Jack bean α-mannosidase digestion profile of hybrid-type N-glycans: Effect of reaction pH on substrate preference

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ABSTRACT

Jack bean α-mannosidase (JBM) is a well-studied plant vacuolar α-mannosidase, and is widely used as a tool for the enzymatic analysis of sugar chains of glycoproteins. In this study, the JBM digestion profile of hybrid-type N-glycans was examined using pyridylamino (PA-) sugar chains. The digestion efficiencies of the PA-labeled hybrid-type N-glycans were examined using pyridylamino (PA-) sugar chains, GNM5-PA and GalGNM5-PA were significantly lower than that of the oligomannose-type N-glycan trimming probably because of the steric hindrance to the JBM activity caused by GlcNAc1-2Man(α) residues of the hybrid-type N-glycans. We also found that the substrate preference of JBM for the terminal Man1-6Man(α) and Man1-3Man(α) linkages in the hybrid-type N-glycans was altered by the change in reaction pH, suggesting a pH-dependent change in the enzyme–substrate interaction.

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1. Introduction

Jack bean α-mannosidase (JBM) is an exoglycosidase that hydrolyzes α1-2, α1-3-, and α1-6-linked mannose residues. JBM localizes in the vacuole and is abundant in the cotyledon of germinating jack beans, and the purified enzyme has been widely utilized for determining the mannose linkage in glycoproteins. The biochemical properties of JBM were investigated previously [1,2]. JBM is synthesized as a precursor of 110 kDa size and then processed posttranslationally [3]. The mature enzyme consists of four subunits, two each of molecular weight 66 and 44 kDa [4]. The larger subunit is modiﬁed with glucose-containing high-mannose-type N-glycans and small xylose- and fucose-containing complex-type N-glycans [5,6].

The pathway of N-glycan trimming by JBM has been investigated extensively using high-mannose-type N-glycans. In the digestion of M9 (Fig. 1A) by JBM, Man1–2Man(α) linkages are hydrolyzed firstly to produce M5 (Fig. 1A). Next, the Man1–3Man(β) linkage is hydrolyzed. Subsequently, the Man1–6Man(α) linkage is hydrolyzed before the digestion of the Man1–3Man(α) linkage. Finally, the Man1–6Man(β) linkage is hydrolyzed to produce the final product M1 (Fig. 1A and B) [7–9]. However, the pathway of hybrid-type N-glycan processing by JBM remains unclear.

In this study, the digestion profile of hybrid-type N-glycans was examined using pyridylamino (PA-) sugar chains, GNM5-PA and GalGNM5-PA were different from that of M4-PA, suggesting a steric hindrance caused by GlcNAc1-2Man(α) residues. We also found that the substrate preference of JBM for the terminal Man1–6Man(α) and Man1–3Man(α) linkages in the hybrid-type N-glycans was altered by the change in reaction pH, suggesting a pH-dependent change in the enzyme–substrate interaction.

2. Materials and methods

2.1. PA-sugar chains

PA-sugar chains, M5-PA and M4-PA (Fig. 1A), were purchased from Takara Bio Inc. (Ohtsu, Japan). GalGNM5-PA (Fig. 1A) was obtained from Masuda Chemical Industries (Takamatsu, Japan). GNM5-PA (Fig. 1A) was enzymatically prepared from M5-PA using a recombinant tobacco N-acetylglucosaminyltransferase expressed in Escherichia coli as previously described [10].
2.2. JBM reaction

JBM was purchased from Sigma–Aldrich (MO, USA). Unless otherwise stated, PA-sugar chains (15 pmole) were incubated in 20 μl of reaction mixture containing 0.1 M sodium acetate, pH 5.0, 10 mM ZnCl₂, and 0.27 U of JBM at 37 °C (the unit is defined as follows: One unit hydrolyzes 1.0 μmole of p-nitrophenyl α-D-mannoside per min at pH 4.5 at 25 °C). After incubation, the enzyme was inactivated by treatment at 95 °C for 5 min.

2.3. Analysis of digestion products

Reversed-phase (RP) and size-fractionation (SF) high performance liquid chromatography (HPLC) of PA-sugar chains were carried out as previously described [11]. The mass-to-charge ratio (m/z) of PA-sugar chains was determined by liquid chromatography tandem mass spectrometry (LC–MS) using Agilent Technologies 1200 series (Agilent Technologies, DE, USA) equipped with HCT plus (Bruker Daltonics, Bremen, Germany). For the digestion of unbranched α1–6-mannose residues, 2 pmol of purified PA-sugar chains was incubated in 10 μl of reaction mixture containing 50 mM sodium citrate, pH 4.5, 0.1% (w/v) bovine serum albumin, and 2 U of recombinant α1–6-mannosidase from Xanthomonas manihotis (New England Biolabs, MA, USA) at 37 °C for 1–6 h. The digestion of the terminal GlcNAc residue of the purified PA-sugar chain was carried out using β-N-acetylgalactosaminidase from Streptococcus pneumoniae (Sigma–Aldrich) at 37 °C for 2 h according to the manufacturer’s instruction.

3. Results and discussion

3.1. JBM digestion profile of hybrid-type N-glycans

GNM5 is the simplest hybrid-type N-glycan, and the first N-glycan in the process of complex-type N-glycan biosynthesis from M5, a high-mannose-type N-glycan. In this study, we investigated the JBM digestion profile of hybrid-type N-glycans, and compared it with that of M4-PA. Isomeric structures of core α1–6-mannose arm for GNMS-PA and M4-PA are identical.

GNM5-PA and M4-PA were incubated with JBM at 37 °C for 1–8 h and then analyzed by SF-HPLC. The reaction product of GNM5-PA gave four peaks, A1, A2, A3, and A4 (Fig. 2A). Peak A4 corresponded to the initial substrate GNM5-PA. Peak A1 was fractionated to a single peak by RP-HPLC, and shifted to the position corresponding to M2b-PA (Fig. 1A) after β-N-acetylgalactosaminidase treatment. In addition, peak A1 gave a signal at m/z 1030.4, which corresponded to HexHexNAc3-PA, in the LC–MS analysis. Therefore, peak A1 was deduced as GNM2-PA (Fig. 1A). Peak A2 was...
GalGNM5-PA. PA-sugar chain substrates (10 pmole) were digested with JBM for 1 h at the initial substrates. The deduced structure of Fig. 2.

(A) Typical SF-HPLC profiles of JBM digestion products of GNM5-PA, M4-PA, and GalGNM5-PA. PA-sugar chain substrates (10 pmole) were digested with JBM for 1 h at 37°C before the HPLC analysis. Arrow indicates the elution position corresponding to the initial substrates. The deduced structure of N-glycan in each peak was shown in the (Fig. 2A). Peak A3 was fractionated to two peaks, A3a and A3b, in the RP-HPLC analysis (Fig. 2B). Both A3a and A3b gave a signal at m/z 1354.5, which corresponded to HexHexNAc2-PA (Fig. 2C). This indicates that A3a and A3b are isomers. Peak A3a but not A3b shifted after the treatment with recombinant α-1–6 mannosidase derived from Xanthomonas manihotis, which specifically removes unbranched α-1–6-linked mannose residues from oligosaccharides [12]. Therefore, A3a and A3b were deduced as GNM4a-PA and GNM4b-PA (Fig. 1A), respectively. The trimming pathway of GNM5 is summarized in Fig. 3A.

On the other hand, the digestion product of M4 was fractionated by SF-HPLC into four peaks, B1, B2, B3, and B4 (Fig. 2A). Peak B4 corresponded to the initial substrate M4-PA. In the LC–MS analysis, B1 and B2 gave signals at m/z 664.8 and 827.3, which corresponded to HexHexNAc2-PA and Hex3HexNAc2-PA, respectively. Thus, B1 and B2 were deduced as M1-PA and M2a-PA, respectively. Peak B3 gave a signal at m/z 989.3, which corresponded to HexHexNAc2-PA in the LC–MS analysis. In RP-HPLC analysis, we found that peak B3 was fractionated to a minor peak B3a and a major peak B3b. Peak B3a but not B3b was digested by α-1–6 mannosidase. And the retention times of peak B3a and B3b in RP-HPLC analyses corresponded to those of M3a-PA and M3b-PA (Fig. 1A), which were prepared from GNM4a-PA (A3a) and GNM4b-PA (A3b) by enzymatic digestion, respectively. Therefore, B3a and B3b were deduced as M3a-PA and M3b-PA, respectively. The trimming pathway of M4-PA is summarized in Fig. 3B. No M3a-PA (Fig. 1A) was detected in the digestion product of M4. This agrees with the previous reports showing the digestion pathways of high-mannose-type N-glycans [7–9].

The time course of the JBM digestion of GNM5-PA and M4 was investigated for 128 h (Fig. 4). The percentage of the sugar chains at each time point was calculated from the peak area of the JBM digestion products of GNM5-PA and M4-PA in the SF-HPLC analysis. The digestion profiles showed that the hydrolysis of GNM5-PA by JBM was significantly slower than that of M4-PA: After 1 h of digestion of M4-PA, M3-PA was approximately 50% of the total sugar chains. On the other hand, it took about 4 h before GNM5 was reduced to approximately 50% of the total sugar chains. After 64 h of incubation, the digestion products of M4-PA were mostly converted to the final product, M1-PA, whereas the intermediate product GNM3-PA comprised more than half of the products of the GNM5-PA digestion.

The above results indicate that the GlcNAcβ1–2Man(α) residue at the nonreducing end of GNM5-PA reduced the efficiency of GNM5-PA digestion by JBM, and suggest that a steric hindrance due to the GlcNAcβ1–2Man(α) residue more strongly inhibits the hydrolysis of the Manα1–6Man(α) linkage than that of the Manα1–3Man(α) linkage.

To investigate the effect of residues bulkier than GlcNAcβ1–2Man(α) on steric hindrance, GalGNM5-PA was used as the substrate for JBM. The digestion products of GalGNM5-PA were fractionated by SF-HPLC into four peaks, C1, C2, C3, and C4 (Fig. 2A). Peak C4 corresponded to the initial substrate GalGNM5-PA. C1 and C2 gave signals at m/z 1192.4 and 1354.5, which corresponded to HexHexNAc2-PA and Hex3HexNAc2-PA in the LC–MS analysis, respectively, and were deduced as GalGNM2-PA and GalGNM3-PA (Fig. 1A). Peak C3 contained two isomers fractionated by RP-HPLC (Fig. 2B). Peaks C3a and C3b were deduced as GalGNM4a-PA and parenthesis (see Fig. 1A for notation of N-glycans). (B) RP-HPLC profiles of the SF-HPLC fractions A2 and C2. (C) LC–MS spectra of the RP-HPLC fractions A2a (upper panel) and A2b (lower panel). Diamonds indicate the peaks corresponding to HexHexNAc2-PA.
GalGNM4b-PA (Fig. 1A), respectively, on the basis of results of the LC–MS analysis (m/z 1516.4) and the digestion experiments with α1–6 mannosidase. The trimming pathway of GalGNM5 is summarized in Fig. 3C.

The digestion profile of GalGNM5-PA showed that its trimming is significantly slower than that of M4-PA, as observed in GNM5-PA digestion (Fig. 4). These results indicate that the Galβ1–4GlcNAcβ1–2Man(α) residue of GalGNM5-PA also caused the steric hindrance preventing the hydrolysis of the terminal α-linked mannose residue of the GalGNM5-PA by JBM, and differently affected the efficiency of the digestion of the Manz1–3Man(α) and the Manz1–6Man(α) linkages. The RP-HPLC analysis revealed that the molar ratios of GalGNM4a-PA and GalGNM4b-PA were 36% and 64%, respectively (Fig. 2B). These were approximately equal to the molar ratios of GNM4a-PA (35%) and GNM4b-PA (65%) in the GNM5-PA digestion experiment (Fig. 2B). This suggests that the terminal galactose of GalGNM5-PA has no additional effect on the steric hindrance observed here.

3.2. Effect of reaction pH on the substrate preference of JBM

The digestion profile of GalGNM5-PA showed that its trimming is significantly slower than that of M4-PA, as observed in GNM5-PA (Fig. 4). These results indicate that the Galβ1–4GlcNAcβ1–2Man(α) residue of GalGNM5-PA also caused the steric hindrance preventing the hydrolysis of the terminal α-linked mannose residue of the GalGNM5-PA by JBM, and differently affected the efficiency of the digestion of the Manz1–3Man(α) and the Manz1–6Man(α) linkages. The RP-HPLC analysis revealed that the molar ratios of GalGNM4a-PA and GalGNM4b-PA were 36% and 64%, respectively (Fig. 2B). These were approximately equal to the molar ratios of GNM4a-PA (35%) and GNM4b-PA (65%) in the GNM5-PA digestion experiment (Fig. 2B). This suggests that the terminal galactose of GalGNM5-PA has no additional effect on the steric hindrance observed here.

3.2. Effect of reaction pH on the substrate preference of JBM

The effects of the reaction conditions during GNM5-PA digestion by JBM on the ratio of GNM4a-PA and GNM4b-PA products were investigated. GNM5-PA was incubated with JBM at various temperatures, and the resulting products were examined by SF- and
RP-HPLC analyses. The amounts of digestion products were highest at 47, 52, and 57 °C, but decreased to more than half at lower (27 °C) and higher (>62 °C) temperatures (Fig. 5A). The ratio of GNM4a-PA to total GNM4-PA was approximately constant from 27 to 67 °C (Fig. 5B). This indicates that reaction temperature affected the enzyme activity but not the substrate preference between the Man\(x_1–3\)Man(\(\alpha\)) and Man\(x_1–6\)Man(\(\alpha\)) linkages in GNM5-PA. Next, we investigated the ratio of M3a-PA and M3b-PA in digestion products of M4-PA. The relative ratio of digestion products of M4-PA at various temperatures had a similar pattern to that of GNM5-PA (Fig. 5A). On the other hand, in the digests of M4-PA, detailed RP-HPLC analysis revealed that the ratio of M3b-PA was approximately 90% of the M3-PA isomers in digestion products of M4-PA, and was approximately constant from 27 to 67 °C as observed in GNM4-PA (Fig. 5B). This indicates that reaction temperature did not significantly affect the substrate preference between the Man\(x_1–3\)Man(\(\alpha\)) and Man\(x_1–6\)Man(\(\alpha\)) linkages in M4-PA. Subsequently, GNM5-PA was digested with JBM under various pH conditions. The amount of the digestion products from GNM5-PA was highest at pHs 4.5–5.5, but decreased at lower (pH 4) and higher (pH 6.5 or above) pHs (Fig. 6A). On the other hand, the ratio of GNM4a-PA to total GNM4-PA linearly increased with increasing pH (Fig. 6B). The ratios of GNM4a-PA were 21% and 48% of the total GNM4-PA at pHs 4.0 and 6.5, respectively. Similar results were obtained from the digestion of GalGNM5-PM (data not shown). These results indicate that JBM prefers the Man\(x_1–3\)Man(\(\alpha\)) linkage to the Man\(x_1–6\)Man(\(\alpha\)) linkage as the substrate under high pH conditions. Note that the pH-dependent change in the GNM4a-PA ratio did not correlate with the efficiency of GNM5-PA digestion (Fig. 6), indicating that the ratio of GNM4a-PA products and the efficiency of GNM5-PA digestion are independently affected by reaction pH. On the other hand, in the digests of M4-PA, the ratio of M3b-PA was only slightly decreased with increase of reaction pH (Fig. 6B). This shows that reaction pH does not significantly affect the trimming pathway of M4-PA, implying that reaction pH mainly affects the extent of the steric hindrance caused by the GlcNAc\(\beta_1–2\)Man(\(\alpha\)) residues of the complex-type N-glycans, thereby altering the substrate preference. The trimming pathway of high-mannose-type N-glycans was also investigated in other plant \(\alpha\)-mannosidases derived from amond [13] and Ginkgo [14]. However, no pH-dependent change in the substrate preference as observed here in the case of JBM has been reported yet.

The above results suggest that reaction pH affects the interaction between JBM and complex-type N-glycan substrates. Reaction pH may affect the conformation of PA-labeled complex-type N-glycans used as substrates. Alternatively, reaction pH may alter the tertiary structure of JBM, which affects the substrate preference for the terminal Man\(x_1–6\)Man(\(\alpha\)) and Man\(x_1–3\)Man(\(\alpha\)) linkages in complex-type N-glycans. JBM is unstable at lower (less than pH 5.0) pHs without \(\text{Zn}^{2+}\) supplementation [2], implying the pH-dependent conformational change of JBM. The primary structure of the JBM subunits has not been reported yet. Information on the primary, secondary, and tertiary structure of JBM should be required for understanding the mechanism of the pH-dependent change in the substrate preference and substrate recognition of JBM.

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