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CHARACTERIZATION AND PARTIAL PURIFICATION OF THREE GLYCOSIDASES FROM CASTOR BEAN ENDOSPERM

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Key Word Index—*Ricinus communis*; Euphorbiaceae; castor bean; α-mannosidase; β-galactosidase, β-*N*-acetylhexosaminidase

Abstract—α-Mannosidase and β-*N*-acetylhexosaminidase, which could function in the cleavage of glycosidic linkages in the native *Ricinus communis* lectins, and β-galactosidase were purified some 100-fold from the endosperm tissue of castor bean seedlings. The procedure used ammonium sulphate precipitation followed by chromatography on CM-cellulose, hydroxyapatite and Sephacryl S-300 to separate the three activities. All three glycosidases were present, with the lectins, in the protein bodies of dry seed and increased in activity during the time that lectins are broken down in the vacuoles. The enzymes show optimal activity in the range pH 3–5.5. The α-mannosidase had a K_m of 0.77 mM for *p*-nitrophenyl-α-D-mannopyranoside. The β-galactosidase showed a K_m of 1.39 mM for *p*-nitrophenyl-β-D-galactopyranoside. The β-*N*-acetylhexasominidase had a K_m of 0.47 mM for *p*-nitrophenyl-*N*-acetyl-β-*N*-glucosamide and a K_m of 0.33 mM for *p*-nitrophenyl-*N*-acetyl-β-D-galactosamide. Effects of competitive inhibitors and cations were described.

INTRODUCTION

The castor bean lectins, ricin and *Ricinus communis* agglutinin are glycoproteins, with M_rs of 60000 and 120000, respectively [1, 2]. They each contain 4.5% carbohydrate [3], present as mannose and *N*-acetylglucosamine [1, 4]. The oligosaccharide chains are *N*-linked to asparagine residues in the polypeptide chains [1]. Binding studies with concanavaline A have shown that the mannose residues are terminal [5, 6]. The oligosaccharide structures have not been completely determined, but from their composition it can be concluded that they are of the 'simple' or 'high mannose' type chains [7]. Therefore, the mannose linkages are probably α and the *N*-acetylglucosamine are β. The lectins are part of the water soluble matrix proteins of the castor bean endosperm [8, 9] and are the only glycoproteins detectable in the protein bodies [8, 10]. During germination and early growth, lectin levels decrease, presumably because the lectins are hydrolysed to their component amino acids and carbohydrates for transport to the growing castor bean seedling [11].

The present study concerns the glycosidases α-mannosidase (EC 3.2.1.24) and β-*N*-acetylhexosaminidase (EC 3.2.1.52), which could function in the breakdown of the oligosaccharide chains. Activity of these two glycosidases is high in extracts of the castor bean endosperm. A third glycosidase, β-galactosidase (EC 3.2.1.23), is also present in high activity [12]. The β-galactosidase was also purified, while separating it from the two glycosidases of interest. Three other glycosidases have very low activity in the extracts [12] and they were not monitored during the purification. The enzyme that cleaves the bond linking the *N*-acetylglucosamine of the oligosaccharide chain to the asparagine of the peptide chain, peptide: *N*-glycosidase (aspartylglycosylamine amido hydrolase) was also assayed.

RESULTS AND DISCUSSION

Glycosidase activity during early growth of seedlings

All three glycosidases were present in the dry seed. Both total and specific activities (units/mg soluble protein) increased during germination and early growth (Fig. 1). Total glycosidase activities increased gradually during the first 2 days and then more sharply, reaching levels some four-fold greater than those originally present (Fig. 1A). Specific activities rose gradually until day 4 and more quickly thereafter (Fig. 1B).

Purification of the glycosidases

The extraction of the glycosidases was done with a buffer that solubilized the cytoplasmic enzymes leaving most of the cell wall associated glycosidase activity in the discarded pellet [12]. About 60% of the total activities of each of the three glycosidases was recovered in the ammonium sulphate precipitate (Tables 1–3) and when the redissolved and dialysed preparation was applied to a CM-cellulose column all three enzymes eluted with the void volume, which contained one-third of the total protein applied. A small amount of β-galactosidase activity eluted with most of the protein in the sodium chloride gradient; since this activity had a less acidic pH optimum it represented contamination from glycosidases from the cell walls [12].

The combined fractions from the void volume were

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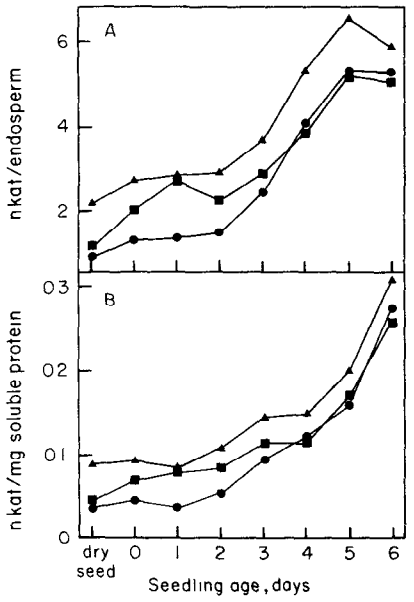

Fig 1 Changes in total (A) and specific activities (B) of glycosidases in endosperm of castor bean seedlings (●) α-Mannosidase, (▲) β-*N*-acetylhexosaminidase, (■) β-galactosidase

dialysed, concentrated and applied to a hydroxyapatite column as described in the Experimental. As shown in Fig 2, the three glycosidases were eluted in the phosphate gradient and partial separation was achieved. Fractions enriched in α-mannosidase, β-*N*-acetylhexosaminidase and β-galactosidase were pooled separately, as indicated by the horizontal bars in Fig. 2, and each of these was applied to a Sephacryl gel filtration column The elution profiles (Fig. 3) show that the enzyme peaks were well separated. The peak fractions of each activity were combined as indicated in Fig 3. The final α-mannosidase preparation contained 5% β-*N*-acetylhexosaminidase activity, the β-*N*-acetylhexosaminidase contained 5% α-mannosidase and 4% β-galactosidase, and the β-galactosidase contained 4% β-*N*-acetylhexosaminidase Tables 1–3 summarize the purification stages for each enzyme and show that overall, purifications of 100–160-fold were achieved

Properties of the glycosidases

The M_r of the α-mannosidase was found to be 280 000 by gel filtration (Fig. 4) and 200 000 from sedimentation behaviour (Table 4) The α-mannosidase had a pH optimum range of 3.0–4.4 The K_m for *p*-nitrophenyl-α-mannopyranoside at pH 4 2 was 0 77 mM. Mannose and glucose were competitive inhibitors, with K_i values of 9.9 and 100 mM, respectively The β-galactosidase showed an M_r of 72 000 by gel filtration (Fig 4) and 90 000 by sedimentation (Table 4). The enzyme was maximally active at pH 3 3–4.3. The K_m for *p*-nitrophenyl-β-D-galactopyranoside was 1 39 mM at pH 4.2 Galactose, arabinose and lactose were competitive inhibitors, with K_i values of 2.2, 35 and 90 mM, respectively

Many β-*N*-acetylhexosaminidases act on both *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide [13], and the enzyme from castor bean is no exception The K_m for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was 0.47 mM at pH 5 0 while that for *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide was 0 33 mM at pH 4 2 The ratio of

Table 1 Purification of α-mannosidase from endosperm tissue of 5-day-old castor bean seedlings

Purification step	Vol (ml)	Total act (nkat)	Total protein (mg)	Sp act (nkat/mg protein)	Purification factor*	Recovery* (%)
Crude extract	270	220	1950	0 113	1	100
33–70% ammonium sulphate cut	53 5	135	350	0 386	3 4	61 4
CM-cellulose	86	120	205	0 587	5 2	54 5
Hydroxyapatite	205	88 6	19	4 66	41 3	40 3
Sephacryl S-300	22	28 7	1 5	19 1	170	13 0

*Calculated with respect to the crude extract

Table 2 Purification of β-*N*-acetylhexosaminidase from endosperm tissue of 5-day-old castor bean seadlings

Purification step	Vol (ml)	Total act (nkat)	Total protein (mg)	Sp act (nkat/mg protein)	Purification factor*	Recovery* (%)	GlcNAc–GalNAc
Crude extract	270	263	1950	0 135	1	100	1 81
33–70% ammonium sulphate cut	53 5	138	350	0 393	2 9	52 3	1 87
CM-cellulose	86	133	205	0 649	4 8	50 4	1 84
Hydroxyapatite	57.7	85 7	24 2	3 54	26 2	32 4	1 93
Sephacryl S-300	28.5	36.4	2 6	14 0	104	13 8	1 88

*Calculated with respect to the crude extract

Table 3 Purification of β-galactosidase from endosperm tissue of 5-day-old castor bean seedlings

Purification step	Vol. (ml)	Total act (nkat)	Total protein (mg)	Sp act (nkat/mg protein)	Purification factor*	Recovery* (%)
Crude extract	270	213	1950	0 109	1	100
33–70% ammonium sulphate cut	53 5	135	350	0 387	3 5	63 3
CM-cellulose	86	127	205	0 622	5.7	59 6
Hydroxyapatite	67 5	66 3	17 6	3.77	34 6	31 1
Sephacryl S-300	25	34 3	3 3	10 4	95 3	16 1

*Calculated with respect to the crude extract

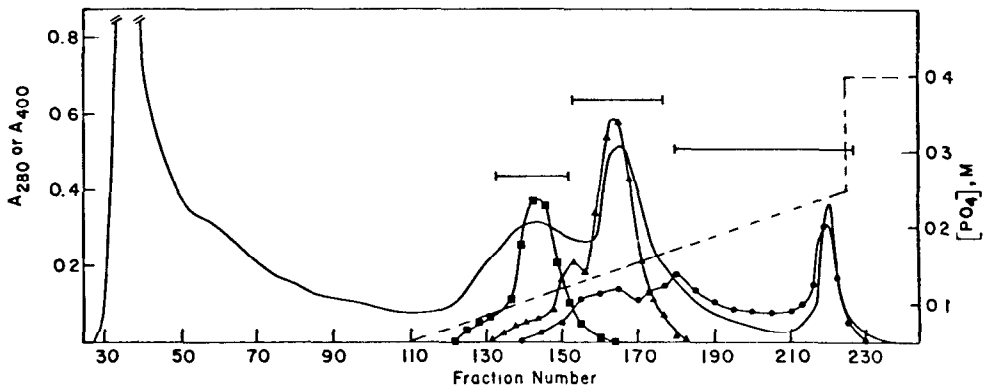

Fig 2 Hydroxyapatite chromatography of glycosidases at pH 7 2 (——) $A_{280\,nm}$, (●) α-mannosidase, (▲) β-*N*-acetylhexosaminidase, (■) β-galactosidase, (– – – –) the phosphate gradient

activities on the two substrates remained constant during purification (Table 2). With *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate at pH 5.0, acetate, *N*-acetylgalactosamine, glucosamine, *N*-acetylglucosamine and mannose were all competitive inhibitors with K_i values of 8 4, 0 79, 6.2, 6.0 and 22 mM, respectively The pH optima were 4.0–5.3 and 3.2–4.9 for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, respectively. The β-*N*-acetylhexosaminidase showed an M_r of 150000 by gel filtration (Fig. 4) and 120000 by sucrose density centrifugation (Table 4).

As shown in Table 5, Hg^{2+} had no effect on the α-mannosidase, indicating that sulphydryl groups are not required for activity or protein stability. The Hg^{2+} ion (2 mM) completely inhibited the β-galactosidase. β-Hexosaminidinase was less strongly inhibited by Hg^{2+}. β-Galactosidase was stimulated by Mn^{2+} and Zn^{2+} stimulated α-mannosidase. β-Hexosaminidase was not affected by either of these cations None of the glycosidases responded to Mg^{2+}.

Peptide:N-*glycosidase*

No peptide *N*-glycosidase was detected in endosperm extracts from 4-day-old seedlings with aspartic acid–glucoseNAc as substrate. This negative result (also noted by others with different materials [14–16]) does not preclude hydrolysis of endogenous substrates. Amido hydrolase from almond will remove the oligosaccharide from a stem bromelian glycopeptide, but does not hydrolyse aspartic acid–glucoseNAc [16], and an enzyme from jack bean, while active on glycopeptides with three or more amino acid residues, does not hydrolyse aspartic acid–glucoseNAc [15]

Localization of the glycosidases

The three glycosidases are present in the proteins solubilized from isolated protein bodies and show the same pH optima and chromatographic properties on hydroxyapatite as those purified from the endosperm tissue. Thus, the three glycosidases described here apparently originate from the protein bodies and are present in the vacuoles of 5-day-old endosperm tissue [11] where the potential lectin substrates for α-mannosidase and β-*N*-acetylhexosaminidase are also located. The pH of the vacuolar fluid is 5.8 [17] and, although this is not the optimal pH for the glycosidases, the enzymes show 17% and 72%, respectively, of their maximum activity at this pH and the levels of activity are far in excess of those required to account for the actual rate of lectin breakdown [11]

EXPERIMENTAL

Plant material Castor bean seeds (*Ricinus communis* L cv Hale) were soaked overnight in running tap water, sown in moist vermiculite and germinated in the dark at 30° The time of planting was taken as day 0 Seedlings were harvested at the desired age and the endosperm tissue collected for extraction

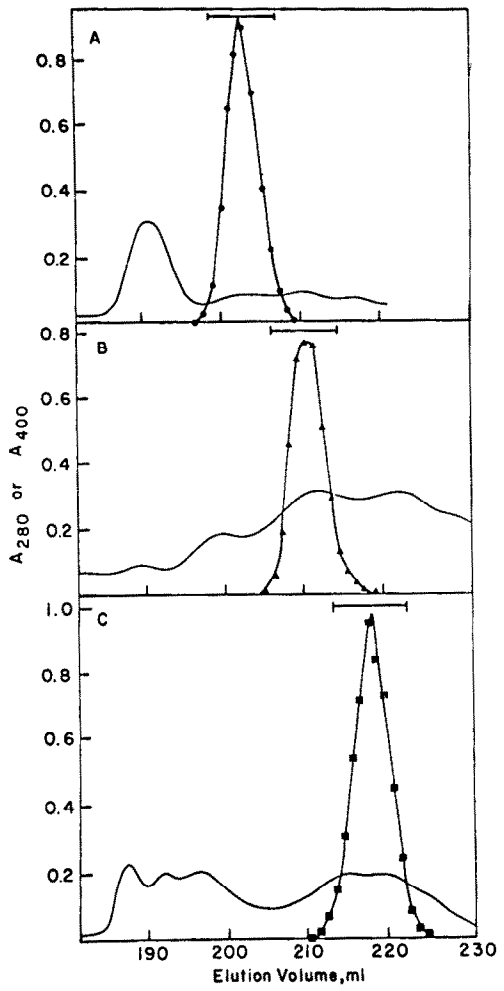

Fig. 3. Elution profiles of glycosidases during gel filtration on Sephacryl S-300. (——) $A_{280\,nm}$; (●) α-mannosidase; (▲) β-*N*-acetylhexosaminidase; (■) β-galactosidase.

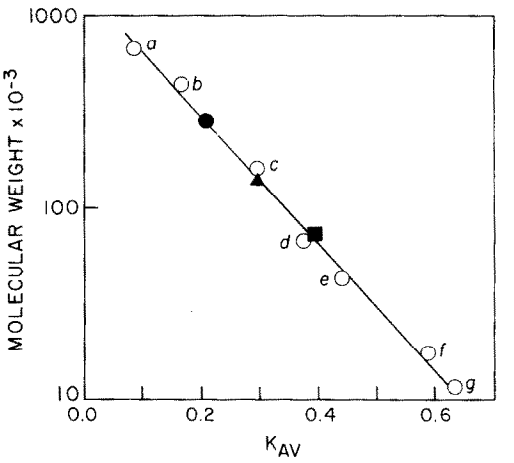

Fig. 4. Determination of M_rs of glycosidases by chromatography on Sephacryl S-300. Closed symbols indicate the positions of: (●) α-mannosidase; (▲) β-*N*-acetylhexosaminidase; (■) β-galactosidase. Open symbols (○) indicate the positions of M_r standards: (a) thyroglobulin (M_r 669 000); (b) ferritin (M_r 440 000); (c) aldolase (M_r 158 000); (d) BSA (M_r 68 000); (e) ovalbumin (M_r 43 000); (f) myoglobin (M_r 17 200) and (g) cytochrome *c* (M_r 11 700).

Enzyme assays. The glycosidase activities were determined by measuring release of *p*-nitrophenol (PNP) from PNP-conjugated substrates. Assays containing 50 μl enzyme and 0.5 ml 5 mM substrate in citrate–Pi buffer [18] were incubated at 25°. After an appropriate time, the reaction was stopped by the addition of 2.5 ml 0.5 M Na_2CO_3–$NaHCO_3$, pH 10. The reactions were linear for at least 60 min and were directly proportional to the amount of enzyme present. The $A_{400\,nm}$ was measured and the amount of PNP released was determined from a standard curve.

α-Mannosidase and β-galactosidase were assayed using PNP-α-D-mannopyranoside and PNP-β-D-galactopyranoside, respectively, at pH 4.2. β-Acetylhexosaminidase was assayed using PNP-*N*-acetyl-β-D-glycosaminide at pH 5.0. This enzyme also hydrolysed PNP-β-*N*-acetylgalactosamine at pH 4.2. Peptide: *N*-glycosidase was assayed as in ref. [14], using 1 mM 2-acetamido-1-β-(L-aspartamido)-1,2-dideoxy-D-glucose (Asp–GlcNAc) in citrate–Pi buffer [18].

Glycosidase activity in the endosperm during germination. The endosperms from 20 seedlings were extracted at 4° with 20 ml 100 mM Tris–HCl, pH 7.5, by grinding with a mortar and pestle. The homogenate was centrifuged at 25 000 *g* for 15 min. After decanting through two layers of Miracloth (Calbiochem), the supernatant was used for measurements of glycosidase activities and soluble protein.

Separation and purification of the glycosidases. All operations were carried out at 4–6°. The initial extraction was made by placing 100 g endosperm tissue from 5-day-old seedlings and 200 ml buffer [0.1 M NaPi (pH 7.0), 1 mM EDTA, 3 mM NaN_3] in a blender and homogenizing for 1 min. The extract was centrifuged at 25 000 *g* for 15 min and the supernatant decanted through two layers of Miracloth. Solid $(NH_4)_2SO_4$ was added to the supernatant and the material that ppted between 33% and 70% satn was collected by centrifugation. The $(NH_4)_2SO_4$ pellet

Table 4. Calculations of M_rs of glycosidases from their sedimentation behaviour during sucrose gradient centrifugation

Standard	α-Mannosidase	β-*N*-Acetylhexosaminidase	β-Galactosidase
Malate dehydrogenase	196 000	119 000	88 000
Yeast alcohol dehydrogenase	205 000	124 500	92 000
Catalase	203 000	123 400	91 000
Average	201 300	122 300	90 300

Table 5. The effects of cations on the activities of castor bean glycosidases

	Glycosidase act. (% control)		
	α-Mannosidase	β-Galactosidase	β-*N*-Acetylhexosaminidase
Control	100	100	100
$MgCl_2$, 5 mM	105	99	98
$MnCl_2$, 5 mM	N.D.*	142	98
$ZnCl_2$, 5 mM	110	107	103
$HgCl_2$, 2 mM	100	0	80
$HgCl_2$, 5 mM	95	0	55

The assays were conducted at 25° for 12 min, using 4 mM substrate.
*N.D., Not determined.

was suspended in 50 mM NaPi, pH 6.0, and dialysed against three 2 l. changes of the same buffer. The material that ppted was removed by centrifugation and discarded.

The dialysed material was loaded onto a CM-cellulose column, 2.6 × 30 cm, equilibrated with 50 mM NaPi, pH 6.0. The column was washed with 400 ml of the same buffer, followed by a 500 ml linear NaCl gradient, from 0 to 0.5 M. Fractions (6 ml each) were collected and those containing glycosidase activity were pooled and concd, dialysed against 60 mM KPi, pH 7.2, and loaded onto a hydroxyapatite column, 2.6 × 30 cm, equilibrated with the same buffer. The column was washed with 400 ml 60 mM KPi (pH 7.2), a 550 ml linear Pi gradient (from 60 to 250 mM KPi, pH 7.2) and a final wash of 400 mM KPi (pH 7.2). Fractions (5 ml each) were collected. Most of the proteins, not containing glycosidase activity, eluted with the void vol. and the glycosidases were eluted in the Pi gradient. Three separate fractions, enriched for a particular glycosidase, were collected as indicated in Fig. 2.

The three glycosidase fractions from the hydroxyapatite column were run separately on a Sephacryl S-300 column, 2.5 × 88 cm, equilibrated with 50 mM Tris–HCl (pH 7.5), 50 mM NaCl and 3 mM NaN_3 and eluted with the same buffer. The peaks of the three activities were separated and collected as shown in Fig. 3.

M_r *determinations.* The M_rs of the glycosidases were determined by gel filtration [19] on Sephacryl S-300 as described above and their sedimentation behaviour during sucrose gradient centrifugation [20]. The Sephacryl S-300 column was calibrated and a standard curve of K_{AV} vs log M_r for the calibration proteins was used to determine the M_r of the glycosidases.

For calculation of M_r by sucrose gradient sedimentation, malate dehydrogenase (EC 1.1.1.37; M_r 70000), yeast alcohol dehydrogenase (EC 1.1.1.1; M_r 141000) and catalase (EC 1.11.1.6; M_r 240000) were used as the M_r standards. The glycosidases and the three M_r standards in 0.5 ml 5% sucrose in 50 mM Tris–HCl (pH 7.5) were layered onto a 13.5 ml linear sucrose gradient, from 10 to 30% in 50 mM Tris–HCl (pH 7.5), on a 1.5 ml 40% sucrose cushion. The gradients were spun in a Beckman SW-27 rotor at 25000 rpm (125000 *g*) for 39 hr in a Beckman L2-65B ultracentrifuge. The gradients were fractionated and the fractions assayed for glycosidases (described above), malate dehydrogenase [21], alcohol dehydrogenase [21] and catalase [22]. The M_rs of the glycosidases were calculated as described [20].

Kinetic studies. The K_m values were determined from Hanes plots $[S]/V$ vs $[S]$ [23]. The K_i values for the various inhibitors were determined from Dixon plots, $1/V$ vs $[I]$ [23] and the type of inhibition from plots of $[S]/V$ vs $[I]$ [23].

Glycosidase activity in protein bodies. Protein bodies were isolated from dry castor beans using the non-aq. glycerol method [24]. The glycosidases were solubilized by adding buffer (50 mM NaPi, pH 7.2) to the protein bodies, centrifuging the extract to remove the insoluble proteins and dialysing the supernatant to remove excess glycerol. The glycosidases were assayed as described above.

Protein measurement. Protein was ppted from the samples to be assayed by the addition of an equal vol. of 12% TCA. The ppted protein was dissolved in 0.5 M NaOH, and 200 μl of an appropriate dilution was assayed [25], using bovine serum albumin as the standard.

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REFERENCES

1. Funatsu, M. (1972) in *Proteins: Structure and Function* (Funatsu, M., Hiromi, K., Imahori, K., Murachi, T. and Narita, K., eds) Vol. 2, p. 103. John Wiley, New York.
2. Olsnes, S. and Pihl, A. (1976) in *The Specificity and Action of Animal, Bacterial and Plant Toxins* (Cuatrecasas, P., ed.) p. 129. Chapman & Hall, London.
3. Olsnes, S. (1978) *Meth. Enzymol.* **50**, 330.
4. Olsnes, S., Refsnes, K., Christensen, T. B. and Pihl, A. (1975) *Biochim. Biophys. Acta* **105**, 1.
5. Lin, T. T. S. and Li, S. S. L. (1980) *Eur. J. Biochem.* **105**, 453.
6. Podder, S. K., Surolia, A. and Bachhawat, B. K. (1974) *Eur. J. Biochem.* **44**, 151.
7. Kornfield, R. and Kornfield, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.) p. 1. Plenum Press, New York.
8. Tully, R. E. and Beevers, H. (1976) *Plant Physiol.* **58**, 710.
9. Youle, R. J. and Huang, A. H. C. (1976) *Plant Physiol.* **58**, 703.
10. Gifford, D. J., Greenwood, J. S. and Bewley, J. D. (1982) *Plant Physiol.* **69**, 1471.
11. Harley, S. M. and Beevers, H. (1985) *Plant Physiol.* (in press).
12. Tully, R. E. (1976) Ph.D. Thesis. University of California, Santa Cruz.
13. Kobata, A. (1979) *Analyt. Biochem.* **100**, 1.
14. Neely, R. S. and Beevers, L. (1980) *J. Exp. Botany* **31**, 299.

15. Sugiyama, K., Ishihara, H., Tejima, S. and Takahashi, N. (1983) *Biochem. Biophys. Res. Commun.* **112**, 115.
16. Takahashi, N. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1194
17. Nishimura, M. (1982) *Plant Physiol.* **70**, 742.
18. Gomori, G. (1955) *Meth. Enzymol.* **1**, 138.
19. Andrews, P. (1970) *Meth. Biochem. Anal.* **18**, 1.
20 Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372.
21. Decker, L. A. (1971) *Worthington Enzyme Manual.* Worthington Biochemical Corporation, Freehold, New Jersey.
22. Luck, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) p. 885. Academic Press, New York.
23. Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics.* Butterworth, London.
24. Mettler, I. J. and Beevers, H. (1979) *Plant Physiol.* **64**, 506.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.