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Purification and Properties of Aryl-α-mannosidase from Microsomal Fraction of Developing *Ricinus communis* Endosperms

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An α-mannosidase, which would be involved in *N*-linked glycoprotein metabolism, was purified and characterized from microsomal fraction of developing *Ricinus communis* endosperms. The purified enzyme with 43 kDa on SDS-PAGE showed maximal activity at pH 5.0 and 50°C, when p-nitrophenyl-α-mannopyranoside was used as a substrate. α-Mannosidase activity was inhibited by EDTA and the reduced activity was rescued by addition of Zn^{2+} or Ca^{2+}, suggesting this α-mannosidase should be a metal enzyme. *Ricinus* aryl-α-mannosidase was able to convert the Man6GlcNAc2-PA and Man5GlcNAc2-PA to Man4GlcNAc2-PA but was completely inactive toward Man4GlcNAc2-PA, Man4Xyl1GlcNAc2-PA and GlcNAc1Man5GlcNAc2-PA.

Key words : plant α-mannosidase, plant glycoprotein, *N*-glycan metabolism, *Ricinus communis*

Introduction

During the course of purification of the glycoprotein processing α-1,2-mannosidase from developing *Ricinus communis* endosperms,[1] we detected two peaks of arylmannosidase activity on DEAE-cellulose column that could react with the *p*-nitrophenyl-α-D-mannospyranoside substrate. In order to elucidate the relationship of these activities to the processing α-1,2-mannosidase and the *N*-glycan processing in general, we started to purify the aryl-α-mannosidase from developing *Ricinus* endosperms to analyze the substrate specificity in detail.

In plant cells, two different classes of α-mannosidase that differ in their pH optima and in their substrate specificities occur.[2,3] One class is the processing α-mannosidase (mannosidase I and II) involved in trimming mannosyl residues of Man9GlcNAc2-Asn structure. These enzymes have pH optima in the near neutral region (between 6 to 7) and occur in the ER and/or Golgi apparatus.[1,3,4] By the cooperative action of these processing α-mannosidases, Man9GlcNAc2-protein is converted to GlcNAc1Man3GlcNAc2-protein via Man5GlcNAc2-protein.[5] The second class of mannosidases comprises the aryl-α-mannosidases that have pH optima in acidic

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Abbreviations
SF-HPLC, size-fractionation HPLC; PA, pyridylamino; PNP-α-Man, p-nitrophenyl-α-mannopyranoside; MES, 2-morpholinoethanesulfonic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acd; OTG, *n*-octyl-β-D-thioglucoside; M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; M4X, Manα1-3Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M4C, Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M5A, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; GNM5, Manα1-6(Manα1-3)Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M6B, Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA.

region (from 4 to 5) showing a broad *in vitro* substrate specificity. The acidic α-mannosidase accumulates in vacuoles or in the protein body and is probably involved in the catabolism and turnover of *N*-linked glycoproteins.[6]

In our previous report,[7] we found a structure of Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M4C) among the *N*-glycans linked to *Ricinus communis* agglutinin, suggesting that a specific α-mannosidase, which is able to convert Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M5A) to the M4C structure, could reside in *Ricinus* endosperms. In this report we have purified an aryl-α-mannosidase, which has the ability to convert the M5A structure to the Man4GlcNAc2 structure, from the microsomal fraction of developing *Ricinus communis* endosperms. Having determined the various properties of the aryl-α-mannosidase, it seems likely that the enzyme might be vacuolar enzymes, and should be solubilized by disruption of the organelle during the extraction of proteins from *Ricinus* endosperm.

Materials and Methods

Material — Plants of *Ricinus communis* were grown during the summer season of 1990 at the farm of Okayama University. Fluorescamine was purchased from Polyscineces. 1-Deoxymannojirimycin and swaisonine were from Sigma Co. Authentic pyridylaminated sugar chains (M6B, M5A, M4C, and M4X) were prepared as described in our previous reports.[1] GNM5 was a generous gift from Professor S. Hase (Osaka University). Mannan-Sepharose 4B was prepared from CNBr activated Sepharose and yeast mannan (Sigma Co.) as described by Axen *et al.*[8]

Buffers — The following buffers were used in this report: A_1, 20mM HEPES/NaOH (pH 7.4) containing 5% glycerol, and 12% sucrose; A_2, 20 mM HEPES/NaOH (pH 7.4) containing 5% glycerol, and 1% Triton X-100; A_3, 20 mM HEPES/NaOH (pH 7.4) containing 5% glycerol, and 0.1% OTG; B_1, 80mM Na-acetate buffer, pH 4.5 containing 0.5% Triton X-100, 1mM $ZnCl_2$; B_2, 80mM Na-acetate buffer, pH 4.5; B_3, 80mM Na-acetate buffer, pH 4.5 containing 0.1% OTG, 10mM $ZnCl_2$.

Preparation of microsomal fraction from Ricinus communis endosperms — Developing *Ricinus communis* endosperms (720g) were homogenized in buffer A_1. From the resulting suspension the microsomal fraction was prepared by ultracentrifugation as described in our previous report.[1]

Solubilization of enzyme from the microsomal fraction — The microsome pellet obtained above was suspended in buffer A_2 and sonicated for 15 min at 0°C. From the resulting extract, the solubilized enzyme was prepared by ultracentrifugation as described in our previous report.[1]

Protein assay — Protein concentrations were determined by a fluorescence method with fluorescamine as described in our previous report.[1]

Assay of α-mannosidase activity — For the assay of α-mannosidase activity, two kinds of substrates were used. The assay of aryl-α-mannosidase activity using *p*-nitrorphenyl-α-D-mannopyranoside (PNP-α-Man, 2mM) as a substrate and the buffer B_1 was carried out as described in our previous report.[1] One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 nmole of PNP-α-Man. When the PA-sugar chains (M6B, M5A, GNM5, M4C, M4) were used as semi-naturally occurring substrates, these PA-sugar chains (about 50pmole) was incubated with enzyme solution (0.67unit, 10 μl) in buffer B_3 (50μl) at 40°C for 16hr. After boiling the reaction mixture for 3min and centrifugation, an aliquot (10μl) of the resulting supernatant was analyzed by HPLC using an Asahipak NH2P-50 column (0.46 × 25cm, Showa Denko Co.). The chromatographic conditions used for separation of PA-sugar chains were the same as in the method previously described.[1]

Results and Discussion

Purification of an aryl-α-mannosidase from Ricinus endosperms — The solubilized material was applied to a DEAE-cellulose column (2.0 × 25cm) which was equilibrated with buffer A_3. The column was washed with the same buffer, and mannosidase activity was then eluted with 50 ml of a linear elution from 0 to 0.5M NaCl in buffer A_3. Two peaks (F-I and F-II) of aryl-α-mannosidase activity were obtained (Figure 1 in our previous report[1]). The first fraction (F-I) was used in the following purification procedure, since this mannosidase fraction was also active on a PA-sugar chain (Man6GlcNAc2-PA) to produce Man5GlcNAc2-PA. The F-I fraction was concentrated to 15.2ml by ultrafiltration and applied onto a column of Sephacryl S-300 (2.2 × 172cm) equilibrated with buffer A_3, and developed with the same buffer. A fraction containing the aryl-α-mannosidase activity (elution volume 250 ~ 320ml) was concentrated to 7ml and then applied onto a mannan-Sepharose 4B column (1.8 × 12.5cm). The absorbed fraction, which contained the aryl-α-mannosidase activity, was eluted with buffer A_3 containing 0.1M NaCl. After the active fractions were pooled, concentrated (6ml) and dialyzed against buffer A_3, the resulting dialyzate was applied to Mono-Q column (0.5 × 5cm) equilibrated with the same buffer and connected to an FPLC system (Pharmacia LKB Biotechnology Inc.). The column was washed with the same buffer, and mannosidase activity was then eluted by a linear gradient of NaCl concentration from 0 to 0.1M in buffer A_3 at a flow rate of 0.7ml/min. The *Ricinus* aryl-α-mannosidase was purified about 120-fold with a yield of 1.3%. The purification procedure is summarized in Table I.

Some properties of Ricinus aryl-α-mannosidase — When *Ricinus* aryl-α-mannosidase was subjected to SDS-PAGE it gave one major band, with estimated molecular mass of 43 kDa by comparing with the relative mobility of protein standards (*Ricinus communis* agglutinin, 130kDa; ricin D, 63kDa; ovalbumin, 43kDa; ricin D B-chain, 32kDa; ricin D A-chain, 31 kDa) (Fig. 1).

The pH optimum of the *Ricinus* aryl-α-mannosidase obtained with PNP-α-Man as a substrate in MES and HEPES buffer was 4.5 to 5.0 and the activity was markedly diminished above pH 6.0. This optimum pH value was very similar to jack bean α-mannosidase[9] and mung bean aryl-α-mannosidase,[10] suggesting that the *Ricinus* aryl-α-mannosidase might also be one of the vacuolar acid glycosidases involved in catabolism of *N*-linked glycoproteins. The *Ricinus* aryl-α-mannosidase is most active from 50 to 60°C suggesting that it is stable at relatively hightemperature, although this optimum temperature might be physiologically meaningless.

Km values of *Ricinus* aryl-α-mannosidase for PNP-α-Man and Man5GlcNAc2-PA were 1.3 mM and 0.4mM (Fig. 2-A and -B), suggesting that affinity of this enzyme toward *N*-glycan is much higher than that for the synthetic substrate.

Table 1 Purification procedure for *Ricinus* aryl-α-mannosidase

Step	protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)
Solubilized extract	1,830	1,599.5	0.87	1.0
DEAE-cellulose	29.8	63.8	2.14	2.5
Sephacryl S-300	3.14	32.3	10.29	11.8
Mannan Sepharose 4B	1.08	30.1	27.87	32.0
Mono Q	0.19	20.2	106.32	122.2

One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1nmole of PNP-α-Man per minute.

Cu^{2+} ion ($CuCl_2$, 4mM) and EDTA (4mM) inactivated the *Ricinus* aryl-α-mannosidase by 70% and 40%, respectively. On the contrary, Ca^{2+} ($CaCl_2$, 4mM) and Zn^{2+} ($ZnCl_2$, 4mM) ions slightly stimulated enzyme activity (113%). Interestingly, addition of Ca^{2+} or Zn^{2+} ions (8mM) rescued the inactivation of the α-mannosidase activity by EDTA up to 77% and 65%, respectively. These results suggest that the *Ricinus* aryl-α-mannosidase is a metal glycosidase requiring Ca^{2+} or Zn^{2+}. The requirement of zinc ion for enzyme activity is very similar to jack bean aryl-α-mannosidase.[9]

It is noteworthy that *Ricinus* aryl-α-mannosidase activity was inhibited by swainsonine (95% inhibition with 0.005mM), which is a good inhibitor for mannsidase II or jack bean aryl-α-mannosidase. Conversely, 1-deoxymannojirimycin, which is a typical inhibitor for mannosidase I (α-1,2-mannosidase), was inactive towards the *Ricinus* aryl-α-mannosidase.

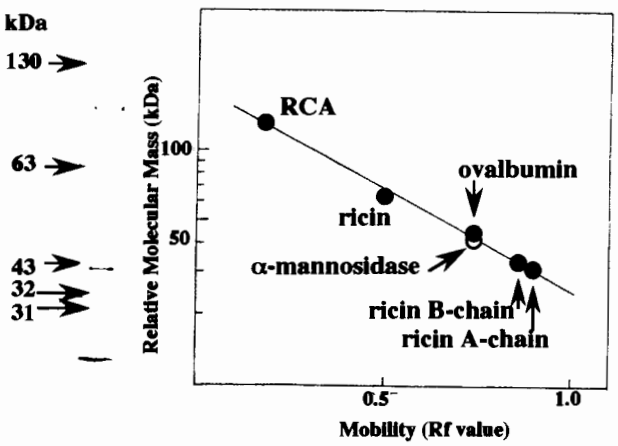

Fig. 1 SDS-PAGE and relative molecular mass of *Ricinus* aryl-α-mannosidase.

Substrate specificity of Ricinus aryl-α-mannosidase — The substrate specificity of the *Ricinus* aryl-α-mannosidase was examined by using various PA-sugar chains including high-mannose type, hybrid type, and xylose-containing type sugar chains. As shown in Fig.3-A, the *Ricinus* enzyme converted M6B having an α-1,2-mannosyl residue to Man5GlcNAc2-PA (80%) and Man4GlcNAc2-PA (11%). However, another high-mannose type *N*-glycans M5A having no α-1,2-mannosyl residue was a rather poor substrate; only 20% of the high-mannose type *N*-glycan was converted to Man4GlcNAc2-PA (Fig. 3-B). This difference in the reactivity of *Ricinus* aryl-α-mannosidase towards these high-mannose type *N*-glycans clearly suggested that this enzyme can hydrolyze α-1,2-mannosyl linkage more easily than α-1,3- or α-1,6-mannosyl linkages. Conversely the *Ricinus* aryl-α-mannosidase was completely inactive with M4C structure [Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA] and M4X structure [Manα1-3Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc-PA] (Fig. 3-C and -D), suggesting the α-1,3-mannosyl linkage in

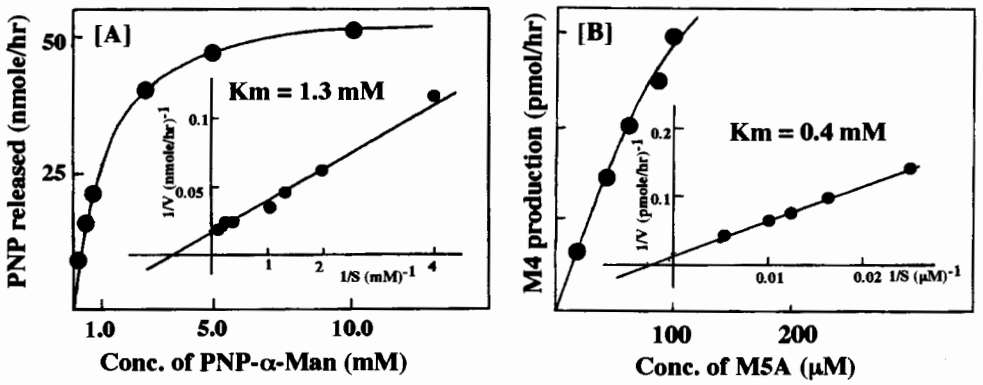

Fig. 2 Effects of p-nitrophenyl-α-D-mannopyranoside and M5A concentration on reaction rate. A, PNP-α-Man; B, M5A.

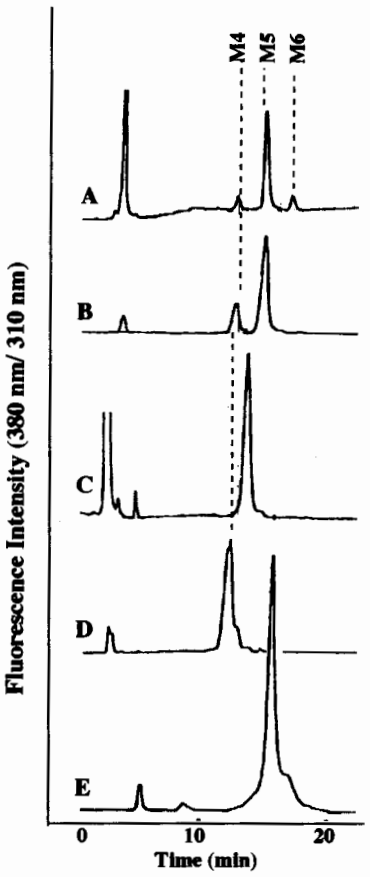

Fig. 3 SF-HPLC of various PA-sugar chains treated with *Ricinus* α-mannosidase.
A, M6B; B, M5A; C, M4X; D, M4C; E, GNM5. M4 -M6: Man4GlcNAc2-PA-Man6GlcANc2-PA.

Manα1-3Manα1-6 Manβ1- structural unit is resistant to the hydrolytic reaction. Furthermore, the *Ricinus* aryl-α-mannosidase was also completely inactive with a hybrid structure; GNM5 [Manα1-6(Manα1-3)Manα1-6(GlcNAcμ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA], suggesting the α-1,6-mannosyl linkage in Manα1-6Manα1-6 Manβ1- structural unit is also resistant to the enzyme reaction. Taking account of this substrate specificity of the *Ricinus* aryl-α-mannosidase, the structure of Man4GlcNAc2-PA derived from M6B and M5A by this enzyme (Fig. 3-A, and -B) might be Manα1-6 Manα1-3Manα1-6Manβ1-4GlcNAcβ1 -4GlcNAc-PA instead of M4C [Manα1-3Manα1 -6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA].

In this report, we have purified and charcterized an α-mannosidase from the microsomal fraction of developing *Ricinus communis* endosperms to elucidate the mannose trimming mechanism in *N*-glycan processing pathway working in plant cells. Since in our previous report[1] we already purified and characterized an α-1,2-mannosidase (mannosidase I) involved in formation of the M5A structure, we tried to purify another processing α-mannosidase (mannosidase II) that derives GlcNAc1Man3GlcNAc2 structure from GNM5 in this report. The *Ricinus* aryl-α-mannosidase purified in this report could derive Man4GlcNAc2 structure from M6B or M5A; however, this enzyme was inactive with GNM5. Furthermore, the pH optimum for this *Ricinus* aryl-α-mannosidase was from 4.5 and 5.0. Swaisonine and EDTA, which are good inhibitors for vacuolar aryl-α-mannosidase from jack bean, inhibited the *Ricinus* enzyme, on the whereas Zn^{2+} ion rescued the reduced enzyme activity by EDTA. These results suggest that this *Ricinus* aryl-α-mannosidase is vacuolar acid α-mannosidase involved in the metabolism of *N*-linked glycoprotein rather than processing α-mannosidase involved in *N*-glycan processing in the ER or Golgi apparatus. Analysis of substrate specificity showed that this *Ricinus* aryl-α-mannosidase is very active with α-1,2-mannosyl residue(s) in *N*-glycans and less active with α-1,3-mannosyl residue in the Manα1-3Manβ1- structural unit.

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登熟期ヒマ種子ミクロゾーム画分からのアリル-α-マンノシダーゼの精製と酵素学的諸性質

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登熟期ヒマ種子のミクロゾーム画分から *p*-nitrophenyl-α-D-mannopyranoside (PNP-α-Man) 及び蛍光標識 *N*-グリカンに対して活性を示すα-マンノシダーゼを精製後，酵素学的諸性質を解析した．精製酵素は還元条件下の SDS-PAGE では43kDa の相対分子量を示し，PNP-α-Man を基質とした場合の至適反応条件は50—60℃，pH 4.5—5.0であった．本酵素は EDTA により阻害を受けたが，Zn^{2+} 及び Ca^{2+} 添加により活性が回復することから金属イオン要求性の酵素であると思われる．また，PNP-α-Man に対する Km 値は1.3mM，Man 5 GlcNAc 2 -PA に対する Km 値は0.4mM であった．本酵素は Man 5 GlcNAc 2 -PA に作用し Man 4 GlcNAc 2 -PA を誘導するものの，Man 6 GlcNAc 2 -PA 対してむしろ強い活性を示し，Man 5 GlcNAc 2 -PA (88%) 及び Man 4 GlcNAc 2 -PA (12%) を誘導した．しかしながら，Man 4 GlcNAc 2 -PA, GlcNAc 1 Man 5 GlcNAc 2 -PA, Man 4 Xyl 1 GlcNAc 2 -PA は本酵素の有効な基質とはなり得なかった．これらの結果から，本酵素はα-1，2-マンノース残基を優先的に加水分解し，その後，β-結合マンノースに結合するα-1，3-マンノース残基を遊離することが示唆された．