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# β-*N*-ACETYLGLUCOSAMINIDASE AND β-GALACTOSIDASE FROM ALEURONE LAYERS OF RESTING WHEAT GRAINS*

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**Key Word Index**—*Triticum aestivum*; Gramineae; wheat; β-*N*-acetylglucosaminidase; aleurone layers; β-galactosidase; wheat grains; wheat germ agglutinin–Sepharose.

**Abstract**—We have examined the characteristics of binding to wheat germ agglutinin–Sepharose of β-*N*-acetylglucosaminidase and β-galactosidase from aleurone layers of resting wheat grains. Although the enzymes interacting with wheat germ agglutinin–Sepharose could be extracted by a procedure which did not involve any solubilizing treatments, the highest activity of these enzymes was obtained by extracting and sonicating the tissues in the presence of 0.5% Triton X-100. The pH optimum and time-course of binding as well as the effect of some divalent ions on the binding were studied. The largest part of the bound enzymes was eluted at low concentration of *N*-acetyl-D-glucosamine (0.05 M), although smaller amounts were still eluted at higher molarities (0.1 and 0.2 M). D-Mannose, D-glucose and L-fucose failed to replace *N*-acetyl-D-glucosamine in eluting the enzymes bound to wheat germ agglutinin–Sepharose, whereas *N*-acetyl-D-galactosamine was much less effective than *N*-acetyl-D-glucosamine. The catalytic properties of the enzymes remained unchanged after the binding to wheat germ agglutinin–Sepharose, although the $K_m$ values of the free and lectin-bound enzymes were slightly different. A rapid and easy three-step procedure of purification, mainly based on affinity chromatography on wheat germ agglutinin–Sepharose, is described. It allows purification of β-galactosidase and β-*N*-acetylglucosaminidase over 200-fold. β-*N*-Acetylglucosaminidase has been further purified to electrophoretic homogeneity and also characterized.

## INTRODUCTION

Wheat germ agglutinin (WGA), the lectin located in the wheat embryo [1], interacts specifically with *N*-acetyl-D-glucosamine (Glc*N*Ac), its β-(1–4)-linked oligomers and *N*-acetylneuraminic acid, either free or glycosidically-linked to glycoconjugates [2].

Like other plant lectins, WGA has been widely used as a probe in the study of cell surface components [3] and, attached to an insoluble support, to purify many animal glycoproteins, including lysosomal hydrolases [4]. Recently [5], we have studied the ability of acid phosphatase and glycosidases from the aleurone layers of resting wheat grains to interact with concanavalin A (Con A–) and WGA–Sepharose as a means of examining their glycoprotein nature. We observed that aliquots of two enzymes, β-*N*-acetylglucosaminidase (EC 3.2.1.30) and β-galactosidase (EC 3.2.1.23), were recovered from WGA–Sepharose with the highest specific activity compared with that of the crude extract, indicating that affinity chromatography on WGA–Sepharose might be a useful tool in the purification of these enzymes.

In the present study we have examined in more detail the characteristics of binding of these two enzymes to WGA–Sepharose with the aim of optimizing the conditions for isolating them from the aleurone layers. In addition, we describe a rapid three-step procedure, mainly based on affinity chromatography on WGA–Sepharose, that allows an over 200-fold purification of these enzymes. β-*N*-Acetylglucosaminidase has been further purified to electrophoretic homogeneity and characterized.

## RESULTS AND DISCUSSION

### *Effect of solubilizing treatments on the rate of the enzymes binding to WGA–Sepharose*

We have previously found [5] that a fraction of β-*N*-acetylglucosaminidase and β-galactosidase which interacted with WGA–Sepharose could be extracted from the aleurone layers by a procedure that did not involve solubilizing treatments (13% and 21% of total activity of β-*N*-acetylglucosaminidase and β-galactosidase, respectively). However, when the tissues were homogenized and sonicated in the presence of 0.5% Triton X-100, the rate of β-*N*-acetylglucosaminidase and β-galactosidase interacting with WGA–Sepharose was enhanced, respectively, to 20% and 27% of total activity. Sonication alone was ineffective and Triton X-100 treatment had a poor effect. These results indicate that the species of β-*N*-acetylglucosaminidase and β-galactosidase able to interact with WGA–Sepharose occurred in the aleurone layers both in an easily removable and in a more strongly bound form.

### *Optimal conditions of enzyme binding to WGA–Sepharose*

The pH optimum of binding was *ca* 6.5–7. About 50% of binding maximum was at pH values of 4.5 and 8.0. The binding increased with time and reached a maximum after

*Part 2 in the series. "WGA-Binding Hydrolases from Wheat Grains." For Part 1 see ref. [5].

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40 min. A similar time-course profile was observed when incubation was carried out at 20° and at 4°.

Since it has been shown that the binding of glycoproteins to Con A–Sepharose may be influenced by divalent ions [4], the effect of $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Ca^{2+}$ (tested at concentrations between 1 $\mu$M and 1 mM) was studied. The ions $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ were ineffective, whereas $Ca^{2+}$ at 1 mM slightly increased the binding rate (23% increase).

*Elution of the enzymes bound to WGA–Sepharose*

Step-wise elution of the affinity gel with Glc*N*Ac solutions of increasing molarities gave the profiles shown in Fig. 1. The largest part of the bound enzymes was eluted by 0.05 M Glc*N*Ac solution, although smaller amounts were still eluted at higher molarities. These results suggest that the enzymes may occur in multiple forms showing varying affinity for WGA.

*Binding specificity to WGA–Sepharose*

D-Glucose, D-mannose and L-fucose were unable to elute the enzymes bound to WGA–Sepharose, whereas *N*-acetyl-D-galactosamine was much less effective than Glc*N*Ac. About 20% and 90% of bound enzymes was eluted by *N*-acetyl-D-galactosamine and Glc*N*Ac, respectively.

*$K_m$ of free and WGA–Sepharose bound enzymes*

The catalytic properties of the enzymes remained unchanged after binding to WGA–Sepharose although the $K_m$ values of the free and lectinbound enzymes were slightly different ($K_m$ values of free and bound enzyme were $5.5 \times 10^{-4}$ and $9.0 \times 10^{-4}$ M for $\beta$-*N*-acetylglucosaminidase and $1.8 \times 10^{-3}$ and $2.3 \times 10^{-3}$ M for $\beta$-galactosidase). This indicates that the lectin binding site and substrate binding site of the enzymes were different. However, the slight increase in the $K_m$ values of the WGA–enzyme complex over the free enzymes may be because of the particulate nature of the complex or it may be due to the fact that the binding of WGA to the enzymes somehow reduces the affinity of the enzyme for the substrate. This type of phenomenon has been observed with enzymes immobilized on solid support [6, 7], including Con A–Sepharose [4], the effect being either to an increase or decrease the substrate binding capacity.

*Purification of $\beta$-N-acetylglucosaminidase and $\beta$-galactosidase*

Table 1 shows the purification of the two enzymes with a procedure involving affinity chromatography on WGA–Sepharose of the 20–60% ammonium sulphate precipitate from the crude extract followed by DEAE–cellulose chromatography. Step-wise elution of

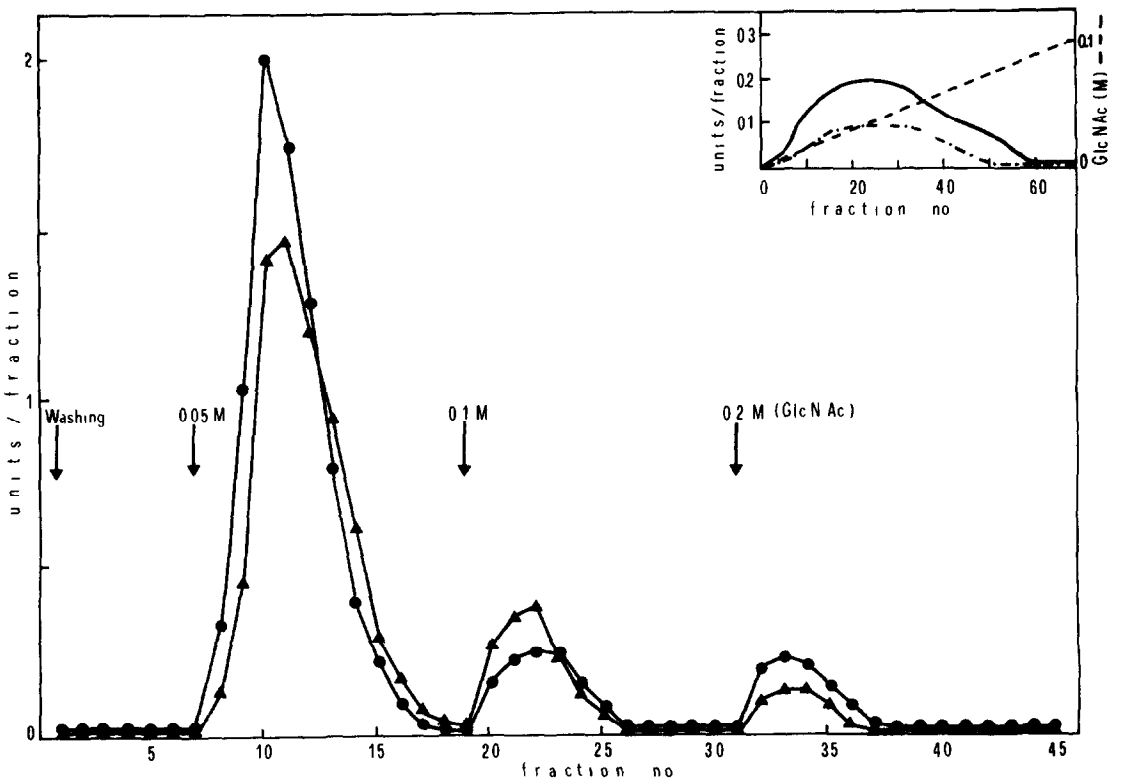


Fig. 1. Elution of the enzymes bound to WGA–Sepharose. The procedure was the same as reported in the Experimental, except that the WGA–Sepharose column, after extensive washing, was eluted step-wise with the indicated increasing concentrations of Glc*N*Ac. Fractions (1 ml) were collected. (●) $\beta$-*N*-Acetylglucosaminidase; (▲) $\beta$-galactosidase. Inset: the experimental conditions were the same as above except that the column of WGA–Sepharose was eluted with a 40 ml linear gradient of Glc*N*Ac (from 0 to 0.1 M) and 0.55 ml fractions were collected. (——) $\beta$-*N*-Acetylglucosaminidase; (– – ·–) $\beta$-galactosidase.

Table 1. Enzyme purification (see Experimental for details)

| Step | Protein (mg) | β-Galactosidase | | | β-N-Acetylglucosaminidase | | |
|---|---|---|---|---|---|---|---|
| | | Total activity (units) | Specific activity (units/mg protein) | Purification (fold) | Total activity (units) | Specific activity (units/mg protein) | Purification (fold) |
| Crude extract | 2900 | 1900 | 0.65 | 1.0 | 4200 | 1.44 | 1.0 |
| 20–60% ammonium sulphate precipitate | 1940 | 1790 | 0.92 | 1.4 | 3900 | 2.01 | 1.4 |
| WGA–Sepharose chromatography | 12 | 520 | 43.3 | 66.6 | 826 | 68.8 | 47.7 |
| DEAE–cellulose | | | | | | | |
| 0.1 M sodium chloride | 3.0 | 400 | 133.3 | 205.0 | 0 | 0 | — |
| 0.2 M sodium chloride | 2.1 | 0 | 0 | — | 670 | 319.0 | 221 |
| Sephadex G-150 CC (eluate from DEAE–cellulose with 0.2 M sodium chloride) | 0.67 | — | — | — | 505 | 754 | 523 |
| Preparative disc-gel electrophoresis | 0.33 | — | — | — | 418 | 1270 | 879 |

DEAE–cellulose, with increasing ionic strength solutions, resolved β-galactosidase from β-*N*-acetylglucosaminidase; the former eluted at 0.1 M sodium chloride, and the latter at 0.2 M sodium chloride. With this three-step procedure, both β-galactosidase and β-*N*-acetylglucosaminidase were purified over 200-fold. After DEAE–cellulose CC, β-galactosidase was very unstable, since the enzyme activity was completely lost after 24 hr at 4°. On the contrary, β-*N*-acetylglucosaminidase was stable and, therefore, could be further purified by Sephadex G-150 filtration and preparative disc-gel electrophoresis.

*Purification extent of β-N-acetylglucosaminidase*

After Sephadex G-150 filtration, the enzyme preparation showed three protein bands by disc-gel electrophoresis which stained by the periodic acid–Schiff method. Only the major band ($R_f$ 0.5) exhibited β-*N*-acetylglucosaminidase activity. The preparation from Sephadex G-150 filtration could be further purified by preparative disc-gel electrophoresis as reported in the Experimental. After this step the enzyme preparation showed a single glycoprotein band ($R_f$ 0.5) on polyacrylamide electrophoresis that was coincident with the β-*N*-acetylglucosaminidase activity.

*Properties of the purified β-N-acetylglucosaminidase*

Unless otherwise stated, the studies on the enzyme properties were performed with the preparation eluted from Sephadex G-150 filtration.

Under the standard assay conditions, the enzyme showed maximal activity between pH 4.8 and 5.2. Half maximal activity was observed at pH 6.2 and 3.4. The activity was linear with time for 45 min. The temperature optimum was *ca* 54°.

The following salts were ineffective at 1 mM: sodium fluoride, magnesium chloride, manganese chloride and zinc chloride. Calcium chloride slightly stimulated the enzyme activity (7% at 1 mM and 13% at 4 mM), whereas $Ag^{2+}$ and $Hg^{2+}$ were potent inhibitors; inclusion of either $2.5 \times 10^{-5}$ M silver nitrate or $2.5 \times 10^{-5}$ M mercuric chloride in the standard assay caused 95% and 40% inhibition, respectively. Glc*N*Ac (0.1 M) inhibited the enzyme activity by 14%. Swainsonine, an indolizidine alkaloid from *Swainsona canescens*, which has been reported to be a potent inhibitor of lysosomal α-mannosidase as well as Jack bean α-mannosidase [8, 9], was ineffective at 2.5 mM and 0.25 μM. The $K_m$ was $5.5 \times 10^{-4}$ M and the $V_{max}$ 1.3 μmol/mg protein. No loss of activity was observed when the enzyme was pre-incubated without substrate at 37° (up to 24 hr). When pre-incubated for 15 min at 50°, 60° and 70°, the enzyme lost 70%, 84% and 100% of its activity, respectively.

All of the following experiments were performed with an enzyme preparation purified by disc-gel electrophoresis, as described in the Experimental. The $M_r$ of the β-*N*-acetylglucosaminidase was determined by Sephadex G-150 filtration by interpolation from a plot of log $M_r$ vs $K_{av}$ of the protein standards. The apparent $M_r$ of the β-*N*-acetylglucosaminidase was $88\,000 \pm 3000$ (mean ± s.d. from three determinations). Isoelectric focusing of the purified enzyme showed a single protein band with an isoelectric point of 4.5. β-*N*-Acetylglucosaminidase activity was coincident with this band.

As reported for β-*N*-acetylglucosaminidase from other higher plant sources ([10–12] and for review [13]), the purified enzyme exhibited β-*N*-acetylgalactosaminidase activity as well, since it caused the hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide. The ratio of β-*N*-acetylglucosaminidase–β-*N*-acetylgalactosaminidase activity was 2.18. The enzyme appeared to be specific for β-glycosidic bonds, since it failed to hydrolyse *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide and nitrophenyl-*N*-acetyl-α-D-galactosaminide.

The *p*-nitrophenyl phosphate derivatives of the following sugars were ineffective as substrates for the purified enzyme: α-D- and β-D-glucopyranoside, α-D- and β-D-mannopyranoside, α-L- and β-D-fucopyranoside, and α-D- and β-D-galactopyranoside. The enzyme was also devoid of sialidase activity. It was found to liberate Glc*N*Ac from *N*,*N*′-diacetylchitobiose.

*Glycoprotein nature of the enzymes*

By their ability to interact with WGA, both β-galactosidase and β-*N*-acetylglucosaminidase appear to be of a glycoprotein nature. Direct proof of this for β-*N*-acetylglucosaminidase has been obtained by the periodic acid–Schiff method as well as by preliminary sugar analysis of the enzyme molecule that revealed the presence of mannose and hexosamines in the enzyme molecule. We could not characterize the individual hexosamines since the amount was too low.

## EXPERIMENTAL

Chemicals, plant material and preparation of aleurone layers were the same as reported previously [5]. Swainsonine was kindly supplied by Dr P R. Dorling, Murdoch University, Western Australia, to Professor A. Ballio, Gruppo di Chimica Biologica e Strutturistica Chimica, Facoltà di Scienze, Roma, Italy.

*Extraction procedure and affinity chromatography.* All operations were carried out at 4° Usually, 0.6 g aleurone layers was extracted in a glass–glass homogenizer with 12 ml 10 mM NaPi buffer, pH 6 5, containing 0.2 M NaCl (buffer A). The homogenate was centrifuged at 105 000 *g* for 1 hr The supernatant (10 ml) was incubated under agitation with 1 ml bed vol. of WGA–Sepharose which had been equilibrated with buffer A. The suspension was kept under agitation for 1 hr at 4° and then transferred within a column to pack the gel. The column was washed until to β-*N*-acetylglucosaminidase and β-galactosidase activities were detected in the washes and they were then eluted with 0.2 M Glc*N*Ac in buffer A. Fractions (1 ml) were collected. Active fractions (2–6) were pooled, dialysed against 10 mM NaPi buffer, pH 6 5, and concd to 0.2 ml by ultrafiltration with a PM 10 membrane filter (Amicon). Under these experimental conditions the binding capacity of the lectin–Sepharose was not exceeded; in fact, when the unbound enzyme fraction was re-incubated with 1 ml WGA–Sepharose, no binding of β-*N*-acetylglucosaminidase and β-galactosidase was observed.

*Effect of solubilizing treatments on the rate of β-N-acetylglucosaminidase and β-galactosidase binding to WGA–Sepharose.* Aleurone layers (0 6 g) were utilized for each extraction condition The procedure was the same as described above except that either: (1) the tissue was sonicated, after homogenization, six times for 10 sec at maximum current on an MSE sonicator; or (2) the tissue was homogenized in the presence of 0 5% Triton X-100; or (3) the tissue was homogenized as in (2) and sonicated as in 1. Affinity chromatography on WGA–Sepharose was carried out as described above.

*Elution of the enzymes bound to WGA–Sepharose with different sugars.* Extraction and incubation of the extract with WGA–Sepharose were carried out as above The extraction buffer contained 0.5% Triton X-100 and the homogenate was sonicated as above. After a 1 hr incubation, the suspension was transferred to a column to pack the gel. The column was washed until no enzyme activities were detected in the washings and then eluted with one of the indicated sugar solns (0 2 M sugar in 10 mM NaPi buffer, pH 6.5, containing 0.2 M NaCl)

*Enzyme purification.* Routinely, 60 g aleurone layers was extracted with 600 ml 10 mM NaPi buffer (pH 7) containing 0.2 M NaCl and 0 5% Triton X-100. The homogenate was sonicated and centrifuged at 20 000 *g* for 30 min. The supernatant was brought to 20–60% satn with $(NH_4)_2SO_4$ and the ppt dissolved in 180 ml 10 mM NaPi buffer, pH 7, containing 0.2 M NaCl (buffer A) and dialysed extensively against the same buffer. Aliquots (18 ml) of the dialysed preparation were each incubated under agitation for 1 hr at 4° with a 5 ml bed vol. of WGA–Sepharose equilibrated with the same buffer. The suspension was then transferred within a column to pack the gel, washed extensively with buffer A and eluted with 0.1 M Glc*N*Ac in the same buffer. Fractions (5 ml) were collected. After use, the 5 ml bed vol. of WGA–Sepharose gel was regenerated according to the manufacturer's instructions and re-used for another 18 ml aliquot of the preparation. This procedure was repeated until the preparation was exhausted. The eluates from WGA–Sepharose were pooled and dialysed extensively against 10 mM NaPi buffer, pH 7. The dialysed material (12 mg protein) was applied to a 30 ml column of DEAE–cellulose which had previously been equilibrated with the same buffer. Following adsorption of the protein, the column was washed with 300 ml of the same buffer and then eluted step-wise with this buffer containing increasing concns of NaCl (0.1 and 0.2 M). β-Galactosidase and β-*N*-acetylglucosaminidase eluted with 0.1 M and 0.2 M NaCl, respectively. To further purify the β-*N*-acetylglucosaminidase, the fraction eluted from the DEAE–cellulose column with 0.2 M NaCl was concd by Diaflo ultrafiltration to 0.5 ml and chromatographed on a Sephadex G-150 column (2.1 × 108 cm) previously equilibrated with NaPi buffer, pH 7, containing 0.2 M NaCl Elution was performed with the same buffer at an upward flow rate of *ca* 2 ml/hr. Fractions (5 ml) were collected. The β-*N*-acetylglucosaminidase emerged as a single peak eluting in fractions 29–38. The active fractions were pooled, concd by Diaflo ultrafiltration and kept at 4° in the same buffer containing 0.02% $NaN_3$ as preservative. The enzyme could be stored for 2 months under these conditions without any appreciable loss of activity. Before use, the enzyme was dialysed against 10 mM NaPi buffer (pH 7) to remove $NaN_3$ and NaCl. When not stated, the properties of the enzyme were studied with this preparation For some studies, a preparation that had been further purified by electrophoresis was used. Preparative electrophoresis was performed essentially as reported previously [14]. Protein (*ca* 100 μg) was applied to each gel. Electrophoresis was performed at 2 mA/gel for *ca* 3 hr until the dye (bromophenol blue) front was at 9 cm from the origin. Some gels were stained with Coomassie blue or periodic acid–Schiff reagent [15], the others were sliced laterally in 3 mm thick sections by a mechanical slicer and each section was eluted with 300 μl buffer B for 12 hr at 4° The elution buffer was separated from the gel section by aspiration and tested for β-*N*-acetylglucosaminidase activity. This enzyme was found as a single peak coincident with a protein band migrating at $R_f$ 0.5. When subjected again to electrophoresis according to the procedure described above, a single glycoprotein band at $R_f$ 0.5 was visible which was coincident with the β-*N*-acetylglucosaminidase activity.

*Enzyme assay.* β-*N*-Acetylglucosaminidase and β-galactosidase activities were assayed as previously reported [5] using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-galactopyranoside as substrates. One unit is defined as the amount of enzyme which hydrolysed 1 μmol substrate/30 sec at 37° When *N,N'*-diacetylchitobiose was used as substrate it was added to the reaction mixture at a concn of 5 mg/ml. The enzymatic release of hexosamine was followed by the Morgan–Elson reaction according to ref [16]. Sialidase was assayed by using mucin as substrate (5 mg/ml) in 50 mM citrate buffer (pH 5). The enzymatic release of sialic acid was measured according to ref. [17]. The $K_m$ value of purified β-*N*-acetylglucosaminidase was obtained from the plot of $[S]/V$ vs $[S]$ [18] by using 1–2 μg purified β-*N*-acetylglucosaminidase. The $K_m$ values of free and WGA–Sepharose bound enzymes were obtained by using 100 μl of the eluate from WGA (containing 10 μg protein). WGA–Sepharose bound enzymes were obtained by prior incubation (1 hr at 4°) of the eluate from WGA with a 50 μl bed vol. of WGA–Sepharose. There was 100% of the enzyme activity bound

to WGA–Sepharose. The enzyme assays were carried out under constant agitation to keep the affinity gel in suspension.

*Isoelectric focusing.* Pre-cast polyacrylamide plates (purchased from LKB) containing Ampholine, pH 3.5–9.5, were used for electrofocusing of purified β-*N*-acetylglucosaminidase. The enzyme was detected by cutting the gel in 0.3 cm pieces, which were extracted with 0.3 ml 10 mM citrate buffer (pH 6) for 12 hr at 4°. The enzyme was assayed as described above.

*Sugar analysis.* A β-*N*-acetylglucosaminidase preparation from disc-gel electrophoresis, containing *ca* 500 μg protein was made 2 M with 11 M HCl. The acid soln was heated in a sealed tube at 100° for 4 hr and evaporated to dryness under red. pres. An aliquot of the residue was assayed for hexosamines by the Morgan–Elson reaction [16]. A second aliquot was deionized with Dowex 50-X8 ($H^+$, 200–400 mesh) and the neutral sugar fraction was assayed enzymatically for galactose and mannose [19]. Protein concn was determined by the method of ref. [20] using bovine serum albumin as standard.

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