Characterization of Carbohydrate–Peptide Linkage of Acidic Heteroglycopeptide with Immuno-Stimulating Activity from Mycelium of *Phellinus linteus*

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The carbohydrate–peptide linkage in acidic heteroglycopeptide from *Phellinus linteus* was characterized. Amino acid analysis showed large amounts of serine and threonine. β-Elimination results in the reduction of serine and threonine and a subsequent increase in alanine after reduction. These results indicated the presence of *O*-type linkage in the polymer.

Key words *Phellinus linteus*; *O*-type linkage; β-elimination

Many polysaccharides with anti-tumor and immuno-stimulating activity have been purified from the mycelium, fruiting body, and culture medium of fungi.[2)] Most of them were found to be β-1,3-glucans with β-1,6-glucose as a side chain.[3)] Other polysaccharides have been identified as polysaccharide–protein complexes.[4)] *Phellinus linteus* is a member of Basidiomycetes, which belongs to the family Polyporaceae. It was reported that a hot-water extract of the fungus showed 96.7% growth inhibition of Sarcoma 180 transplanted in mice.[5)] After our success in cultivation of the fungus, we purified an active polysaccharide from the cultured mycelium.[6)] Partial characterization showed that the polysaccharide is composed of polysaccharide and peptide. A detailed study of its structure is in progress. Here we report the carbohydrate–peptide linkage type.

Materials and Methods

Isolation of Polysaccharide *P. linteus* was cultivated in peptone-yeast-extract-glucose (PYG) medium and mycelial polysaccharide was purified as described previously.[6)] Fraction III (F-III) showed the highest immuno-stimulating activity in plaque-forming cell assay. This fraction was used for the study of the carbohydrate–peptide linkage.

Gel Filtration Native F-III (20 mg) was applied to a column (2 × 52 cm) of Toyopearl HW-65F gel and eluted with 0.1 M NaCl. The polysaccharide after β-elimination treatment (see below) was loaded on a column (2 × 52 cm) of Toyopearl HW-40S and eluted with distilled water. Carbohydrate and protein were assayed by the phenol–sulfuric acid method[7)] and the Bradford method,[8)] respectively.

Amino Acid Analysis The sample was treated with constant-boiling HCl for at 110 °C for 24 h. The HCl was removed by repeated evaporation to dryness at 35 °C. Remained HCl was removed by addition of MeOH–water–triethylamine (2 : 2 : 1, v/v) followed by evaporation to dryness under vacuum. The hydrolyzed amino acids were derivatized with phenyl isothiocyanate and analyzed on a Pico Tag free amino acid analysis column (3.9 × 300 mm) (Waters, U.S.A.).

β-Elimination A sample of F-III (20 mg) was incubated with 5 ml of 0.3 M $NaBH_4$ in 0.4 M NaOH. The reaction was carried out at 25 °C in an atmosphere of N_2. After 48 h, the reaction was terminated by adjusting the pH to 4.5 with acetic acid and the mixture was evaporated to dryness.

Results and Discussion

In gel filtration chromatography of the intact F-III, polysaccharide and protein were coeluted (Fig. 1). This result suggested that the F-III is a glycopeptide. Also, the amino acid analysis of F-III indicated the absence of asparagine. Instead, large amounts of serine and threonine, which are the most common amino acids in *O*-glycosyl proteins, were found in this fraction (Table 1). This result suggested that the carbohydrate moieties might be linked to the peptide through the hydroxyl groups of these amino acids.

Glycoproteins may contain *N*-type or *O*-type linkages.[9)] The only *N*-glycosidic bond presently known in glycoproteins is *N*-acetylglucosaminyl-asparagine (GlcNAc(β1-*N*)Asn), whereas *O*-glycosidic moieties have a wide variety of linkages.[10)] *O*-Substituted seryl and threonyl residues are known to undergo β-elimination in the presence of strong alkali, resulting in the formation α-aminoacrylic acid and α-aminocrotonic acid, respectively.[11)] In order for β-elimination to occur, both the amino group and the carboxyl groups of the hydroxyl-substituted seryl or threonyl residue must be substituted.[12)]

A known procedure[13)] was employed to investigate the involvement of serine and threonine in the carbohydrate–peptide linkages. The dehydro-amino acids, which result

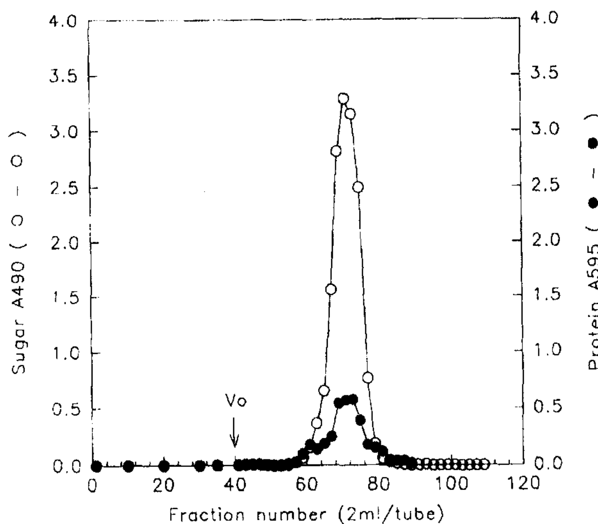

Fig. 1. Gel Filtration of the Intact F-III on a Toyopearl HW-65F Column

The column was eluted with 0.1 M NaCl. Sugar and protein were assayed by the phenol–sulfuric acid method and the Bradford method, respectively.

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Table 1. Amino Acid Composition of the Glycopeptide from *P. linteus*

Amino acid	Amount (%)
Gly	13.07
Glu	12.20
Ser	11.32
Thr	10.76
Ala	9.51
Asp	9.16
Pro	7.09
Lys	5.41
Val	5.07
Leu	4.67
Ile	2.87
Tyr	2.68
His	2.47
Phe	1.86
Met	0.89
Trp	0.42
Cys	0.39
Arg	0.17

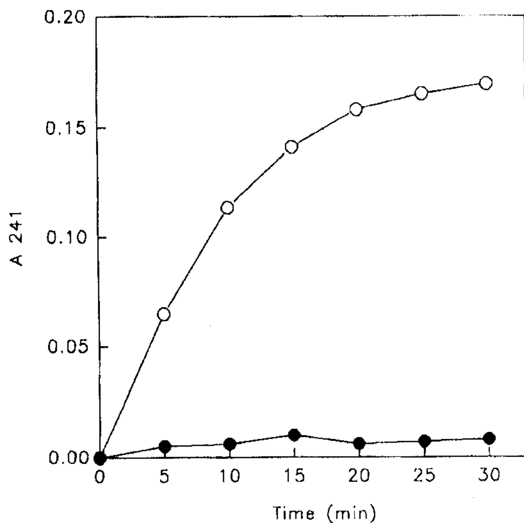

Fig. 2. Changes in A_{241} upon Alkaline Borohydride Treatment of F-III

○, F-III after β-elimination; ●, control (serine and threonine only).

from β-elimination, absorb strongly at 241 nm and have the same extinction coefficient at this wavelength.[11] The absorbance at 241 nm increased sharply when the fraction was subjected to β-elimination (Fig. 2). No increase in absorbance at 241 nm was observed when either serine or threonine was treated in the same manner. The formation of α-aminoacrylic acid and α-aminocrotonic acid from β-elimination of hydroxyl-substituted seryl and threonyl residues can also be demonstrated after reduction of the dehydro-amino acids to alanine and α-aminobutyric acid, respectively.

The change in the amino acid composition of the polymer after this treatment was determined by amino acid analysis. The reduction of serine and threonine were observed (Table 2), accompanied by increases in alanine and α-aminobutyric acid. The lack of stoichiometry may be attributed to incomplete reduction and losses resulting from destruction during hydrolysis.

Table 2. Changes in Amino Acid Composition Following Alkaline Borohydride Treatment of Glycopeptide (F-III)

Amino acid	Untreated polymer	Alkaline borohydride-treated polymer	Increase or decrease
Serine	11.32	3.88	(−) 7.44
Threonine	10.76	0.98	(−) 9.78
Alanine	9.51	16.63	(+) 7.12
α-Aminobutyric acid	0	4.88	(+) 4.88

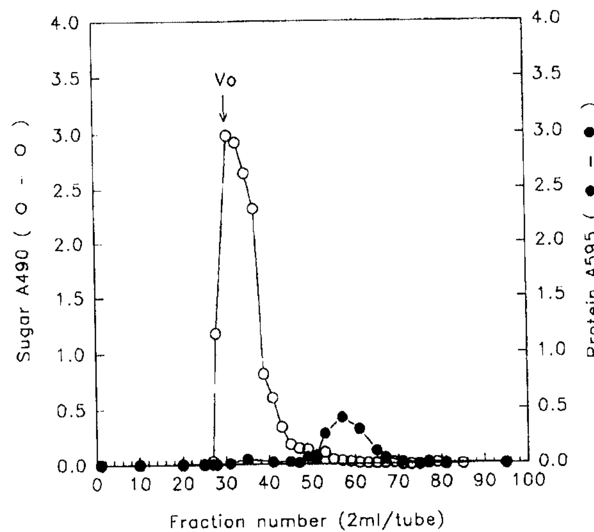

Fig. 3. Gel Filtration of the β-Elimination-Treated F-III on a Toyopearl HW-40S Column

The column was eluted with water. Sugar and protein were assayed by the phenol-sulfuric acid method and the Bradford method, respectively.

The alkali-labile nature of carbohydrate peptide linkage was further demonstrated by the release of the peptide from the carbohydrate by alkaline borohydride treatment. After β-elimination, the residue was dissolved in 2 ml of water, then the solution was subjected to Toyopearl HW-40S gel permeation chromatography. The column was eluted with distilled water. The protein portion was eluted separately from the polysaccharide (Fig. 3). This fact indicated that the peptide had been released from the carbohydrate. On the basis of the elution time, the peptide seemed to be a small molecule, though backbone cleavage of the protein can occur in β-elimination.[14] The protein content in the polysaccharide moiety of the alkali-treated F-III was as low as that in a protease E-treated fraction (data not shown). Thus, most of the protein might be removed by this β-elimination treatment. After the hydrolysis and acetylation of the treated F-III, the sugar alditol acetate was analyzed by gas chromatography. A small amount of mannitol acetate was detected (data not shown). The result indicated that the reducing terminal sugar is mannose.

The previous result showed that the peptide moiety did not contribute to the immuno-stimulating activity.[6] Instead, the carbohydrate moiety is essential for the activity. The peptide could act as a pyrogen and an inducer of shock. The removal of the peptide from the F-III is

thus a prerequisite for intravenous administration of the fraction as an injection. It would also afford a better-defined compound.

Two cases have been reported in which a peptide moiety is necessary for the activity. In the case of a β-1,6-glucan containing protein from *Agaricus blazei*, the anti-tumor activity was expressed as a result of binding of the polysaccharide and peptide portions in a polysaccharide-protein complex.[15] In *Coriolus versicolor*, the protein component of PSK is reportedly essential for anti-tumor activity.[16] The structure of the polysaccharide component in PSK was elucidated,[17] but the chemical bond between the protein component and polysaccharide, and its role in the anti-tumor activity remain unclear.

In conclusion, the immuno-stimulating glycopeptide from *P. linteus* is of *O*-linked type. Serine and threonine were found to be involved in the linkage. Alkaline treatment of glycopeptide could remove the peptide, which is not required for the activity.

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References and Notes

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