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Characterization of Oligosaccharide Moieties of Rat Intestinal Phytase

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Phytase of Rat Intestine had a large amount of oligosaccharides; The enzyme consisted of two different subunits with the molecular weights of 90 KDa and 70 KDa in its intact form, whereas the apparent molecular weights turned to 72 KDa and 52 KDa, respectively, after deglycosylation. The treatment with glycopeptidase F reduced the molecular weights from 90 KDa and 70 KDa to 83 KDa and 52 KDa, respectively, while endoglycosidase H caused no change in their molecular weights. These results indicate that the 70 KDa subunit contains only the N-linked oligosaccharide chains, while the 90 KDa subunit contains O-linked oligosaccharides as well as N-linked ones. Enzyme-linked lectin assays suggested that bisecting *N*-acetyl-D-glucosamine and galactose 1-4 *N*-acetyl-D-glucosamine structures were present and that fucose was included in these oligosaccharide moieties. Sialic acid was not found in either subunit.

Key words: Phytase, Rat intestine, Enzyme-linked Lectin assay

INTRODUCTION

Phytase is important for digesting phytic acid which is abundant as a primary storage form of phosphoric acid in grains. In the previous report (Yang *et al.*, 1991) we represented that phytase of rat intestine was attributed to intestine-specific alkaline phosphatase, and that it was a glycoprotein. Recently, some knowledge on the contributions of specific carbohydrate moieties to the function and structure of enzyme proteins has been accumulated. Komoda and Sakagishi (1978) demonstrated that human hepatic alkaline phosphatase became more heat-labile and more sensitive to protease digestion, when it was desialylated. When intestinal isozyme of alkaline phosphatase was treated with various glycosidases, such as α-mannosidase, exo-*N*-acetyl-D-glucosaminidase, and endo-*N*-acetyl-D-glucosaminidase, it showed a significant decrease in activity. They also found that the treatments with sialidase and/or glycosidases accelerated the turnovers of both the hepatic and intestinal isozymes. On the other hand, Lehmann (1980) and Koyama *et al.* (1986) established the structural differences between sugar chains of alkaline phosphatase isozymes of human and rat using serial lectin affinity technique. Koyama *et al.* (1987) also described that sugar chains of alkaline phosphatase were replaced with those peculiar to tumors, when malignant transformation took place. These findings suggest the significance of sugar chains in the enzyme. In the present report, we analyzed the structural characteristics of carbohydrate moieties of rat intestinal phytase/alkaline phosphatase by an enzyme-linked lectin assay and glycosidase treatments.

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MATERIALS AND METHODS

Reagents

Goat IgG fraction of anti-rabbit IgG and peroxidase-anti-peroxidase (PAP) complex were purchased from Cappel (USA) and Zymed (USA), respectively. Endoglycosidase H, glycopeptidase F, and neuraminidase were from Boehringer-Mannheim (Germany). Peroxidase-Lectin Kit was from Hohnen (Japan). Freund's complete and incomplete adjuvants were from Difco (USA). DE-52 was from Whatman (UK). Other reagents were of analytical grade and obtained from local sources.

Preparation of Rabbit Anti-phytase Antiserum

The enzyme was purified from the intestinal mucosa

of rat, as described previously (Yang *et al.*, 1991). The 90 KDa subunit band on SDS-PAGE of the purified enzyme preparation was extracted from the polyacrylamide gel by the method of Hager and Burgess (1980). A rabbit was immunized with 0.2 mg of the extracted protein emulsified in Freund's complete adjuvant. A booster of the same dose of the enzyme protein emulsified in Freund's incomplete adjuvant was given 4 weeks later, and then the rabbit was bled every 7 days. IgG fraction was purified from the antiserum by DE-52 column chromatography, as described by Sano *et al.* (1989).

Western Blotting

Immunochemical analyses of the enzyme by Western blotting were performed, as described by Sano *et al.* (1989). SDS-PAGE was carried out on 7% polyacrylamide gel, according to the procedure of Laemmli (1970) and proteins were transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979) with a slight modification that a semi-dry blotting apparatus was used. Rabbit anti-phytase antiserum was diluted 4,000-fold with 3% BSA in phosphate-buffered saline (PBS), and loaded on the membranes overnight. Then, the immunoreactive bands were detected with goat anti-rabbit IgG (400-fold dilution) and peroxidase anti-peroxidase (PAP) complex (400-fold dilution).

Chemical Deglycosylation

A slight modification by Wolff *et al.* (1987) was adopted. The intestinal mucosa was homogenized in 4 vol of Tris-HCl (pH 7.4) containing 0.5 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF), centrifuged at 105,000×g for 60 min, and the resulting pellet was suspended in the same buffer. Proteins in the particulate fraction of the intestinal mucosa were precipitated with 5% (w/v) trichloroacetic acid, and washed with cold acetone at −20℃. Then, the precipitate was dried to remove acetone, and incubated with trifluoromethanesulfonic acid (TFMS)/anisole (2 : 1, v/v) for 3 h at 0℃. The reaction was terminated by addition of 2 vol of 50% pyridine and 1% SDS. For control, pyridine-SDS solution was added on the protein precipitate prior to the addition of TFMS/anisole. The reaction mixtures were subjected to extraction with diethylether three times and heating in a boiling water bath to remove organic solvents, and dialyzed against disulled water.

Enzymatic Deglycosylation

The particulate fraction of the intestinal mucosa was boiled in the presence of 1% SDS and 1% 2-mercaptoethanol for 3 min, and then subjected to enzymatic deglycosylation. The reaction mixture for endoglycosi-

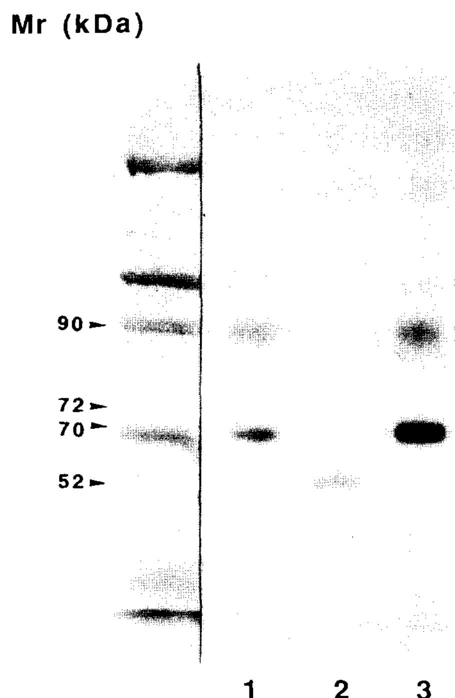

Fig. 1. Chemical deglycosylation of rat intestinal phytase. An aliquot of the particulate fraction of rat intestinal mucosa was treated with TFMS, as described in Materials and Methods. Ten μg sample was subjected to SDS-PAGE, and phytase molecule was detected by Western blotting. Lane 1: control sample, lane 2: deglycosylated sample, and lane 3: untreated sample. The marker proteins stained with 0.1% Amido Black in 10% acetic acid/45% methanol were represented in the left end lane; myosin (200 KDa), β-galactosidase (116.3 KDa), phosphorylase b (92.5 KDa) bovine serum albumin (66.2 KDa), and ovalbumin (45 KDa).

dase H (EC 3.2.1.96) consisted of 50 mM sodium acetate (pH 5.5), 50 mM EDTA, 1% 2-mercaptoethanol, 2 mM PMSF, 1% Triton X-100, and 1 mU/ml enzyme, and that for glycopeptidase F (EC 3.2.2.18) was 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% 2-mercaptoethanol, 2 mM PMSF, 1% Triton X-100, and 2 U/m enzyme. The reaction was carried out at 37℃ for 14 h at a protein concentration of 1 mg/ml.

The treatment with neuraminidase (EC 3.2.1.18) was performed as follows; the purified phytase in 100 mM sodium acetate (pH 6.5) containing 40 mM $CaCl_2$ was boiled for 3 min, and then incubated with 1 U/ml of neuraminidase at 37℃ for 14 h.

Enzyme-linked Lectin Assay

The binding profile of phytase with various lectins was analyzed using peroxidase-conjugated lectins. The purified phytase was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was washed with 4 changes of 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20

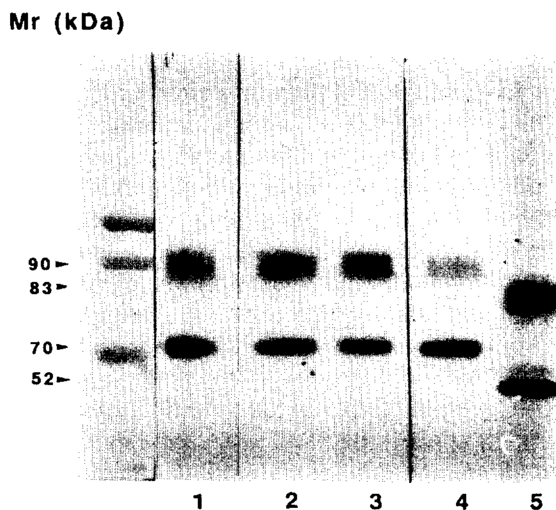

Fig. 2. Enzymatic deglycosylation of rat intestinal phytase. An aliquots of the particulate fraction of rat intestinal mucosa were incubated with (lane 3) and without (lane 2) endoglycosidase H, and with (lane 5) and without (lane 4) glycopeptidase F. Samples of 10 μg digests and the same amount of the untreated sample (lane 1) were subjected to Western blot analysis, as described in Fig. 1.

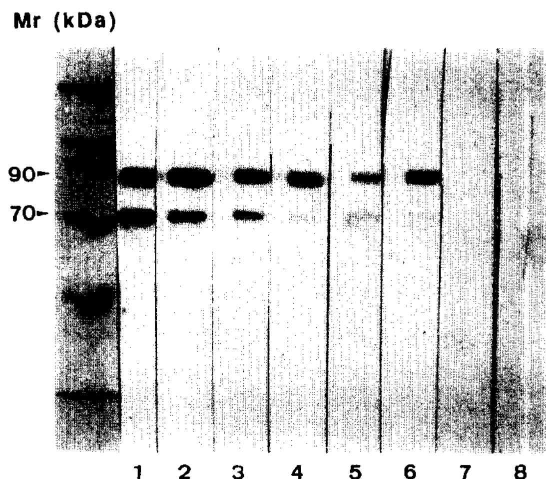

Fig. 3. Binding of various lectins to rat intestinal phytase. Phytase was purified as described previously (Yang *et al.*, 1991), and 0.5 μg of the purified enzyme preparation was subjected to analysis by the enzyme-linked lectin assay. The lectins examined were as follows; lane 1: PHA-E_4, lane 2: WGA, lane 3: RCA_{120}, lane 4: UEA-I, lane 5: LCA, lane 6: DBA, lane 7: PNA, and lane 8: Con A. Full names of the lectins were given in Table I.

(Tween-TBS), and 5 μg/m*l* of peroxidase-conjugated lectin in Tween-TBS was loaded on the membrane for 1 h at room temperature. Then the membrane was washed with Tween-TBS 4 times, and put into the substrate solution consisting of 0.1 M Tris-HCl (pH 7.4), 10 μg 3, 3'-diaminobenzidine (DAB) hydrochloride and 10 μl of 30% H_2O_2.

RESULTS AND DISCUSSION

In order to determine the content of oligosaccharides in the enzyme protein, chemical deglycosylation was carried out, by which all the oligosaccharides are decomposed. As shown in Fig. 1, the apparent molecular weights of the 90 KDa and 70 KDa subunits were reduced to 72 KDa and 52 KDa, respectively, by this treatment, indicating that the sugar moieties account for as much as 18KDa each. This result also suggests that the difference of molecular weight between the two subunits are due to the difference of size in polypeptide moieties.

Next, we examined what oligosaccharide residues were contained in the enzyme by enzymatic deglycosylation. It is known that glycopeptidase F hydrolyzes all types of N-linked (asparagine-linked) oligosaccharide chains (Plummer et *al.*, 1984), but that endoglycosidase H removes only high mannose type and hybrid type of N-linked oligosaccharide chains (Tarentino and Maley, 1974; Tai et *al.*, 1977). As shown in Fig. 2, the treatment with glycopeptidase F converted the apparent molecular weight of the 90 KDa subunit to 83 KDa and that of the 70 KDa subunit to 52 KDa, suggesting that the 70 KDa subunit contained only N-linked oligosaccharides, and that the 90 KDa subunit had both N-linked and O-linked (serine, threonine-linked) oligosaccharides. On the other hand, the treatment with endoglycosidase H caused no change in the molecular weight of either subunit, indicating that high mannose type and hybrid type were not contained.

With peroxidase-conjugated lectins we examined the structure of oligosaccharide portions of the en-

Table I. Binding profiles of rat intestinal phytase with various lectins

Lectin	90 KDa subunit	70 KDa subunit
PHA-E_4	+++	+++
WGA	+++	++
RCA_{120}	++	++
UEA-I	++	+
LCA	+	+
DBA	++	±
PNA	−	−
Con A	−	−

The results in Fig. 3 were summarized with arbitrary units. The numbers of "+" represent the intensities of bands detected. The symbol "−" represents the absence of binding. Abbreviations used are as follows: PHA-E4, *Phaseolus vulgaris* lectin; WGA, Wheat germ lectin; RCA_{120}, Caster bean lectin; UEA-I, *Ulex europaeus* lectin; LCA, Lentil lectin; DBA, *Dolichos biflorus* lectin; PNA, Peanut lectin; Con A, Concanavalin A.

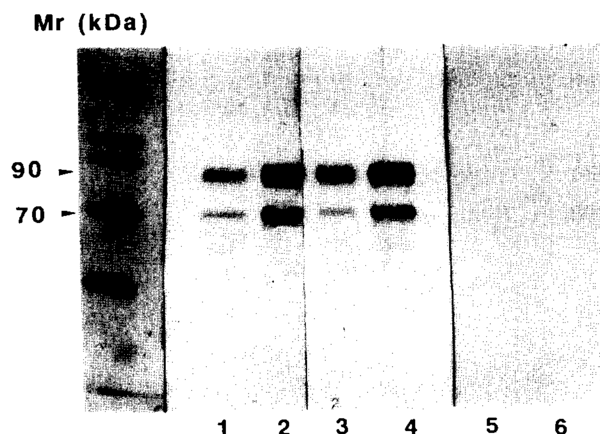

Fig. 4. Effect of Neuraminidase treatment on rat intestinal phytase. The purified enzyme preparation was incubated with (lane 1, 3, 5) or without (lane 2, 4, 6) neuraminidase. Samples of 0.5 μg protein were subjected to enzyme-linked lectin assay with WGA (lanes 1, 2), RCA_{120} (lanes 3, 4), and PNA (lanes 5, 6).

zyme. As shown in Fig. 3, the binding profiles of the 90 KDa and 70 KDa subunits with various lectins were almost identical. The results were represented with arbitrary units in Table I. From these data the structural characteristics of the oligosaccharide chains can be summarized as follows; 1) *Phaseolus vulgaris* lectin, PHA-E_4 and wheat germ lectin (WGA) showed high affinities for both the subunits, indicating that the bisecting *N*-acetyl-D-glucosamine structure is present. 2) Caster bean lectin (RCA_{120}) was tightly bound to both the subunits, indicating that galactose 1-4 *N*-acetyl-D-glucosamine structures are contained at the terminals of oligosaccharide chains. 3) *Ulex europaeus* lectin (UEA-I) and lentil lectin (LCA) were also bound, suggesting the presence of fucose in both the subunits. 4) *Dolichos biflorus* lectin (DBA) showed an affinity for the 90 KDa subunit, but peanut lectin (PNA) did not react with it, suggesting that *N*-acetyl-D-galactosamine is present but galactose is absent at the terminals of O-linked oligosaccharide chains.

Since the enzyme was found in the markedly acidic region in isoelectric focusing (around pH 4, data not shown), we examined whether the enzyme molecule contained sialic acid. Contrary to our expectation, however, as shown in Fig. 4, neither the molecular weights nor the reactivity to the antiserum was affected by the treatment with neuraminidase. In addition, this treatment did not give any effects on the binding of lectins. These results suggest that no sialic acid is contained in the enzyme.

Our finding of the absence of sialic acid in phytase is contrasted to most proteins located on the plasma membrane. Besman and Coleman (1985) analyzed the contents of various carbohydrate moieties of bovine intestinal alkaline phosphatase isozymes and detected small amounts of sialic acid, while Fosset *et al.* (1974) reported its absence. On the other hand, Mulivor *et al.* (1978) demonstrated a marked decrease in sialic acid content of human intestinal alkaline phosphatase during development. Komoda and Sakagishi (1978) also showed that the intestinal alkaline phosphatase from adults contained little sialic acid, whereas the fetal enzyme contained it. Furthermore, McKenna *et al.* (1979) found that in high resolution two-dimensional electrophoresis migration of the intestinal alkaline phosphatase was unaffected by neuraminidase treatment. The present result on the intestinal phytase/alkaline phosphatase from adult rats coincides with the above findings.

The physiological significance of the oligosaccharide structures in the phytase function must await further investigation.

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