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Structure of asparagine-linked oligosaccharides of an aspartic proteinase from the zygomycete fungus *Rhizomucor pusillus*

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The zygomycete fungus *Rhizomucor pusillus* (previously called *Mucor pusillus*) secretes an aspartic proteinase containing two asparagine-linked, high-mannose type oligosaccharide chains at Asn^{79} and Asn^{188}. For structural elucidation of the carbohydrate moieties, the protein was divided into two portions, an N-terminal portion containing Asn^{79} and a C-terminal portion containing Asn^{188}, by a specific autocatalytic cleavage under alkaline conditions. Each of the asparagine-linked oligosaccharides was then released by peptide-*N*-glycosidase F digestion and pyridylaminated with a fluorescent reagent, 2-aminopyridine, at the reducing end. High-performance liquid chromatography analyses showed that the structure of the asparagine-linked oligosaccharide chain attached to residue Asn^{79} was $Man_5GlcNAc_2$, and that bound to residue Asn^{188} was $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$. These observations suggest that the processing of mannose residues in asparagine-linked oligosaccharides in the Golgi apparatus of *Rhizomucor* resembles that in mammalian cells.

Keywords: asparagine-linked oligosaccharide, *Rhizomucor pusillus*, zygomycete, aspartic proteinase

INTRODUCTION

The zygomycete fungus *Rhizomucor pusillus* (previously called *Mucor pusillus*) secretes a characteristic aspartic proteinase with high milk-clotting activity along with relatively low proteolytic activity (Arima *et al.*, 1967, 1968). This proteinase, called *R. pusillus* pepsin (MPP), is widely used as a milk coagulant in industrial cheese production. We previously reported the cloning and sequencing of the MPP structural gene from *R. pusillus* IFO4578 (Tonouchi *et al.*, 1986) and its expression in *Saccharomyces cerevisiae* (Yamashita *et al.*, 1987) and *Aspergillus oryzae* (Murakami *et al.*, 1993). Studies with these heterologous expression systems revealed that two asparagine residues, Asn^{79} and Asn^{188}, among the three possible *N*-linked glycosylation sites (Asn^{79}-Ile-Thr, Asn^{113}-Val-Ser and Asn^{188}-Asn-Thr in MPP) were actually glycosylated in *S. cerevisiae* (Aikawa *et al.*, 1990) and *A. oryzae* (Murakami *et al.*, 1993). In addition, we found that the MPPs produced by both recombinant *S. cerevisiae* (Aikawa *et al.*, 1990) and *A. oryzae* (Murakami *et al.*, 1993) were more glycosylated than that produced by *R. pusillus* due to different processing of mannose residues. The extra mannosylation of MPPs caused a decrease in milk-clotting activity along with an increase in proteolytic activity. Furthermore, deglycosylation of MPP by glycosidase treatment or by amino acid replacement of the glycosylation sites caused an increase in milk-clotting activity along with a decrease in proteolytic activity (Murakami *et al.*, 1993). The glycosylation of MPP thus causes distinct modulation of the enzymic properties, which indicates the functional importance of *N*-linked oligosaccharide chains of MPP. In addition to the effect of *N*-linked glycosylation on the properties of this industrially important enzyme, a slight difference in

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Abbreviations: PA, pyridylaminated; MPP, *R. pusillus* pepsin; Endo H, endo-β-*N*-acetylglucosaminidase H; PNGase F, peptide-*N*-glycosidase F; GU, glucose unit.

processing of *N*-linked glycans among various organisms, and even among the filamentous fungi, prompted us to determine the structure of *N*-linked oligosaccharides in MPP. In the present study we have characterized the carbohydrate moieties of MPP produced by *R. pusillus* IFO4578.

METHODS

Purification of MPPs. MPPs from *R. pusillus* strains IFO4578 and F27 were purified as previously described (Murakami *et al.*, 1993, 1994). Endo-β-*N*-acetylglucosaminidase H (Endo H; Seikagaku Kogyo)-treated MPP and recombinant yeast MPPs were purified as previously described (Aikawa *et al.*, 1990). Protein content of the purified MPP samples was estimated by measuring their absorbance at 280 nm with $A^{1\%}_{1\,cm} = 10$ (Arima *et al.*, 1968). Purified MPP was digested with Endo H and peptide-*N*-glycosidase F (PNGase F) (Takara Shuzo) by the method of Tarentino *et al.* (1974) and Chu (1986), respectively.

Carbohydrate composition. Purified MPP was hydrolysed at 100 °C for 12 h in 2·5 M trifluoroacetic acid. Carbohydrate compositions were determined by high-pH anion-exchange chromatography with a pulsed amperometric detection system (Dionex BioLC) (Hardy *et al.*, 1988). Mannose (Man), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose and galactose can be measured by this method.

N-terminal amino acid sequencing. Purified MPP was subjected to automated Edman degradation on an Applied Biosystems gas-phase sequencer equipped with an on-line amino acid phenylthiohydantoin analyser.

Pyridylamination. The oligosaccharides released from glycopeptides by PNGase F digestion were labelled with 2-aminopyridine by using a commercially available reagent kit (Takara Shuzo). The pyridylaminated derivatives (PA-oligosaccharides) were purified by Sephadex G-15 column chromatography.

High-performance liquid chromatography. PA-oligosaccharides were separated by HPLC using a chromatography system equipped with a fluorescence spectromonitor (Shimadzu, RF-550). For size-fractionation HPLC, PA-oligosaccharides were loaded onto a TSK gel amide-80 column (0·46 × 25 cm, Tosoh Corp.) equilibrated with a mixture of 3% acetic acid/triethylamine buffer (pH 7·3) and acetonitrile (35:65, v/v). The column was eluted at a flow rate of 1·0 ml min^{-1} at 40 °C by increasing the ratio of acetic acid/triethylamine buffer to acetonitrile from 35:65 to 50:50 over 60 min.

The reverse-phase HPLC was performed with a Nakanopak ODS-A column (6 × 150 mm) equilibrated with 10 mM sodium phosphate buffer (pH 3·8) containing 0·1% 1-butanol. The column was eluted at a flow rate of 1·0 ml min^{-1} at 55 °C by linearly increasing the 1-butanol concentration from 0·1% to 0·25% over 60 min. PA-oligosaccharides were detected by fluorescence using the excitation and emission wavelengths of 320 and 400 nm respectively. The glucose units were determined by comparing the retention time of each PA-oligosaccharide on both columns with those of the standard PA-glucose oligomers (Takara Shuzo) and plotting on a two-dimensional sugar map according to the method of Tomiya *et al.* (1988).

Partial acetolysis was carried out by the method of Hase *et al.* (1985). The partial acetolysates obtained from the PA-oligosaccharides as well as the standard oligomannose-type oligosaccharides (Takara Shuzo) were analysed by size-fractionation HPLC.

RESULTS

Carbohydrate composition of MPP

MPP contains two sugar chains at Asn^{79} and Asn^{188} (Aikawa *et al.*, 1990). Our previous studies using the MPP gene (Tonouchi *et al.*, 1986) in the hosts *Saccharomyces cerevisiae* (Aikawa *et al.*, 1990) and *Aspergillus oryzae* (Murakami *et al.*, 1993) showed that MPP also contains two high-mannose-type glycans at the same asparagine residues. To examine the presence of *O*-linked glycosylation in MPP in *R. pusillus* IFO4578, we removed the two *N*-linked oligosaccharides by Endo H treatment and prepared the protein by Mono Q anion-exchange column chromatography followed by Superose 12 gel-filtration column chromatography as described previously (Aikawa *et al.*, 1990). We then analysed the carbohydrate composition of the Endo H-treated MPP. Native MPP produced by strain IFO4578 contained nearly four (4·4 mol per mol protein) GlcNAc and ten (9·8 mol per mol protein) mannose residues, consistent with the previous data (Murakami *et al.*, 1994). On the other hand, the Endo H-treated MPP contained nearly two (2·3 mol per mol protein) GlcNAc residues and no mannose residues. Other sugars such as fucose, galactose and GalNAc were not detected in either the native or Endo H-treated MPP. These data show that MPP produced by *R. pusillus* IFO4578 contains two high-mannose-type glycans and no *O*-linked oligosaccharides.

Autocatalytic digestion of MPP

To analyse the Asn^{79}-linked and Asn^{188}-linked oligosaccharide chains separately, we first tried to digest the MPP protein produced by *R. pusillus* IFO4578 with restriction proteinases, such as arginyl-endopeptidase and lysyl-endopeptidase, to obtain peptides containing one of the two sugar chains. This approach did not give good separation of fragments. However, during these studies, we found that MPP was autocatalytically digested into two glycopeptides when concentrated MPP (1 mg ml^{-1}) was incubated at 37 °C for 12 h in 100 mM Tris/HCl buffer, pH 8·0. The autocatalytic cleavage of MPP also occurred with MPPs produced by the recombinant yeast and with the Endo H-digested MPP produced by *R. pusillus* under the same conditions (Fig. 1a). The glycosylation of MPP in two different *R. pusillus* strains, IFO4578 and F27, was assumed to be the same since the sizes of undigested MPP and digested fragments were the same.

To determine whether each of the two autolysed fragments contained a sugar chain, we further investigated these two glycopeptides by using MPPs produced by the recombinant yeast as references, because these were easily obtained in large quantities. The mutated MPPs used were: 79Q, in which one (Asn^{79}) of the glycosylation sites was replaced by Gln; 188Q, in which one (Asn^{188}) of the two glycosylation sites was replaced

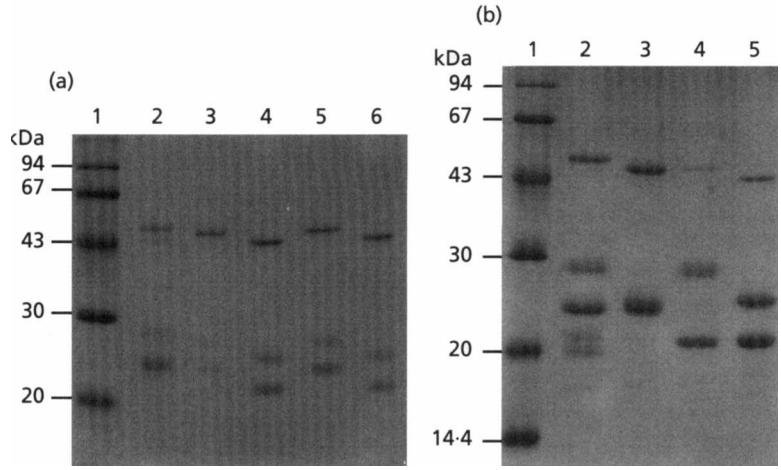

Fig. 1. Autocatalytic digestion of MPPs produced by recombinant yeast and *R. pusillus* (a) and mutated MPPs produced by recombinant yeast (b). (a) Each purified MPP (1 mg ml^{-1} in 100 mM Tris/HCl, pH 8·0) was incubated at 37 °C for 12 h and subjected to SDS-PAGE. Gels contained 1 μg protein per track, 12·5% acrylamide gels were stained with Coomassie brilliant blue R250. In addition to undigested MPP (43–46 kDa in size) in each lane, two autolysed fragments (between 22 and 28 kDa) are seen. Lane 1, size markers; lane 2, yeast MPP; lane 3, F27 MPP; lane 4, F27 MPP (Endo H treated); lane 5, IFO4578 MPP; lane 6, IFO4578 MPP (Endo H treated). (b) The mutated MPPs (1 mg ml^{-1} in 100 mM Tris/HCl, pH 8·0) were autodigested and subjected to SDS-PAGE as in (a). Lane 1, size markers; lane 2, yeast MPP (native); lane 3, yeast MPP (79Q); lane 4, yeast MPP (188Q); lane 5, yeast MPP (79Q188Q).

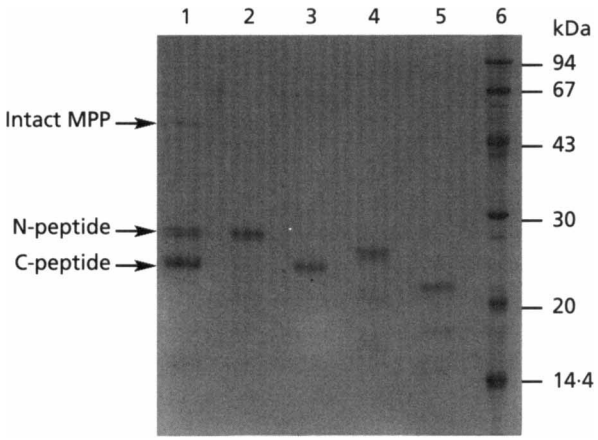

Fig. 2. Purification of N- and C-peptides of MPP produced by *R. pusillus* IFO4578. Both purified peptides and PNGase F-digested peptides were subjected to SDS-PAGE. Gels contained 1 μg protein per track, 12·5% acrylamide gels were stained with Coomassie brilliant blue R250. Lane 1, IFO4578 MPP (autolysed at pH 8); lane 2, N-peptide; lane 3, C-peptide; lane 4, N-peptide (PNGase F digested); lane 5, C-peptide (PNGase F digested); lane 6, size markers.

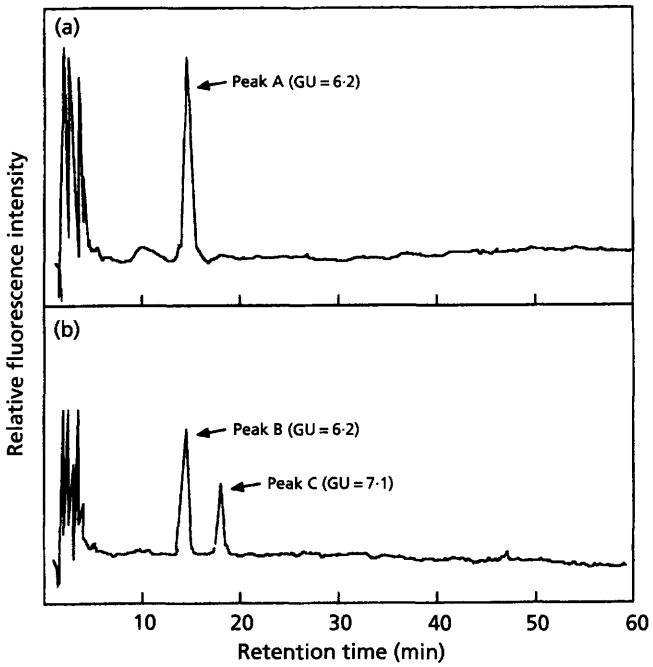

Fig. 3. Size-fractionation HPLC of PA-oligosaccharides from the N-peptide (a) and the C-peptide (b). GUs were determined using a PA-oligomer mixture as the standard.

by Gln; and 79Q188Q, in which both Asn residues were replaced by Gln. These were autocatalytically digested into two glycopeptides under alkaline conditions, as determined by SDS-PAGE (Fig. 1b). Comparison of the autolysed patterns of native MPP and 79Q predicted that the 28 kDa band (N-peptide) derived from native MPP contained a sugar chain attached to Asn^{79}. Similarly, the 22 kDa glycopeptide (C-peptide) was predicted to contain the Asn^{188}-linked oligosaccharide chain.

Isolation of glycopeptides from MPP

We purified the two glycopeptides (N-peptide and C-peptide) by Mono Q anion-exchange column chromatography followed by Superose 12 gel-filtration column chromatography. The purified glycopeptides and the peptides deglycosylated with PNGase F were analysed by SDS-PAGE (Fig. 2). We determined the N-terminal amino acid sequence of both glycopeptides and found that N-peptide had the sequence Ala-Glu-Gly-Asp-Gly, which was identical to the N-terminal amino acid sequence of the mature MPP produced by *R. pusillus* IFO4578 (Hiramatsu *et al.*, 1991). On the other hand, the C-peptide gave two N-terminal amino acid sequences, Ser^{170}-Val-Tyr-Met-Asn and Met^{173}-Asn-Tyr-Asn-Asp, which showed that MPP can be cleaved at

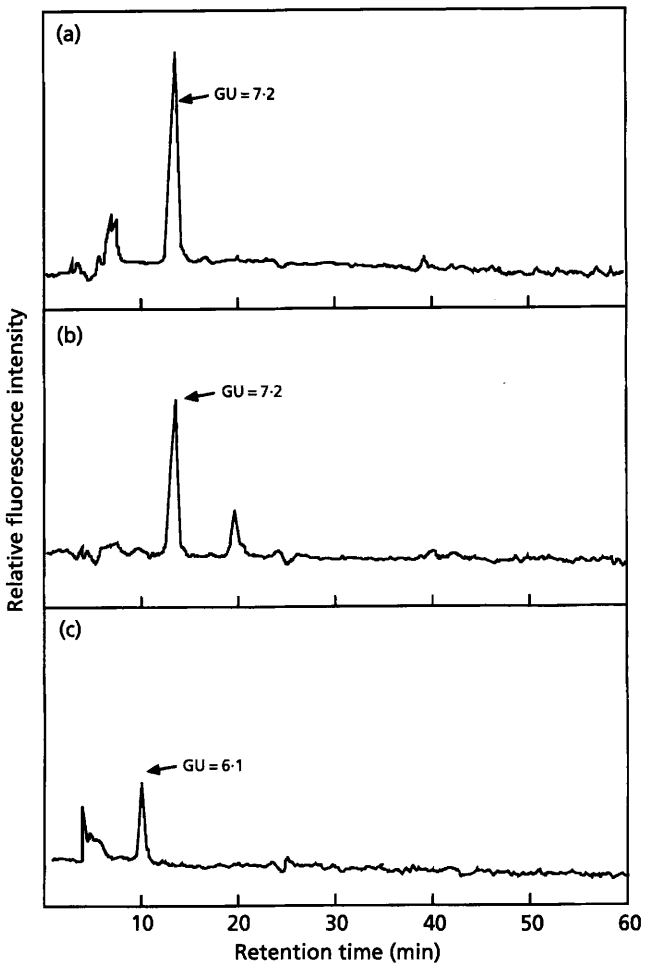

Fig. 4. Reverse-phase HPLC of peaks A (a), B (b) and C (c) obtained by size-fractionation HPLC. Peak A is derived from the N-peptide, peaks B and C are derived from the C-peptide. GUs were determined using a PA-oligomer mixture as the standard.

two sites, between Phe[169] and Ser[170] and between Tyr[172] and Met[173].

Structural analysis of the sugar chains in MPP

To elucidate the structure of the asparagine-linked oligosaccharide chains of MPP, the oligosaccharides were released from each of the N- and C-peptides by PNGase F digestion and modified by 2-aminopyridine at the reducing ends. Fig. 3 shows the elution profiles of PA-oligosaccharides from an amide silica column. The PA-oligosaccharides derived from the N-peptide showed a single peak (peak A) on size-fractionation HPLC (Fig. 3a). The PA-oligosaccharide in peak A showed a single peak on reverse-phase HPLC, which demonstrated the presence of a single component (Fig. 4). The glucose unit (GU) value was determined by comparing the retention time of peak A with those of the standard PA-glucose oligomers (Takara Shuzo). From the GU value of peak A on both the size-fractionation (GU = 6·2) and the reverse-phase HPLC (GU = 7·2), plotted on a two-

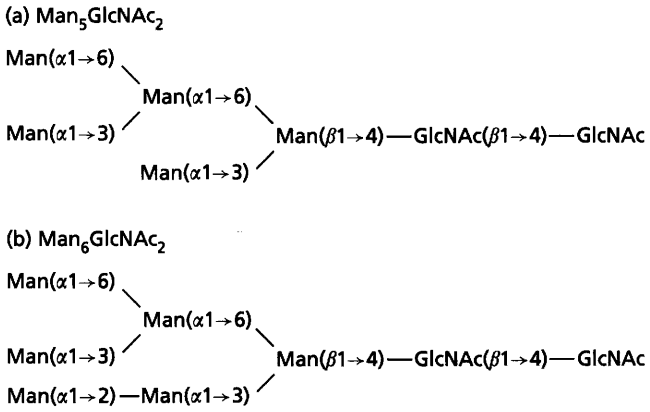

Fig. 5. Structures of the standard PA-oligosaccharides, $Man_5GlcNAc_2$ (a) and $Man_6GlcNAc_2$ (b), used as references for determination of GU values.

dimensional sugar map according to the method of Tomiya *et al.* (1988), the PA-oligosaccharide from the N-peptide corresponds to $Man_5GlcNAc_2$-PA. Furthermore, the elution position of peak A on both columns was the same as that of the standard oligomannose-type oligosaccharide, $Man_5GlcNAc_2$-PA. On the other hand, the PA-oligosaccharides from the C-peptide showed two major peaks [peaks B (GU = 6·2) and C (GU = 7·1)] on size-fractionation HPLC (Fig. 3b). These two peaks were collected as separate fractions and analysed by reverse-phase HPLC (Fig. 4). The GU values of peaks B and C on both the size-fractionation and reverse-phase HPLC were identical to that of $Man_5GlcNAc_2$-PA and $Man_6GlcNAc_2$-PA respectively. The elution positions of peak B and C were the same as those of standard oligomannose-type oligosaccharides $Man_5GlcNAc_2$-PA and $Man_6GlcNAc_2$-PA, respectively. The structures of the standard oligosaccharides are shown in Fig. 5. In addition, digestion of the PA-oligosaccharides eluted in peaks B and C by α-mannosidase yielded a new peak which appeared at the elution position of $Man_1GlcNAc_2$-PA on both reverse-phase and size-fractionation HPLC (data not shown).

The oligosaccharides in peak A and peak C on the reverse-phase HPLC (Fig. 4) were collected and further analysed by partial acetolysis. The fraction of the acetolysis product containing the reducing end residue from peak A, as well as a standard oligomannose-type oligosaccharide, $Man_5GlcNAc_2$-PA, were analysed by size-fractionation HPLC. The partial acetolysate obtained from peak A gave two major peaks, M2 and M4 (Fig. 6a, b). M2 and M4 corresponded to a tetrasaccharide and a hexasaccharide respectively, the retention times of which were identical to those of the partial acetolysate obtained from the standard oligosaccharide $Man_5GlcNAc_2$-PA (Fig. 5). The structures of sugars in peak M2 and M4 were thus assumed to be Manα1-3Manβ1-4GlcNAc$_2$-PA and Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAc$_2$-PA respectively. The partial acetolysates obtained from peak C yielded two

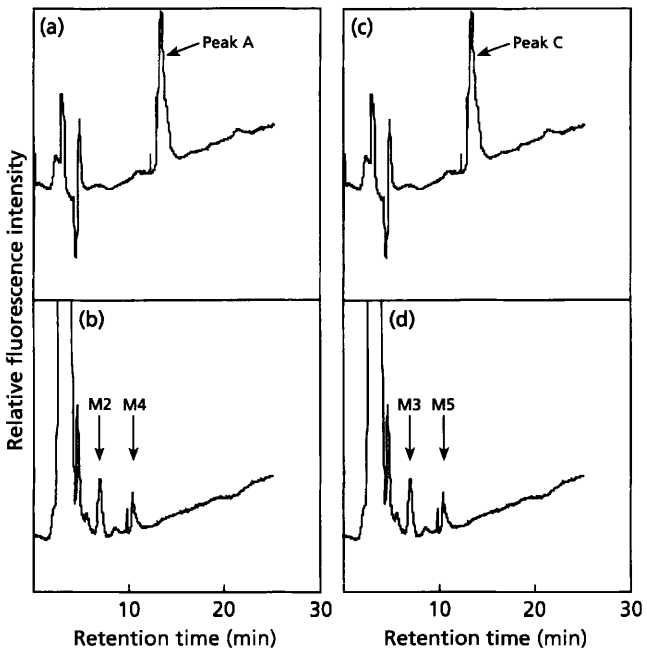

Fig. 6. Size-fractionation HPLC of PA-oligosaccharide products derived from peaks A and C of fig. 4 by partial acetolysis. (a) PA-oligosaccharide obtained from peak A of fig. 4. (b) Partial acetolysates of PA-oligosaccharides obtained from peak A. (c) PA-oligosaccharide obtained from peak C. (d) Partial acetolysates of PA-oligosaccharides obtained from peak C.

major peaks, M3 and M5 (Fig. 6c, d). M3 and M5 showed sizes corresponding to a pentasaccharide and a heptasaccharide, respectively, the elution positions of which were identical to those from the standard oligosaccharide $Man_6GlcNAc_2$-PA (Fig. 5). The sugars in peaks M3 and M5 were therefore assumed to be Manα1-2Manα1-3Manβ1-4GlcNAc$_2$-PA and Manα1-3Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc$_2$-PA respectively. All of these data indicate that the *N*-linked oligosaccharide chain bound to residue Asn^{79} was $Man_5GlcNAc_2$ and that bound to residue Asn^{188} was $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$.

DISCUSSION

In this study, we characterized the carbohydrate moiety of MPP produced by *R. pusillus* IFO4578. Consistent with the carbohydrate composition of MPP, Asn^{79} was found to bind a sugar chain of $Man_5GlcNAc_2$ and Asn^{188} was found to bind two different sugar chains of $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$. The synthesis of the *N*-linked oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$-dolichol) and the initial processing of the oligosaccharide in the endoplasmic reticulum are similar in fungi and most other eukaryotes (Kukuruzinska *et al.*, 1987), while the later trimming steps in the Golgi apparatus are different. Our preliminary analysis using oligosaccharide processing inhibitors, such as castanospermine, 1-deoxynojirimycin and N-methyldeoxynojirimycin, suggest that a sugar chain larger than $Man_{5-6}GlcNAc_2$ is transferred to MPP in *R. pusillus*, because MPP accumulated in the presence of these processing inhibitors had a lower mobility than mature MPP on PAGE (unpublished data). In the Golgi apparatus of mammalian cells, $Man_8GlcNAc_2$ is trimmed to $Man_5GlcNAc_2$ by Golgi α-mannosidase I and further processed by a series of glycosidases and glycosyltransferases. On the other hand, in *S. cerevisiae*, $Man_8GlcNAc_2$ is thought to be the ultimate product of trimming (Kukuruzinska *et al.*, 1987), suggesting that yeast lacks an enzyme like Golgi α-mannosidase I. The processing of mannose residues in *N*-linked glycans in *R. pusillus* resembles that in mammalian cells (Kornfeld & Kornfeld, 1985), suggesting that the zygomycete fungus *R. pusillus* possesses a specific α-mannosidase similar to the mammalian Golgi α-mannosidase I.

We previously showed that MPP produced by the recombinant *Aspergillus oryzae* was more highly glycosylated than that produced in the original *R. pusillus* strain (Murakami *et al.*, 1993), indicating that the processing of *N*-linked glycans in *A. oryzae* was different from that in *R. pusillus*. Salovuori *et al.* (1987) reported that the structures of *N*-linked glycans produced by the filamentous fungus *Trichoderma reesei* were $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$. Takegawa *et al.* (1989) reported that the most abundant *N*-linked glycan attached to the glucoamylase of *Rhizopus niveus* was $Man_8GlcNAc_2$. It is therefore apparent that the distribution and substrate specificity of the mannose-trimming enzymes in the Golgi apparatus are different among the filamentous fungi. The processing enzymes of filamentous fungi such as Golgi α-mannosidase are useful for studying the processing of glycoproteins. Cloning of a processing enzyme, α-1,2-mannosidase, from *R. pusillus* is now in progress in our laboratory.

We found that MPP was cleaved autocatalytically into two major glycopeptides under alkaline conditions. Analysis of the N-terminal amino acid sequences of both glycopeptides identified the cleavage sites to be between Phe^{169}-Ser^{170} and Tyr^{172}-Met^{173}. MPP is a member of the acid protease family in which two aspartate residues are involved in the catalysis (MacKinlay & Wake, 1971; Pitts *et al.*, 1992). It therefore has an acidic optimum pH and has relatively high substrate specificity with extremely low proteolytic activity (Arima *et al.*, 1967; Iwasaki *et al.*, 1967). MPP, which is known to recognize aromatic amino acid residues and cleave peptide bonds at its COOH side, preferentially cleaves the peptide bond Phe^{105}-Met^{106} in κ-casein and induces destabilization and clotting of κ-casein dispersed in milk (Etoh *et al.*, 1982). Since the autocatalytic cleavage sites, between Phe^{169}-Ser^{170} and between Tyr^{172}-Met^{173}, are similar to Phe^{105}-Met^{106} of κ-casein, the substrate specificity of MPP may be slightly changed in some unknown way under the alkaline conditions.

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