

Note

Identification of the Glycosylation Site of a Major Soybean Allergen, *Gly m* Bd 30KNoriko BANDO, Hideaki TSUJI,[†] Rintaro YAMANISHI, Noriki NIO,* and Tadashi OGAWA

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A major soybean allergen, *Gly m* Bd 30K, is a glycoprotein. A peptide containing a sugar chain was isolated from an α -chymotrypsin hydrolyzate of the allergen. The amino acid sequence analysis of the peptide showed that its sugar chain binds to the Asn¹⁷⁰ residue. Furthermore, the sugar moiety of the allergen and peptide was shown to consist of mannose, *N*-acetylglucosamine, fucose, and xylose at a molar ratio of 3:2:1:1. These results indicate that *Gly m* Bd 30K is a *N*-linked glycoprotein.

Key words: soybean protein; soybean allergen; *Gly m* Bd 30K; 34 kDa oil-body-associated protein; soybean glycoprotein

In the systematic investigation of allergens in soybeans, we have found 16 allergenic proteins.¹⁾ Among them, a protein with a molecular mass of 30 kDa, named *Gly m* Bd 30K, was shown to be one of the major soybean allergens and to be identical with soybean 34-kDa oil-body-associated protein or P34.²⁾ The amino acid sequence of P34 had been deduced from the nucleotide sequence of a cDNA encoding P34.^{3,4)}

In the course of the studies on the allergenicity of this protein, we demonstrated that the protein was positive to periodate Fuchsin reagent.²⁾ As the amino acid sequence of Asn-X-Thr (or Ser) is generally thought to be a *N*-glycosylation site of proteins, a sugar chain may link to the Asn¹⁷⁰ residue on P34 (Asn¹⁷⁰-Cys¹⁷¹-Thr¹⁷²). However, no characterization of the sugar components of P34 had been done. Lately, Komatsu *et al.*⁵⁾ described that the allergen could not be detected with peroxidase-conjugated concanavalin A, and might not contain any *N*-linked sugar chain. To analyze the allergenicity of *Gly m* Bd 30K, we must characterize the sugar moiety of the allergen. In this paper, we describe the identification of the glycosylation site of *Gly m* Bd 30K and its sugar composition.

Gly m Bd 30K was prepared in the same manner as described previously.²⁾ For the isolation of the sugar-chain-containing peptide fragment(s), the allergen in 0.5 M Tris-HCl buffer (pH 8.5) including 6 M guanidine and 10 mM EDTA was reduced with

dithiothreitol and carboxymethylated with iodoacetic acid. The carboxymethylated *Gly m* Bd 30K (CM-*Gly m* Bd 30K) was heated for 5 min in a boiling water bath and then digested at 30 °C for 5 h with α -chymotrypsin (Sigma, U.S.A.). The ratio of the protease to CM-*Gly m* Bd 30K was 1:200 (w/w). The hydrolyzates were separated by HPLC with a Inertsil C4 column (GL Sciences, Japan) by elution with a linear gradient of acetonitrile (0–50%) in 0.05% trifluoroacetic acid. The eluate was fractionated at a interval of 2 min and the fractions were lyophilized by a centrifugal evaporator. Each fraction was examined with respect to sugar components by the phenol-sulfuric acid method, and a glycopeptide was isolated. The N-terminal amino acid sequence of the glycopeptide was analyzed by automated Edman degradation with an Applied Biosystems 477 protein sequencer.²⁾ The enzymatic deglycosylation of the glycopeptide was done in 50 mM citrate buffer (pH 5.0) at 30 °C for 5 h with glycopeptidase A from almond (Seikagaku Kogyo, Japan) and examined by the above-mentioned HPLC and amino acid sequencing. The sugar contents of *Gly m* Bd 30K and the isolated glycopeptide were measured as follows. Both of the materials were hydrolyzed in a mixture of 4 M trifluoroacetic acid and 4 M hydrochloric acid at 100 °C, for 4 h. After hydrolysis, the sugar components were *N*-acetylated and further converted to pyridylaminated derivatives. Authentic monosaccharides were pyridylaminated in the same manner as described above, and D-ribose was used as an internal standard during the acetylation and pyridylation of the sample and the authentic compounds. The pyridylaminated sugar components were analyzed by HPLC on a PALPAK column (Takara, Japan).

As described above, *Gly m* Bd 30K is a glycoprotein. Therefore, the carbohydrate components of the allergen were investigated. The allergen was shown to consist of mannose, *N*-acetylglucosamine, fucose, and xylose at a molar ratio of 3:2:1:1 (Table). These results suggest that the sugar chain might link to an Asn residue of the allergen. To identify the glycosylation site(s) on *Gly m* Bd 30K, the reduced and carboxymethylated allergen was digested with α -chymotrypsin. The peptides formed were fractionated by HPLC and the only peptide-containing sugar moiety was shown to be eluted at a retention time of 40.6 min (Fig. A). The carbohydrate composition of the glycopeptide was identical with that of the original allergen (Table). Furthermore, the amino acid sequence of the glycopeptide was TGGIYDGE-CTSPY, which corresponds to the sequence Thr¹⁶²-Tyr¹⁷⁵ of the allergen. The amino acid residue of the isolated peptide corresponding to Asn¹⁷⁰ could not be detected, but the Asn¹⁷⁰ residue was confirmed to exist in the deglycosylated peptide. Whether the sugar chain of the glycopeptide is eliminated by glycopeptidase A was investigated. While the original glycopeptide was eluted at a retention time of 40.6 min by HPLC (Fig. B), the peak of the enzyme-treated peptide was observed at a retention time of 44.6 min (Fig. C) suggesting that the hydrophilic sugar

Table Carbohydrate Compositions of *Gly m* Bd 30K and the Isolated Glycopeptide

Monosaccharide	<i>Gly m</i> Bd 30K	Glycopeptide
Mannose	3 ^a	3
<i>N</i> -Acetylglucosamine	2	2
Xylose	1	1
Fucose	1	1
<i>N</i> -Acetylgalactosamine	n.d. ^b	n.d.
Glucose	n.d.	n.d.

^a The values express the ratios of sugar components.

^b Not detected.

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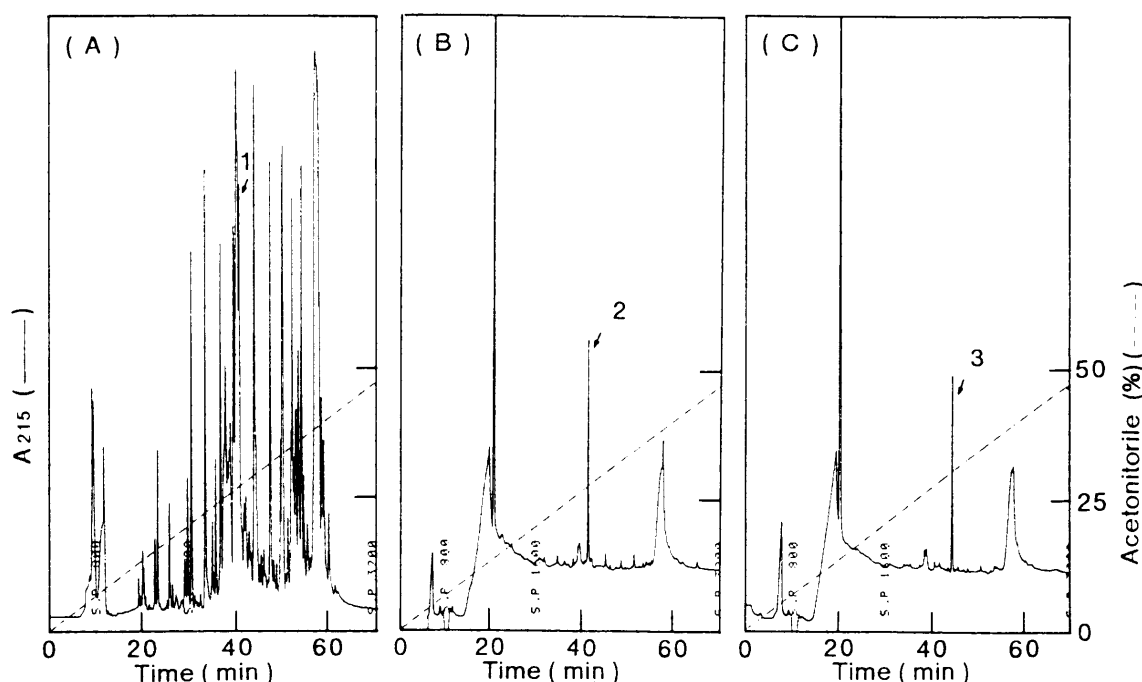


Fig. Elution Patterns of the Peptides (A) Obtained by Digestion of the Allergen with α -Chymotrypsin, the Isolated Glycopeptide (B), and the Peptide (C) Derived from the Glycopeptide by the Treatment with Glycopeptidase A on an Inertsil C4 Column.

1, a glycopeptide in the peptides obtained from the allergen; 2, the isolated glycopeptide; 3, the deglycosylated peptide.

moiety was removed from the peptide. The carbohydrate components derived from the glycopeptide were eluted in a fraction at retention times between 8 and 10 min, and no carbohydrate component could be detected in the other fractions including the deglycosylated peptide. The above findings show that the glycosylation site of the allergen is only one and that the sugar chain links to Asn¹⁷⁰. Komatsu *et al.*⁵⁾ reported the failure to detect *N*-linked sugar components by staining with peroxidase-conjugated concanavalin A and supposed that the allergen might not contain any *N*-linked sugar component. We tried to stain the allergen with the peroxidase-conjugated concanavalin A and observed a very weak reactivity of the allergen with concanavalin A. Our observations clearly verify the idea that the glycosylation site of P34 may be the Asn¹⁷⁰ residue. The analysis of the carbohydrate compositions of the allergen and the isolated glycopeptide suggests that the structure of the sugar chain moiety of the allergen may be similar to those of phytohemagglutinin, *Ricinus communis* agglutinin, horseradish peroxidase, and carrot cell wall β -fucosidase.⁶⁾ However, the exact structure of the sugar chain of the allergen should be further analyzed.

Recently, the α 1,3-fucosylation of the asparagine-bound *N*-acetylglucosamine of bromelain and phospholipase A₂ has been demonstrated to be an important epitope that is recognized by IgE antibodies obtained from the sera of allergic patients.⁷⁾ It is of interest to examine whether the fucosyl group in *Gly m Bd* 30K

can act as an epitope recognized by IgE antibodies in sera of the soybean-sensitive patients. At present, the studies on the allergenicity of the sugar chain moiety of the allergen are in progress.

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