Physicochemical and Immunochemical Studies on Similarities of Acid Proteases *Mucor pusillus* Rennin and *Mucor miehei* Rennin[†]

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Physicochemical and immunochemical comparisons were made on the two acid proteases *Mucor pusillus* rennin (MPR) and *Mucor miehei* rennin (MMR). The molecular weight and isoelectric points of the two enzymes were similar: MPR (M.W., 38,500 and I.P., 3.9) vs. MMR (M.W., 42,000 and I.P., 4.1). D-Galactose and D-glucosamine, which had been found in MMR, were also detected in the carbohydrate moiety of MPR with gas-chromatography and amino acid analyses. The structural similarity between MPR and MMR was investigated utilizing peptide mapping techniques, which showed a close correlation between the two enzymes. Furthermore, immunodiffusion test using antisera prepared against each enzyme suggested the presence of a common antigenic structure. The results confirm that a high similarity exists between MPR and MMR, which has been supposed from the morphological identity of the two enzyme-producing fungal strains.

Microbial rennin has been produced commercially only by two fungi belonging to the genus *Mucor*, although a number of microorganisms have been surveyed for rennin activity. *Mucor pusillus* rennin* was found by Arima *et al.*¹⁾ as the first successful microbial milk-clotting enzyme commercially substituting for calf rennin, and its physicochemical and enzymatic properties were clarified.^{2~5)} A similar type of milk-clotting enzyme originating from *Mucor miehei** has recently become commercially available and its properties have been reported independently by Rickert *et al.*^{6~9)} and by Sternberg.^{10,11)} These two enzymes have similar molecular weight, isoelectric point, amino acid composition and other properties. Furthermore, these two microorganisms *M. pusillus* and *M. miehei* develop morphologically in the same way at the asexual stage, although some differences were observed at the zygosporic stage.^{12,13)} *M. miehei* had been defined as a synonym of *M. pusillus* according to

Zycha¹⁴⁾ until the difference in zygosporic stage was found. In the present paper, we made comparative studies on the properties of *Mucor pusillus* rennin and *Mucor miehei* rennin in order to show the close relationship between two enzymes.

MATERIALS AND METHODS

Purification of enzymes. *Mucor pusillus* rennin (MPR) produced by *Mucor pusillus* var. Lindt, and *Mucor miehei* rennin (MMR) produced by *Mucor miehei* Cooney et Emerson were purified to homogeneity by published methods^{4,9)} from two commercial milk-clotting preparations, Meito Rennet (Meito Co., Japan) and Rennilase (Novo Industry A/S, Denmark). The purity of the enzymes was confirmed by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. The procedure was similar to that described by Wever and Osborn.¹⁵⁾ Electrophoresis was carried out using 7.5% gel at room temperature at 8 mA per tube for four hours using 10 μ g of protein per tube containing a trace amount of pyronine G. After electrophoresis, gels were extruded from tubes, stained in 0.2% Coomassie brilliant blue and destained in several changes of 7% acetic acid. The gels were scanned at 565 nm. Marker proteins, such as egg albumin, chymotrypsinogen A and cytochrome c, were used for determination of molecular weight.

[†] Milk-clotting Enzyme from Microorganisms. Part XII.

* These two enzymes are usually called *Mucor*-rennin (*Mucor pusillus* rennin) and *Mucor* Miehei Protease (*Mucor miehei* rennin).

Isoelectric focusing electrophoresis. Isoelectric point was determined by isoelectric focusing electrophoresis with a 110 ml capacity ampholine column as described in the instruction manual. The carrier ampholite was selected to give a pH gradient from 3.5 to 5.0 at an average final concentration of 1%. Electrofocusing was performed for 42 hr at 500 V at 4°C.

Analysis of carbohydrate composition in MPR

Neutral hexose. Neutral hexoses in MPR were determined with its acid hydrolysate. MPR (5 mg) was hydrolyzed with 2 ml of 4 N HCl in an evacuated sealed tube at 100°C for 6 hr. The hydrolysate was evaporated to remove the acid. The residue was dissolved in distilled water. The neutral hexose fraction was isolated by linked columns of Dowex 50W×2, hydrogen-form resin (1 cm by 15 cm). The effluent was collected and evaporated completely. The residue was treated at 80°C for 5 min in a small volume of pyridine-hexamethyldisilazane (HMDS)-trimethylchlorosilane (TMCS) (10:2:1, by volume).¹⁸⁾ The ester derivatives of neutral hexoses were extracted with chloroform by mixing the reaction mixture with 2 ml of chloroform-water (1:1, by volume), and analyzed by gas liquid chromatography (Hitachi GC-063). The analytical column was 1 m glass packed with 3% SE-30 on Chromosorb W (Johns-Man Ville). The esters were determined by comparisons of retention times with those of glucose, mannose and galactose.

Hexosamine. Hexosamine moieties were determined by an amino acid analyzer.¹⁷⁾ MPR was hydrolyzed as described above. The hydrolysate evaporated was applied to a 1 cm by 15 cm column of Dowex 50W×8 (hydrogen-form) and eluted with 2 N HCl after washing with a quantity volume of water. The eluates were pooled, evaporated and dissolved in a small volume of 0.2 M citrate buffer (pH 2). An aliquot of the sample was analyzed for hexosamine components with an amino acid analyzer (Hitachi KLA-5/Short Column #2611). Hexosamines were identified by comparing the retention times with those of standard hexosamines (glucosamine and galactosamine).

Peptide mapping technique

Digestion of proteins. Prior to trypsin digestion, 20 mg of each enzyme was denatured in 5 ml of solution (1.5 ml; 1.66 M tris, pH 8.6, 0.15 ml; 5% EDTA, 2.31 g; urea, 0.05 ml; β -mercaptoethanol per 5 ml) by incubation for two hours at room temperature and further incubation with 0.1 g of monoiodoacetate for one hour at room temperature under a stream of nitrogen gas. These denatured samples were dialyzed against distilled water, filled to 10 ml with distilled water and adjusted to pH 8.0 with NH_4OH in the presence of 200 mg of NH_4HCO_3 . These samples were digested with 0.2 mg of trypsin (Sigma) for 10 hr at

37°C and lyophilized. Each sample (1 mg) was dissolved in a small volume of water containing a trace amount of methylorange, as marker dye for paper chromatography.

Paper chromatography and high voltage paper electrophoresis.¹⁸⁾ Samples digested with trypsin were subjected to paper chromatography (60×60 cm, Toyo Roshi No. 51A), which was developed downwards for 16 hr at room temperature in the upper phase of *n*-butanol-acetic acid-water (4:1:5, by volume). After the chromatograms were air dried, high voltage paper electrophoresis was carried out at right angle to the direction of the paper chromatography at 3500 V for 40 min at 20°C. The electrophoresis buffer was pyridine-acetic acid-water (10:0.4:90, by volume), pH 6.5. Lysine, alanine and glutamic acid were electrophoresed at the same time as the standard markers. Peptides were detected by staining the chromatograms with ninhydrin reagent. The relative *R_f* values of ninhydrin positive spots were calculated against both the *R_f* value of methylorange at the first direction and that of lysine at the second direction.

Immunological methods

Preparation of antisera against MPR and MMR. Both antisera were kindly prepared at the Osaka Laboratory of Fujisawa Pharmaceutical Co., as follows; Three rabbits were immunized with purified MPR and two with purified MMR by intravenous injection for seven weeks according to the schedule shown in Table I. The total dose of MPR and MMR was 15 mg and 17.5 mg, respectively. Antisera were treated at 56°C for 30 min to inactivate the complements after the animals were bled. The titer of both antisera was measured by the precipitin test and expressed at maximum dilution of immune serum where 10 $\mu\text{g/ml}$ of each antigen solution formed precipitin (Fig. 1). Normal rabbit serum was generously supplied from the Tokyo Laboratory of Sankyo Pharmaceutical Co.

TABLE I. INJECTION SCHEDULE FOR PRODUCTION OF ANTI-MPR AND ANTI-MMR SERA

Day	MPR (mg)	Day	MMR (mg)
0	5	0	6.5
21	2.5	21	2.5
35	2.5	35	2.5
50	2.5	48	3
76	2.5	78	3
91	Bled	89	Bled
Total	15 mg	Total	17.5 mg

Double gel diffusion test. Immunodiffusion in agar gel (J. agar) was carried out at room temperature in micro scale as described by Ouchterlony.¹⁹⁾ The gel

FIG. 1. The Rise in Antiserum Titers of Rabbits Immunized with MPR (a) and MMR (b).

The titer is expressed at maximum dilution by the procedure described in the text.

was prepared on slide glass plating of about 3 ml of 1.4% agar solution containing 0.15 M NaCl, 0.01 M Na-phosphate (pH 7.0) and 0.1% azide. Into each well (2 mm diameter) arranged as shown in Fig. 5, 5 μ l of antigen or antiserum solution was added. After incubating the gels at room temperature for 2 days, the gels were washed with saline for one day and stained by amido black.

Neutralization test. Ten milliliters of anti-MPR serum, anti-MMR serum and normal serum were separately dialyzed against two liters of 0.01 M Na-phosphate buffer-0.15 M NaCl (pH 7.0) at 4°C for one day to remove excessive NaCl which was detrimental to milk clotting activity.²⁰ These antisera were concentrated to the initial volume (10 ml) by Diaflo, and series of two fold dilutions with 0.01 M Na-phosphate buffer (pH 7.0) were prepared. After 1 ml of antigen solution (0.05 mg MPR or MMR/ml) was incubated with 1 ml of each diluted serum (*i.e.*, anti-MPR serum, anti-MMR serum and normal serum *vs.* MPR or MMR) at 35°C for 30 min, aliquots (0.5 ml) of sample were measured for residual enzymatic activity, as described in the previous paper.²⁾

Preparation of culture filtrate. Five strains shown in Fig. 7 were inoculated on wheat bran medium (5 g, wheat bran; 3.5 ml, distilled water) in 100 ml Erlenmeyer flask (capped with cotton plugs) and incubated at 30°C for 3 days. The enzyme was extracted with 50 ml of distilled water containing 0.5 ml toluene for 6 hr at room temperature, and then filtrated through Toyo No. 2 filter paper. The filtrate was adjusted to pH 7.0 with 1 N NaOH and centrifuged to remove the precipitate. The clear filtrates were used for the double gel diffusion test.

RESULTS

Molecular weight and isoelectric point of MPR and MMR

The molecular weight of both MPR and MMR was determined by SDS-polyacrylamide gel electrophoresis as described in METHODS AND MATERIALS. The molecular weight of MPR and MMR was estimated to be 38,500 and 42,000, respectively. The isoelectric point was 3.9 for MPR and 4.1 for MMR when determined by isoelectric focusing electrophoresis, as described in METHODS AND MATERIALS. These values were similar to those reported by Rickert *et al.*^{7,9)}

Determination of carbohydrate composition in MPR

Neutral hexose. Sililated carbohydrates were prepared from MPR and analyzed by gas-liquid chromatography as described in METHODS AND MATERIALS. As shown in Fig. 2, three peaks were detected with retention times of 1.2, 2.8 and 7.5 min. The former two peaks may not be carbohydrates, although they were not identified. The peak at 7.5 min was identified as galactose compared with the standard carbohydrate derivatives. According to Rickert *et al.*,⁸⁾ MMR contains glucose and mannose, in addition to galactose as neutral hexose components.

FIG. 2. Gas-chromatogram of TMS Derivatives Obtained from Hydrolysate of MPR.

Preparation of TMS derivatives and chromatographic conditions are described in the text. Column temperature, 160°C; detector temperature (flame ionization), 200°C. The peak at 7.5 min was identified as galactose.

Hexosamine. The elution pattern of the hydrolysate of MPR is shown in Fig. 3.

FIG. 3. Elution Pattern in Chromatography of the Basic Fraction with the Amino Acid Analyzer.

Conditions were the same as the standard amino acid analysis with elution buffer of pH 5.28 at 55°C. —, absorbance at 570 nm; ----, absorbance at 440 nm.

Standard hexosamines, glucosamine and galactosamine, were separately eluted between aromatic amino acids and lysine with a retention time of 23.5 min (glucosamine) and 26.5 min (galactosamine). Compared with the standard elution pattern, the peak with the retention time of 23.5 min was identified as glucosamine. The glucosamine content was calculated to be 0.56 moles per mole of enzyme from HW values (height $\times$ width). Since the hydrolysis condition required for the hexosamine release from glycoproteins was milder than that required for complete protein hydrolysis, peptide components were included in hydrolysates, resulting in a rise of baseline. On the other hand, MMR contained two moles of glucosamine per protein moiety (Rickert *et al.*⁸¹).

Peptide mapping of MPR and MMR

Both MPR and MMR were digested to peptide fragments with trypsin, and analyzed by paper chromatography, followed by high voltage paper electrophoresis, as described in METHODS AND MATERIALS. Ninhydrin positive spots (16 and 13) were detected in trypsin digests of MPR and MMR, respectively (Fig. 4). When comparing the *R_f* values of each spot against methylorange and lysine at the first and the second direction of chromatography, seven spots, about half of total peptides, were identical. These results indicate that MPR and MMR share partially the primary structure.

FIG. 4. Peptide Maps of the Trypsin Digests of MPR (a) and MMR (b).

Samples were exposed to paper chromatography (1) followed by high voltage electrophoresis (2). Spots observed at the same positions in the two maps are shaded.

Immunological properties of MPR and MMR

Double gel diffusion test. The antisera against MPR and MMR were prepared, as described in METHODS AND MATERIALS. The similarity of these two enzymes was determined immunologically by the double gel diffusion test, according to Ouchterlony.²¹ Anti-MPR serum formed precipitin lines with both MPR and MMR. Anti-MMR serum also reacted with both MPR and MMR (Fig. 5, a, b). Normal serum, however, did not show any precipitin lines against either MPR or MMR (data, not shown). When anti-MPR serum or anti-MMR serum was used in the experiments, a spur was formed at the meeting point of the two precipitin lines against MPR and MMR. These results suggest that the two enzymes share some antigenically common structure in their molecules.

Neutralization of enzyme activity with anti-serum. Antiserum prepared against MPR or

FIG. 5. Immunodiffusion Test of Antisera against MPR (a) and MMR (b).

Reactions were performed in 1.4% agar gel using antisera prepared against each enzyme, as described in MATERIALS AND METHODS. Precipitin lines were stained for protein by Amido black. P, purified MPR; M, purified MMR; AP, anti-MPR serum; AM, anti-MMR serum.

MMR contained antibody to precipitate both MPR and MMR (Fig. 6, a, b). Inhibition of enzyme activity was determined with these antisera, as described in METHODS AND MATERIALS. Both antisera clearly inhibited the milk-clotting activity of these two enzymes. Anti-MPR serum inhibited 50% of MPR and MMR activity at a dilution of approximately $1/2^2$ and $1/2^1$, respectively, of the original. The inhibition curves by anti-MMR serum shifted to the right (higher dilution) compared with those by anti-MPR serum. This is due to the titer of anti-MMR initially being 4 fold higher than that of anti-MPR serum (Fig. 1). This inhibition of enzyme activity by antiserum should be specific, since normal serum did not significantly inhibit enzymatic activities of the two enzymes (Fig. 6, c). The inhibition of enzymatic activity by normal serum at higher concentration seems to be nonspecific, due to un-

FIG. 6. Effects of Anti-MPR (a), Anti-MMR (b) and Normal (c) Sera on the Activities of the Two Enzymes. ●, MPR; ▲, MMR.

known factors detrimental to milk-clotting activity. Therefore, these results also suggest that MPR and MMR contain common molecular structure.

DISCUSSION

Many works have demonstrated characteristics and properties of two enzymes MPR and MMR, such as physicochemical properties, substrate specificity,²²⁾ essential groups in the active center.^{23, 24)} These results suggest the possibility that these two enzymes bear a close structural resemblance to each other. It was therefore most important and interesting to investigate the structure of the two enzymes by other methods which had not been applied. Results of comparative studies by immunological techniques, peptide mapping and other analyses described here clearly indicate that there exists a common structure in the molecule of two enzymes MPR and MMR.

Precipitin patterns of complete identity

should not be formed when there exists a partial difference between antigens tested. For example, the antiserum prepared against purified prorennin (calf) gave lines of partial identity against prorennin and rennin,* just as in the case of present results shown in Fig. 5. Even in such a case (between an enzyme and its zymogen), the small difference in structure is detected by the method. It is therefore concluded that MPR and MMR share large parts of molecular structure. Two precipitin lines were formed between MPR and anti-MMR serum in the double diffusion test (Fig. 5). One possible explanation for the results is that heterogeneity exists in MPR which could be detected only immunologically and not by other methods, like SDS-polyacrylamide gel electrophoresis.

FIG. 7. Immunodiffusion Test of Antisera with Culture Filtrates of Five Strains of *Mucor*.

Each well was filled with 5 μ l of antiserum or clear filtrate prepared by the procedure described in MATERIALS AND METHODS. AP, anti-MPR serum; AM, anti-MMR serum. Culture filtrate from *Mucor pusillus* IAM 6122 (1); IAM 6154 (2); IFO 4578 (4); IFO 4579 (5); *Mucor miehei* CBS 182.67 (3), (6).

* H. Uchiyama (personal communication).

Culture filtrates of the four strains of *M. pusillus* and one strain of *M. miehei* showed precipitin lines against anti-MPR and anti-MMR serum which were similar to those obtained with purified antigens (Fig. 7). Furthermore, the milk-clotting activity in these filtrates was strongly inhibited by anti-MPR or anti-MMR serum. According to Iwasaki *et al.*,²⁵⁾ culture filtrates of several strains of *Mucor*, except *M. pusillus* and *M. miehei*, did not react with serum prepared against MPR. These results suggest that either anti-MPR or anti-MMR serum might be useful to immunologically identify the two fungi *M. pusillus* and *M. miehei*. As no protein produced by other *Mucor* species showed a reaction with antiserum against either MPR or MMR, it is likely that *M. pusillus* and *M. miehei* belong to the same species.

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REFERENCES

- 1) K. Arima, S. Iwasaki and G. Tamura, *Agric. Biol. Chem.*, **31**, 540 (1967).
- 2) S. Iwasaki, G. Tamura and K. Arima, *ibid.*, **31**, 546 (1967).
- 3) S. Iwasaki, T. Yasui, G. Tamura and K. Arima, *ibid.*, **31**, 1421 (1967).
- 4) K. Arima, J. Yu, S. Iwasaki and G. Tamura, *Appl. Microbiol.*, **16**, 1727 (1968).
- 5) J. Yu, G. Tamura and K. Arima, *Biochim. Biophys. Acta*, **171**, 138 (1969).
- 6) M. Ottensen and W. Rickert, *Compt. Rend. Trav. Lab. Carlsberg*, **37**, 301 (1970).
- 7) W. S. Rickert, *Biochim. Biophys. Acta*, **271**, 93 (1972).
- 8) W. S. Rickert and P. A. McBride-Warren, *ibid.*, **336**, 437 (1974).
- 9) W. S. Rickert and J. R. Elliott, *Can. J. Biochem.*, **51**, 1638 (1973).
- 10) M. Z. Sternberg, *J. Dairy Sci.*, **54**, 159 (1971).

- 11) M. Sternberg, *Biochim. Biophys. Acta*, **285**, 383 (1972).
 - 12) D. G. Cooney and R. Emerson, "Thermophilic Fungi," W. H. Freeman and Company, San Francisco, 1964, p. 17.
 - 13) A. K. Sarbhoy, *Trans. Br. Mycol. Soc.*, **51**, 25 (1968).
 - 14) H. Zycha, "Kriptogamenflora der Mark Brandenburg und Angrenzender Gebiete," *Vla*, **2** (1935).
 - 15) K. Wever and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
 - 16) T. Yamakawa, N. Ueta and I. Ishizuka, *Jpn. J. Exp. Med.*, **34**, 231 (1964).
 - 17) E. F. Walborg, Jr., B. F. Cobb, III, M. A. Mayne and D. N. Ward, *Anal. Biochem.*, **6**, 367 (1963).
 - 18) A. M. Katz, W. J. Dreyer and C. B. Anfinsen, *J. Biol. Chem.*, **234**, 2897 (1959).
 - 19) Ö. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949).
 - 20) A. Hamday and D. Edelsten, *Milchwirtschaft*, **25**, 450 (1970).
 - 21) Ö. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **32**, 231 (1953).
 - 22) T. Oka, K. Ishino, H. Tsuzuki, K. Morihara and K. Arima, *Agric. Biol. Chem.*, **37**, 1177 (1973).
 - 23) K. Takahashi, Wen-J. Chang and K. Arima, *J. Biochem.*, **80**, 61 (1976).
 - 24) W. S. Rickert and P. A. McBride-Warren, *Biochim. Biophys. Acta*, **480**, 262 (1977).
 - 25) S. Iwasaki, T. Yasui, G. Tamura and K. Arima, *Agric. Biol. Chem.*, **31**, 1427 (1967).
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