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# STRUCTURAL REQUIREMENTS FOR THE BINDING OF HIGH-MANNOSE-TYPE GLYCOPEPTIDES TO IMMOBILIZED POKEWEED Pa-2 LECTIN*,†

YASUHIRO KATAGIRI, KAZUO YAMAMOTO, TSUTOMU TSUJI, AND TOSHIAKI OSAWA
*Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113 (Japan)*
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ABSTRACT

The structural requirements for the interaction of asparagine-linked glycopeptides with immobilized pokeweed mitogen Pa-2 were investigated. Some high-mannose-type glycopeptides obtained from porcine thyroglobulin were found to have strong affinity for Pa-2–Sepharose, whereas complex- and hybrid-type glycopeptides were shown to have much weaker affinity. The elution profiles of various glycopeptides modified by glycosidase treatment and acetolysis showed that the total structure α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-Glc*p*NAc-(1→4)-β-Glc*p*NAc→Asn was essential for the binding of glycopeptides to a Pa-2–Sepharose column.

## INTRODUCTION

In previous papers, we reported that pokeweed (*Phytolacca americana*) mitogens Pa-1 and Pa-2 recognize a core *N*,*N*′-diacetylchitobiose moiety in asparagine-linked sugar chains of glycoproteins[1], and the receptors for the mitogens on the surface of human erythrocytes[1] and murine lymphocytes[2] were characterized. Since immobilized plant-lectins are useful tools for isolation and structural studies of a variety of glycoconjugates, especially glycoproteins, the binding specificities of several immobilized lectins, such as concanavalin A–Sepharose 4B (refs. 3 and 4), wheat-germ agglutinin–Sepharose 4B (ref. 5), *Phaseolus vulgaris* agglutinin–Sepharose 4B (refs. 6 and 7), *Lens culinaris* agglutinin–Sepharose 4B (refs. 8 and 9), and *Pisum sativum* agglutinin–Sepharose[8,9] have been investigated in detail. In this paper, we describe the structural requirements for the binding of oligosaccharides and glycopeptides to a major pokeweed-mitogen, Pa-2, coupled to Sepharose 4B.

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EXPERIMENTAL

*Preparation of Pa-2-Sepharose 4B.* — A pokeweed mitogen, Pa-2, purified by the method of Waxdal[10], was coupled to Sepharose 4B by the cyanogen bromide method[11] in the presence of 0.1M 2-acetamido-2-deoxy-D-glucopyranose in order to protect the binding site of the mitogen. The amount of Pa-2 bound to Sepharose 4B was estimated to be ~4.0 mg/mL of gel by substracting the amount of unbound protein in the supernatant and washing solutions after the coupling reaction. Protein was determined by the method of Lowry *et al.*[12]. The Pa-2–Sepharose 4B column thus prepared was stable for more than one year at 4°, and its binding capacity was unchanged after repeated uses. Concanavalin A, purified by the method of Agrawal and Goldstein[13] from jack bean meal (Sigma Chemical Co.), was coupled to Sepharose 4B according to the method of Matsumoto *et al.*[14].

*Oligosaccharides and glycopeptides.* — Chitin oligosaccharides were prepared by partial acid hydrolysis of chitin by the method of Rupley[15]. Ovalbumin glycopeptides, GP-I, GP-II-A, GP-II-B and GP-III, were prepared by the methods of Tai *et al.*[16] and Yamashita *et al.*[17]. Porcine thyroglobulin glycopeptides were prepared according to the methods of Tsuji *et al.*[18] and Yamamoto *et al.*[19]. The structures of these glycopeptides are shown in Scheme 1.

*Labeling of oligosaccharides and glycopeptides.* — Oligosaccharides were labeled at the reducing terminal residues by reduction with sodium borotritide (250 mCi/mmol, New England Nuclear, Boston, MA) by the method of Takasaki and Kobata[20]. The radioactive label in the glycopeptides were introduced by acetylation with [$^{14}$C]acetic anhydride (30.0 mCi/mmol, The Radiochemical Center, Amersham, England) by the method of Tai *et al.*[16]. The labeled glycopeptides were purified by gel filtration on a column of Sephadex G-25.

*Affinity chromatography on Pa-2–Sepharose 4B columns.* — A radioactively labeled sample (2000–5000 c.p.m., 0.1–0.25 nmol) in a volume of 20 $\mu$L was applied to a Pa-2–Sepharose 4B column (0.2 × 30 cm) which had been equilibrated with 10mM sodium phosphate buffer, pH 7.2, containing 0.15M sodium chloride. After 1 h at 22°, the column was eluted with the same buffer at a flow rate of 2 mL/h by use of a peristaltic pump, and fractions (0.1 mL) were collected. Recovery of radioactivity was always more than 90%.

*Gel-permeation chromatography.* — Gel-permeation chromatography was performed with a liquid chromatograph (Jasco Tri-rotor; Japan Spectroscopic Co., Japan) equipped with a column (0.8 × 100 cm) of Bio-Gel P-4 (>400 mesh) at a flow rate of 0.3 mL/min by the method of Tsuji *et al.*[18]. During the operation, the column was maintained at 55°. Oligomers of D-glucose and 2-acetamido-2-deoxy-D-glucose were used as standards.

*Enzymes.* — Endo-*N*-acetyl-$\beta$-D-glucosaminidase H from *Streptomyces griseus* was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). $\alpha$-D-Mannosidase was purified from jack bean meal (Sigma Chemical Co., St. Louis, MO.) by the method of Li and Li[21]. Neuraminidase from *Arthrobacter ureafaciens* was

purchased from Nakarai Chemicals Co. (Kyoto, Japan), and α-L-fucosidase from *Charonia lampas* from Seikagaku Kogyo Co. (Japan). *N*-[$^{14}$C]acetylated glycopeptides (5000 c.p.m., ~0.2 nmol) were incubated with 10 milliunits of endo-*N*-acetyl-β-D-glucosaminidase H in 0.15M sodium citrate–0.1M sodium phosphate buffer, pH 5.0 (50 μL) for 24 h at 37°. The products were isolated as described by Yamashita *et al.*[17]. Digestion with α-D-mannosidase was carried out in 50mM sodium acetate buffer, pH 4.0. For digestion with α-L-fucosidase, 0.1M sodium citrate–0.1M sodium phosphate buffer, pH 4.0, containing 0.5M sodium chloride was used.

*Acetolysis.* — Acetolysis of the sodium borotritide-reduced oligosaccharides was carried out by the method of Kocourek and Ballou[22]. The products obtained were analyzed by gel-permeation chromatography on a Bio-Gel P-4 column.

## RESULTS AND DISCUSSION

Chitin oligosaccharides, reduced with sodium borotritide, were unexpectedly found to have low affinity for the column of Pa-2–Sepharose 4B (see Fig. 1). Furthermore, the affinity of these oligosaccharide alditols was independent of the number of 2-acetamido-2-deoxy-D-glucosyl residues.

Next, the interaction between the asparagine-linked glycopeptides **1**–**9** and Pa-2–Sepharose 4B was examined. The sugar chains of these glycopeptides may be classified into high-mannose (**2**–**7**), complex (**8** and **9**), and hybrid type (**1**) (see

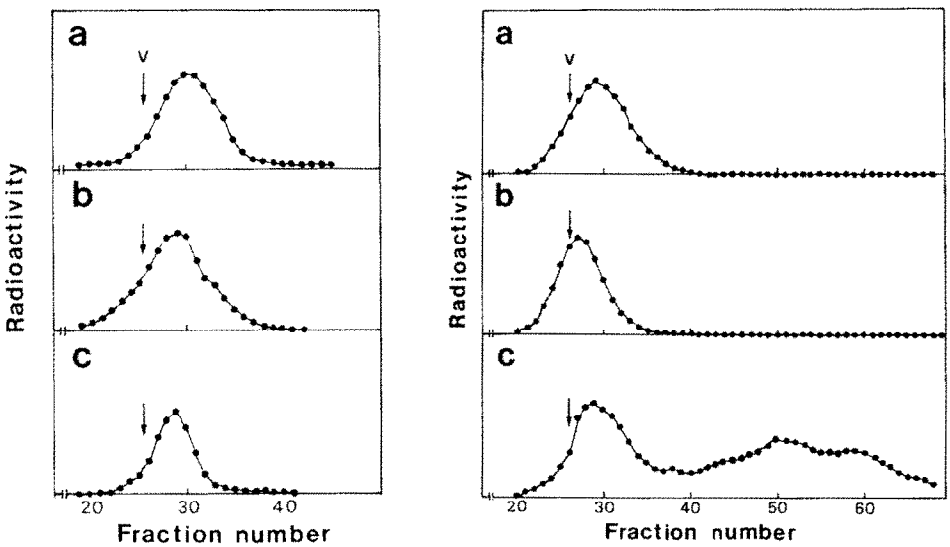
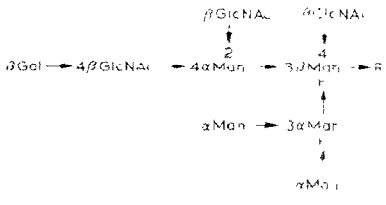


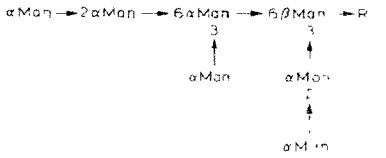

Fig. 1. Affinity chromatography of sodium borotritide-reduced 2-acetamido-2-deoxy-D-glucose oligomers on a Pa-2–Sepharose 4B column. Experimental details are given in the Experimental section. Elution was performed with 10mM sodium phosphate buffer, pH 7.2, containing 0.15M sodium chloride. Arrows (V) indicate the elution volume of Blue dextran. Elution profiles of: (a) β-D-Glc*p*NAc-(1→4)-D-GlcNAcol, (b) β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAcol, and (c) β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAcol.

Fig. 2. Affinity chromatography of asparagine-linked glycopeptides on a Pa-2–Sepharose 4B column. Experimental details and the symbols are the same as in the legend to Fig. 1. Elution profiles of: (a) asialo **2** (asialo PTG UB-II-b); (b) **1** (OA-GP-I); and (c) PTG UA glycopeptides (**3**–**7**).

Glycopeptides obtained from ovalbumin

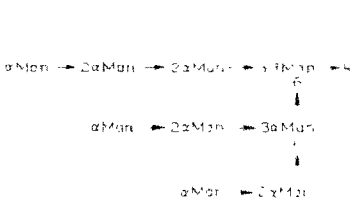


1 (OA-GP-I)

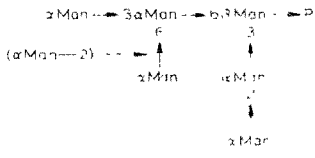


2 (OA-GP-III-B)

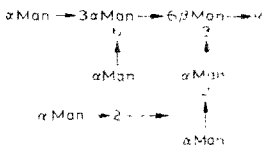
Glycopeptides obtained from porcine thyroglobulin



3 (PTG UA-I)



4 (PTG UA-II)



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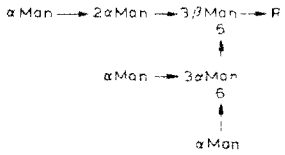
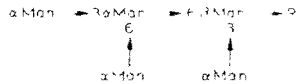
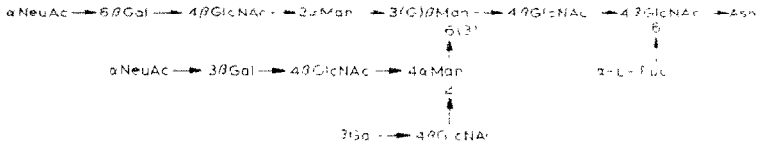


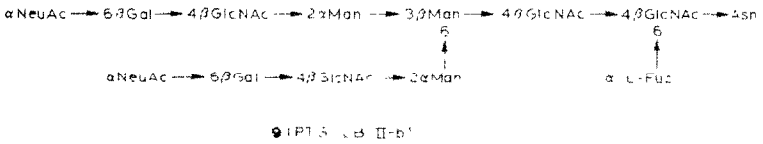
6 (PTG UA-IV)



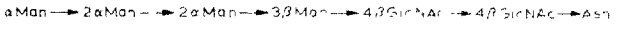
7 (PTG UA-V)



8 (PTG UB II a)



9 (PTG UB II-b)



10

R = 4βGlcNAc→4βGlcNAc→Asn

Scheme 1

Scheme 1). When a complex-type glycopeptide from porcine thyroglobulin (**8** or **9**) was applied to a column of Pa-2–Sepharose, it was recovered with only small retardation (Fig. 2). Similar results were obtained after treatment of this glycopeptide with neuraminidase or a mixture of neuraminidase and α-L-fucosidase (data not shown). Hybrid-type glycopeptides from ovalbumin [OA-GP-I (**1**), OA-GP-II-A, and OA-GP-II-B] gave elution profiles similar to those of complex-type glycopeptides (Fig. 2b; results not shown for OA-GP-II-A and -B). Thus, in these experiments, only very weak interaction was observed between complex- or hybrid-type glycopeptides and Pa-2–Sepharose 4B. In contrast, when a mixture of high-mannose-type glycopeptides [$(Man)_{5-9}(GlcNAc)_2Asn$] obtained from porcine thyroglobulin was applied to a column of Pa-2–Sepharose 4B, a part of the glycopeptides was found to be significantly retarded on the column (Fig. 2c). Thus, some high-mannose-type glycopeptides have higher affinity for the column than the complex- and hybrid-type glycopeptides. We reported previously[18] that the high-mannose-type glycopeptides from porcine thyroglobulin were composed of compounds in which the number of D-mannose units varied from five to nine (**3**–**7**). In order to elucidate which of these glycopeptides had high affinity for the column, **3**–**7** were separately examined on a Pa-2–Sepharose 4B column (Fig. 3). A part of **4** [$Man_8(GlcNAc)_2Asn$] and **5** [$Man_7(GlcNAc)_2Asn$] was found to have much higher affinity (see Fig. 3b and c).

To determine the contribution of sugar residues in the core of high-mannose-type glycopeptides to the interaction with Pa-2–Sepharose 4B, **4** was treated with endo-*N*-acetyl-β-D-glucosaminidase H which is known to cleave the *N*,*N*′-diacetylchitobiosyl residue of glycopeptides. The product, $Man_8GlcNAcol$, was applied to a Pa-2–Sepharose 4B column. As shown in Fig. 4, this oligosaccharide could not interact with the lectin column, thus demonstrating the importance of the core *N*,*N*′-diacetylchitobiosyl residue for interaction. However, when **4** was extensively treated with α-D-mannosidase, the resulting glycopeptide, β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc→Asn, could not bind to the Pa-2 column (Fig. 4c), indicating that peripheral D-mannosyl residues are also important for interaction.

In order to elucidate which glycopeptides of **4** and **5** (each consisting of three structural isomers) had the highest affinity for Pa-2–Sepharose 4B, glycopeptides **4** and **5** were each applied to a Pa-2–Sepharose 4B column and divided into a fraction with stronger and a fraction with weaker affinity for the column. Each fraction was successively digested with endo-*N*-acetyl-β-D-glucosaminidase H, reduced with sodium borotritide to label the terminal 2-acetamido-2-deoxy-D-glucitol residue (**4** giving **11**–**13**; **5** giving **14**–**16**), and then acetolyzed, which preferentially cleaves the α-D-Man*p*-(1→6)-D-Man linkage. Scheme 2 shows the theoretical fragmentation by acetolysis of modified **4** and **5** (**11**–**13** and **14**–**16**, respectively). The products obtained were analyzed by gel-permeation chromatography on a Bio-Gel P-4 column as described previously[18] (Fig. 5). In the case of **5**, $Man_4GlcNAcol$ was obtained from the glycopeptides with stronger affinity as the only radiolabeled, fragment containing 2-acetamido-2-deoxy-D-glucose, but radioactive $Man_3GlcNAcol$ was

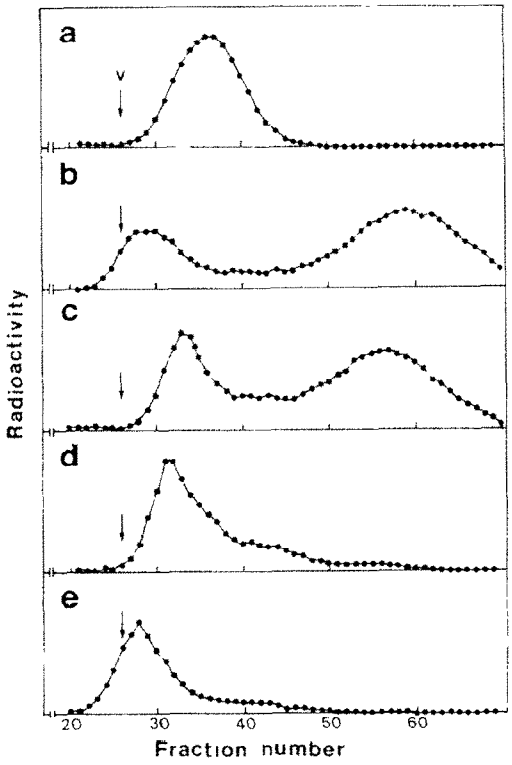



Fig. 3. Affinity chromatography of porcine thyroglobulin unit A glycopeptides on a Pa-2–Sepharose 4B column. Experimental details and the symbol are the same as in the legend to Fig 1 Elution profiles of: (a) **3** $Man_9(GlcNAc)_2Asn$; (b) **4** $Man_8(GlcNAc)_2Asn$; (c) **5**, $Man_7(GlcNAc)_2Asn$, (d) **6**, $Man_6(GlcNAc)_2Asn$, and (e) **7**, $Man_5(GlcNAc)_2Asn$.

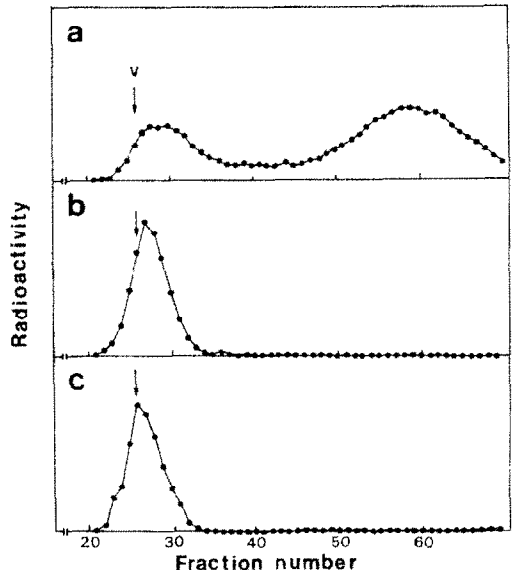



Fig 4. Affinity chromatography of glycopeptides **4** and their glycosidase digests on a Pa-2–Sepharose 4B column Experimental details and the symbol are the same as in the legend to Fig. 1. Elution profiles of: (a) **4**, $Man_8(GlcNAc)_2Asn$, (b) **4** after endo-*N*-acetyl-β-D-glucosaminidase H treatment, $Man_8GlcNAcol$; and (c) **4** after α-D-mannosidase treatment, β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-

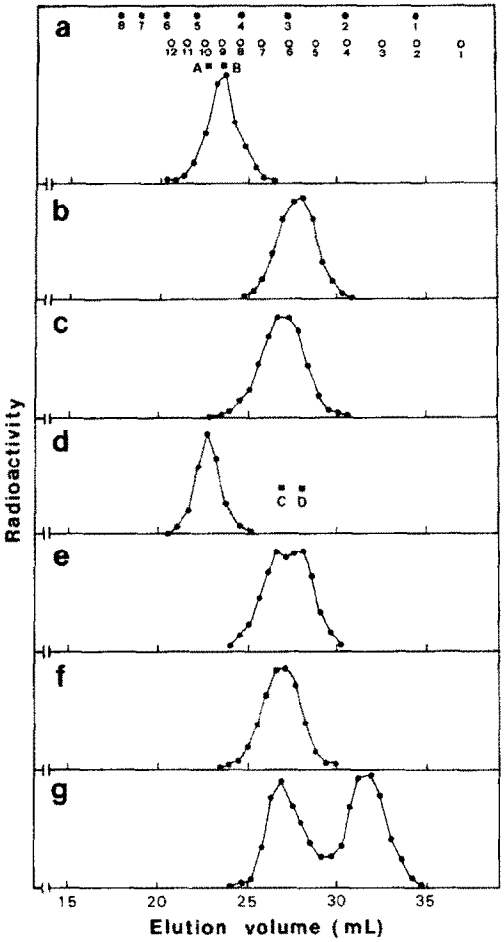



Fig. 5. Gel-permeation chromatography of endo-*N*-acetyl-β-D-glucosaminidase digests and their acetolysis products on a Bio-Gel P-4 column. Experimental details are given in the text: (a) radioactive oligosaccharide alditol, $Man_7GlcNAcol$, released by endo-*N*-acetyl-β-D-glucosaminidase H from **5**; (b) acetolyzate of oligosaccharide alditols derived from glycopeptides **5** with lower affinity; (c) acetolyzate of oligosaccharide alditols derived from glycopeptides **5** with higher affinity; (d) radioactive oligosaccharide alditols ($Man_8GlcNAcol$) released by endo-*N*-acetyl-β-D-glucosaminidase H from **4**; (e) acetolyzate of oligosaccharide alditols from "lower-affinity glycopeptides" in **4**; (f) acetolyzate of oligosaccharide alditols from "higher-affinity glycopeptides" in **4**; (g) acetolyzate of oligosaccharide alditols from "higher-affinity glycopeptides" in **4**, which were reduced with sodium borotritide and isolated after binding to a concanavalin A–Sepharose column. Open circles (1–12) and closed circles (1–8) indicate the elution positions of standard oligomers of D-glucose and 2-acetamido-2-deoxy-D-glucose, respectively. A–D (closed squares) indicate the elution positions of authentic standard oligosaccharides prepared from porcine thyroglobulin unit A glycopeptides[18]: (A) $Man_8GlcNAcol$; (B) $Man_7GlcNAcol$; (C) α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAcol; and (D) α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAcol.

Scheme 2 Sequential degradation by α-Mannosidase followed by reduction of oligosaccharide moieties obtained from 4 and 5

not detected in the acetolyzate (Fig. 5). Thus, the glycopeptide that gave **15** and which contains structure **10** could be easily recognized by Pa-2–Sepharose. In the case of **4**, the presence of radioactive $Man_4GlcNAcol$ in the acetolyzates of the oligosaccharide alditols from both the high- and low-affinity-bound glycopeptides was observed (Fig. 5e and f). Since radioactive $Man_3GlcNAcol$ was observed in the acetolyzate of the oligosaccharide alditol from the low-affinity-bound glycopeptide, it may be assumed that **11** was derived from a low-affinity-bound glycopeptide, but it is uncertain which glycopeptide (that gives **12** or **13**) is the high-affinity-bound one. Therefore, the acetolysis products derived from the high-affinity-bound glycopeptides obtained from **4** were radiolabeled by reduction with sodium borotritide, and the labeled oligosaccharide mixture was subjected to affinity chromatography on a concanavalin A–Sepharose 4B column[13], which binds $Man_2Manol$ but does not bind $Man_1Manol$. The fraction bound to the concanavalin A column was eluted with methyl α-D-mannopyranoside and analyzed by gel-permeation chromatography. As shown in Fig. 5g, this fraction was found to contain $Man_4GlcNAcol$ and $Man_2Manol$. This indicates that the glycopeptide giving **13** has the strongest affinity to Pa-2–Sepharose 4B among **4**. The glycopeptides giving **13** and **16** have in common structure **10**, which is so important for Pa-2–Sepharose 4B recognition by high-mannose-type glycopeptides. This assumption was also supported by the observation that **6** and **7**, which do not contain a common structure, showed much weaker interaction with the Pa-2 column than **4** and **5** (Fig. 3,d,e). Furthermore, another high-mannose-type glycopeptide (**2**), having a carbohydrate composition identical with that of **5** but a structure different from that of the glycopeptide giving **15**, was purified from ovalbumin by Tai *et al.*[16], and applied to the Pa-2 column. It was recovered without retardation, possibly because this glycopeptide does not contain the structure that the glycopeptides giving **13** and **16** share.

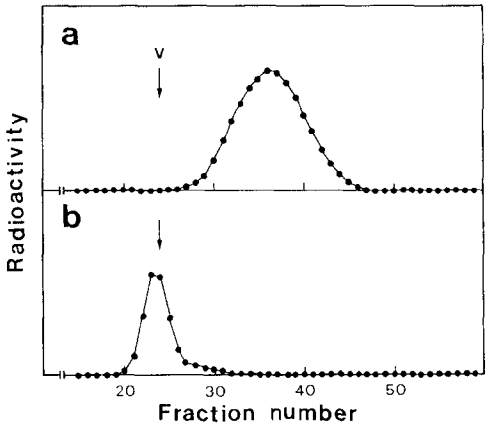



Fig. 6. Affinity chromatography of glycopeptide **3** and its acetolyzate on a Pa-2–Sepharose 4B column. Experimental details and symbols are the same as in the legend to Fig. 1. Elution profile of: (a) **3**, and (b) acetolyzate of **3**.

The reason for **3** and the glycopeptide giving **11**, both of which contain structure **10**, cannot interact with the Pa-2–Sepharose 4B column as strongly as the glycopeptides giving **13** or **16** has not yet been elucidated. A possible explanation is that a certain steric hindrance prevents their interaction with the column.

Finally, in order to test whether **10** itself could bind to Pa-2–Sepharose 4B or not, **3**, radiolabeled at the asparagine residue by acetylation with [$^{14}$C]acetic anhydride, was subjected to acetolysis and the resulting **10** applied to a column of Pa-2–Sepharose 4B. As shown in Fig. 6, no retardation of the radioactive product on the column was observed, indicating that **10** alone cannot bind to the column.

Recently, many studies have suggested that cell-membrane glycoproteins play an important role in many biological phenomena. In most cases, however, it is difficult to investigate the structure of the glycoproteins because only limited amounts of material are available. Various combinations of lectin-affinity chromatography will be useful for the purification and for structural determination of these glycoproteins.

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