DEMONSTRATION OF A NEW AMIDASE ACTING ON GLYCOPEPTIDES

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SUMMARY

An enzyme preparation from almond emulsin cleaved the peptide-carbohydrate linkage of stem bromelain glycopeptide, Asn-Asn(oligosaccharide)-Glu-Ser-Ser. The resulting products were determined to be an intact peptide, Asn-Asp-Glu-Ser-Ser, and an intact oligosaccharide unit with two moles of N-acetylglucosamine. So far as tested the enzyme hydrolyzed glycopeptides with 3 to 10 amino acids, while both asparagine-oligosaccharide from ovalbumin and Asn-GlcNAc were not. Thus, the enzyme is a new amidase capable of hydrolyzing aspartylglycosylamine linkage in glycopeptides with multiple amino acid residues.

In many glycoproteins, the carbohydrate moiety is attached to the protein moiety by β-aspartylglycosylamine linkage. A kind of amidase is known to hydrolyze the linkage and is termed as 4-L-aspartylglycosylamine amidohydrolase [EC 3.5.1.37]. The reaction catalyzed by the enzyme is as follows (1):

Asn-GlcNAc + H_2O = Aspartic acid + 1-amino-N-acetylglucosamine

The 1-amino-N-acetylglucosamine is subsequently hydrolyzed non-enzymatically to yield N-acetylglucosamine and ammonia. The enzyme is specific to the substrates which contain both the free α-amino and the free α-carboxyl groups of aspartic acid involved in the linkage. In other words, the amidase cleaves the linkage in asparagine-oligosaccharides, but can not act on glycopeptides which have more than two amino acid residues.

The present report demonstrates, for the first time, novel type of amidase that cleaves β-aspartylglycosylamine linkage in

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Abbreviation : Asn-GlcNAc, 2-Acetamido-1-N-(4-L-asparty1)-2-deoxy-β-D-glucopyranosylamine.

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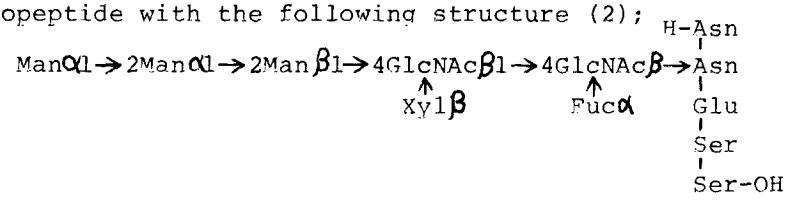
glycopeptides with both oligosaccharide and oligopeptide moieties. The enzyme is expected to be a valuable tool for structural analysis of glycoprotein.

The substrate used to detect the enzyme was stem bromelain glycopeptide with the following structure (2);



MATERIALS AND METHODS

Glycopeptides from stem bromelain were prepared in our laboratory as described previously (3). One of the glycopeptides was N-[^{14}C]acetylated in its amino-terminal residue with [^{14}C]-acetic anhydride (29 mCi/mmol, Radiochemical Centre, Amersham) according to Koide and Muramatsu (4). Asparagine-oligosaccharide from ovalbumin was prepared by the method of Yamashina and Makino (5). The average composition was as follows: Asp, 1; GlcNAc, 3; and Man, 5. Asn-GlcNAc was kindly donated by Dr. I. Yamashina. Almond emulsin (β-glucosidase) was purchased from Sigma Chemical Co.

Amino acid analysis of peptides was carried out with a Hitachi KLA-3B amino acid analyzer (6). Asparagine was determined also using the amino acid analyzer without acid hydrolysis by lithium citrate buffer method (7). The amount of N-acetylglucosamine in the oligosaccharide was determined by Morgan-Elson reaction after hydrolysis in 2N HCl at 100 °C for 12 h (8). Paper electrophoresis was carried out at a potential of 50 volts per cm for 1 h with pyridine-acetic acid-water (5 : 0.2 : 95 v/v), pH 6.5 as a buffer. For descending paper chromatography, 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12 v/v) was used as a solvent. Periodate-benzidine method was used for detection of sugars on papers (9).

For the enzyme preparation, 50 mg of almond emulsin was dissolved in 1 ml of 0.01 M acetate buffer, pH 5.0, containing 0.1 M NaCl. The crude extract was applied to a column of Sephadex G-200 (2.2 x 30 cm) equilibrated with 0.01 M acetate buffer, pH 5.0, containing 0.1 M NaCl, and was eluted with the same buffer. Most of the amidase activity was eluted in the preceding fractions of major protein peak (from 60 ml to 68 ml). It was pooled and was concentrated to 1.8 mg/0.9 ml by dialysis against polyethylene glycol 20,000.

The enzyme preparation could be stored at 4 °C for at least several weeks without loss of activity.

In order to determine the amidase activity, 0.1 μmol of a stem bromelain glycopeptide [$(Man)_3(Xyl)_1(Fuc)_1(GlcNAc)_2(Asn)_2(Glu)_1(Ser)_2$] was digested with the amidase preparation (4 to 20 μg of protein) in 30 μl of 0.2 M acetate buffer, pH 5.0, for 20 h. The products were analyzed by paper electrophoresis.

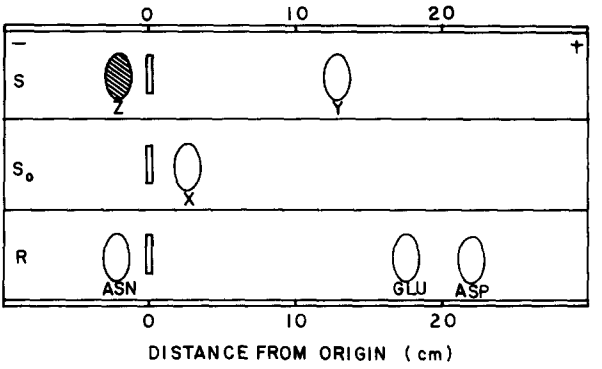


Fig. 1. Analysis of the enzymatic hydrolysate of stem bromelain glycopeptide.
S: The bromelain glycopeptide (0.1 μmol) digested with 10 μg of the amidase as described in "Materials and Methods".
S_0: The glycopeptide treated with 10 μg of the boiled enzyme.
R: Reference amino acids.
The samples were applied to a sheet of paper and were analyzed by paper electrophoresis at pH 6.5 at a potential of 50 volts/cm for 1 h.
X: The original glycopeptide.
Y: The peptide product detected by ninhydrin staining.
Z: The oligosaccharide detected with periodate-benzidine.
O: The origin.

RESULTS AND DISCUSSION

A partially purified preparation of an amidase was obtained from almond emulsin as described in "Materials and Methods". This amidase preparation cleaved the aspartylglycosylamine linkage in a stem bromelain glycopeptide as follows.

Asn-Asn(oligosaccharide)-Glu-Ser-Ser ⟶ Asn-Asp-Glu-Ser-Ser + oligosaccharide

This conclusion was based on the following experimental facts. After the reaction mixture was subjected to paper electrophoresis at ph 6.5, ninhydrin staining revealed the release of a peptide component (Fig. 1). The product (designated as "Y" in the figure) showed a mobility different from that of the starting material. Amino acid composition of "Y" was determined after extraction from the electrophoretogram and hydrolysis with 2N HCl at 110 °C

Table I

Content of amino acid and glucosamine in the substrate and the product

	Molar ratio			
	Asp	Ser	Glu	GlcN
Substrate (bromelain glycopeptide)	2.1	1.9	1.0	2.0
Product (peptide Y)	1.9	2.1	1.0	0

Glutamic acid was taken as reference 1.0.

for 20 h. Although peptide Y lacked any glucosamine residue, its peptide composition was indistinguishable from that of the original substrate (Table I). The result confirmed that the enzyme was of novel specificity, since the amidase previously described could not release peptides (1) and the peptide-rich products released by an endo-β-N-acetylglucosaminidase should contain at least one glucosaminyl residue (4).

The next question was whether the asparagine residue involved in the linkage was conserved as asparagine or was converted to aspartyl residue after the reaction. In order to answer the question, 1 mg of peptide Y was digested with 40 μg of Pronase-P* in 100 μl of reaction mixture at pH 8.0 and 37 °C for 24 h. This procedure released about equimolar amounts of asparagine and aspartic acid from peptide Y. By end group analysis according to Iwanaga et al. (10), peptide Y was confirmed to have one asparagine as amino-terminal residue. The combined results indicated the amino acid sequence of peptide Y to be Asn-Asp-Glu-Ser-Ser. The

*The enzyme preparation contained no asparaginase activity.

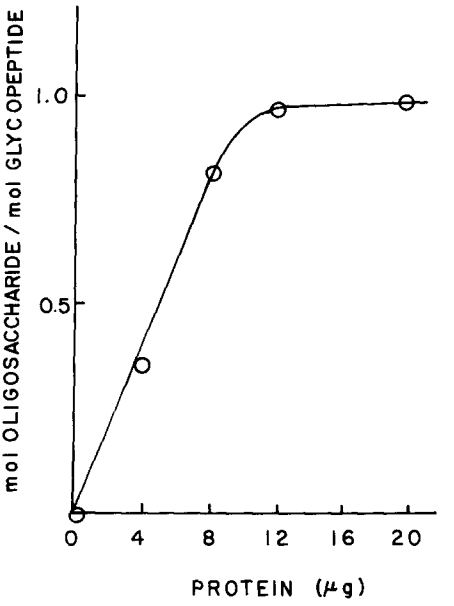


Fig. 2. Relationship between the amount of the enzyme and the enzymatic activity. The amidase and the stem bromelain glycopeptide were incubated as described in "Materials and Methods". The amount of the oligosaccharide released was determined by measuring the content of glucosamine in component Z after extraction from the electrophoretogram and hydrolysis with 2N HCl at 100 °C for 20 h.

original glycopeptide has two asparagine residues, one at the amino terminal and the other at the branching point to the carbohydrate moiety. Therefore, the asparagine residue involved in protein-carbohydrate linkage was concluded to be converted into aspartic acid concurrently with the removal of the carbohydrate moiety.

The other part of the products, namely the sugar moiety, was recovered as neutral material (component "Z" in Fig. 1) after paper electrophoresis. When this material was extracted and was analyzed by paper chromatography, it behaved as an oligosaccharide scarcely migrating from the origin. No release of monosaccharides

Table II

Substrate specificity of the amidase

Expt no.	Substrates	Per cent hydrolysis of the substrates
1	oligosaccharide[a] Asn-Asn-Glu-Ser-Ser	100
2	oligosaccharide[a] Asn-Glu-Ser	107
3	Ala-Arg-Val-Pro-Arg-oligosaccharide[a] Asn-Asn-Glu-Ser-Ser	4.3
4	N-[^{14}C]acetyl-oligosaccharide[a] Asn-Glu-Ser	0
5	Asn-oligosaccharide[b]	1
6	Asn-GlcNAc	0.3

The amidase (10 μg) and the substrates were incubated as described in "Materials and Methods". The amount of substrates was 0.1 μmol, except for the case of Expt 4 (0.68 nmol, 4 x 10^3 cpm). The amount of released products was measured as follows. In Expt 1, 2 and 3, the released peptides were isolated by paper electrophoresis and were determined by amino acid analysis after acid hydrolysis. In Expt 4, the reaction mixture was analyzed by paper electrophoresis at pH 6.5, and the possible release of the [^{14}C]acetylated peptide was surveyed by liquid scintillation counting. In Expt 5 and 6, the possible release of asparagine or aspartic acid was determined by amino acid analysis.
a): $(Man)_3(Xyl)_1(Fuc)_1(GlcNAc)_2$, from stem bromelain (2).
b): $(Man)_5(GlcNAc)_3$, from ovalbumin (11).

were detected except for trace amounts of xylose liberated by contaminating β-xylosidase.

The amount of the oligosacchride was determined by measuring the content of glucosamine in component Z after hydrolysis with 2N HCl at 100 °C for 12 h, and the amount of peptide Y by amino acid analysis. By such procedures it was confirmed that 10 μg of the

enzyme completely hydrolyzed 0.1 μmol of the substrate at the assay condition described in "Materials and Methods", and yielded equimolar amount of the oligosaccharide and the peptide.

Although the evidence is not obtained yet, that 1-amino-N-acetylglucosamine is formed at the reducing terminal of the oligosaccharide produced, these experimental data support the contention that the enzyme is not an N-glycosidase but an amidase.

Dose-response curve of the enzymatic reaction was obtained by the measurement of the released oligosaccharide (Fig. 2). Linear relationship between the amount of the enzyme and the enzymatic activity was observed up to 80 % hydrolysis. The maximum activity was at pH 5.0.

The enzyme also hydrolyzed the glycopeptide with three residues of amino acids and that with ten residues of amino acids, although the velocity was low in the latter case (Table II). The enzyme did not hydrolyze the acetylated glycopeptide, asparagine-oligosaccharide from ovalbumin and Asn-GlcNAc. Thus, the enzyme appeared to have some sort of specificity at least with respect to the structure of the peptide moiety of the substrates. The structural basis of the specificity is now under investigation, using various glycopeptide preparations as substrates.

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