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ERYTHRO- AND LYMPHOAGGLUTININS OF *PHASEOLUS ACUTIFOLIUS*

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Key Word Index—*Phaseolus acutifolius*; Leguminosae; lectin; lymphoagglutinin; erythroagglutinin; isolation; characterization.

Abstract—A potent lymphoagglutinin which had low affinity for red cells or fetuin and another lectin which reacted strongly with red cells and fetuin but was a poor agglutinin for lymphocytes were isolated from seeds of *Phaseolus acutifolius*. A number of other lectin components with intermediate activity towards these cells was also isolated. All the lectins had very similar amino acid and carbohydrate composition, sedimentation patterns, partial specific volume and molecular weight values of about 116600 and were thus smaller than the related *Phaseolus vulgaris* lectins (M_r = 119000). The lectins contained four subunits with only minor size and charge differences between the lympho- and erythroagglutinating subunits and their electrophoretic mobility in SDS gel electrophoresis was anomalously high. The existence of lympho- and erythroagglutinating subunits in two members of the genus *Phaseolus* supports their close morphological similarity.

INTRODUCTION

There is an increasing body of evidence [1, 2] to indicate that lectins of different legumes may be structurally related homologous proteins. Lectins with specificity for D-mannose (D-glucose) residues, irrespective of whether they belong to the one-chain lectins from the tribe Diocleae, e.g. concanavalin A and *Dioclea grandiflora* lectin [1, 3], or to the two-chain lectins from Vicieae [1], are all highly conserved [1] with respect to sequence, metal-binding sites, hydrophobic cavity and carbohydrate-binding site. Similarly lectins from Phaseoleae, including those from *Phaseolus vulgaris* [4, 5] and *Glycine max* [6], have a high degree of conservation of structural features implicated in agglutination. Although the function(s) of lectins in plants is still largely unknown, these structural homologies suggest that lectins have performed beneficial and similar roles for the plants during their evolution [7]. Thus comparative studies assume a great importance in lectin research.

Lectins from the seeds of *Phaseolus acutifolius* L. (PAL), despite their smaller molecular size, show extensive immunochemical cross-reactivity to the much more widely studied PHA lectins from *Phaseolus vulgaris* [7]. In the present paper, the isolation and properties of the erythro- and lymphoagglutinating lectins from the seeds of *Phaseolus acutifolius* are described.

RESULTS AND DISCUSSION

Based on different affinities for fetuin, and thus having slightly different sugar specificities, a number of closely related lectins have been isolated from the seeds of *Phaseolus acutifolius*. These lectins are similar in agglutinating properties to those of the well-studied lectins from *Phaseolus vulgaris* [8–11]. Thus the lectin components which are poorly bound to fetuin–Sepharose 4B have high lymphocyte-agglutinating activities, while those strongly bound to and eluted from the column only at low pH values are mainly erythroagglutinating (Table 1, Fig. 1). Those with intermediate affinity for fetuin have intermediate values for their activities against erythrocytes and lymphocytes. However while there is some experimental evidence to support the existence of separate erythrocyte-(E)- and a lymphocyte-(L)-agglutinating subunits in PHA (Fig. 2a) and consequently the occurrence of five tetrameric isolectins E_4, E_3L, E_2L_2, EL_3 and L_4 in the seeds, no such simple scheme can account for the similarly diverse biological activities of PAL lectins (Fig. 2a, b). Both T_2 and F_2 lectins were resolved by SDS gel electrophoresis into three or more slightly different components of appreciably faster mobilities and thus probably of smaller molecular size than the corresponding PHA E- or L-type subunits (Fig. 2a). Isoelectric focusing in non-dissociating media indicated the presence of a great number of isoelectric species in the pH range of 4.5–5.5 (results not shown). As the presence of tightly bound metal ions might produce such a charge-heterogeneity, isoelectric focusing on polyacrylamide gels was carried out in 8 M urea solutions with samples directly dissolved in 9.5 M urea solutions and also with samples which have been dialysed first against 8 M urea containing 0.05 M Na-EDTA and then against several changes of 8 M urea (Fig. 2b). Both isoelectric focusing and, even more, two-dimensional electrophoresis experiments (Fig. 2a) of de-metallized T_2 and F_2 lectins produced complex and dissimilar patterns, thus demonstrating genuine size and charge heterogeneity for the PAL subunits.

The very similar amino acid composition (Table 2) of the two lectins reflected well the close similarities in their electrophoretic properties. The amino acid composition was characteristic of that generally found with a number of other lectins and contained little methionine and probably no cysteine. Both lectins contained appreciable amounts of carbohydrates: the total neutral sugar content [12] was 7.0% for T_2 and 5.2% for F_2. As the main

Table 1. Overall recovery of material and cell agglutinating activity from *Phaseolus acutifolius* seeds at various stages of purification including affinity chromatography of the albumin fractions by fetuin–Sepharose 4B. The recovered fractions were tested for cell agglutination by rabbit erythrocytes or by human lymphocytes (Ficoll-Paque). The results are given for the dry weight of the various fractions

Fraction		Amount (g)	H.U./mg	Total H.U. ($\times 10^{-8}$)	L.U./mg	Total L.U. ($\times 10^{-6}$)
Seed meal		98.3	3300	3.27	30	3.0
Residue		69.4	50	0.03	n.d.*	n.d.*
Crude extract		n.d.*	n.d.*	2.33	n.d.*	n.d.*
Globulins (pH 5 precipitate)		9.5	500	0.05	n.d.*	n.d.*
Albumins		4.1	67000	2.68	600	2.46
Fractions† obtained by affinity chromatography	T_1	2.21	20	—	20	0.05
	T_2	0.25	9000	0.02	6600	1.65
	T_3	0.17	270000	0.44	1250	0.21
	T_4	0.12	270000	0.34	1250	0.15
	F_1	0.03	130000	0.03	10	—
	F_2	0.29	540000	1.54	80	0.02
	F_3	0.14	270000	0.39	20	—
	U	0.08	33000	0.06	0	—

*n.d. = not determined.
†The designation of the fractions is as in Fig. 1.

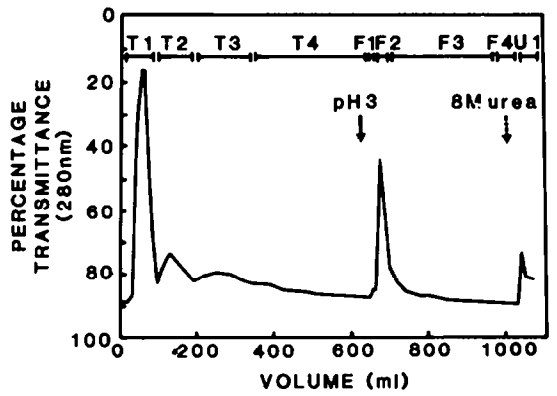

Fig. 1. Fractionation of proteins from the seeds of *Phaseolus acutifolius* by affinity chromatography on fetuin–Sepharose 4B (15 g; column size: 14.5 cm long, 2.2 cm diameter) of albumins (500 mg). Buffer flow rate was 50 ml/hr and fractions of 9.8 ml were collected. The initial elution buffer B was changed first to 0.05 M glycine–HCl, 0.5 M NaCl, pH 3.0 and then to 8 M urea as indicated by arrows. The pooling of effluents into designated fractions is given in the diagram.

carbohydrate components are glucosamine and mannose, these lectins are glycoproteins of the high-mannose-type widespread in the Plant Kingdom. Of the other monosaccharide components xylose amounted to about one residue per lectin molecule and there were smaller amounts of fucose, rhamnose, and glucose. Thus the PAL lectins are clearly different from PHA [13]. However as glycosylation of PHA does not appear to be a prerequisite for its biological activity or even for processing by the cotyledonary cells [14], the significance of this difference is not clear.

Sedimentation experiments

The T_2 lectin sedimented as a single component with an $S_{20,w}$ value of 6.3 S at 1.2 g/dl concentration in buffer B (pH 8.0). The sedimentation coefficient was dependent on protein concentration (0.19–1.22 g/dl) and all results could be fitted into the following equation with 95% confidence: $S_{20,w} = 6.61\ (\pm 0.02) - [0.42\ (\pm 0.04) \times \text{concentration}]$. The F_2 lectin preparations showed very similar sedimentation patterns and concentration dependence: $S_{20,w} = 6.65\ (\pm 0.01) - [0.27\ (\pm 0.02) \times \text{concentration}]$. The difference between the $S_{20,w}$ values for T_2 and F_2 lectins, respectively, was not statistically significant ($P = 0.05$) although the steeper concentration dependence of T_2 indicated a more flexible molecular conformation for the lymphoagglutinin. Neither of the two lectins appeared to dissociate into subunits at low pH values although the $S_{20,w}$ value at pH 2.6 (buffer A) for the lectins was reduced to 5.7 S because of charge effects.

Partial specific volume

From the regression equations based on the results obtained from density measurements in the concentration range of 0.163 to 2.082 g/dl a V_{av} value of 0.734 $\pm$ 0.003 ml/g was obtained for T_2. The corresponding value for F_2 was 0.727 $\pm$ 0.007 mg/g. There was no significant difference ($P < 0.05$) between the two regression equations or the partial specific volume values calculated from these equations.

Molecular weight

M_r values obtained at pH 8.0 (buffer B) from sedimentation equilibrium studies were also close for the two lectins. All data could be combined into one overlapping and essentially constant plot within experimental error of

Fig. 2a

Fig. 2b

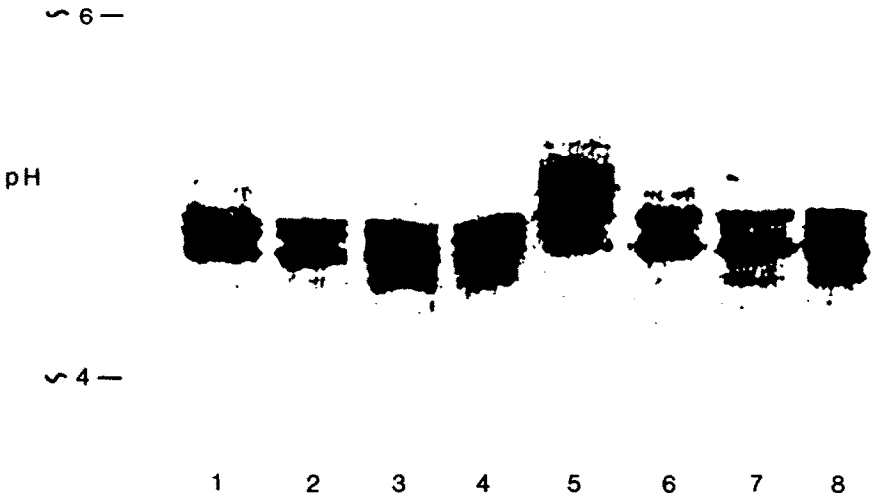

Fig. 2. Electrophoretic patterns of erythro- (F_2) and lymphoagglutinating (T_2) lectins of *Phaseolus acutifolius*. (a) In the two-dimensional part of the gel slab (lanes 4, standard preparation of T_2 and 5, standard preparation of F_2) appropriately prepared (see under Experimental) strips from first dimension of isoelectric focusing in 8 M urea of PAL preparations were embedded with warm agar on top of the central part of the pre-cast vertical SDS gel slab. Subsidiary wells for applying standards (run in SDS only) to run parallel with the lectins on the strips from the first dimension were also cast with agarose on top of the gel slab and the whole assembled gel was then electrophoresed in SDS for 6 hr. The position of known standards and the first dimension gel strips used are indicated. Standards: lane 1, *Phaseolus vulgaris* extract; 2, F_2; 3, T_2; 6, F_2 and 7, *Phaseolus vulgaris* extract. (b) Isoelectric focusing patterns of PAL preparations before and after dialysis against 0.05 M EDTA in 8 M urea. Lane 1, standard F_2; 2, de-metallized standard F_2; 3, second F_2 preparation; 4, same after de-metallization; 5, second T_2 preparation; 6, same after de-metallization; 7, standard T_2; 8, de-metallized second F_2 preparation. The lectins designated as second preparations were obtained by size-exclusion chromatography (by HPLC) on a TSK-3000 SW instead of the conventional AcA-34 column used in the standard method (see under Experimental). Fractions T_2 and F_2, unless otherwise designated, always refer to the two main lectin fractions obtained by affinity chromatography (Fig. 1) and subsequent chromatography on an AcA-34 column.

Table 2. Amino acid and carbohydrate composition of the lympho- and erythroagglutinating lectins (T_2 and F_2) from *Phaseolus acutifolius*. The values are given in g anhydrous amino acids or carbohydrates per 100 g protein and represent a recovery of total N (excluding NH_3) in excess of 95% for both lectins

Amino acid or carbohydrate	Quantity T_2	F_2
Lysine	4.18	4.36
Histidine	0.80	1.01
Arginine	3.85	3.46
Aspartic acid	15.15	14.55
Threonine	6.59	7.10
Serine	8.76	7.94
Glutamic acid	6.36	6.46
Proline	3.36	3.11
Glycine	3.61	3.66
Alanine	3.86	3.97
Valine	7.06	7.21
Methionine*	0.26	0.47
Isoleucine	4.61	4.47
Leucine	8.88	9.00
Tyrosine	1.76	2.22
Phenylalanine	7.01	7.45
Tryptophan†	2.40	2.68
Cysteine*	0	0
	88.5%	89.12%
Glucosamine	1.02	1.40
Rhamnose	0.06	0.02
Fucose	0.09	0.06
Arabinose	0.02	0
Xylose	0.12	0.13
Mannose	2.60	2.24
Galactose	0	0
Glucose	0.09	0.07

*Determined after oxidation with performic acid [19].

†Determined after hydrolysis with 4 N methane sulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 115° for 22 hr [20].

the various point average molecular weight values vs concentration in the cell, thus confirming the absence of smaller components, fast association–dissociation reactions and non-ideality. The average of all values ($M_{w,av}$) gave 116 900 ± 100 (932 observations) for T_2 and 116 400 ± 100 (904 observations) for F_2 and the two values were not significantly different ($P < 0.05$). Again the only slight difference between the two lectins is the finding of a steeper concentration dependence of the molecular weight values of T_2. In the general equation of $M = M^\circ - [a \times$ net fringe displacement (in μm)] the a value was 1.24 for T_2, while it was only 0.87 for F_2 (significant at $P = 0.05$).

The electrophoretic patterns and mobilities in SDS gels (Fig. 2a) indicate that the M_r values of PAL subunits are approximately 2000 smaller than those of PHA. However this difference is too large when compared with a value calculated from the thermodynamically correct M_r values of 119 000 for PHA [15] and of 116 600 for PAL in the present paper. Thus based on four subunits per lectin molecule of 119 000 the M_r value calculated for the PHA subunits is 29 750. This value is probably correct as it is also independently supported by lectin sequence data based on isolated PHA lectin genes [5]. As the SDS runs indicated subunit M_r values of about 28 000 or less for the PAL components in contrast to the value of 29 150 calculated from the results of sedimentation experiments it is very likely that the mobility of PAL subunits in SDS electrophoresis is anomalously too fast. Moreover as even the E and L subunits of PHA separate well in SDS runs (Fig. 2a) despite their nearly identical M_r value estimated from sedimentation studies [10] and similar carbohydrate content [15] most *Phaseolus* lectins apparently have some degree of anomaly in their behaviour in SDS. However as the reason for this is likely to be a conformation-dependent hydrophobic behaviour and SDS-binding it will have to await the results of sequence and other structural studies.

Finally, the finding of lympho- and erythroagglutinating lectins and their hybrid tetramers in two members of the genus *Phaseolus* highlights their closeness in morphology. Although tetrameric structure appears to be very general for lectins from Phaseoleae and indeed for a number of lectins generally, such clear division of biological activity in two types of subunits is rarer and so far has been demonstrated only for the seeds of *Phaseolus vulgaris*, *Griffonia simplicifolia* [16] and in this paper for *Phaseolus acutifolius*.

EXPERIMENTAL

Seeds of *Phaseolus acutifolius* (tepary bean) were a gift from Dr. L. Telek of Mayaguez Institute of Tropical Agriculture (Mayaguez, Puerto Rico).

Agglutination tests. For these rabbit erythrocytes and human lymphocytes (purified by Ficoll-Paque; Pharmacia Fine Chemicals) were used. Fractions or lectins were dissolved in 0.9% NaCl solution and serially diluted before the addition of cells. The extent of clumping was observed under a microscope and expressed as agglutination units (H.U. or L.U./mg of material). One unit was taken as the amount of material/ml in the test tube giving a strong 2+ reaction.

Analytical isoelectric focusing in 8 M *urea.* Gels (11 cm long, 8 cm wide and 0.025 cm thick) were cast containing 4% acrylamide (30:1 acrylamide to bis-acrylamide), 8 M urea and 3.12% total ampholyte (2.6% of pH 4–6 and 0.52% pH 3–10; LKB, GB Ltd.) on Gel-Fix support (Serva Feinbiochimica, Heidelberg, F.R.G.). Samples were dissolved in 9.5 M urea at concentrations of 3–5 mg/ml and applied (4–10 μl) to the gels on wicks of Whatman 3 MM or No. 1 papers placed between 1.5 and 2.0 cm from the anode end of the gel. Strips of paper (1% H_2SO_4, anode and 1% ethanolamine, cathode) were used for connecting to electrodes. Most experiments were run at 10° with a maximum of 10.8 W and 2000 V for 3 hr giving an average of about 1270 watt mins. After focusing the gels were fixed in 20% TCA for 30 min and stained in 0.003% w/v Coomassie Blue Type R.

SDS polyacrylamide-gel electrophoresis. This was carried out by the vertical slab gel (0.075 cm thick) system (LKB, GB Ltd.) with a separation gel of 17.6% acrylamide (pH 8.8) and a stacking gel of 6% acrylamide (pH 6.8). Conditions: 6 hr at 20 mA and room temperature.

Two-dimensional electrophoresis. Samples (2 mg/ml) were first run in isoelectric focusing in 8 M urea and the resolved bands were stained with 0.015% w/v Coomassie Blue Type G250,

0.05% w/v $CuSO_4$; in HOAc–H_2O–MeOH (2:9:9), destained with 0.1% w/v $CuSO_4$ in HOAC–H_2O–MeOH (2:13:5) and dried. Suitable size strips of gels were then cut, washed in H_2O for about 45 min and incubated at 20° for approximately 10 min in an equilibrating buffer (0.6 M Tris, 2% SDS, 10% glycerol and 2% 2-mercaptoethanol). The incubated strips were placed on top of the pre-cast vertical SDS gel and embedded with a warm (about 50°) solution of 1% agarose (dissolved in the pH 6.8 stacking-gel buffer). Subsidiary wells for applying standards to run parallel with the strips from the first dimension were also cast from the agarose solution. Electrophoresis in SDS was then carried out as above for 6 hr. The gels, after fixing in 20% TCA (20 min) were first stained with 0.1% w/v Coomassie Blue Type R, de-stained (HOAc–H_2O–MeOH, 2:16:7) and then silver-stained [17].

Amino acid analysis. These were performed after hydrolysis with constant boiling HCl as before [18]. Cysteine and methionine were estimated according to Moore [19]. Tryptophan was determined after hydrolysis with 4 N methane sulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 115° for 22 hr [20].

Carbohydrate analysis. Total neutral sugar was estimated as described in ref. [12]. Hexosamines were estimated after hydrolysis with 2 N HCl at 100° for 4 hr [21]. Individual carbohydrates were measured after hydrolysis with 2 N trifluoroacetic acid at 100° for 3 hr, conversion to alditol acetates and separation by GC.

Sedimentation studies. Lectin samples were examined at various concentrations and pH values in buffers (A) 10-times diluted citrate–phosphate buffer [22] containing 0.1 M NaCl of pH 2.6, or (B) 0.05 M Tris, 0.025 M acetic acid, 0.1 M NaCl, 5 mM $CaCl_2$, 0.1 mM $MnSO_4$ and 0.02% sodium azide, pH 8.0. Sedimentation coefficients and concentration of the various components were determined as before [10]. Sedimentation equilibrium experiments and density measurements were done at various protein concentrations in buffer B as before [10].

Isolation of the lectins. Seed meal was extracted by stirring with 0.2 M borate buffer, pH 8.0 with a meal to buffer ratio of 1:8 at 0° for 90 min. The extract was centrifuged (Sorvall RC-2, GSA rotor) at 9000 rpm for 1 hr. The sediment was re-extracted with half of the original volume of buffer. After centrifugation the two supernatants were combined, dialysed against 0.02 M borate buffer, pH 8.0 overnight and then against 4 changes of 0.033 M acetate, pH 5.0 for 72 hr. The precipitate was removed by centrifugation. The soluble part after dialysis against water was recovered by freeze-drying. This contained about 85% of the haemagglutination activity of the seed and was further fractionated by affinity chromatography on fetuin-Sepharose in buffer B [23]. After four not completely resolved peaks (T_1–T_4) were eluted with the starting buffer the more strongly absorbed lectin components (F_1–F_3) were eluted at pH 3.0 (0.05 M glycine–HCl, 0.5 M NaCl). Finally the column was cleared of any aggregated material with 8 M urea (Fig. 1). The overall recovery and agglutinating activity of materials (Table 1) indicated that components T_2, T_3 and T_4 had higher lymphocyte-agglutinating activity than those eluted with the pH 3 buffer (F_1, F_2 and F_3) which in turn had very high haemagglutinating activity. Both the most active components, F_2 and T_2 were re-chromatographed on fetuin–Sepharose and finally chromatographed on an AcA-34 column. Both gave mainly one component, the protomer, with small amounts of a dimer. Both however had the same specific activity.

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REFERENCES

1. Strosberg, A. D., Lauwereys, M. and Foriers, A. (1983) in *Chemical Taxonomy, Molecular Biology and Function of Plant Lectins* (Goldstein, I. J. and Etzler, M. E., eds) pp. 7–20. Alan R. Liss, New York.
2. Shannon, L. M. (1983) in *Chemical Taxonomy, Molecular Biology and Function of Plant Lectins* (Goldstein, I. J. and Etzler, M. E., eds) pp. 47–61. Alan R. Liss, New York.
3. Richardson, M., Campos, F. D. A. P., Moreira, R. A., Ainouz, I. L., Begbie, R., Watt, W. B. and Pusztai, A. (1984) *Eur. J. Biochem.* **144**, 101.
4. Hoffman, L. M., Ma, Y. and Barker, R. F. (1982) *Nucl. Acids Res.* **10**, 7819.
5. Hoffman, L. M. and Donaldson, D. D. (1985) *EMBO J.* **4**, 883.
6. Vodkin, L. O. (1983) in *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins* (Goldstein, I. J. and Etzler, M. E., eds) pp. 87–98. Alan R. Liss, New York.
7. Pusztai, A., Croy, R. R. D., Grant, G. and Stewart, J. C. (1983) in *Seed Proteins* (Daussant, J., Mosse, J. and Vaughan, J., eds) pp. 53–82. Academic Press, London.
8. Yachnin, S. and Svenson, R. H. (1972) *Immunology* **22**, 871.
9. Leavitt, R. D., Felsted, R. L. and Bachur, N. R. (1977) *J. Biol. Chem.* **252**, 2961.
10. Pusztai, A. and Stewart, J. C. (1978) *Biochim. Biophys. Acta* **536**, 38.
11. Manen, J. F. and Pusztai, A. (1982) *Planta* **155**, 328.
12. Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
13. Chrispeels, M. J. (1983) *Planta* **157**, 454.
14. Bollini, R., Ceriotti, A., Daminati, M. G. and Vitale, A. (1985) *Physiol. Plant.* **65**, 12.
15. Vitale, A., Warner, T. G. and Chrispeels, M. J. (1984) *Planta* **160**, 256.
16. Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 127.
17. Görg, A., Postel, W., Weser, J., Schiwara, H. W. and Boesken, W. H. (1985) *Sci. Tools* **32**, 5.
18. Pusztai, A. and Watt, W. B. (1974) *Biochim. Biophys. Acta* **365**, 57.
19. Moore, S. (1963) *J. Biol. Chem.* **238**, 235.
20. Simpson, R. J., Neuberger, M. R. and Liu, T. Y. (1976) *J. Biol. Chem.* **251**, 1396.
21. Rondle, C. J. M. and Morgan, W. T. J. (1955) *Biochem. J.* **61**, 586.
22. McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183.
23. Pusztai, A. and Palmer, R. (1977) *J. Sci. Food Agric.* **28**, 620.