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Separation of yeast asparagine-linked oligosaccharides by high-performance anion-exchange chromatography*

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ABSTRACT

Oligosaccharides obtained from *Saccharomyces cerevisiae* mannoproteins by digestion with endo-*N*-acetyl-β-D-glucosaminidase H were fractionated by anion-exchange chromatography, by elution with 50–100mM NaOH without or with a sodium-acetate gradient, and detected with a pulsed amperometric detector (PAD). The elution times of homologous oligosaccharides fell on a straight line having a slope characteristic of the structural type. The response of the PAD detector per mole of oligosaccharide increased about 2-fold going from $Man_3GlcNAc$ to $Man_{13}GlcNAc$, and appeared to depend primarily on the oxidation of the reducing-end *N*-acetylglucosamine unit common to all the oligosaccharides. The digestion of a $Man_{10}GlcNAc$ with jack-bean α-mannosidase was monitored by injecting portions of the crude reaction mixture, and the intermediates were characterized by their elution positions and n.m.r. spectra in the anomeric proton region. One commercial jack-bean α-mannosidase preparation contained a novel endolytic activity that released *N*-acetylglucosamine from the reducing ends of the oligosaccharides and was shown to convert P→^{6}αMan→^{6}αMan→^{6}βMan→^{4}αβGlcNAc to P→^{6}αMan→^{6}αMan→^{6}αβMan plus free *N*-acetylglucosamine. Another commercial jack-bean α-mannosidase converted the $Man_{10}GlcNAc$ to a $Man_3GlcNAc$ having the structure αMan→^{6}βMan→^{4}αβGlcNAc,

$$\begin{array}{l} \\ \uparrow^3 \\ \alpha\text{Man} \end{array}$$

whereas the *Oerskovia* sp. α-mannosidase converted the same oligosaccharide to a $Man_4GlcNAc$ having the structure

$$\begin{array}{l} \alpha\text{Man}\rightarrow{}^6\alpha\text{Man}\rightarrow{}^6\beta\text{Man}\rightarrow{}^4\alpha\beta\text{GlcNAc.} \\ \quad\uparrow^3 \\ \quad\alpha\text{Man} \end{array}$$

INTRODUCTION

Anion-exchange separation of complex oligosaccharides at high pH has been shown to have exceptional resolving power for closely related compounds[1–5]. Because we have available many isomeric and homologous high-mannose oligosaccharides from our studies on yeast mannoproteins[3,4,6,7], we have used them to investigate the utility of this methodology for their separation. The results are as impressive as those reported for other oligosaccharides[1,2], with good separations being obtained for neutral oligosaccha-

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rides up to $Man_{24}GlcNAc$ and for mono- and di-phosphorylated oligosaccharides having 10–14 mannose units. In addition, the method is useful for monitoring the enzymic digestion of the oligosaccharides and for detecting intermediates in the reaction; and, because the analysis is rapid, it is possible to stop the reaction before completion and recover such intermediates.

EXPERIMENTAL

Materials. — Jack-bean α-mannosidase (EC 3.2.1.24) was obtained from V-labs, Covington, LA, Boehringer Mannheim, and Sigma. Exo-(1→2)-α-D-mannosidase was prepared from *Aspergillus phoenicis* ATCC 14332 (ref. 8) and a nonspecific α-mannosidase was isolated from *Oerskovia* sp. ATCC 33522 (ref. 9). Bacterial alkaline phosphatase (Type III-N) (EC 3.1.3.1) was from Sigma. Oligosaccharides (Fig. 1) were from previous studies[3,4,7,10] or were obtained during the course of this work.

Methods. — The Dionex BioLC Carbohydrate System, fitted with a CarboPac PA1 Column (4 × 250 mm) and pulsed amperometric detector (PAD), was operated according to the manufacturer's directions. The pumping rate and the post-column pumping rate were each 1 mL/min. The sensitivity was 1000–3000 nA. The elution conditions varied and are given in each figure legend. Gel filtration was done on a Bio-Gel P-4 (−400 mesh) column (2 × 200 cm) by elution with water in 1-mL fractions, and carbohydrate was monitored by the phenol–sulfuric method[11]. ^{1}H-N.m.r. spectra were determined at 40° in ^2H_2O on a Bruker 500-MHz spectrometer in the Department of Chemistry. Chemical shifts are referenced to internal acetone at δ 2.217 relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS)[3,12,13].

The successful use of empirical chemical shift values for assigning the structures of high-mannose oligosaccharides derives from the facts that (*a*) the structural types are limited to α-(1→2)-, α-(1→3)-, and α-(1→6)-linked mannose, in which linkages the anomeric protons have the very different chemical shifts of δ 5.03 ±0.02, δ 5.13 ±0.02, and δ 4.91 ±0.02, respectively; and (*b*) the substitution of such mannoses at position 2 causes a downfield shift of about 0.25 p.p.m. while substitution at positions 3 or 6 causes an upfield shift of 0.01–0.02 p.p.m.[12].

Mannosidase digestion. — For digestion of $Man_{10}GlcNAc$ with bacterial exo-(1→2)-α-D-mannosidase, 1 mg of oligosaccharide was dissolved in 0.1 mL of acetate buffer, pH 5.0, containing 10 mU of enzyme, and the mixture was incubated for 6 h at 30°. For isolation of the $Man_8GlcNAc$ intermediate, 1 mg of oligosaccharide was digested in 0.08 mL of the same buffer with 2 mU of enzyme for 10 h at 30°. For digestion with *Oerskovia* sp. α-mannosidase, 5 mg of oligosaccharide was dissolved in 0.5 mL of 0.1M potassium phosphate, pH 6.8, containing 0.3 mM $CaCl_2$ and 25 U of enzyme, and the mixture was incubated for 47 h at 37°.

Digestion of $Man_{10}GlcNAc$ (3 mg) with V-labs jack-bean α-mannosidase was done in 0.3 mL of 0.04M acetate buffer, pH 4.5, containing 5 mM $ZnCl_2$, by incubation for 54 h at 37° with the addition of 1 U of enzyme at 0, 12, 24, and 44 h. For jack-bean α-mannosidase from Boehringer (Lot 11300220), 5 mg of oligosaccharide in 0.5 mL of

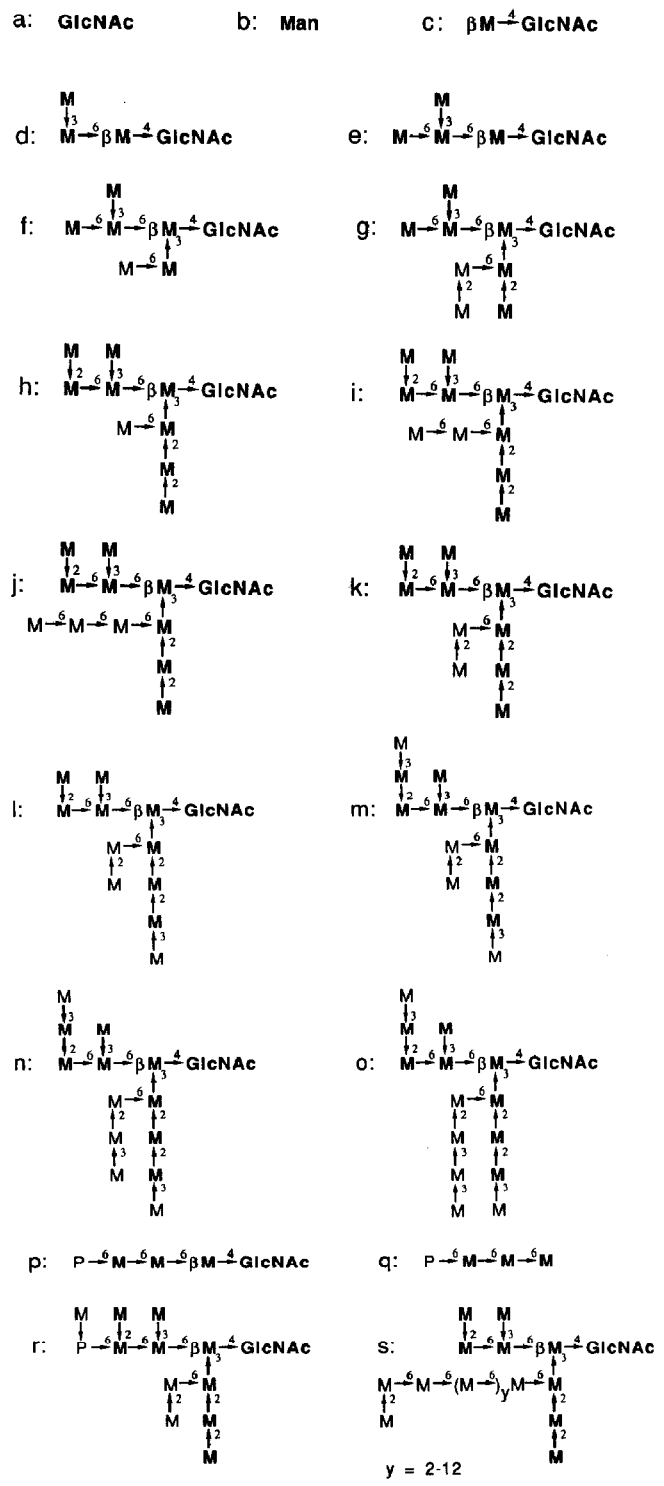

Fig. 1. Structures of compounds analyzed by anion-exchange chromatography. Symbols: M or Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; P, phosphate. All mannose is α-linked except the one attached to GlcNAc.

the same buffer was treated with 2.5 U of enzyme at zero time, and incubated for 30 h at 37° with additions of 2 U at 12 and 24 h. Digestion of the *mnn1 mnn9* oligosaccharide phosphate (0.6 mg) was done in 0.06 mL of acetate buffer by adding 1 U of V-labs α-mannosidase at zero time and incubating 48 h at 37° with the addition of a further 1 U of enzyme at 24 h. The same oligosaccharide (4 mg) was digested with the Boehringer jack-bean α-mannosidase in 0.4 mL of buffer by incubation for 60 h at 37°, with the addition of 2.5 U of enzyme at 0, 12, 24, 36, and 48 h.

For product analysis the digest was heated to inactivate the enzyme, centrifuged, and then applied to a Bio-Gel P-4 (−400 mesh) column (2 × 200 cm) which was eluted with water. Fractions of 1 mL were collected and assayed for carbohydrate by the phenol–sulfuric method[11]. The carbohydrate-containing peaks were collected, lyophilized, and characterized by their anomeric proton spectra.

RESULTS AND DISCUSSION

Separation of high-mannose N-*linked oligosaccharides.* — In Fig. 2A is shown the separation of a synthetic mixture of oligosaccharides from $Man_9GlcNAc$ to $Man_{14}GlcNAc$, the letters identifying each peak with one of the structures in Fig. 1. Two homologous sets were studied, one in which the sizes increase by the addition of α-(1→6)-linked mannose units, and another in which the components have increasing

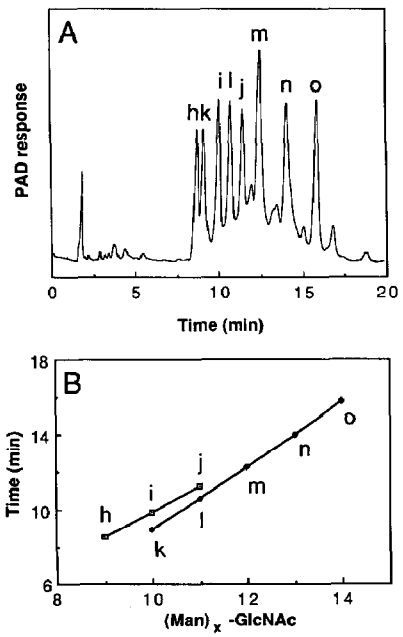

Fig. 2. Relation of retention time to oligosaccharide structure. A, pattern for oligosaccharides that differ in numbers of α-(1→3)- or α-(1→6)-linked mannose units; B, plots of retention times against the number of mannose units in the oligosaccharides that differ in α-(1→6)-linked mannose (**h-i-j**) and in α-(1→3)-linked mannose (**k-l-m-n-o**). The eluent was 100mM NaOH with 50–130mM sodium acetate (25-min gradient).

numbers of α-(1→3)-linked mannose units. A plot of the retention times against size (Fig. 2B) gave two distinct lines that correlated with structural type. Oligosaccharides **i** and **k** both have 10 mannoses, but they differ in that **i** has an $\alpha M \rightarrow {}^{6}\alpha M \rightarrow {}^{6}$ unit while **k** has an $\alpha M \rightarrow {}^{2}\alpha M \rightarrow {}^{6}$ unit. It is known that the 2-hydroxyl of a hexose is the most acidic, and since oligosaccharide **i** has one more free 2-hydroxyl group than **k** we attribute its longer retention time to that fact[2]. A similar explanation applies to oligosaccharides **j** and **l**, and this rationalizes the two curves **h-i-j** and **k-l-m-n-o** since the two sets represent structurally different groups.

The *S. cerevisiae mnn1 mnn2 mnn10* mutant[14] makes oligosaccharides with structure **s** in Fig. 1, and the anion-exchange system resolves the mixture into 11 peaks that represent a homologous series in which the individuals differ by a single α-(1→6)-linked mannose residue[4]. A plot of retention time against size gave a straight line, which confirms that the homologs are closely related structurally (not shown).

The response of the PAD detector is structure-dependent[5] but, for the oligosaccharides studied here, all of which have an *N*-acetylglucosamine unit at the reducing end, the response varies only 2-fold on a molar basis over the range $Man_3GlcNAc$ to $Man^{14}GlcNAc$ (data not shown). Since the responses for free mannose, *N*-acetylglucosamine, and $Man_3GlcNAc$ are almost identical, we conclude that most of the response can be attributed to the reducing-end sugar.

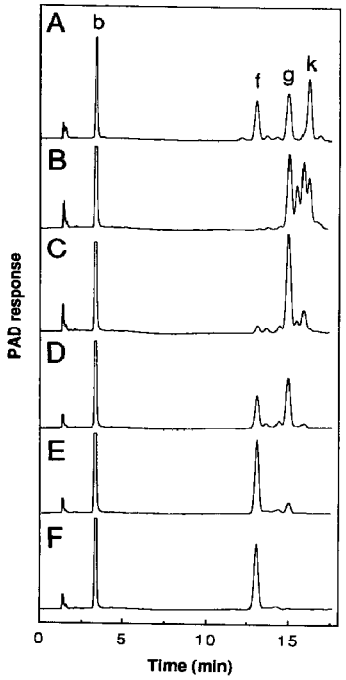

Fig. 3. Digestion of $Man_{10}GlcNAc$ (**k**) with bacterial exo-(1→2)-α-D-mannosidase. A, reference oligosaccharides, designated by letters corresponding to structures shown in Fig. 1; B-F, reaction mixture after 0.5, 1, 2, 4, and 6 h of digestion. The final products are mannose (peak **b**) and $Man_6GlcNAc$ (peak **f**). The eluent was 100mM NaOH with 10–60mM sodium acetate (20-min gradient).

Digestion of $Man_{10}GlcNAc$ with mannosidases. — Figure 3 shows the time course of the digestion of the oligosaccharide with exo-(1→2)-α-D-mannosidase. The oligosaccharide has four mannose residues that should be susceptible to the action of this enzyme, and the patterns indicate the initial removal of one and two mannoses to give a mixture of what is probably two $Man_9GlcNAc$ isomers and a $Man_8GlcNAc$. Although three $Man_9GlcNAc$ isomers are possible, we believe that removal of the two mannoses leading to $Man_8GlcNAc$ **g** occurs preferentially so that only two intermediates are formed. At later times, the $Man_8GlcNAc$ accumulates and is more slowly converted into the final product, $Man_6GlcNAc$, without the appearance of significant amounts of intermediates. When the digestion was done with a small amount of enzyme, the $Man_8GlcNAc$ could be recovered from the digest by gel filtration. Its anomeric-proton spectrum (Fig. 4C) supported the structure **g** shown in Fig. 1. The structure of the substance of peak **f** in Fig. 3 has been reported[3].

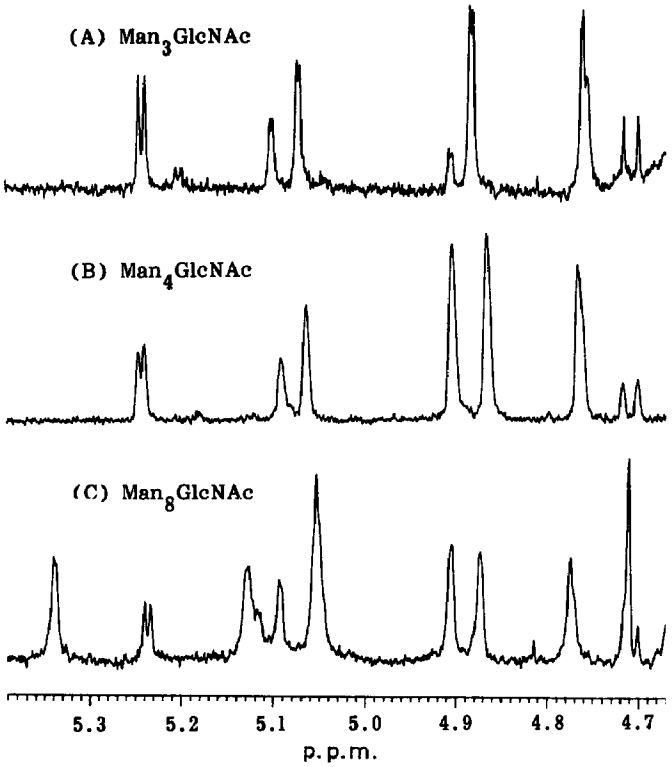

Fig. 4. Anomeric-proton spectra of intermediates in mannosidase digestions of $Man_{10}GlcNAc$. A, $Man_3GlcNAc$ produced by Sigma jack-bean α-mannosidase, showing signals for the GlcNAc anomers (δ 4.70 and 5.24), for the β-mannose (δ 4.77), for a 3-substituted (1→6)-linked α-mannose (δ 4.89), and for the (1→3)-linked α-mannose (δ 5.08 and 5.10, split by interaction with the GlcNAc anomers[13]); B, $Man_4GlcNAc$ produced by *Oerskovia* sp. α-mannosidase, showing all of the signals of A plus a signal at δ 4.91 for a terminal (1→6)-linked α-mannose; and C, $Man_8GlcNAc$ produced by partial exo-(1→2)-α-D-mannosidase digestion, showing the signals of B plus a signal at δ 5.04 for two terminal (1→2)-linked-mannoses, a signal at δ 5.12 for a (1→6)-linked-α-mannose substituted at position 2, and at δ 5.33 for a (1→3)-linked α-mannose substituted at position 2. The signal at δ 4.71 is an artifact. The conditions of digestion are given in the *Methods* section.

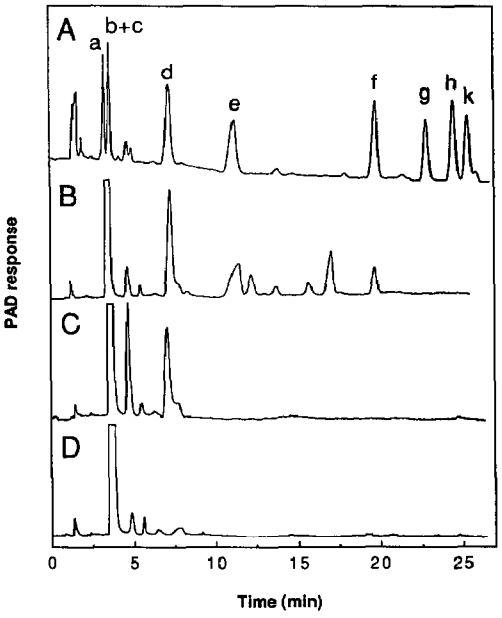

Fig. 5. Digestion of $Man_{10}GlcNAc$ (**k**) with V-labs jack-bean α-mannosidase. A, reference oligosaccharides; B-D, reaction mixture after 12, 30, and 54 h of digestion. The major final product is $\beta Man \rightarrow {}^{4}\alpha\beta GlcNAc$ (peak **b**), whereas *N*-acetylglucosamine (peak **a**) is not formed. The eluent was 100mM NaOH containing 10mM sodium acetate for the first 3 min, followed by a 10–60mM salt gradient for the next 22 min, and a gradient from 60–100mM salt for the last 10 min.

Jack-bean α-mannosidase is expected to convert the $Man_{10}GlcNAc$ to $\beta Man \rightarrow {}^{4}\alpha\beta GlcNAc$, and the patterns in Fig. 5 show that this process involves a number of intermediates, including $Man_6GlcNAc$ **f**, $Man_4GlcNAc$ **e**, and $Man_3GlcNAc$ **d**. The final product (Fig. 5D) is an unresolved peak containing mannose and $\beta Man \rightarrow {}^{4}\alpha\beta GlcNAc$ (**b** + **c**). A second commercial jack-bean mannosidase preparation gave an unexpected result (Fig. 6) in which free *N*-acetylglucosamine appeared early during the digestion (peak **a**, trace C). Although this could have occurred by the action of a contaminating β-mannosidase on the $\beta Man \rightarrow {}^{4}\alpha\beta GlcNAc$, we found that the same enzyme preparation acted on the phosphorylated oligosaccharide **r** to produce a phosphorylated trisaccharide having the structure **q**, whereas digestion of **r** with V-labs jack-bean mannosidase gave the expected product $P \rightarrow {}^{6}\alpha Man \rightarrow {}^{6}\alpha Man \rightarrow {}^{6}\beta Man \rightarrow {}^{4}\alpha\beta GlcNAc$ (Fig. 7). Both products were characterized by their anomeric-proton spectra (Fig. 8). Thus, it is clear that jack-bean meal must contain an endolytic activity that releases *N*-acetylglucosamine from the reducing ends of $Man_nGlcNAc$ oligosaccharides. It is probable that this activity is not expressed until much of the mannose has been released from $Man_{10}GlcNAc$, however, because free *N*-acetylglucosamine is not apparent in the pattern of Fig. 6B, at which time the digestion has proceeded to and beyond the $Man_6GlcNAc$ stage. In a separate digestion of the $Man_{10}GlcNAc$ with Sigma jack-bean mannosidase, a small amount of fragment **d** was recovered by gel filtration, and it gave the anomeric-proton spectrum in Fig. 4A.

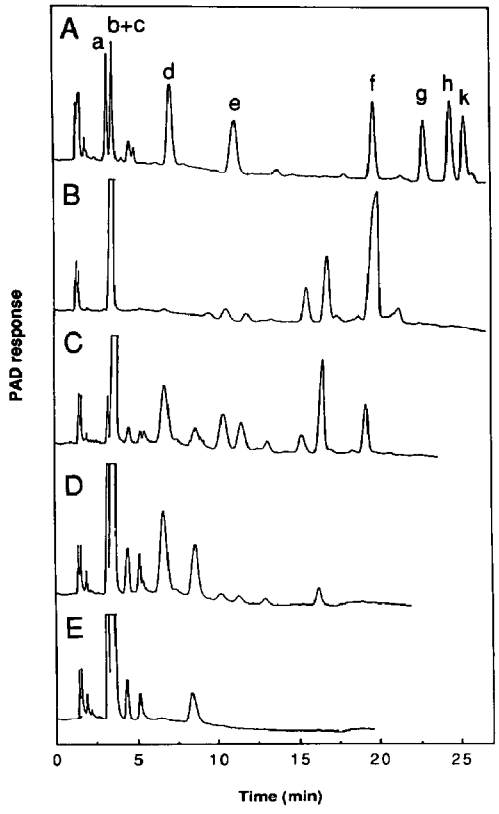

Fig. 6. Digestion of $Man_{10}GlcNAc$ (**k**) with Boehringer jack-bean α-mannosidase. A, reference oligosaccharides; B-E, reaction mixture after 1, 4, 12, and 24 h of digestion. Peak **a**, which begins to appear after 4 h (panel C), is *N*-acetylglucosamine. The elution was done as described for Fig. 5.

Finally, the bacterial (*Oerskovia* sp.) α-mannosidase converted the $Man_{10}GlcNAc$ to $Man_4GlcNAc$ **e** (see Fig. 4B for the anomeric-proton spectrum), with $Man_6GlcNAc$ **f** and an uncharacterized $Man_5GlcNAc$, between **e** and **f**, as detectable intermediates (Fig. 9). It is apparent that the reaction could be interrupted at the stage shown in Fig. 9B to allow isolation of the $Man_5GlcNAc$ intermediate.

Digestion of the oligosaccharide phosphate monoester from carboxypeptidase Y with alkaline phosphatase. — The oligosaccharide phosphate diester[10] was treated with 0.01M HCl for 30 min at 100° to hydrolyze the glycosyl-phosphate bond[3], and the phosphate monoester was recovered by gel filtration on a Bio-Gel P-4 column. The samples, before and after digestion with alkaline phosphatase, were fractionated on the anion-exchange system (results not shown). Several homologs of the phosphate monoester were eluted from 29 to 33 min, with separation based on differences in the number of α-(1→3)-linked mannose units. Following digestion with alkaline phosphatase, about 80% of the material was eluted from 6 to 15 min, which times are characteristic of the neutral oligosaccharides. The similarity of the two elution patterns suggests that in both instances the separations were based on the oligosaccharide structures and that the presence of phosphate only increased the retention times.

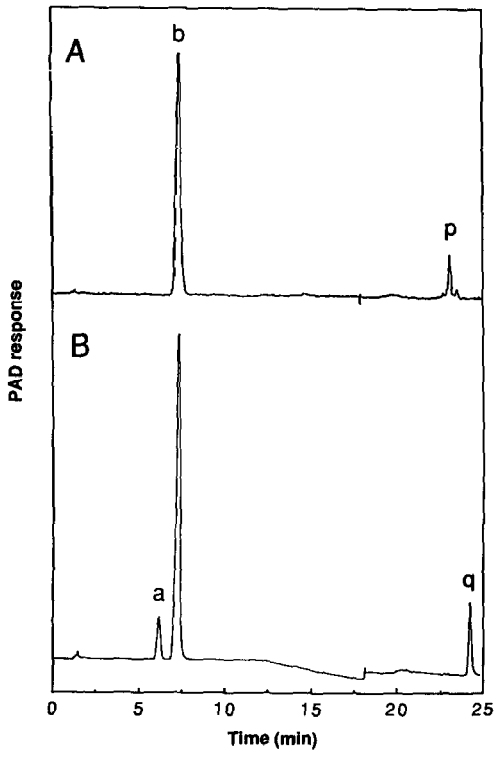

Fig. 7. Digestion of the major *mnn1 mnn9* oligosaccharide monophosphate with jack-bean α-mannosidase. A, V-labs mannosidase, which produces mannose (**b**) and P-Man_3GlcNAc (**p**); B, Boehringer mannosidase, which produces *N*-acetylglucosamine (**a**), mannose (**b**) and P-Man_3 (**q**). The eluent was NaOH, held at 50mM for the first 8 min, then increased to 100mM during the next 2 min, after which a 30-min gradient of 0–750mM sodium acetate in 100mM NaOH was applied.

Comments. — Anion exchange of carbohydrates, as carried out with the Dionex BioLC Carbohydrate System with pulsed amperometric detection, has greatly facilitated the separation of closely related oligosaccharides having d.p.s in excess of 25 hexose units. Although it is not always easy to rationalize the separations, and thereby infer structure from elution position, there are predictable patterns in the behavior of closely related homologs[1]. Owing to the nature of the ion exchange process, it is possible to inject crude enzyme digests without damage to the column, which makes it easy to monitor the reaction and interrupt the process for the isolation of intermediates[1,15]. Finally, by following the formation of intermediates, one can learn something about the sequence by which an enzyme acts on a complex substrate or about the presence of contaminating enzyme activities.

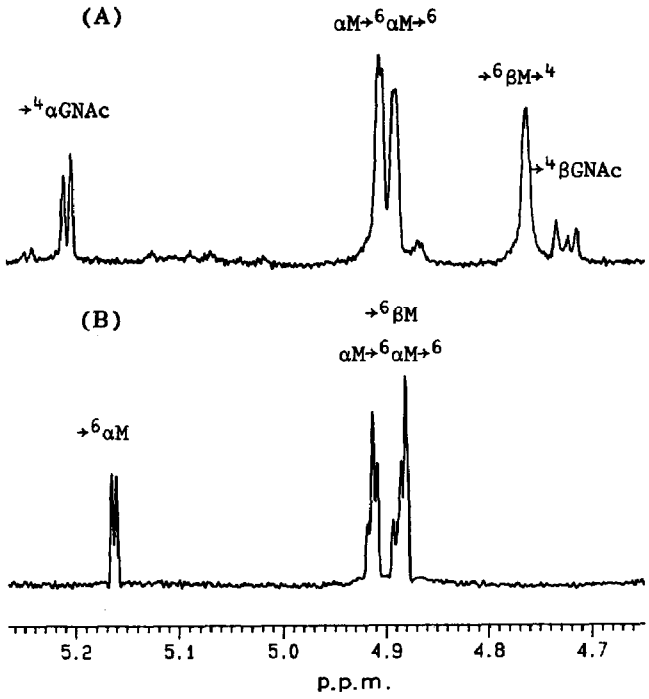

Fig. 8. Anomeric-proton spectra of products **p** and **q** from the oligosaccharide phosphate of Fig. 7. A, product from the digestion with jack-bean α-mannosidase from V-labs, and B, with Boehringer mannosidase. The two signals in both spectra in the region of δ 4.9 represent the nonreducing terminal α-(1→6)-linked mannose (δ 4.91), and the internal α-(1→6)-linked mannose, which resonates at δ 4.89 owing to the shielding effect of substitution at position 6. The reducing-end hexoses show signals for both anomers and the β-linked mannose gives a signal at δ 4.77.

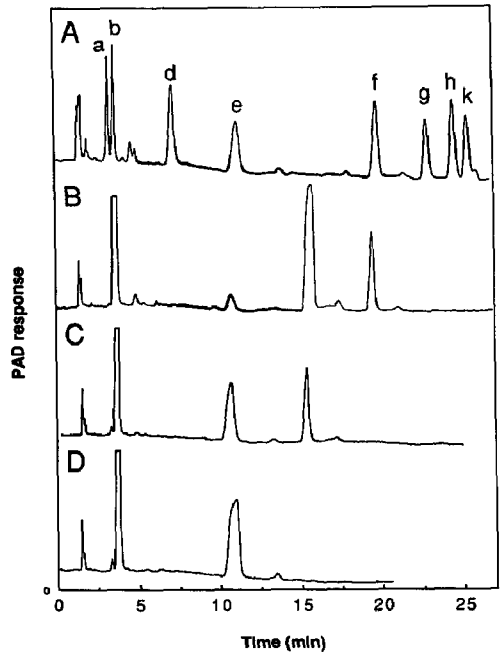

Fig. 9. Digestion of $Man_{10}GlcNAc$ (**k**) with *Oerskovia* sp. α-mannosidase. A, reference oligosaccharides; B-D, reaction mixture after 1, 6, and 47 h of digestion.

ACKNOWLEDGMENT

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