Effects of Glycosylation on the Secretion and Enzyme Activity of
Mucor Rennin, an Aspartic Proteinase of Mucor pusillus, Produced by
Recombinant Yeast*

Jun-ichi Aikawa†, Takashi Yamashita‡, Makoto Nishiyama, Sucharu Horinouchi,
and Teruhiko Beppu

From the Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo, Yayoi 1-1-1, Bunkyo-ku,
Tokyo 113, Japan

The Mucor rennin gene encoding a prepro form of the fungal aspartic protease from Mucor pusillus was expressed under the control of the yeast GAL7 promoter in Saccharomyces cerevisiae. The mature M. pusillus rennin secreted efficiently by yeast was a highly glycosylated protein. Analysis by a combination of site-directed mutagenesis of each of the three possible glycosylation sites and treatment of the secreted M. pusillus rennins with endo-β-N-acetylglucosaminidase H revealed that the mature yeast M. pusillus rennin contained two asparagine-linked glycosylation sites among the three possible glycosylation sites. A mutation of the 2 glycosylated asparagine residues of M. pusillus rennin resulted in significant decreases in the level of secretion by yeast cells. In addition, the extent of glycosylation of M. pusillus rennin was found to affect the enzyme properties such as milk-clotting and proteolytic activities.

The aspartic proteinases produced extracellularly by the two closely related species of zygomycete fungi, Mucor pusillus (1) and Mucor miehei (2), possess relatively high milk-clotting activity along with low proteolytic activity. These are called Mucor rennins and are widely used as milk coagulants in industrial cheese production. We reported previously the cloning and sequencing of the structural gene for M. pusillus rennin (3). The gene encodes a prepro enzyme composed of 361 amino acids of the mature M. pusillus rennin and an additional NH₂-terminal sequence of 66 amino acid residues. When the gene was expressed in Saccharomyces cerevisiae cells under the control of the yeast GAL7 promoter, a highly glycosylated form of the mature M. pusillus rennin which was excreted into the medium efficiently (4). We also confirmed that the primary secretion product of the enzyme was a pro-M. pusillus rennin possessing a pro-sequence of 44 amino acids; the pro-sequence was removed by autocatalytic processing at acidic pH (5).

The amino acid sequence of M. pusillus rennin contains three possible N-glycosylation sites, i.e. Asn⁷⁰, Asn¹⁵⁸, and Asn¹⁸⁸. Analysis of the mature M. pusillus rennin secreted from the recombinant yeast indicated that the enzyme was highly glycosylated mainly with mannose moieties (about 37 residues/mol) (4). In contrast, the commercial preparation of M. pusillus rennin produced by M. pusillus contains only a few glucosidic moieties in its molecule (6). Comparison of the catalytic properties of both the preparations has revealed that the ratio of milk-clotting activity to proteolytic activity of the recombinant yeast M. pusillus rennin is distinctly lower than that of the commercial M. pusillus rennin.

The present study was undertaken to determine the exact glycosylation sites of yeast M. pusillus rennin together with the effect of glycosylation on the catalytic properties of the enzyme. We also found that mutant M. pusillus rennins lacking the glycosylation sites obtained by means of site-directed mutagenesis were secreted in decreased levels from the yeast cells.

MATERIALS AND METHODS

Strains and Plasmids—S. cerevisiae MC16 (α, leu2, his4, ade2) (7) was used as the host for production of M. pusillus rennins. Escherichia coli JM105 (Δlac pro thi rpsL endA sbcB15 hsdR1 F'TR pRD proAB lacP lacZAM15) purchased from Amersham Corp. was used as the host for site-specific mutagenesis. Plasmid JPl (see Fig. 1), in which the cloned M. pusillus rennin gene was placed under the control of the GAL7 promoter (8), has been described previously (4).

Culture Conditions—Synthetic SD medium (4) supplemented with adenine and histidine (20 mg/liter each), YPD medium containing 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco Laboratories), and 2% glucose, and YPGal medium, containing 3% galactose instead of glucose in YPD medium, were used for cultivation of yeast strains carrying plasmid JPl. The strains were cultured in the synthetic medium for 2 days, and the cells were then centrifuged and resuspended in the same volume of YPD medium. After cultivation for 1 day, the harvested cells were resuspended in 10 volumes of YPGal medium and cultured for 2 or 4 days. All strains were cultured aerobically at 30°C. For purification of M. pusillus rennin, the strains were first cultured to early stationary phase in YPD medium for 1 day, and the harvested cells resuspended in 100 volumes of YPGal medium were cultured for 3 days.

Mutagenesis—Site-directed mutagenesis was carried out using synthetic oligonucleotides (Table I) by Kunkel's method (9) using a Bio-Rad Mutagen kit. All the mutations were checked by nucleotide sequencing using the M13 dye-dye chain termination method (10).

Analysis of Extracellular and Intracellular M. pusillus Rennins—S. cerevisiae transformants carrying the M. pusillus rennin genes were cultured in YPGal medium at 30°C for 2 days. Culture broths (25 μl each) were obtained by centrifugation at 6000 x g for 5 min, and the precipitates were precipitated by addition of 3 volumes of ethanol. The precipitates were dissolved in 9 μl of distilled water and subjected to SDS-polyacrylamide gel electrophoresis. The harvested cells were

* The abbreviations used are: SDS, sodium dodecyl sulfate; endo H, endo-β-N-acetylglucosaminidase H.
Glycosylation and the lysate was electrophoresed. Proteins were detected by West-endo H (0.04 units/ml) at 37 °C, and the samples were subjected to blotting with anti-M. pusillus rennin antibody (15). SDS-polyacrylamide gel electrophoresis. Lane M, molecular weight transformant broth was centrifuged at 6000 × g for 15 min to remove purified commercial M. pusillus rennin (3 pg). b, purified commercial the cells. The supernatant was adjusted to pH 5.5 and then applied M. pusillus rennin (260 pg/ml) was treated with endo H. The method been washed with 500 ml of the same buffer, the absorbed proteins applied to a Superose 12 gel filtration column (Pharmacia LKB concentration using a PM-10 filter (76 mm, Amicon). The sample was contained in M. pusillus rennin (MPR) (450 pg/ml) was treated with endo-p-N-acetylglucosaminidase H (endo H) (Seikagaku Kogyo) by the method of Tarentino et al. (14). Two chromogenic oligopeptides I and II (Table II) were used as substrate to determine kinetic parameters, as described previously (14).

RESULTS

Glycosylation of Yeast M. pusillus Rennin—We observed previously that the M. pusillus rennin protein secreted efficiently from the cells of S. cerevisiae containing plasmid JP1 (Fig. 1) had a larger molecular mass (46 kDa) than that of the commercial M. pusillus rennin (40 kDa) produced by the industrial strain of M. pusillus (4). This seemed to be due to the different extents of asparagine-linked glycosylation in S. cerevisiae transformants, as detected by the periodic acid-Schiff staining method. We confirmed these observations and also found that the decrease in size of the yeast M. pusillus rennin by the endo H treatment proceeded in two steps, as shown in Fig. 2a. During the endo H digestion, the initial mass of 46 kDa was reduced first to an intermediate one of 43 kDa, and then the final product of 40 kDa appeared upon prolonged incubation. This suggested that the yeast M. pusillus rennin contained two glycosylated sites although there were three possible asparagine-linked glycosylation sites in the M. pusillus rennin sequence, i.e. AsnT9-Ile-Thr, AsnT9-Val-Ser, and AsnT9-Asn-Thr. These were confirmed by means of site-directed mutagenesis, as will be described later. The endo H treatment of the commercial M. pusillus rennin caused no decrease in molecular mass (Fig. 2b), suggesting that the commercial M. pusillus rennin had almost no sugar modification.

The treatment of the yeast M. pusillus rennin with endo-α-N-acetylglucosaminidase (Boehringer Mannheim) for removing Thr/Ser-linked glycosyl mass moieties (O-glycosylation) did not cause any change in molecular mass on SDS-polyacrylamide gel electrophoresis (data not shown).

Site-directed Mutagenesis to Remove the N-Glycosylation Sites—In order to determine the glycosylated sites, we constructed mutant M. pusillus rennin genes in which each of the 3 asparagine residues described above was exchanged to glutamine by site-directed mutagenesis.

S. cerevisiae transformants carrying the mutated M. pusillus rennin genes were cultured in YPGal medium for 4 days, and

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**TABLE I**

| Position in M. pusillus rennin | Created mutation | Mutagenic oligonucleotide |
|-------------------------------|-----------------|---------------------------|
| AsnF50 | GlnF50 | 5' TAGGTGATTGGGCAAGGGTTG 3' |
| AsnF113 | GlnF113 | 5' ACGTGGCACAGCTACACCCGC 3' |
| AsnF159 | GlnF159 | 5' GTCGGTCAACCAACTACCTC 3' |

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**TABLE II**

| Substrate | Amino acid sequence |
|-----------|---------------------|
| x-Casein | Leu-Ser-Phe-Met-Ala-Ile- |
| I | Leu-Ser-Phe(NO2)-Met-Ala-Leu-OMe |
| II | Lys-Pro-Ile-Glu-Phe(NO2)-Arg-Leu |

* OMe, methoxy. lyed by successive treatment with 0.2 mg/ml zymolase (Seikagaku Kogyo) at 30 °C for 1.5 h and then with 0.5% SDS at 37 °C for 1 h, and the lysate was electrophoresed. Proteins were detected by Western blotting with anti-M. pusillus rennin antibody (15). Purification of Yeast M. pusillus Rennin—One liter of the yeast transformant broth was centrifuged at 6000 × g for 15 min to remove the cells. The supernatant was adjusted to pH 5.5 and then applied directly to a DEAE-Toyopearl 650M column (TOSOH, 4 × 12 cm) equilibrated by 50 mM acetate buffer, pH 5.5. After the column had been washed with 500 ml of the same buffer, the absorbed proteins were eluted with 150 mM NaCl in the same buffer. The fractions containing M. pusillus rennin activity (about 100 ml) were pooled, and the protein solution was concentrated to about 20 ml by ultrafiltration using a PM-10 filter (76 mm, Amicon). The sample was applied to a Superose 12 gel filtration column (Pharmacia LKB Biotechnology Inc., 1.6 × 50 cm). Milk-clotting activity was eluted coincidentally with a single protein peak. The peak was used as a purified preparation of yeast M. pusillus rennin. The purity of the sample was checked by SDS-polyacrylamide gel electrophoresis. Commercial M. pusillus rennin (Meito) was purified in an essentially similar way and used as a commercial M. pusillus rennin preparation.

**Fig. 1.** Construction of plasmid JP1. The blank, dotted, and dark areas in the upper straight line represent the regions of the pre-, pro-, and mature parts of M. pusillus rennin (MPR), respectively. The striped area and the solid line in the circle represent the DNA sequence regions of pBR322 and yeast plasmid pDB246, respectively. The M. pusillus rennin gene is placed under the GAL7 promoter.

**Fig. 2.** Endo H treatment of yeast M. pusillus rennin. a, purified yeast M. pusillus rennin (MPR) (450 µg/ml) was treated with endo H (0.04 units/ml) at 37 °C, and the samples were subjected to SDS-polyacrylamide gel electrophoresis. Lane M, molecular weight standards (67,000, bovine serum albumin; 43,000, ovalbumin); lane C, purified commercial M. pusillus rennin (3 µg); b, purified commercial M. pusillus rennin (260 µg/ml) was treated with endo H. The method was the same as that above.
the culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, both the Gln\textsuperscript{79} and Gln\textsuperscript{168} M. pusillus rennins in the culture supernatant had a reduced molecular mass of about 43 kDa whereas the molecular mass of Gln\textsuperscript{133} M. pusillus rennin was the same as that of nonmutated M. pusillus rennin (46 kDa) (Fig. 3). This is consistent with the observations on the time course of endo H digestion of yeast M. pusillus rennin. When both Asn\textsuperscript{79} and Asn\textsuperscript{168} were changed to glutamine, the mass of the M. pusillus rennin with the double mutation was the same as that of the commercial M. pusillus rennin. All these data clearly showed that the number of glycosylation sites of yeast M. pusillus rennin was two and that they were Asn\textsuperscript{79} and Asn\textsuperscript{168}.

Effect of Glycosylation on Secretion of M. pusillus Rennin—All the lanes of SDS-polyacrylamide gel shown in Fig. 3 contained M. pusillus rennin samples prepared from equal volumes (100 \( \mu l \)) of the culture supernatants. The secreted amounts of Gln\textsuperscript{79} and Gln\textsuperscript{168} M. pusillus rennins were apparently reduced. Secretion of the double mutant Gln\textsuperscript{79}/Gln\textsuperscript{168} M. pusillus rennin was reduced to about 50\% of that of the nonmutated M. pusillus rennin. In order to determine whether the lack of glycosylation in the mutated M. pusillus rennins caused a decrease in intracellular to extracellular transport, we examined the amounts of M. pusillus rennin in both the culture supernatants and the cells. In this experiment, the yeast transformants were induced in YPGal medium for 2 days because prolonged cultivation for 4 days caused extensive decrease in the intracellular M. pusillus rennins, probably due to proteolytic activity of the host cells. As shown in Fig. 4, most of the extracellular proteins cross-reactive with the anti-M. pusillus rennin antibody was present in two forms. From the observation that the conversion from pro-M. pusillus rennin to mature M. pusillus rennin occurred in the culture broth (5), we concluded that the upper band (asterisked in Fig. 4) was pro-M. pusillus rennin, and the lower band was mature M. pusillus rennin. When the cultivation was prolonged for 2 more days, the pro-M. pusillus rennins (upper band) were converted to the mature M. pusillus rennin (lower band), and the pattern of SDS-polyacrylamide gel electrophoresis was identical to that shown in Fig. 3. The Gln\textsuperscript{79}/Gln\textsuperscript{168} M. pusillus rennin gave a single band of pro-M. pusillus rennin due to a very small extent of self-processing at a low concentration of the protein (5). Prolonged cultivation resulted in yielding a single band of mature M. pusillus rennin that had the same mass as that shown in Fig. 3. The mutations of Gln\textsuperscript{79}, Gln\textsuperscript{168}, and Gln\textsuperscript{79}/Gln\textsuperscript{168} caused a marked decrease in the extracellular M. pusillus rennins whereas Gln\textsuperscript{133} M. pusillus rennin was excreted as efficiently as the nonmutated M. pusillus rennin. In contrast, distinct intracellular accumulation of the immunoreactive proteins was observed only with the mutations at the actual glycosylation sites. A major band detected in the cells of each Gln\textsuperscript{79}, Gln\textsuperscript{168}, and Gln\textsuperscript{79}/Gln\textsuperscript{168} transformant showed a larger molecular mass than that of the non-glycosylated pro-M. pusillus rennin, suggesting that they were pro-M. pusillus rennins glycosylated at the residual asparagine residues in the secretory apparatus. Several degradation products were also detected with Gln\textsuperscript{168} and Gln\textsuperscript{79}/Gln\textsuperscript{168}. All these data clearly indicated that glycosylation at the two sites of M. pusillus rennin was required for efficient secretion from yeast cells.

Effect of Glycosylation on Enzyme Activity—The catalytic properties of the endo H-treated yeast M. pusillus rennin and the mutated M. pusillus rennins were compared with those of the nonmutated yeast M. pusillus rennin and the commercial M. pusillus rennin. The results are shown in Table III.

The nonmutated yeast M. pusillus rennin showed distinctly lower milk-clotting activity with relatively higher proteolytic activity than those of the commercial M. pusillus rennin. As a consequence, the clotting activity/proteolytic activity ratio of the yeast M. pusillus rennin was only one-fourth that of the commercial M. pusillus rennin. The endo H treatment of the yeast M. pusillus rennin improved the ratio to a value very similar to that of the commercial M. pusillus rennin mainly because of an increase of clotting activity. Similar changes were observed with the mutated M. pusillus rennins lacking one or both of the N-glycosylation sites. As the number of glycosylated residues was reduced, their clotting activity increased, and their proteolytic activity decreased. Gln\textsuperscript{79} and Gln\textsuperscript{168} had almost the same clotting activity, and Gln\textsuperscript{79}/Gln\textsuperscript{168} showed the most improved clotting activity/proteolytic activity ratio, being almost equal to those of the commercial M. pusillus rennin and endo H-treated yeast M. pusillus rennin but slightly different from that of the commercial M. pusillus rennin.

We analyzed further the kinetic properties of these mutants using synthetic oligopeptides I and II (Table IV). Although the \( K_m \) and \( k_{cat} \) values of the yeast M. pusillus rennin for both the substrates were slightly different from those of the commercial nonglycosylated M. pusillus rennin, they still did not coincide after endo H treatment or site-directed mutagenesis.

**DISCUSSION**

The present work has revealed that the 2 asparagine residues, Asn\textsuperscript{79} and Asn\textsuperscript{168}, among the three possible N-linked glycosylation sites in the M. pusillus rennin polypeptide are actually glycosylated during the secretory processes whereas the Asn\textsuperscript{133} residue is not glycosylated in yeast cells. Although x-ray crystallographic analysis of M. pusillus rennin has not...
TABLE III
Clotting and proteolytic activities of M. pusillus rennin and its mutants

| No. of glycosylated residues | Clotting activity | Proteolytic activity | Clotting activity/proteolytic activity ratio |
|------------------------------|------------------|----------------------|---------------------------------------------|
| Yeast M. pusillus rennin     |                  |                      |                                             |
| Nonmutated (-endo H)         | 2 units/µg       | 3.04 (45.1)          | 0.790 (22.3)                                |
| Nonmutated (+endo H)         | 0 units/µg       | 5.61 (83.2)          | 2.28 (65.7)                                |
| Gln<sup>79</sup>             | 1 units/µg       | 4.30 (65.1)          | 1.96 (60.0)                                |
| Gln<sup>79</sup>-Gln<sup>188</sup> | 1 units/µg       | 4.08 (60.5)          | 1.44 (41.5)                                |
| Commercial M. pusillus rennin | 0 units/µg       | 5.95 (88.3)          | 2.73 (78.7)                                |

*Values in parentheses are percentages of activity.

TABLE IV
Kinetic parameters of M. pusillus rennin and its mutants

| No. of glycosylated residues | Synthetic peptide I | Synthetic peptide II |
|------------------------------|---------------------|---------------------|
|                              | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
| Yeast M. pusillus rennin     |              |                   |                     |              |                   |                     |
| Nonmutated (-endo H)         | 2 0.214 15.7  | 73.4              | 5.88 73.4 12.5      |              |                   |                     |
| Nonmutated (+endo H)         | 0 0.168 16.5  | 98.2              | 4.29 77.4 18.0      |              |                   |                     |
| Gln<sup>79</sup>             | 1 0.106 14.6  | 74.0              | 10.1 10.5 10.4      |              |                   |                     |
| Gln<sup>79</sup>-Gln<sup>188</sup> | 1 0.201 14.1  | 74.0              | 10.1 10.5 10.4      |              |                   |                     |
| Commercial M. pusillus rennin | 0 0.194 12.4  | 63.9              | 8.67 76.7 8.85      |              |                   |                     |

Removal of the glycosylation sites of M. pusillus rennin resulted in a significant decrease in the amount of secreted M. pusillus rennin along with accumulation of M. pusillus rennin in the yeast cells. A relationship between N-linked glycosylation and protein secretion by yeast has also been reported for human tissue-type plasminogen activator expressed in S. cerevisiae (19). In Chinese hamster ovary cells, a mutant tissue-type plasminogen activator lacking possible N-linked glycosylation sites remained in the cell as a stable complex with a protein GRP78, which regulates the transport of secretory proteins (20). It is possible that a similar protein is associated with the accumulation of the mutant M. pusillus rennins in S. cerevisiae cells (21, 22).

The clotting activity of yeast M. pusillus rennin was distinctly low but recovered after endo H treatment. Increase in the relative milk-clotting activity was also observed with the mutated M. pusillus rennins lacking 1 or both of the 2 glycosylated asparagine residues. These results indicate that glycosylation of M. pusillus rennin causes distinct modulation of its enzymatic activity, probably due to a change in the specificity for scissile peptide bonds in the high molecular weight protein substrates. A similar result was obtained when the tissue-type plasminogen activator secreted from S. cerevisiae was treated with endo H (19). However, only a slight change in the kinetic parameters was observed with the synthetic substrates examined. Similar results were obtained upon mutagenesis of the glycosylation site of another aspartic proteinase, renin (23). The difference in the lengths of substrates probably accounts for this inconsistency. For example, various aspartic proteinases show different pH optima for acid-denatured hemoglobin but almost identical optima for shorter peptide substrates (14, 24). A longer synthetic peptide with an appropriate sequence will be required for further analysis of the effect of glycosylation on catalytic activity.

One of the glycosylation sites, Asn<sup>79</sup>, is located in the "flexible flap region" that partially covers the substrate binding cleft. Several residues on the flap may interact with a bound substrate. One of the flap residues in chymosin, Tyr<sup>77</sup>,

yet been done, the highly conserved tertiary structure among several aspartic proteinases allows speculation on the possible locations of these residues in the M. pusillus rennin molecule. The side chain of Asn<sup>79</sup> seems to exist in the exposed region of the molecule, as depicted in the structure of endothiapepsin (Fig. 5) (16). The specificity of glycosylation may therefore not be due to the accessibility of the asparagine residues to glycosyltransferase. Another possible explanation is the relative rate of glycosylation of asparagine residues in Asn-X-Thr and Asn-X-Ser. Kaplan et al. (17) reported that the threonine-containing sequence was glycosylated more rapidly than the serine-containing one both in vivo and in vitro (17). Consistent with this, the Asn<sup>79</sup> and Asn<sup>188</sup> residues of M. pusillus rennin are in the Asn-X-Thr sequence whereas Asn<sup>113</sup> is in the Asn-X-Ser sequence. Recently, the same group discovered a glycosylation site binding protein, a component of the oligosaccharide transferase (18). The glycosylation site binding protein might play an important role in sorting the glycosylation of M. pusillus rennin.
corresponding to Tyr in M. pusillus rennin, seems to be involved in both substrate binding and catalytic activity (14). It seems possible that Asn-linked glycosylation causes perturbation of the flap structure, thus influencing the enzyme activity. Another glycosylation site, Asn, is located at the junction of the NH2-terminal and the COOH-terminal domains of the enzyme. We may assume that Asn-linked glycosylation influences the global tertiary structure of the enzyme. However, such a possible effect should be examined carefully by several means such as NMR spectroscopy.

The clotting and proteolytic activities of the endo H-treated yeast M. pusillus rennin and the mutant M. pusillus rennin (Gln/Gln) lacking the N-glycosylation sites were not completely identical to those of commercial M. pusillus rennin. The reason for this is still unknown, but the difference of the host is one possible explanation; the process of protein folding or some other modification might differ slightly between S. cerevisiae and M. pusillus.

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