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DEMONSTRATION OF A NEW GLYCOPEPTIDASE, FROM JACK-BEAN MEAL, ACTING ON ASPARTYLGLUCOSYLAMINE LINKAGES*

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An enzyme preparation from jack-bean meal hydrolyzed β-aspartylglucosylamine linkages in glycopeptides. The enzyme could release sialic acid-containing complex-type oligosaccharides as well as high-mannose-type and hybrid-type oligosaccharides. The products were equimolar amounts of ammonia, oligosaccharide and peptide. The enzyme cleaved glycopeptides with three or more amino acid residues, whereas it did not hydrolyze GlcNAc-Asn. The mechanism of action of the enzyme and substrate specificity so far tested were similar to those of the glycopeptidase from almonds.

Glycopeptidase that cleaves β-aspartylglucosylamine linkages in glycopeptides was found in almond emulsin by Takahashi et al. (1-3). Since almond glycopeptidase quantitatively releases intact oligosaccharide moiety from the glycopeptides, we have demonstrated that the enzyme is useful for structural analyses of asparagine-linked oligosaccharides (4-6). Furthermore, we applied the enzyme to the functional study of glycoprotein (6, 7). The presence of such an enzyme was later confirmed by Plummer and Tarentino (8).

This paper demonstrates the presence of another glycopeptidase in jack-bean meal. The possibility was shown that glycopeptidases with a similar substrate specificity distributed ubiquitously in seeds or beans. The present enzyme (jack-bean glycopeptidase) acted as an amidase and not as an N-glycosidase. The situation is the same as with almond glycopeptidase, although Plummer and Tarentino reported that almond enzyme is peptide:N-glycosidase(8, 9).

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The abbreviations used are: CHO, oligosaccharide moiety; TLC, thin layer chromatography; Xyl, xylose; Fuc, fucose.

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MATERIALS AND METHODS

Chemicals and enzymes---Jack-bean meal was purchased from Sigma Chemical Co. Human fibrinogen (Grade L) was from AB Kabi, Sweden. Glucose oligomers used as a standard for thin-layer chromatography (TLC) were prepared by acid hydrolysis of dextran (10). Almond glycopeptidase was prepared from almond nuts as described previously (3).

Substrates---Ovalbumin glycopeptides, Glu-Glu(or Gln)-Lys--Tyr-Asn(CHO)-Leu-Thr-Ser-Val, were isolated from pepsin-digested ovalbumin as described previously. Each glycopeptide contained either the high-mannose-type or the hybrid-type oligosaccharide (4). Human fibrinogen glycopeptides containing sialylated biantennary oligosaccharide chains were isolated from pepsin-digested fibrinogen as described previously (6). Stem bromelain glycopeptide, Asn-Asn(CHO)-Glu-Ser-Ser, was prepared as described previously (2). The composition of the oligosaccharide chain was decided to be $Man_{2-3}GlcNAc_2Xyl_1Fuc_1$ (5).

Enzyme Preparation---Jack-bean meal (3 g) was dissolved in 15 ml of 0.01 M sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl and the crude extract was centrifuged at 10,000 rpm for 30 min. The supernatant was concentrated by dialysis against polyethylene glycol 20,000 and was applied to a column (2 X 150 cm) of Sephacryl S-200 equilibrated with the above buffer and eluted with the same buffer. The major active fractions (from 160 ml to 220 ml) were pooled and concentrated with an Amicon PM-10 filter. 50 µl of the enzyme solution corresponded to 25 mg of jack-bean meal. The enzyme preparation did not contain asparaginase activity. It showed α-mannosidase and β-galactosidase activity slightly toward p-nitrophenyl derivatives. However, these glycosidase activities did not affect the results of the present study.

Assay of the glycopeptidase---The amount of the released ammonia was determined. Ovalbumin glycopeptides (0.06 µmol) were incubated with 50 µl of the enzyme solution in 0.01 M sodium-phosphate buffer (pH 6.5) containing 0.1 M NaCl, at 37°C for 5 h. After terminating the reaction by adding 20 µl of 1 M HCl, the reaction mixture was incubated for an additional 5 h at 37°C. Three ml of freshly prepared phosphate-buffered o-phthalaldehyde-mercaptoethanol were added to the reaction mixture, and the fluorescence was measured as described previously (11).

RESULTS AND DISCUSSION

An enzymatic activity that cleaved β-aspartylglucosylamine linkages in glycopeptides was found in an extract of jack-bean meal. The experimental facts so far obtained suggested the following two step reactions, as in the case of amidases (15) and almond glycopeptidase previously investigated (2), although we have not yet confirmed the formation of an intermediate, 1-amino-N-acetylglucosamine-oligosaccharide.

Oligosaccharide-GlcNAc—Asn-peptide + H_2O ⟶
oligosaccharide-(1-amino-GlcNAc) + Asp-peptide (1)

Oligosaccharide-(1-amino-GlcNAc) + H_2O ⟶
oligosaccharide-GlcNAc + NH_3 (2)

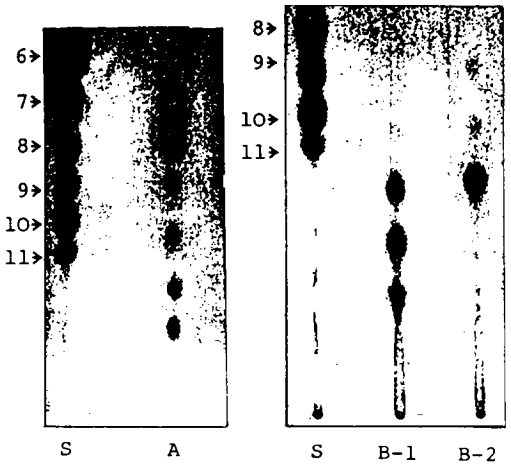

Fig. 1. Thin-layer chromatogram of the oligosaccharides released by jack bean glycopeptidase digestion. Ovalbumin or fibrinogen glycopeptides (0.2 μmol) were incubated with 100 μl of the glycopeptidase preparation in 0.01 M sodium-phosphate buffer (pH 6.5) containing 0.1 M NaCl, at 37°C for 5 h. Each one tenth of sample was applied to a silica gel 60 plate. The plate was developed at room temperature for 20 h with the following solvent system: n-propanol/acetic acid/water (3:3:2, v/v). Oligosaccharides were visualized with orcinol-H_2SO_4 reagent (12). A, from ovalbumin glycopeptide; B-1, from fibrinogen glycopeptide; B-2, from desialylated fibrinogen glycopeptide; S, mixture of standard glucose oligomers (vertical numbers indicate glucose units).

Step (1) is catalyzed by the enzyme and step (2) proceeds non-enzymatically at acidic conditions. The above equation was based on the following experimental facts.

1. Liberation of the Intact Oligosaccharides---When ovalbumin or fibrinogen glycopeptides were used as substrates, Fig. 1 shows a typical liberated oligosaccharide profile. Both high-mannose-type and hybrid-type oligosaccharides of ovalbumin (Fig. 1, lane A) were confirmed to be in accord with the standard ovalbumin oligosaccharide markers (4). Three spots of fibrinogen oligosaccharides on TLC (Fig. 1, lane B-1), corresponded to di-, mono-, and non-sialylated biantennary sugar chains, respectively (13, 14). Thus, both sialic-acid-containing sugar chains and desialylated sugar chains of fibrinogen were released.

2. Conversion of Asparagine Residue Involved in the Linkage Into Aspartyl Residue---When the stem bromelain glycopeptide (Asn-Asn(CHO)-Glu-Ser-Ser) was used as a substrate, the reaction mixture was subjected to paper electophoresis (Fig. 2). In the control experiment, almond glycopeptidase prepara-

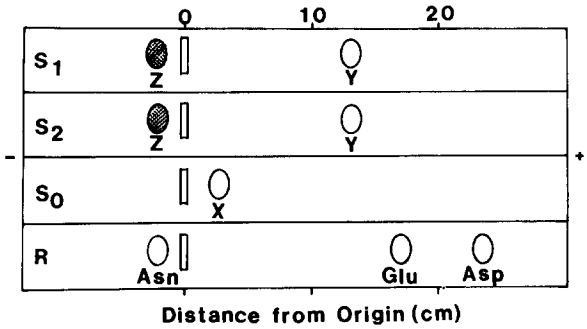

Fig. 2. Electorophoretogram of the glycopeptidase hydrolysate of stem bromelain glycopeptide. Stem bromelain glycopeptide (0.2 μmol of pentapeptide) was incubated with 50 μl of jack-bean glycopeptidase preparation in 0.01 M sodium-phosphate buffer (pH 6.5) containing 0.1 M NaCl, at 37°C for 5 h. The products were separated by paper electrophoresis at a potential of 50 V per cm for 1 h. The buffer was pyridine/acetic acid/water (5:0.2:95, v/v), pH 6.5 (1). The materials obtained were stained with the following reagents: dotted, periodate-benzidine; white, ninhydrin. S_1, stem bromelain glycopeptide incubated with jack-bean glycopeptidase; S_2, stem bromelain glycopeptide incubated with almond glycopeptidase (10 μl) under the same conditions; S_0, the glycopeptide treated with 50 μl of the boiled enzyme; R, reference amino acids. X, substrate glycopeptide. Y, peptide obtained. Z, oligosaccharide obtained.

tion was used instead of jack-bean enzyme solution. The product "Y" produced by jack-bean enzyme migrated to the anode further than the starting material "X" and showed a mobility equal to that of the peptide product of almond glycopeptidase. Strong acidity of the peptide "Y" means that the asparagine residue involved in cabohydrate-peptide linkage of the starting material was converted into aspartic acid concurrently with the removal of the carbohydrate moiety as elucidated previously in the case of almond enzyme (1). Peptide "Y" did not contain any glucosamine residue although its amino acid composition was identical with that of the original substrate. The neutral product "Z" was the oligosaccharide moiety. The oligosaccharide was composed of $GlcNAc_2Man_{2.5}Xyl_1Fuc_1$. It was confirmed that the enzyme solution yielded equimolar amounts of the peptide and the oligosaccharide.

3. Ammonia Release During the Incubation---In our preliminary experiment, ammonia production was observed in the reaction mixture containing the enzyme solution and glycopeptide. We investigated the time course of the production of both carbohydrate-free Asp-peptide and ammonia from stem bromelain

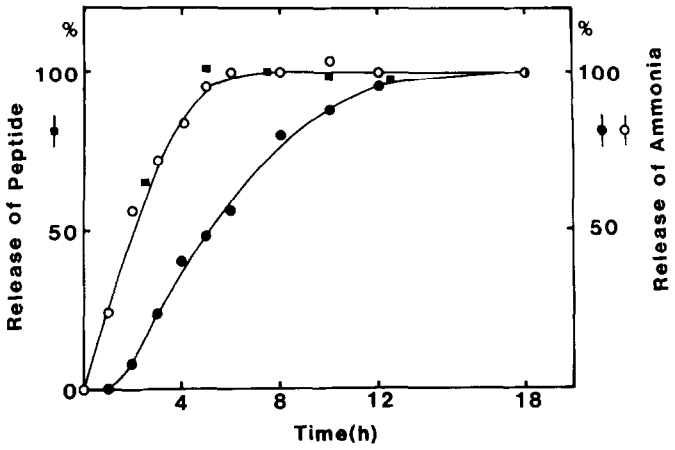

Fig. 3. Time course of the production of carbohydrate-free peptide and ammonia from stem bromelain glycopeptide by jack-bean glycopeptidase. Glycopeptide (0.06 μmol of penta-peptide) was incubated with 50 μl of the enzyme preparation in 0.01 M sodium-phosphate buffer (pH 6.5) containing 0.1 M NaCl. The amounts of released ammonia were determined as described in the text. Acid treatment was performed after the termination of the reaction, by a further 5 h of incubation at pH 2.0. The amount of released peptide was determined after paper electrophoresis of the reaction mixture, as described in the text. ■, peptide; ●, ammonia, immediately after termination of the reaction; o, ammonia, after acid treatment.

glycopeptide by glycopeptidase digestion (Fig. 3). Spontaneous ammonia liberation was delayed as compared to the production of Asp-peptide. After incubation for 18 h, however, equimolar amounts of Asp-peptide and ammonia were obtained. In another experiment, after terminating the reaction by adding 20 μl of 1 M HCl, the reaction mixture was incubated for an additional 5 h at pH 2.0 at 37°C. The released ammonia was then estimated. The amounts of ammonia liberated after the treatment were in good agreement with the amounts of Asp-peptide. A delay in ammonia liberation together with the fact that no activity of asparaginase was detected in the enzyme solution, supports the view that the enzyme is not an N-glycosidase, but an amidase.

Properties of the Jack-bean Glycopeptidase---The optimum pH of the glycopeptidase was at pH 6.5. The glycopeptidase activities towards several glycopeptides, such as from stem bromelain, ovalbumin and human fibrinogen, were investigated. The enzyme hydrolyzed glycopeptides with three or more amino acid residues, but not GlcNAc-Asn.

Jack-bean is a good source not only of glycopeptidase but also of several glycosidases. The elimination of the

contaminating α-mannosidase and β-galactosidase from jack-bean glycopeptidase preparation, therefore, is essential in the purification of the glycopeptidase. It remains to be demonstrated by more detailed examination, whether the enzyme acts on intact glycoproteins and whether the enzyme preparation shows a multiple form like almond enzyme. Works along these lines are in progress.

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