Porcine Pancreatic α-Amylase Shows Binding Activity toward N-Linked Oligosaccharides of Glycoproteins

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Porcine pancreatic α-amylase was shown by interaction analyses using a resonance mirror detector and α-amylase-immobilized Sepharose to bind with glycoproteins possessing N-glycans but not O-linked mucin-type glycans. Direct binding of three types of N-glycans to the α-amylase was demonstrated by surface plasmon resonance. Binding with biotin-polymer sugar probes revealed that the α-amylase has affinity to α-mannose, α-N-acetylleucaminic acid, and β-N-acetyllactosamine, which are components of N-glycans. The binding of glycoproteins or carbohydrates enhanced the enzyme activity, indicating that the recognition site for N-glycans is different from its catalytic site. The binding activity was unique to porcine pancreatic α-amylase and was not observed for α-amylase from saliva, wheat, and fungus.

α-Amylase (EC 3.2.1.1) is a well known starch hydrolase discovered in 1830 that has important roles in energy acquisition in animals, plants, and microbes. It is an endo-type enzyme that typically cleaves α,1,4-glucose linkages in starch which is further processed by the exoenzymes. Because in vivo modulation of α-amylase activity in pancreatic secretions is important in nutrition and for developing therapies for diabetes mellitus, interaction between porcine pancreatic α-amylase (PPA) and its inhibitors, including antinutrients, has been investigated (1–3).

Legumes contain large amounts of antinutrients such as lectins, tannins, phytates, and enzyme inhibitors that may be investigated (1–3). Most plant lectins and α-amylase inhibitors are glycoproteins. Because PPA possesses three potential glycosylation sites, we studied the possibility that carbohydrate-dependent interaction between α-amylase and various glycoproteins occurs in the physiological processes. PPA specifically recognizes N-linked glycans. The novel carbohydrate binding activity of PPA may provide new insight into the secretion, targeting, and modulation mechanism of this long known enzyme, and the antinutritive activities of plant lectins in the digestive tract.

EXPERIMENTAL PROCEDURES

Materials—PPA was purchased from Elastin Products Co., Inc. (Owensville, MO), for use in most experiments and from Roche Molecular Biochemicals and Sigma for comparison. Disopropyl fluorophosphate-treated PPA, α-amylase from barley (a mixture of α- and β-amylase; the β-amylase was inactivated by heat treatment at 70 °C for 15 min) and human saliva, wheat α-amylase inhibitor, ovalbumin, calf test, bovine submaxillary gland mucin (BSM), ribonuclease B, yeast mannan, and human apotransferrin were purchased from Sigma. Bacillus subtilis α-amylase and bovine serum albumin (BSA) were purchased from Roche Molecular Biochemicals Co. Biotinyl polymer (BP-) sugar probes were purchased from Seikagaku Co. (Tokyo, Japan). Cleodorodon trichotomum lectin (CTA) was purified as described previously (14). Streptavidin-biotin peroxidase complex was from Amersham Biosciences. Pyridylaminophosphate-Sepharose and α-mannosidase were purchased from Seikagaku Co. (Tokyo, Japan).

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Affinity Chromatography on PPA-Sepharose—PPA-Sepharose was prepared by coupling 15 mg of PPA with formyl-Sepharose (2 g) (15) in 20 ml of 0.15 M NaCl, 15 mM phosphate buffer (pH 7.4) containing 60 mg of NaCNBH4 at 4 °C for 2 days. Excess formyl groups were blocked with 40 ml of 1 M Tris-HCl (pH 7.4) containing 124 mg of NaCNBH3 at room temperature for 1 h. About 5 mg of protein per ml of gel was immobilized. A PPA-Sepharose column (0.75 × 6 cm) was equilibrated with 15 mM sodium succinate buffer containing 20 mM CaCl2 and 0.5 M NaCl (pH 5.6). 200 μl of each glycoprotein solution (1 mg/ml) was applied to the column and washed with the same buffer. The bound glycoproteins were eluted with 0.2 μM methyl α-D-mannoside, methyl β-D-galactoside in the same buffer, or 0.1 μM citrate buffer (pH 3.0) and monitored by absorbance at 280 nm.

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Affinity chromatography of glycoproteins on a PPA column. Chromatogram of bovine fetuin, ovalbumin, and RNase B (Chromatogram BIAcore AB, Uppsala, Sweden) was used. After washing with the same buffer, the bound fetuin was successively eluted with 0.2 M methyl \( \beta \)-galactoside (400 \( \mu l \)) (a), 0.2 M methyl \( \alpha \)-mannoside (400 \( \mu l \)) (b), 0.2 M methyl \( \beta \)-galactoside (800 \( \mu l \)) (c), and 0.2 M methyl \( \alpha \)-mannoside (800 \( \mu l \)) (d). For ovalbumin and RNase B, 0.2 M methyl \( \alpha \)-mannoside (1.5 ml) (e) and 0.2 M methyl \( \alpha \)-mannoside (4 ml) (f) were used as eluants.

Quantitative Interaction Analyses between PPA and Various Glycoproteins by Resonance Mirror Detector (IAssys)—IAssys (Affinity Sensors, Cambridge, UK), a biosensor based on optical resonance, was used for kinetic analyses of interactions between PPA and glycoproteins. Immobilization was performed with 200 \( \mu l \) of PPA solution (1 mg/ml) in 10 mM sodium acetate buffer (pH 5.5) containing 0.2 M methyl \( \beta \)-mannoside, and PPA (3 \( \mu g/ml \)) in 10 mM sodium succinate buffer containing 150 mM NaCl and 2 mM CaCl\(_2\) was applied to a PPA-Sepharose column (0.75 \( \times \) 6 cm). After equilibration with HEPES- and PA-oligosaccharides, a BIAcore 2000 SPR apparatus (BIACORE—IAssys) was used. After washing with the same buffer, the bound fetuin was successively eluted with 0.2 M methyl \( \beta \)-galactoside (400 \( \mu l \)) (a), 0.2 M methyl \( \alpha \)-mannoside (400 \( \mu l \)) (b), 0.2 M methyl \( \beta \)-galactoside (800 \( \mu l \)) (c), and 0.2 M methyl \( \alpha \)-mannoside (800 \( \mu l \)) (d). For ovalbumin and RNase B, 0.2 M methyl \( \alpha \)-mannoside (1.5 ml) (e) and 0.2 M methyl \( \alpha \)-mannoside (4 ml) (f) were used as eluants.

To measure binding curves, various concentrations of glycoproteins in 10 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and 5 mM CaCl\(_2\) were added to the cuvette, and after 200 \( \mu l \) the cuvette was washed with the same buffer. Glycoproteins that bound to the cuvette were eluted with 0.1 M methyl \( \alpha \)-mannoside to regenerate the cuvette or 3 mM NaCl in the same buffer or diluted NaOH (pH 10) for extensive washing. Binding constants were calculated from their binding curves using connected software, according to the equation, \( \frac{d[R]}{dt} = k_a [S]_0 - k_d [R]_0 \). Where \( R \) is the response (arc seconds), \( [S]_0 \) is the initial concentration of ligand, and \( [R]_0 \) is maximum response that will be seen at concentrations of ligand high enough to saturate the binding site of \( \alpha \)-amylase. On introducing \( k_a = k_b [S]_0, k_d, k_m \) is obtained from the slope of \( \frac{d[R]}{dt} \) versus \( R \) plot. The association constant, \( K_a \), is calculated according to the equation, \( \frac{k_a}{k_d} = \frac{k_m}{k_m} \). Where \( k_m, k_m \) is obtained from the slope and the intercept of \( k_m \) versus \( [S]_0 \) under various concentrations of ligands.

Interaction Analyses between PPA and PA-oligosaccharides by Surface Plasmon Resonance (BIACore)—For binding studies between PPA and PA-oligosaccharides, a BIACORE 2000 SPR apparatus (BIACORE AB, Uppsala, Sweden) was used. After equilibration with HEPES-buffered saline on a CM5 sensor chip, the surface of the sensor chip was activated with an amine coupling kit, and PPA (3 mg/ml) in 10 mM sodium acetate buffer (pH 4.3) containing 0.2 M methyl \( \alpha \)-mannoside was injected onto the activated surface, and then the remaining N-hydroxysuccinimide esters were blocked by the addition of 1.0 M ethylenediamine hydrochloride (pH 8.0). Each step was performed for 14 min at a constant flow rate of 10 \( \mu l/min \) to immobilize PPA maximally. The reference flow cell was prepared with BSA as a ligand. The PA-oligosaccharides were separately injected onto a PPA-immobilized sensor chip at concentrations of 25.0 \( \mu M \) Man, GlcNAc-PA and 25.8 \( \mu M \) each of NeuAc, Gal, Man, GlcNAc-PA and NeuAc, Gal, Man, GlcNAc-PA in 10 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and 5 mM CaCl\(_2\) for 150 s at a flow rate of 2 \( \mu l/min \) at 25°C.

Measurement of Enzyme Activity—Enzyme activity was measured according to Bernfeld (17). PPA solution in PBS (0.1 ml) was preincubated at 25°C for 5 min, and various concentrations of glycoproteins (10 \( \mu l \)) were added. After incubation for 1 h, 0.2 ml of 1% starch in 20 mM phosphate buffer (pH 6.9) containing 0.006 M NaCl was added, and the mixture was reacted at room temperature for 30 min. To stop the reaction, 0.2 ml of 3,5-dinitrosalicylic acid was added, and it was boiled in a waterbath for 5 min to develop the color. The reaction mixture was cooled to room temperature, diluted with 2 ml of water, and measured at 540 nm. Maltose was used as a standard for reducing sugar.

Carbohydrate Analyses of PPA—Neutral carbohydrate analysis was carried out from blotted polyvinylidene difluoride membranes according to the method described previously (18). Lectin reactivity was examined on the membrane according to the method described previously (16) using biotinyl lectins, concanavalin A, Paathrella velutina lectin, and P. vulgaris leukoagglutinin.

RESULTS

Affinity Chromatography of Glycoproteins on a PPA-Sepharose—Fig. 1 shows the affinity of glycoproteins on a PPA-immobilized Sepharose chromatography column. As shown in Fig. 1A, fetuin bound to the column and was dissociated from it by pulse elution with 0.2 M Me \( \alpha \)-Man (arrows, b and d) but not with Me \( \beta \)-Gal (arrows, a and c). As shown in Fig. 1, B and C, the bound ovalbumin and RNase B, respectively, were eluted with methyl \( \alpha \)-mannoside but not with methyl \( \beta \)-galactoside (data not shown). On the contrary, \( \alpha \)-amylase inhibitor bound to the column and eluted with 0.1 M sodium citrate buffer (pH 3.0) as reported previously (8), but it was not eluted.
with 0.2 M methyl α-D-mannoside, in contrast to other glycoproteins tested here (data not shown).

Interaction between PPA and Various Glycoproteins Analyzed by IAsys—For immobilization of PPA in the cuvette, methyl α-D-mannoside was necessary to protect the carbohydrate-binding site; otherwise, fetuin did not bind to the PPA-immobilized cuvette (data not shown). Changes of the resonance angle caused by the immobilization or interaction were measured in arc seconds, a response unit of IAsys (163 arc s/nm2), and plotted versus time in the binding curves. The total amount of immobilized PPA was about 2,000 arc s.

The optimum condition for interaction analyses was examined using fetuin. As shown in Fig. 2A, fetuin bound to PPA-immobilized cuvettes at pH 4.0–7.4 with optimal binding at pH 4.5. Between pH 4.5 and 5.0, however, binding occurred too quickly to produce a biphasic curve, and pH 5.5 was adapted for quantitative interaction analyses because it is closer to the pH of pancreatic fluid. As shown in Fig. 2B, transferrin and fetuin bound to the PPA cuvette at pH 5.5, but BSM and BSA did not. Asialofetuin, CTA, and ovalbumin also bound to the PPA cuvette (data not shown). The bound glycoproteins were eluted with 0.2 M methyl α-D-mannoside, but when the elution was incomplete, 3 M NaCl was used to elute the cuvette with 0.2 M d-galactose, indicating that the binding was not caused by the carbohydrate recognition of CTA.

Quantitative Parameters for Interaction between PPA and Glycoproteins—Binding constants were measured for each glycoprotein from the plots of $k_{on}$ versus $[S]_0$ from the binding curves at various ligand concentrations, as shown in Fig. 2C. Association rate constants ($k_{a}$), dissociation rate constants ($k_{d}$), and affinity constants ($K_a$) were calculated as described under “Experimental Procedures” and are summarized in Table I. Transferrin bound best among the samples, with a $k_a$ of $2.3 \times 10^4$ M$^{-1}$ s$^{-1}$ and a $K_a$ of over $2.3 \times 10^2$ M$^{-1}$. Desialylation of fetuin markedly decreased $k_a$ and consequently $K_a$ to 1/30 of fetuin, suggesting that sialyl residues contribute to the binding. On the contrary, the $k_f$ values of all the samples tested were close. All the bound glycoproteins have N-linked oligosaccharides of various types, i.e., complex types (transferrin and fetuin), plant complex types with β-1,2-linked xylose and α-1,3-linked fucose (CTA), oligomannose, and hybrid types (ovalbumin) as summarized in Table I. On the contrary, of the unbound glycoproteins, BSM possesses only O-linked oligosaccharides, and BSA does not contain any glycans. These results suggest that a common trimannosyl moiety of N-glycans may play an essential role in the interaction of glycoproteins with PPA.

Interaction between PPA and RNase B by IAsys—Except RNase B, the glycoproteins examined here bound to PPA optimally at a pH lower than 5, whereas RNase B bound optimally at pH 7–7.5 as well as at pH 5 (data not shown). As shown in Fig. 2D, RNase B bound to PPA better than its unglycosylated isoform RNase A, especially at pH 5.5, suggesting that a high mannose type N-glycan is involved in the interaction. Because RNase B and A adsorbed significantly to underivatized CM-dextran cuvettes, probably due to electrostatic interaction, quantitative parameters could not be obtained.
units = 1 ng/mm²), respectively. The changes of resonance units induced by binding of analytes to PPA-immobilized flow cell were corrected for bulk effect by subtracting the changes on the BSA-immobilized reference cell. As shown in Fig. 3, the binding and dissociation occurred rapidly at the start and end of the injection of PA-oligosaccharides, demonstrating the specific binding of the PA-oligosaccharides to PPA with quick association and dissociation rates. From the changes in resonance units induced by binding of analytes to PPA-immobilized flow cells corrected for bulk effect by subtracting the changes on the BSA-immobilized reference cell, the bound amounts were calculated to be 0.22, 0.18, and 0.044 pmol/mm² for NeuAc2Gal2Man3GlcNAc4-PA, NeuAc3-Gal or NeuAc2-6; △, NeuAc α2→3; ○, Fuc; □, Xyl. Interactions between PPA and glycoproteins are measured in 10 mM acetate buffer (pH 5.5) containing 150 mM NaCl and 5 mM CaCl₂ using IAsys. $k_a$, shows association rate constant; $k_d$, dissociation rate constant; and $K_a$, association constant. $K_a = k_a/k_d$, calculated from the slope and the intercept of the $k_a$ versus $[S]$ in Fig. 2 C as described in the text.

| Major Oligosaccharides          | $k_{ass}$ [10⁶ M⁻¹ s⁻¹] | $k_{diss}$ [10⁶ s⁻¹] | $K_a$ [10⁷ M⁻¹] |
|---------------------------------|--------------------------|---------------------|-----------------|
| Biantennary complex-type        |                          |                     |                 |
| Triantennary complex-type       |                          |                     |                 |
| Oligomannose-type hybrid-type   |                          |                     |                 |

### Interaction between PPA and Sugar-BP Probes

As shown in Fig. 4A, PPA bound best with α-mannose and α-mannose 6-phosphate-BP probes among the probes examined. The binding was not restricted to the mannosyl residue, but Galβ1-4GlcNAc (LacNAc), α-NeuAc-α, and α-GalNAc-BP probes bound to a lesser extent than α-mannose-BP. β-GlcNAc-, β-Lac-, β-Gal-3-sulfate-, or 3-O-sulfO-Galβ1,4(Fucα1,3)GlcNAc-BP probes did not bind, suggesting that the binding is not caused merely by N-acetyl-specific or electrostatic interaction. α-GalBP probe did not bind, suggesting that catalytic subsites of PPA (19) are not involved in the interaction with the sugar-BP probes.

In the inhibition test, 50 mM mannan inhibited the interaction between PPA and the α-mannose-BP probe by about 75%, but methyl α-d-mannoside, methyl β-d-galactoside, or methyl α-d-glucoside did not, even at the concentration of 0.1 M (data not shown). From these results, the glycoprotein binding is considered to be due to the affinity of PPA for multiple carbohydrate residues that are components of N-linked glycans.

### M.A.N-N-BP Binding Activity of α-Amylases of Different Origins

As shown in Fig. 4B, PPA from three manufacturers all exhibited the mannose-BP binding activity. In contrast, α-amylases of barley, Bacillus, and human saliva origins did not exhibit obvious binding activity toward the mannose-BP probe.

### Carbohydrate Analyses of PPA

Carbohydrate analyses indicated that PPA contained 0.04 mol each of D-mannose and D-glucosamine per 1 mol of PPA, and a trace amount of α-d-galactose, which is lower than the reported carbohydrate contents for PPA (20). 1-Fucose, N-acetylated-galactosamine and α-d-xylose were not detected. The results indicate that although it has three potential glycosylation sites, most parts of PPA are unglycosylated, and a very small amount (<1% of potential sites) is N-glycosylated. PPA electroblotted onto the membrane after SDS-PAGE was very slightly stained with concanavalin A and only weakly stained with P. velutina lectin and P. vulgaris leukoagglutinin, suggesting that the glycosylated molecules contain sialylated tri- or tetra antennary branched N-glycans.

### Effect of Glycoproteins on an Enzyme Activity of PPA

As shown in Fig. 5, glycoproteins that interacted with PPA did not inhibit the enzyme activity but rather enhanced it to various degrees with the exception of the wheat PPA inhibitor only. When transferrin was added at 5-, 30-, and 100-fold excess in molar ratio to PPA, it enhanced the enzyme activity by 125, 134, and 145%, respectively (Fig. 5A). Fetuin similarly enhanced the enzyme activity by 110% at 30-fold excess in molar ratio.
ratio to PPA, whereas ovalbumin and BSA did not affect the enzyme activity, and wheat PPA inhibitor inhibited it to 80% at the same concentration (Fig. 5B).

**DISCUSSION**

This paper demonstrates that PPA binds to glycoproteins by carbohydrate-specific interaction. The affinity chromatography
and quantitative analyses with IAsys indicated that immobilized PPA interacted with glycoproteins possessing N-glycans at a $K_d$ of $10^{-7}$ M$^{-1}$ at acidic pH, whereas mucin did not bind at all. The binding studies of PA-oligosaccharides to PPA with BIAcore indicated that sialylated complex-type oligosaccharides bind better than high mannose oligosaccharides at pH 5.5. Microplate assay using sugar-BP probes indicated that the interaction is due to the affinity of PPA for component saccharide residues of the N-linked complex type, i.e. for α-mannose, α-NeuAc, and for β-LacNAc. Supportingly, the glycoproteins that bound to immobilized PPA were most effectively eluted with methyl α-mannoside among the saccharides tested. Because the sugar-BP probe is a multivalent probe exhibiting an affinity constant higher than free sugar by $10^2$–$10^5$ M$^{-1}$ (21, 22), it was successfully used to reveal the binding specificity of PPA. The affinity of PPA toward free mono- or disaccharide would not be enough to elute completely the bound glycoproteins from a PPA-immobilized cuvette.

The glycoprotein binding was inhibited in the presence of EDTA, although once bound, glycoproteins were hardly eluted with EDTA. When the PPA-immobilized cuvette was washed with 10 mM NaOH, the carbohydrate binding of PPA was inactivated, but equilibrating the cuvette with the buffer containing Ca$^{2+}$ restored the binding activity of PPA. These observations indicate that the carbohydrate recognition of PPA is Ca$^{2+}$-dependent and that the bound glycoprotein might prevent EDTA from accessing Ca$^{2+}$ located near the lectin site of PPA. The interaction of glycoproteins with PPA did not inhibit its enzyme activity but instead enhanced it to various degrees. On the other hand, the N-glycan binding activity was completely lost in diisopropyl fluorophosphate-treated PPA that had been modified at serine residues to inactivate protease activity while maintaining the amylase activity (data not shown). These observations suggest that the N-glycan recognition is exhibited at a site different from the catalytic subsites of PPA.

The Man-BP binding activity is unique to PPA and was not observed for α-amylase isolated from barley, B. subtilis, or human saliva, indicating that the N-glycan binding activity has been acquired during evolution to adapt PPA to the pancreas-specific environment. Because mature PPA is almost unglycosylated, the receptor glycan for PPA may be present on other glycoconjugates.

The N-glycan-binding site may play a role in targeting PPA to intestinal membrane surfaces after secretion because the surface epithelium of the gut is extensively glycosylated and PPA is localized at the luminal surface. In this case, an oligomannosyl N-glycan, such as that of RNase B, may be a first candidate for the receptor glycan due to the slightly alkaline to neutral pH of pancreatic fluid and the intestinal lumen. The epithelium of the small intestine is organized into crypts and villi; the less differentiated crypt cells usually contain oligomannosyl glycans on the membrane glycoproteins, and upon differentiation of the cells, the glycosylation changes to express complex glycans on the fully mature cells of the villi (23–25). Binding to the membranous glycans on the epithelium would concomitantly protect PPA from proteolysis, stabilize it to extend its life, and/or activate it at the intestinal surface. Furthermore, the binding of PPA to intestinal surface glycans would make the product spatially available as a substrate for the exo-type enzymes that are naturally anchored to the intestinal brush border membranes, e.g., maltase-glucoamylase or sucrose-isoamylase complexes (26), to metabolize starch efficiently. Like many proteins anchored to the brush-border membrane, human maltase-glucoamylase is a glycoprotein with 32–38% carbohydrates that possesses unsialylated complex N-glycans at 19 potential glycosylation sites (27). The binding of PPA to complex glycans is rather weak at neutral pH, but the multivalent glycans on one molecule usually increase the affinity toward carbohydrate-binding proteins. Therefore, maltase-glucoamylase as well as other highly glycosylated membrane enzymes or receptors may also bind pancreatic α-amylase. When the mature epithelial cells are shed from the villus tips into the lumen as part of normal cell turnover, the cellular material is digested, but the liberated pancreatic α-amylase might bind to the next receptor. In this context, the antinutrient activity of plant lectins is primarily explained by the predominant binding of lectins to glycan receptors at the intestinal surface and blocking them from PPA.

Alternatively or compatibly, the carbohydrate binding activity of PPA found here might be involved in the formation of zymogen granules in the exocrine pancreas. PPA in zymogen aggregates may sort the aggregates into zymogen granules via binding to the N-glycans of the intragranular submembranous granules.
glycoprotein matrix and/or to the Man₉-GlcNAc portion of glycosylphosphatidylinositol-anchored proteins located on rafts of the trans-Golgi network or granular membrane for envelopment. This hypothesis is consistent with the report that glycosylphosphatidylinositol-anchored proteins and rafts play an important role in the granule formation and regulated apical secretion of zymogen in rat pancreas and that the inhibition of raft assembly results in missorting of pancreatic amylase to constitutive secretion (28). The binding characteristics of PPA found in this study, i.e. that PPA exhibits the highest binding to various types of N-glycan at around pH 5 but not at alkaline pH in the presence of Ca²⁺, is consistent with the fact that the association events take place Ca²⁺-dependently at mildly acidic pH (29, 30) and that PPA would dissociate from the granular membrane at the alkaline pH of pancreatic fluid. The carbohydrate binding activity found for PPA was not observed for salivary α-amylase in this study, and that may indicate the lack of universality of the aggregation-sorting pathway among secretory tissues. Carbohydrate-binding specificity might be present but different in salivary α-amylase because component glycoproteins are not common to the luminal aspect of all secretory granule membranes (29). The function of carbohydrate-specific binding of PPA is still unknown and needs to be elucidated.

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