ca* 470-fold for BCA synthase with the specific activity of 132 U/mg protein and the yield of 2%, as compared to the specific activity of the extract of acetonized preparation of mitochondria.

Properties of the purified BCA synthase

The M_r of the purified enzyme from *L. latifolius* was estimated by gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of ref [23]. BCA synthase activity was found invariably as a single peak, corresponding to an M_r of 56000 The purified enzyme was subjected to SDS-PAGE on 12% gels to determine its subunit structure, following the method of ref. [24]. A single band with an M_r of 28000 indicated two identical subunits, and that it has been purified to apparent homogeneity

The purified enzyme had absorbance peaks at 280 nm and 410 nm that revealed a typical pyridoxal 5'-phosphate (PLP)-enzyme. The identification of bound PLP in the purified enzyme was demonstrated by direct spectrophotometric measurements [25] Assuming an M_r of 56000, this gave 1.9 mol of PLP indicating BCA synthase in *L latifolius* has one molecule of PLP bound to each subunit

The pH optimum was found to be pH 9.1 with Tris–HCl buffer Lineweaver–Burk plots gave K_m values of 1 6 mM for L-cysteine and 0 51 mM for cyanide The K_m value for L-cysteine was less than that determined for BCA synthases from both spinach [1] and blue lupin [18], while it was higher than that of the enzyme from white lupin [20]. The K_m value for CN^- was also less than the one determined for BCA synthase from spinach [1], while it was almost the same value as the one for the enzyme from blue lupin [18].

The addition of PLP had an accelerating effect on the activity of this enzyme, the increase being about 20% at a concentration of 10^{-2} mM, but was inhibitory at higher concentration, 1 mM causing 75% inhibition. An activation of 20% by 1 mM PLP has been reported for BCA synthase from spinach [1]. The enzyme was sensitive to the PLP-enzyme inhibitors. Sodium borohydride at concentrations of 10^{-4}, 10^{-3} and 1 mM caused 35, 70 and 100% inhibition, respectively, while hydroxylamine had a less inhibitory effect, the decrease being 20% at 0.1 mM.

Substrate specificity

Under standard assay conditions, BCA synthase in *L. latifolius* clearly appears to be specific for L-cysteine as a donor for the amino-substrate. Under identical conditions the activity was 123, 20.4 and 7.3% in the presence of β-chloro-L-alanine, *O*-sulpho-L-serine and L-cystine as compared to L-cysteine at 10 mM, respectively The purified enzyme showed 10.5% activity in the presence of *O*-acetyl-L-serine. No detectable activity was found with *O*-phospho-L-serine or with L-serine. D-Cysteine or *O*-acetyl-D-serine also did not act as substrates.

The purified enzyme also showed distinct substrate specificity when a variety of thiol compounds or *N*-heterocyclic compounds were used as acceptors for the amino-substrates. The relative activities of the purified enzyme with different substrates are shown in Table 2. BCA synthase in *L. latifolius* could synthesize *S*-substituted L-cysteines from both L-cysteine and *O*-acetyl-L-serine. The enzyme could also synthesize β-(pyrazol-1-yl)-L-alanine, β-(3-amino-1,2,4-triazol-1-yl)-L-alanine, L-quisqualic acid and *O*-ureido-L-serine in low activities, while this enzyme could not synthesize L-mimosine, L-willardiine or L-isowillardiine. Thus, the specific activities of the purified BCA synthase towards a variety of substrates are different from those of BCA synthase from spinach [1] and from other sources [17–22], and also from those of cysteine synthases from other sources [2–6]. The different acceptors were tested at pH 9.0 with L-cysteine or at pH 8.0 with *O*-acetyl-L-serine, the optimal pH for the formation of BCA and L-cysteine, respectively.

Amino acid composition

The amino acid composition of the purified enzyme is given in Table 3. The data presented show that BCA synthase in *L. latifolius*, like the BCA synthase in spinach [1], does not contain tryptophan, while BCA synthase purified from blue lupin does contain tryptophan [19]. The results obtained in this study also indicate that the enzyme consists of 526 residues and contains a large amount of glutamic acid (58 residues) and glycine (58 residues) like BCA synthase in spinach. The amino acid composition of BCA synthase obtained in this study is similar to the one purified from spinach [1], but it is very different from the BCA synthase enzymes purified from blue lupin [19] or white lupin [20].

The M_r of BCA synthase, calculated on the basis of the amino acid composition, is 55 900, which corresponds to the value found by gel filtration on Sephadex G-100 (56 000).

DISCUSSION

In previous work we demonstrated that BCA synthase from spinach can dissociate into two identical subunits, each with an M_r about one-half that of the intact enzyme, and that PLP is tightly bound to the enzyme [1]. In this study we have purified an analogous enzyme from the perennial sweet pea *Lathyrus latifolius*. When the physicochemical properties of these two enzymes are compared, they are found to be almost the same, even the amino acid compositions are similar in several aspects. However, the BCA synthases from blue lupin, white lupin and barley are different from the above two enzymes, having no subunits and only one molecule of PLP per enzyme molecule [17–21]. At this point we may consider two separate classes of BCA synthases in higher plants, the lupin and barley enzymes belonging to one class, and

Table 2 Relative synthetic rates of *S*-substituted L-cysteines and β-substituted alanines from L-cysteine or *O*-acetyl-L-serine by β-cyano-L-alanine synthase purified from *L. latifolius*

Substrate	Amino acid synthesized	Relative velocity of synthesis (%)	
		L-Cysteine	*O*-Acetyl-L-serine
NaCN	β-Cyano-L-alanine	100	10.5
H_2S	L-Cysteine	—	3.4
MeSH	*S*-Methyl-L-cysteine	1.26	0.07
CH_2=CH–CH_2–SH	*S*-Allyl-L-cysteine	0.02	0.08
HOOC–CH_2–SH	*S*-Carboxymethyl-L-cysteine	0 5	0.2
Pyrazole	β-(Pyrazol-1-yl)-L-alanine	0.03	0 17
3-Amino-1,2,4-triazole	β-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	0.1	0.07
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0.1
3,4-Dihydroxypyridine	L-Mimosine	0	0
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0.01	0.03

The relative rates of synthesis were compared with that of β-cyano-L-alanine from L-cysteine and NaCN. The reaction conditions are given in the Experimental and are as described before [1–6].

Table 3 Amino acid composition of β-cyano-L-alanine synthase purified from *L. latifolius*

Amino acids	Numbers of residues/ 56 000 g*
Asp	40
Thr	36
Ser	38
Glu	58
Pro	30
Gly	58
Ala	44
Val	40
Cys	4
Met	22
Ile	28
Leu	44
Tyr	14
Phe	14
Trp	0
Lys	32
His	6
Arg	18
Total	526

*Results are expressed as residues /mol and are based on an M_r of 56 000 Values for Thr and Ser are extrapolated to zero-time hydrolysis

The numbers of residues of amino acids were calculated based on the results of analyses after 24, 48 and 72 hr acid hydrolysis of native enzyme Means of duplicate analyses are given Determination of tryptophan was made by alkaline hydrolysis and methanesulphonate hydrolysis [2, 30]

the enzymes from spinach and *L. latifolius* belonging to a second class that may also contain the structurally similar BCA synthase from *Chromobacterium violaceum* [22]. This second class of enzyme also has some structural similarity with cysteine synthases in higher plants, responsible for the formation of some heterocyclic β-substituted alanines [2–6] Also in bacteria the physicochemical properties of cysteine synthases are described as "remarkably similar" to the BCA synthase of *C. violaceum* [22].

In *L. latifolius* BCA synthase and cysteine synthase can be completely separated by gradient elutions with K-Pi buffers from DEAE-Sephadex A-50 and from AH-Sepharose 4B columns

Among the substrates studied so far (Table 2), BCA synthase from *L. latifolius* could catalyse the formation of *S*-substituted L-cysteines from either L-cysteine or *O*-acetyl-L-serine as amino-substrates and corresponding co-substrates. The purified enzyme can also catalyse the synthesis of some heterocyclic β-substituted alanines such as L-quisqualic acid, β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine in low activities, as well as *O*-ureido-L-serine, while the spinach enzyme could not synthesize *O*-ureido-L-serine [1] BCA synthase from *L. latifolius*, like the one from spinach, can thus play a role in the detoxification of endogenous- or eventually exogenous-toxic molecules such as cyanide, hydrogen sulphide, pyrazole or 3-amino-1,2,4-triazole This observation is in line with our previous findings on BCA synthases and cysteine synthases in higher plants [1–6] Both BCA synthases and cysteine synthases may play a similar role as multifunctional enzymes in the biosynthesis of non-protein amino acids as secondary metabolites in higher plants.

The amino acid compositions of BCA synthases from *L. latifolius* and from spinach are very similar· both enzymes contain small amounts of cysteine (both 4 residues) and histidine (6 and 4 residues), and similar amounts of proline (30 and 32 residues), lysine (both 32 residues) and arginine (both 18 residues), and rather large amounts of methionine (22 and 26 residues), glutamic acid (58 and 64 residues) and glycine (58 and 64 residues) Neither enzyme contains tryptophan The similarities between these two enzymes are much greater than between the BCA synthases from blue lupin [19] and white lupin [20] When the known amino acid compositions of the purified enzymes are compared by a mathematical method [26], it is suggested that the BCA synthases from *L. latifolius* and from spinach are closely related, and also that they both are related to cysteine synthases from other plant sources, except the isoenzyme B from pea seedlings [4] All these findings concerning plant BCA synthases and cysteine synthases suggest that all these enzymes may have a common ancestor and perhaps BCA synthase may have arisen by duplication of the gene for cysteine synthase during the course of evolution

The absence of unequivocal information on the biosynthesis of the lathyrotoxins α,γ-diaminobutyric acid and β-aminopropionitrile, which are both present in *L. latifolius* [13], and the absence of BCA in the seedlings and aerial parts of this same plant while BCA synthase is highly active, leads to the assumption that BCA may be a short lived intermediate in the biosynthesis of those lathyrotoxins The decarboxylation of BCA can lead to β-aminopropionitrile, while a reduction of the cyano group into an amino group would lead to α,γ-diaminobutyric acid.

EXPERIMENTAL

Materials The aerial parts of *Lathyrus latifolius* L were used in this work Seeds were supplied by Sakata Seed Corporation (Japan) and they were grown in the medicinal plant gardens of our university After harvest, young aerial parts were collected and then cooled for 1 hr at 0–4° before extraction Sephadex G-25 and G-100, DEAE-Sephadex A-50 and AH-Sepharose 4B were purchased from Pharmacia Ultrogel AcA 44 was obtained from LKB All other chemicals used were of the highest commercial grade available

Activity assay This was performed as described previously [1] The formation of L-cysteine was measured spectrophotometrically according to the method of ref [27] The unit of enzyme activity was equivalent to 1 μmol of BCA or L-cysteine produced per min at 30° in 50 mM Tris-HCl buffer, pH 9 Protein was determined by the method of ref [28]

Purification of BCA synthase from the aerial parts of L latifolius All operations were carried out at 0–4° BCA synthase was prepared from 7 5 kg of fresh aerial parts Portions (100 g) of the aerial parts were repeatedly homogenized in 0 2 M K-Pi

buffer, pH 8, containing 10 mM 2-mercaptoethanol and 0 5 mM EDTA The homogenate obtained was centrifuged at 15000 *g* for 20 min, and the supernatant was discarded and then the ppt was completely suspended in a minimum vol. of 30 mM K-Pi buffer, pH 8, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (buffer A). To the suspension obtained, a 5× vol. of acetone at −20° was added and homogenized for 30 sec in a Waring blendor, followed by centrifugation at 5000 *g* for 10 min. Acetonized preparation of mitochondria was prepared according to a modified method of refs [17, 19] After discarding the acetone, buffer A was added to an acetonized preparation for the solubilization of the enzyme and then homogenized for 10 min in the same buffer. After standing for 2 hr, the ppt which had formed was removed by centrifugation at 15000 *g* for 20 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, and a 40–60% satd $(NH_4)_2SO_4$ fraction was resuspended in buffer A The resulting soln was applied to a column (8 × 25 cm) of Sephadex G-25 (fine) pre-equilibrated with buffer A. The protein fraction was concd by $(NH_4)_2SO_4$ pptn and then applied to a column (4.6 × 90 cm) of Sephadex G-100 pre-equilibrated with the same buffer The active fractions were pooled and then applied to the first DEAE-Sephadex A-50 column (4 6 × 6 cm) pre-equilibrated with buffer A and the enzymes eluted with a linear gradient of K-Pi (30–300 mM) in the same buffer. BCA synthase activity was eluted at 105–130 mM and two cysteine synthase activities were eluted at 140–170 mM and 220–250 mM K-Pi buffer, respectively, and the first BCA synthase active fractions (105–130 mM K-Pi fractions) were concd by $(NH_4)_2SO_4$ pptn The active fraction was applied to a column (3 × 100 cm) of Ultrogel AcA 44 pre-equilibrated with buffer A The eluates were collected and active fractions were concentrated by Immersible CX-10 (Millipore) The resulting soln was applied to a column (1.2 × 8 5 cm) of AH-Sepharose 4B pre-equilibrated with buffer A and the enzyme eluted with a linear gradient of K-Pi (30-300 mM) in the same buffer. BCA synthase was eluted at 110–140 mM and concentrated by Immersible CX-10. The resulting soln was subjected to prep. PAGE on 7 5% gel at pH 8.3 (Tris–glycine buffer). BCA synthase fraction obtained from gel slices was finally applied to a column (1.2 × 2 cm) of DEAE-Sephadex A-50 pre-equilibrated in buffer A The highly purified enzyme fraction (75–85 mM K-Pi fractions) was a pale yellow soln and was stable for at least one month at 0°. This enzyme preparation in 50 mM Tris–HCl buffer, pH 9, was used in all further expts

Properties of BCA synthase were studied by the methods of ref. [29].

Identification of heterocyclic β-substituted alanines and S-substituted L-cysteines as reaction products was performed as described previously [2]

Determination of amino acid composition was also performed as described previously [2]. This was also achieved by the method of ref [30]

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REFERENCES

1. Ikegami, F, Takayama, K., Tajima, C. and Murakoshi, I. (1988) *Phytochemistry* **27**, 2011
2. Murakoshi, I., Ikegami, F and Kaneko, M. (1985) *Phytochemistry* **24**, 1907
3 Murakoshi, I, Kaneko, M., Koide, C. and Ikegami, F (1986) *Phytochemistry* **25**, 2759
4 Ikegami, F., Kaneko, M., Lambein, F., Kuo, Y.-H. and Murakoshi, I (1987) *Phytochemistry* **26**, 2699.
5. Ikegami, F, Kaneko, M., Kamiyama, H. and Murakoshi, I (1988) *Phytochemistry* **27**, 697
6 Ikegami, F., Kaneko, M, Kobori, M. and Murakoshi, I. (1988) *Phytochemistry* **27**, 3379.
7 Attal, H C., Kulkarni, S W, Choubey, B. S., Palkar, N. D. and Deotale, P G. (1978) *Indian J. Med. Res.* **67**, 608.
8 Bell, E A (1980) *Food Chem.* **6**, 213
9 Lambein, F and De Vos, B (1981) *Arch. Int. Physiol. Biochim* **89**, B66.
10. Ressler, C, Nigam, S. N. and Giza, Y -H. (1969) *J Am. Chem. Soc.* **91**, 2758
11 Ressler, C (1962) *J Biol Chem.* **237**, 733.
12, Ressler, C., Nigam, S N., Giza, Y.-H. and Nelson, J. (1963) *J. Am Chem. Soc.* **85**, 3311
13. Lambein, F, De Bruyn, A., Ikegami, F. and Kuo, Y-H. (1986) *Lathyrus and Lathyrism* (Kaul, A K and Combes, D., eds), p 246. Third World Medical Research Foundation, New York
14 Conn, E E (ed.) (1981) *The Biochemistry of Plants* Vol. 7, p. 479 Academic Press, New York.
15 Wurtele, E. S, Nikolau, B J and Conn, E. E. (1985) *Plant Physiol.* **78**, 285
16 Manning, K (1986) *Planta* **168**, 61.
17 Hendrickson, H. R. and Conn, E E (1969) *J Biol. Chem.* **244**, 2632
18. Akopyan, T. N., Braunstein, A. E. and Goryachenkova, E. V. (1975) *Proc Natl Acad. Sci. U.S.A.* **72**, 1617.
19 Akopyan, T N and Goryachenkova, E. V. (1976) *Biokhimiya* **41**, 906
20 Galoyan, S. M, Tolosa, E A., Willhardt, I. G. and Goryachenkova, E. V. (1981) *Biokhimiya* **46**, 1855.
21 Leonova, T G, Goryachenkova, E. V. and Strogonov, B. P. (1983) *Fiziologiya Rastenii* **30**, 130.
22 Macadam, A M and Knowles, C. J. (1984) *Biochim. Biophys. Acta* **786**, 123.
23 Andrews, P (1965) *Biochem J.* **96**, 595.
24. King, J and Laemmli, U. K. (1971) *J Molec. Biol.* **62**, 465.
25 Kumagai, H, Yamada, H., Matsui, H., Ohkishi, H. and Ogata, K (1970) *J Biol. Chem.* **245**, 1773.
26 Chernoff, H. (1973) *J Am Stat Assoc* **68**, 361.
27 Gaitonde, M. K (1967) *Biochem J* **104**, 627
28 Lowry, O. H, Rosebrough, N J, Farr, A L. and Randall, R. J. (1951) *J Biol. Chem* **193**, 265
29. Murakoshi, I, Ikegami, F, Hinuma, Y. and Hanma, Y. (1984) *Phytochemistry* **23**, 973
30. Simpson, R J Neuberger, M. R. and Liu, T.-Y. (1976) *J Biol. Chem* **251**, 1936