A β-MANNOSIDIC LINKAGE IN THE CARBOHYDRATE MOIETY OF OVALBUMIN*

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1. Introduction

The sequence of sugar residues in the oligosaccharide chain of ovalbumin has been determined from chemical and enzymatic studies [1–3]. The anomeric configuration of the mannose residues has also been studied using α-mannosidases from various sources, and it has been claimed that all mannosidic linkages are of the α-type. Of these linkages, however, one of the innermost appears to be unique in that it is resistant to α-mannosidases from jack bean meal [3] and hog kidney [4], while other linkages are cleaved by these well characterized enzymes. Assignment of this innermost mannosidic linkage to the α-type is based on Lee's report [5] that this linkage was cleaved by a newly isolated plant α-mannosidase. However, the characteristics of this α-mannosidase have not been reported.

Using a β-mannosidase preparation from a snail, *Achatina fulica*, we found that the innermost mannosidic linkage is of the β-type. Procedures used in reaching this conclusion are reported in this paper.

2. Materials and methods

Ovalbumin glycopeptide (asparaginyl–oligosaccharide) was prepared according to Yamashina and Makino [6]. The glycopeptide had the following average composition: asparagine, 1; *N*-acetylglucosamine, 3; mannose, 5. However, this glycopeptide is heterogeneous as regards the structure of the carbohydrate moiety as has been shown by Huang et al. [3].

α-Mannosidase having 12 units/mg protein was prepared from hog kidney by the method of Okumura and Yamashina [4]. The specificity of this α-mannosidase seems to be broad as regards the aglycone since it is capable of hydrolyzing α-mannosylmannoses having $1 \rightarrow 2$, $1 \rightarrow 3$, $1 \rightarrow 4$ and $1 \rightarrow 6$ linkages (unpublished results).

β-Mannosidase was prepared from the acetone powder of the viscera of snails (*A. fulica*). Purification procedures consisted of ammonium sulphate fractionation, acetone fractionation, hydroxylapatite chromatography and DEAE-cellulose chromatography (details will be published elsewhere). During preparation, β-mannosidase was assayed in a buffer, pH 4.5, prepared from 0.05 M citric acid and 0.025 M K_2HPO_4, containing 5 mM phenyl β-*D*-mannoside synthesized according to Helferich and Winkler [7]. Liberated phenol was determined according to Kerr et al. [8].

The phenyl β-mannoside used was of high degree of purity, showing a single peak on gas-liquid chromatography (GLC) with a retention time of 24.16 min. For the chromatography, a column of Chromosorb W coated with 1.5% silicone gum, SE 30, was used, and chromatography was carried out at 160° for 2 min and then the temperature was raised to 230° at the rate of 2°/min. The flow rate of N_2 gas as carrier and that of H_2 gas were 20 ml/min, respectively. The sample was trimethylsilated according to Sweeley et al [12]. Under these conditions, phenyl α-mannoside, synthesized also according to Helferich and Winkler [7], had a retention time of 22.12 min.

The purity of the phenyl β-mannoside was further investigated enzymatically. α-Mannosidase from hog kidney did not act on this mannoside whilst β-mannosidase used in the present study cleaved it quantitatively, producing equimolar amounts of mannose and phenol.

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The purified β-mannosidase preparation had 2.74 units/mg protein, and the activities of contaminating α-mannosidase and β-*N*-acetylglucosaminidase were only 0.0067 and 0.016 units/mg protein, respectively, when assayed using *p*-nitrophenyl glycosides under optimum conditions [4, 9]. The preparation showed no enzymatic activity towards the intact ovalbumin glycopeptide. One unit of glycosidase is the amount of enzyme which will liberate one μmole of phenol or *p*-nitrophenol per min.

The amino acid and glucosamine contents of glycopeptides were determined in a Hitachi amino acid analyzer after hydrolysis in 6 N HCl at 100° for 16 hr. Mannose was determined by the orcinol-H_2SO_4 method [10]. Hydrolyses of glycopeptides by α- or β-mannosidases were followed by determining the increase in reducing power using the method of Park and Johnson [11]. The amounts of released mannose were also determined by GLC after conversion into mannitol essentially as by Sweeley et al. [12]. The ninhydrin reaction according to Yemm and Cocking [13] was used to monitor the chromatographic fractions.

3. Results

Ovalbumin glycopeptide (29 mg) was incubated with α-mannosidase (7 units) in 5 ml of a buffer, pH 4.6, prepared from 0.05 M citric acid and 0.1 M Na_2HPO_4, containing 0.5 mM zinc acetate, at 37° for 30 hr, when the increase in reducing power had reached a plateau. The digest was applied to a column of Bio-Gel P-4 (0.9 × 130 cm) and eluted with water. The elution pattern was the same as that previously reported [4]. Of the 2 glycopeptide peaks, Peak II gave on analysis mannose, glucosamine and aspartic acid in a molar ratio of 1.16:1.86:1.00, and was considered to have the following composition: $Man_1(GlcNAc)_2Asn$. In peak I the ratio was approximately 5:4:1. Based on the amounts of aspartic acid contained in these 2 peaks, Peaks I and II were estimated to be nearly equimolar.

The fractions in Peak II were combined and evaporated to dryness. The residue was resubmitted to α-mannosidase digestion under conditions similar to those used in the initial digestion for 60 hr at 37°. No increase in reducing power was observed. The glycopeptide was recovered by gel filtration on a Bio-Gel column.

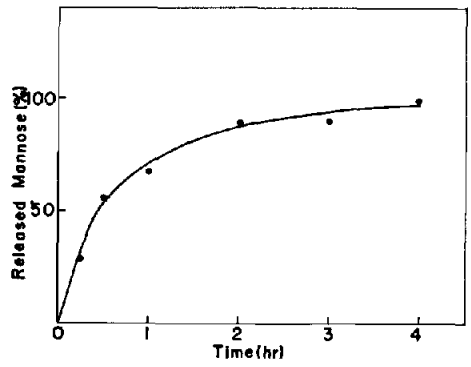

Fig. 1. Release of mannose by the action of β-mannosidase on a glycopeptide isolated from the α-mannosidase digest of ovalbumin glycopeptide. The glycopeptide, $Man_1(GlcNAc)_2$-Asn, corresponding to 345 μg mannose, was incubated in 3 ml of the buffer, pH 4.5, containing 0.12 unit of the enzyme. The reaction was followed by determining the reducing power.

The glycopeptide was then incubated with the β-mannosidase preparation corresponding to 0.12 unit in 3 ml of the pH 4.5 buffer used for enzyme assay. As shown in fig. 1, the increase in reducing power reached a value corresponding to 100% release of mannose after 4 hr of incubation. GLC analysis of

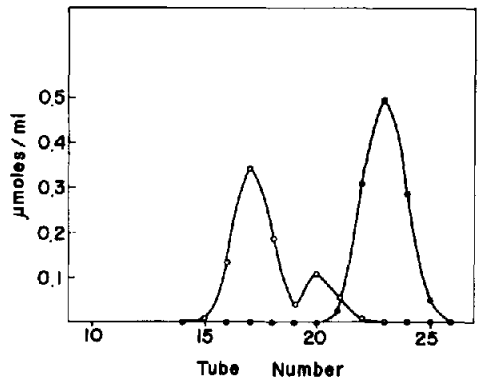

Fig. 2. Gel filtration of the β-mannosidase digest (as shown in fig. 1) on Bio-Gel P-4. The digest was applied to a column (0.9 × 90 cm) and eluted with water at a flow rate of 15 ml/hr. Fractions of 2.2 ml were collected, then aliquots were used for the ninhydrin reaction and for the orcinol-H_2SO_4 reaction. o——o, Aspartic acid equivalents (μmoles/ml); •——•, mannose equivalents (μmoles/ml).

the digest showed a release of mannose in the amount expected from the increase in reducing power. No release of *N*-acetylglucosamine was seen in the chromagram.

The β-mannosidase digest was applied to a column of Bio-Gel P-4 (0.9 × 90 cm) and eluted with water with the results shown in fig. 2. The first peak was positive only in the ninhydrin reaction and on hydrolysis gave aspartic acid and glucosamine in a molar ratio of 1.00:1.97. This indicated that β-mannosidase had split off all the mannose residues from the Peak II glycopeptide, producing a glycopeptide, $(GlcNAc)_2$-Asn. The second peak, which was also positive only in the ninhydrin reaction, gave neither aspartic acid nor glucosamine on hydrolysis, but several other amino acids. This peak must have originated from the enzyme preparation. The third peak , which was positive only in the orcinol-H_2SO_4 reaction, was identified as mannose on GLC.

Peak I glycopeptide was no longer susceptible to α-mannosidase nor to β-mannosidase, suggesting that the non-reducing termini are occupied by *N*-acetylglucosamine residues.

4. Discussion

The sequence of sugar residues in the carbohydrate moiety of ovalbumin presented by Huang et al. [3] , and shown below, seems compatible with many observations from chemical and enzymatic studies on glycopeptides. This sequence

$$\begin{array}{l} (GlcNAc)_{0\ or\ 1\ or\ 2}-Man-GlcNAc-GlcNAc-Asn \\ \qquad\qquad\qquad\qquad\quad |\leftarrow \\ \qquad\qquad\quad Man_{0\ or\ 1}-(Man)_3 \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\quad Man(GlcNAc)_{0\ or\ 1} \end{array}$$

represents the structures of 5 different glycopeptides, A–E.

According to this sequence, it is likely that the Peak II glycopeptide, $Man_1(GlcNAc)_2Asn$, from the α-mannosidase digest was the product of 2 of the 5 glycopeptides, D and E, since these 2 forms have no *N*-acetylglucosamine residues at the non-reducing termini. Mannosidic linkages at the non-reducing termini of these 2 forms could be assigned to the α-type since no release of mannose was observed on incubation of the intact ovalbumin glycopeptide with β-mannosidase. Thus, the mannosidic linkage, shown to be β-type in the present study, would be that indicated by an arrow in the above sequence.

The Peak I glycopeptide would be a mixture of glycopeptides produced from the other 3 glycopeptides, A, B and C, all of which have *N*-acetylglucosamine residues at the non-reducing termini.

It should be noted that the sum of D and E is nearly equimolar to that of A, B and C, according to Huang et al. [3] . This is consistent with the observation that α-mannosidase produced Peaks I and II in nearly equimolar amounts.

The trisaccharide–asparagine structure with a composition of $Man_1(GlcNAc)_2Asn$, which may or may not be identical with the structure of the Peak II glycopeptide from ovalbumin, has been shown to occur also in ribonuclease B [14] , Taka-amylase [4] and stem-bromelain [4] . Since the mannosidic linkage of this structure is resistant to well characterized α-mannosidases, i.e. those from jack bean meal or hog kidney, it seems possible that the linkage would be of the β-type. A study along this line is currently in progress.

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