The Open Biotechnology Journal, **2015,** *9,* **119-126**

Screening and Characterization of a Mutant Fungal Aspartic Proteinase from *Mucor pusillus*

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Abstract: In this study, site-directed mutagenesis was carried out to alter properties of Mucor pusillus rennet (MPR) in order to find a potential substitution of commercial chymosin. Mutant G186D/E13D screened from thousands of mutants showed a significant milk-clotting activity (MCA). Mutant G186D/E13D rennet was purified and characterized. The molecular weight was estimated to be 44 kDa by SDS-PAGE. The maximum enzyme activity was at a wide range of pH (5.0-7.0) and 60ºC. The enzyme was inhibited by metal ions (Fe^{2+}, Fe^{3+}, Cu^{+} and Zn^{2+}), 1.10-Phenantrolin and pepstatin A. Further texture analysis of types of cheddar cheese made by non-mutant rennet, mutant (G186D/E13D) rennet and commercial rennet suggested that the soluble nitrogen content and hardness of cheddar cheese made by chimeric mutant rennet was decreased without any significant change in flavor between these cheeses. The result implicated that, to some extent, the mutant rennet could decrease hydrolysis of protein during ripening of cheese, probably as a candidate for a useful milk coagulant.

Keywords: Aspartic proteinase, *Mucor pusillus* rennin, Mutation, Thermostability, Proteolytic activity.

1. INTRODUCTION

Chymosin is an aspartic proteinase (EC 3.4.23.4) that is responsible for the coagulation of milk in the fourth stomach (abomasum) of unweaned calves in the form of an inactive precursor prochymosin [1], which is used extensively in cheese production because it cleaves κ-casein in a specific manner, at the Phe105-Met106 bond, with low proteolytic activity, and for the production of quality cheeses with good flavor and texture [2]. Unavailability of calf stomach and ethical problems associated with animal slaughtering has necessitated the finding of other alternatives to calf chymosin. In this regard, various plants and microbial proteases alternatives are used for chymosin production. Plant sources for milk-clotting enzymes have been identified from Cynara scolymus [3], Carica papay [4], Streblus asper [5], Centaurea calcitrapa [6] and Albizia [7]. Unfortunately, most of these sources are not suitable for production of quality cheese as they produce a bitter taste [2]. Proposed microbial substitutes for animal proteases include those from fungi and bacteria, such as Basidiomycete [8], Mucor pusillus [9], Bacillus sphaericus [10], Rhizomucor pusillus [11], Rhizopus oryzae [12] and Aspergillus [13]. At present, microbial rennet is used for one third of the entire cheese produced worldwide [14].

Mucor rennins are an aspartic proteinase produced by two closely related strains of Mucorales fungi, Mucor pusillus and Mucor miehei [15, 16]. These enzymes possess similarly characterized milk-clotting characteristics to those of calf chymosin, and they have been used as substituting enzymes for calf chymosin in the cheese industry. However, these enzymes are more proteolytic than bovine chymosin, thus leading to a lower yield in the production of cheese due to continued proteolysis following milk coagulation. Additionally, during cheese ripening, the curd-entrapped enzyme remains active and further degrades casein fractions by extensive non-selective peptide bond attack. This phenomenon may also lead to bitter flavor and structural deficiencies in ripe cheese, even after the heat-treatment step often present in its processing [17]. Moreover, the enzyme fraction in the whey could degrade proteins of economic value [18].

Site-directed mutagenesis is a good tool to research on the relationship of structure and function of proteins, especially for researching on chymosin, to vary systematically the sequence of peptide substrates and also to vary the specificity subsites using site-directed mutagenesis [19-23]. Meanwhile, site-directed mutagenesis is also a good strategy to alteration and modification of sequence. We have cloned the preproRMPP gene, and have developed efficient expression systems for the enzymes as zymogens in Pichia pastoris [24]. By using this system, site-directed mutagenesis of many milk-clotting enzymes was carried out to generate mutant enzymes with amino acid exchanges at position 13, 101 and 186. The mutant enzyme was purified and characterized biochemically, and cheeses made by the enzyme were analyzed for textural parameters and proteolysis after cheese ripening. In this case, replacement of Glu13/Gly186 was found to cause a marked decrease in the proteolytic activity.

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1874-0707/15

2. MATERIALS AND METHOD

2.1. Strains and Plasmids

The MPR gene encoding preproMPR, cloned from the genome of Mucor pusillus consists of a pre-sequence of 22 amino acids for secretion, a pro-sequence of 44 residues and a mature enzyme of 362 residues [24]. The plasmid pPICZα A, containing the MPR gene downstream of the AOX promoter, was introduced into Pichia pastoris GS115 (his4, lacZ) as a host to produce the wild-type and mutated MPR genes.

2.2. Media and Culture Conditions

YPD medium contained 2% Bacto-peptone (Difco, USA), 1% Bacto-yeast extract (Difco). The yeast transformants were pre-cultured in YPD medium at 30°C for 24 h. The cells were then harvested and re-suspended in 100 mL of BMMY medium containing 1.34% (w/v) bacto-yeast nitrogen base, 1% yeast extraction, 2% (w/v) peptone, 0.5% (w/v) biotin and 1% (v/v) methanol. Cultivation was continued at the same temperature for an additional 5 days [25].

2.3. Mutagenesis

Site-directed mutagenesis was carried out using a Polymerase Chain Reaction mediated method. Substitutions were made by overlap extension PCR mutagenesis [20]. Five mutants Ala101Thr (A101T), Glu186Asp (G186D), Glu13Asp (E13D), Glu13Gln (E13Q) and Glu13Ala (E13A) were obtained by reverse overlap primers as shown in Table **1**. All the mutations were checked by nucleotide sequencing and introduced into the corresponding position of pPICZα A by forming single mutants (A101T, G186D, E13D, E13Q, E13A and E13P) and chimeric mutants (A101T/G186D, A101T/E13D, G186D/E13D and G186D/E13Q). Transformation of Pichia pastoris was carried out by the electroporation method of Becher *et al.* 1991 [26].

2.4. Screening

Clotting activity determination of all transformants by microplate assay according to [20] was carried out using 4% (w/v) skim milk tempered to 35^{o}C. Clotting was measured as an increase in absorption at OD800, and the clotting activity calculated from a standard curve was obtained using serially diluted standardized unmutated MPR. Selected transformants were used for the shake flask experiments, which were transformant cultured in BMMY induced by methanol for 5 days at 30^{o}C on orbital shaker at 220 rpm. The cells were harvested by centrifugation at 10,000g for 20 min at 4^{o}C and the supernatant (crude enzyme) was used for purification experiments [10].

2.5. Purification of MPR

The crude enzyme solution was precipitated at 80% saturation of (NH4)2SO4. The active fraction with high milk clotting activity (MCA) was further purified by passing through Sephadex G-100 column (100cm ×1.2cm) pre-washed with 50 mM sodium phosphate buffer at pH 5.8. Fractions of 5 ml each were collected at room temperature at a flow rate of about 20 ml/h. The active fractions were dialyzed against distilled water and concentrated *via* lyophilization [9]. The concentrated enzyme was loaded on to a DEAE-52 (20cm×1.6cm) pre-equilibrated with 50 mM sodium phosphate buffer at pH 5.8. Elution of protein was then carried out by batch-wise addition of 50 ml portions of increasing molarities (0.0 -0.5 M) of NaCl in 50 mM sodium phosphate buffer at pH 5.8. Fractions of 5 ml each were collected at room temperature (25°C) at a flow rate of about 30 ml/h and analyzed for MCA and protein content [12]. The active enzyme fractions were pooled and stored at 4°C for further studies. The purified proteinase was examined for protein by electrophoresis under denaturing conditions in 12% polyacrylamide slab gels [17, 27].

2.6. Assay of Milk-Clotting Activity (MCA)

The MPR was assayed using the method described by Arima *et al*. [11]. A 10% solution of skim milk (Snow Brand

Table 1. Nucleotide sequences used for site-directed mutagenesis of Ala101, Gly186 and Glu13.

Mutant Site	Primers	Sequence of Mutant Primer
101	A101T-F	5'CGGCGGTACGACCGTGAAG3'
	A101T-R	5'CTTCACGGTCGTACCGCCG3'
186	G186D-F	5'GTCTTTGGTGACGTCAACAACACC3'
	G186D-R	5'GGTGTTGTTGACGTCACCAAAGAC3'
13	E13Q-F	5' GACTTGGAGCAGTACGCCATTC3'
	E13Q-R	5' GAATGGCGTACTGCTCCAAGTC3
	E13D-F	5' GACTTGGAGGACTACGCCATTC3'
	E13D-R	5' GAATGGCGTAGTCCTCCAAGTC3'
	E13P-F	5'GACTTGGAGCCGTACGCCATTC3'
	E13P-R	5'GAATGGCGTACGGCTCCAAGTC3'
	E13A-F	5' GACTTGGAGGCATACGCCATTC 3'
	E13A-R	5'GAATGGCGTATGCCTCCAAGTC3'

milk products Co.) containing 10mM CaCl2 was used as the substrate. Substrate solution (5ml) was added to the enzyme solution (0.5ml) at 35^{o}C. The time required for curd particles to form was measured with a stop watch. Under the above assay condition, 1 unit of activity (Soxhlet Unit) was defined as the amount of enzyme that clotted the milk solution in 40 min [28].

2.7. Assay of Proteolytic Activity (PA)

The proteolytic activity was measured using a 1.0% solution of casein (M/10 50 mM sodium phosphate buffer, pH 5.8) as the substrate. Five milliliters of the substrate solution was incubated with 1ml of enzyme solution at 45oC for 30 min and the enzyme reaction was stopped with 5ml of trichloroacetic aci mixture solution. After 30 min of incubation, the reaction mixture was filtered using filter paper and 2 ml of the filtrate was added to 5 ml of 0.55M Na_2CO_3 and 1 ml of Folin's reagent. This mixture was measured. One unit of the activity was defined as the amount of enzyme, which released 1ug of amino acid expressed as the tyrosine concentration per min under the above condition [28].

2.8. Effect of Temperature on Enzyme

The effect of temperature on milk-clotting activity of MPR was studied by measuring the activity of the purified enzyme at different temperatures (30-75°C).

The thermal stability of the purified enzyme was studied by measuring the milk-clotting activity of the residual enzyme after incubation for 0, 10, 20, 30, 40, 50, 60, 90 and 120 min at 55°C [3].

2.9. Effect of pH on Enzyme

The effect of pH on milk-clotting activity of the purified enzyme was studied at pH range of 5.0-8.0. The buffers used were: 0.1M citrate-phosphate (pH5.0-6.0) and 0.1M sodium phosphate (pH 6.0-8.0) [11, 14].

The pH Stability of the enzyme was studied at pH values from 3.0 to 10.0. The buffers employed were 50mM sodium citrate for pH 3.0, 4.0 and 5.0; 50mM sodium phosphate for pH 6.0 and 7.0; 50mM Tris/HCl for pH 8.0, 9.0 and 10.0. After incubation for 16 h at 25°C, the residual enzyme activity was measured [29, 30].

2.10. Effect of Metal Ions and Inhibitors

The effect of some metal ions (Ni^{2+}, K^{+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{+}, $Fe^{2+}$and Fe^{3+}) at 5mM concentration and some inhibitors (o-Phenantrolin, Aprotinin, Leupeptin, phenyl-methylsulphonyl fluoride (PMSF), EDTA and pepstatin A on purified enzyme activity was tested. The concentrations of the inhibitors are listed in Table **3**. The purified enzyme was incubated at room temperature for 30 minutes with metal ions or inhibitors and the residual milk-clotting activity was measured [31].

2.11. Cheese Manufacture and Analysis

Textural and proteolytic properties of cheese made with MPR were analyzed using a standard procedure [32]. Cheddar cheese was made with the mutant rennin (G186D/E13D)

The composition of the cheese was determined in triplicate. Fat content [33] and moisture content were determined [34]. The pH of the cheese was estimated [35].

The proteolysis of cheeses was assayed by determination of total nitrogen (TN), pH 4.6 phosphotungstic acid-soluble nitrogen (PTASN) according to methods described by Christensen *et al*. [36]. Total protein content was then obtained by multiplying the TN value by 6.38 [37].

Texture profile analysis (TPA) was carried out of the cheeses according to the methods described by Bhaskaracharya RK [38]. All analyses were carried out thrice.

2.12. Statistical Analysis

The SPSS package (SPSS 12.0 for Windows, SPSS Inc. Chicago, ILand USA) was used for statistical analysis of the results. Analysis of variance (ANOVA) was undertaken and the mean was established for $P < 0.05$. Mean comparisons were performed according to the Tukey's honest significant differences (HDS) test. Thus, a, b, c superscripts were employed to state significant differences between the lots for the exact same ripening time [37].

3. RESULT AND DISCUSSION

3.1. Screening of Mutant MPR

To test the effect of mutations at positions A101, G186 and E13 on the thermostability and proteolytic activity of MPR, one or two of the residues were substituted in MPR and the chimeric mutants in Pichia pastoris were expressed [11, 24]. The results of measurements for milk-clotting activity, proteolytic activity and thermostability of these mutants are presented in Table **2**. The mutants' enzymes (A101T/G186D, G186D/E13D, G186D/E13Q, G186d/E13Q and A101T /G186) were shown to be more sensitive to thermos than that of the non-mutated enzyme, especially the double mutant A101T/G186D that lost milk-clotting by 50% when incubated for 40min at 55oC, but without any change in the proteolytic activity. When residues were exchanged in position E13, the proteolytic activity of all the mutants (E13P, E13Q, E13D and E13A) decreased remarkably to almost a half of that of non-mutant enzyme, but there was no change in thermostability. Chimeric mutants (G186D/E13D and G186D/E13Q) had a reduction in thermostability along with a sharp decrease in proteolytic activity. The mutant G186D/E13D was selected for further characterization as described below.

Generally, protein cores are typically hydrophobic. Hydrophobic interaction is considered as a dominant force in structural stability and increased packing efficiency is often correlated with increased hydrophobicity [39-41], therefore hydrophobic amino acids always exist in protein cores to keep the proteins stable. Hydrophobic amino acids (Ala and

Table 2. Milk clotting and proteolytic activities of the non-mutant and mutant MPRs.

Enzyme	Clotting Activity (U/μg)	Proteolytic Activity (U/μg)	C/P ratio	Thermostability Relative Activity (%) at 55°C 40 min
Non-mutation	10.19	2.83	3.6	88.6
A101T	13.6	2.72	4.8	75.8
G186D	16.63	2.64	6.3	58.6
E13P	8.63	1.87	4.6	86.2
E13Q	7.97	1.19	6.7	88.2
E13D	8.38	1.18	7.1	89.1
E13A	8.26.	1.23	6.7	85.5
A101T/ G186D	16.24	2.62	6.2	53.5
G186D/E13D	10.47	1.36	7.7	70.2
G186D/E13Q	9.53	1.27	7.5	73.6

Gly) at positions 101 and 186 were substituted by charged amino acids (Thr and Asp) respectively, which made it difficult to maintain hydrogen cores of rennet [42], finally leading to unstable or flexible conformation of MPR.

Site E13 in RMPP plays a critical role in forming the correct hydrogen bond network around the active center and influence catalytic rate of RMPP as reported by Aikawa [12]. Substitutions at position 13 exchanged residue from Glu to Asp to alter space conformation of MPR resulting in decreased affinity of the MPR to substrate. Simulation analysis and molecular docking analyzed by software found possibility that position 13 may be related to increased substrate specificity (Analysis by our colleague, data is not shown), which was later proved in this experiment and also in RMPP Aikawa in 2001.

3.2. Purification of Mutated MPR

The results pertaining to purification of mutated MPRs using a combination of different purification techniques are summarized in Table **3**. After fermentation, the mutated MPR produced by recombinant Pichia pastoris GS115 was fractioned at 70% ammonium-sulphate with 1.3-fold purification and 91.6% recovery. Passage through Sephadex G-75 column resulted in about 5.7-fold purification of the enzyme with specific activity of 17203 U/mg. Finally, the concentrated active fractions passed through DEAE -52 column and the enzyme was purified about 7.1-fold with 5.2% recovery. Figs. (**1**, **2**) show the elution diagrams of the mutant G186D/E13D using Sephadex G-75 and DEAE-52 columns, respectively. Purified MPRs were separated into two peaks of proteins with one activity peak on Sephadex G-75 and one eluted activity peak (elution peak I) with 0.2 mol/L NaCl sodium phosphate buffer, pH 6.0 on DEAE-52. These peaks with MCA of proteins appeared as one band with molecular mass of 44 kDa on SDS-PAGE (shown in Fig. **3**).

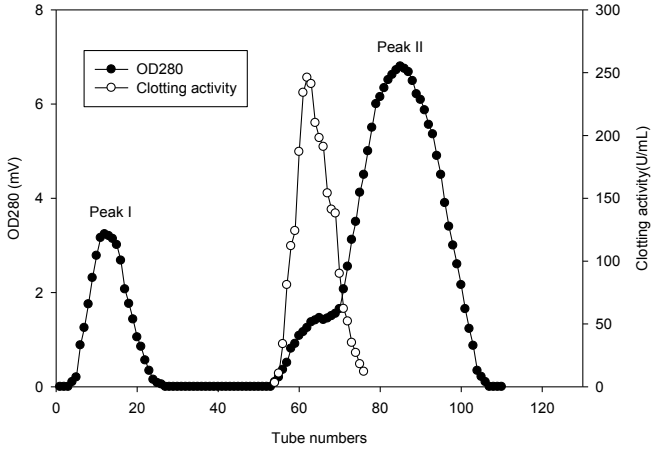

Fig. (1). Elution profile of mutant MPR on sephadex G-75 chromatography.

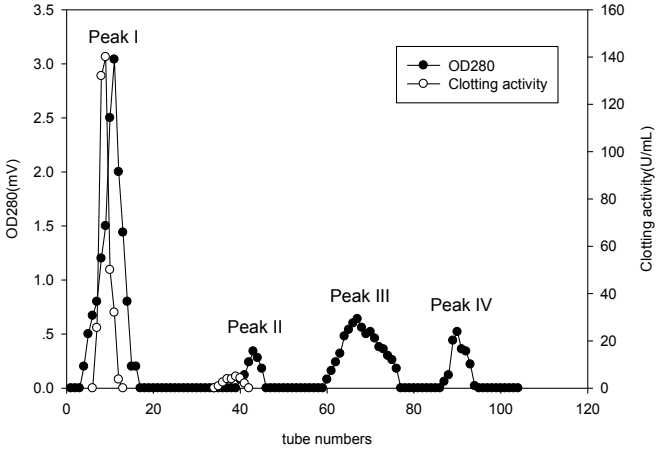

Fig. (2). Elution profile of mutant MPR on DEAE-52 chromatography.

Table 3. Purification scheme of Mutant MPRs produced by *Pichia pastoris.*

	Volume (mL)	Clotting Activity (U/mL)	Total Clotting Activity (U)	Protein Content (ug/mL)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude extract	100	83	8300	27.5	2.75	3018	100	1
$(NH4)_2SO4$	10.0	760	7603	193.7	1.94	3922	91.6	1.3
G-75	3.0	462	1386	26.9	0.081	17203	16.7	5.7
DE-52	3.0	144	432	6.7	0.022	21428	5.2	7.1

3.3. Effect of Temperature

Purified mutant G186D/E13D and non-mutant enzymes acted optimally at 60°C and later, they started losing their activity rapidly leading to complete inactivation at 75°C (Fig. **5**). The thermal inactivation experiments indicated that the mutant enzymes were slightly sensitive to heat than the non-mutant enzymes (Fig. **4**), despite that MPR is a relatively thermostable protein. The non-mutated enzyme at 55ºC remained almost fully active even after 60min of incubation, but G186D/E13D lost 30% of its activity after 50 min at the same temperature.

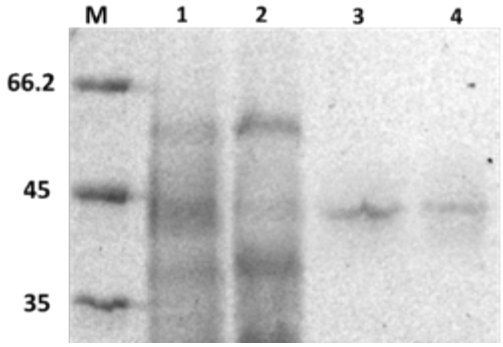

Fig. (3). Electrophoretogram of mutant MPR (G186D/E13D) after various steps of purification on SDS-PAGE of purification steps. Lane M: standard molecular weight markers; Lane1: Supernatant extract; Lane 2: (NH4)2SO4 fractionation; Lane 3: Sephadex G-75, Lane 4: DEAE-52.

Previously, the optimal temperature of MCA produced by Enterococcus faecalis TUA2495L [28] was 70ºC. The maximum MCA of purified enzyme produced by Rhizopus oryza [12] and Bacillus sphaericus [10] was at 60ºC and 55ºC respectively. The crude enzyme from Yeast Extracellular [29] showed maximum activity at 65°C with 10% of clotting activity lost at 60 min incubation at 45°C.

3.4. Effect of pH

Fig. (**6**) shows that the MCA of all of the mutant enzymes decreased as the pH increased from 5.0 to 7.5, and no activity was observed at pH 8.0. The optimum pH for both the non-mutant and mutant enzymes was 5.5 and the same result has been reported by Ashwani for milk clotting protease from Capra hircus. The pH optimum of purified APs from C. calcitrapa cell suspensions was detected at pH 5.1 [43]. The milk-clotting enzyme from glutinous rice wine mash liquor exhibited maximal MCA in milk at a pH of 5.5 [14]. Initial enzyme activity remained stable after treatment at pH 6.0, but there was an almost 50% loss in the activity at pH 5.0 or 7.0 (Fig. **7**). Sushil found that extracellular acid protease from Rhizopus oryzae [12] retained 96% of its activity at pH 5.5-7.5.

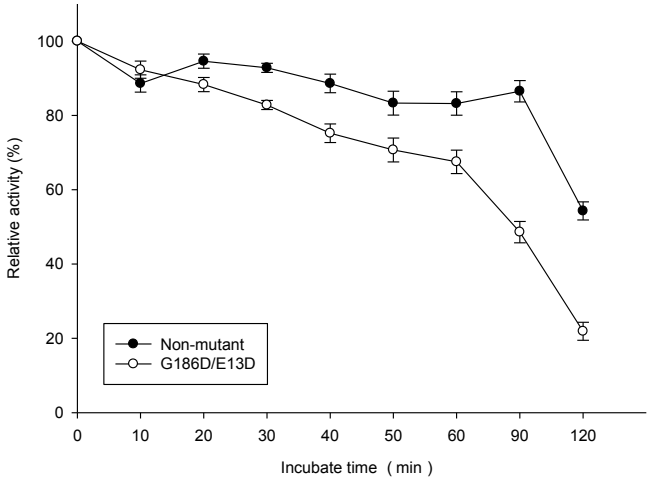

Fig. (4). Thermostability of purified non-mutant and mutant MPRs. Relative milk-clotting activities (%) were determined between 10-120 min at 55°C for non-mutant MPR and mutant MPR G186D/E13D produced by recombinant yeasts.

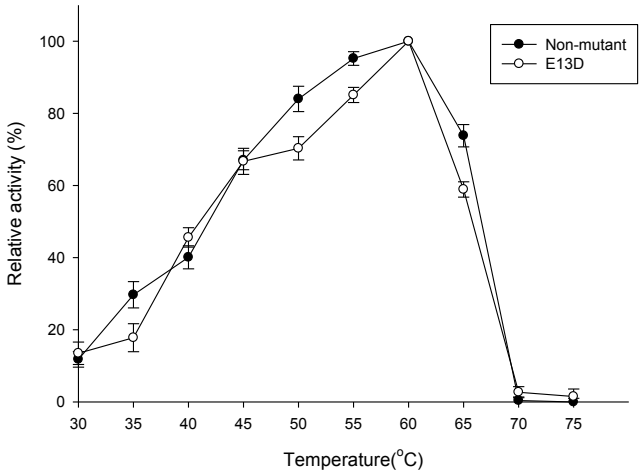

Fig. (5). Effect of temperature on purified proteinase activity. Relative milk-clotting activities (%) were measured using skim milk as substrate from 30°C to 75°C for non-mutant MPR and mutant MPR G186D/E13D produced by recombinant yeasts.

3.5. Effect of Metal Ions and Inhibitors

The effect of various metal ions on the percent residual activity is shown in Table **4**. As shown, Ca^{2+}, Mn^{2+}, and Mg^{2+}were activators, whereas Ni^{2+}, Fe^{3+}, Fe^{2+}, and Zn^{2+} were

inhibitors of the MCA for these enzymes. However, Cu^+, K^+ and Na^+ had no effect on the enzyme activity. Contrary to our results, Wang and others [14] reported that K+ was an inhibitor of the MCA.

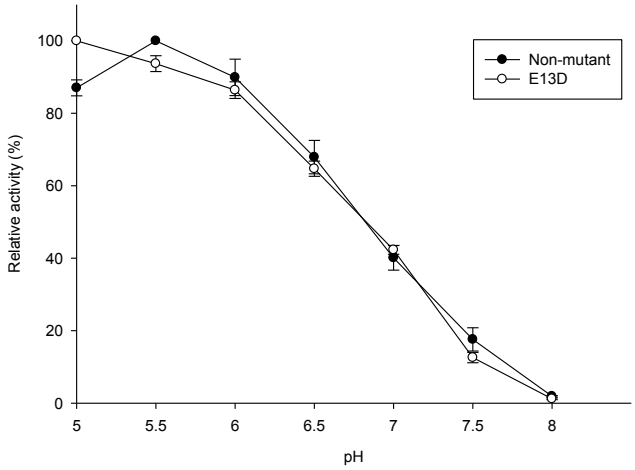

Fig. (6). Effect of pH on purified proteinase activity. Relative milk-clotting activities (%) were measured using skim milk as substrate from 5.0 to 7.0 for non-mutant MPR and mutant MPRsG186D/ E13D produced by recombinant yeasts.

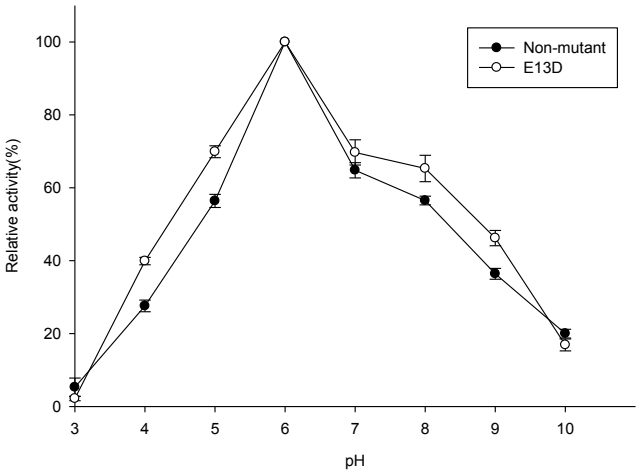

Fig. (7). Stability of proteinase to pH. The non-mutant and mutant MPR were incubated at various pH values for 4h at 25˚C, the residual activities were measured.

Mutant G186D/E13D retained above 90% of relative activity in the presence of PMSF or aprotin (Table **5**), hence serine participation at the active site of the enzyme has also been ruled out. EDTA and aprotinin had little effect on the activity of these enzymes. The protease activity was found to be sensitive to pepstatin, thereby confirming the aspartic protease nature of the activity. But the mutant G186D/E13D was found to be sensitive to 1.10-Phenantrolin, which suggested that these enzymes may be metalloenzyme. However, this result was totally different from other MCPs as reported by Sara [43], Magda [10], Arun [44] and Sushil [12].

3.6. Textural and Proteolytic Properties of Cheese Made with MPR the Mutants

The composition and pH of cheddar cheese made with commercial rennet, non-mutant and mutant rennet (G186D/E13D) with ripening 180d is shown in Table **6**. The values for pH, moisture, protein and fat content were in the range 5.16 to 5.21, 36.68 to 38.37%, 23.25 to 26.72% and 29.25 to 31.53%, respectively. The protein content of non-mutant cheese was marked ($P<0.05$) lower than that of mutant rennet cheese whose protein contenthad no significant ($P<0.05$) difference with commercial rennet cheese. Similar levels of moisture, fat and pH were shown between the three cheeses.

Table 4. Effect of metal ions on MCA of purified non-mutant and mutant enzymes.

Metal Ion (5mM)	Relative Activity (%)	
	G186D/E13D	Non-Mutant
Control	100	100
Ca^{2+}	227.6	218.8
Mn^{2+}	148.3	120.7
Ni^{2+}	28.7	47.8
Mg^{2+}	119.5	106.8
Cu^{+}	96.4	100
K^{+}	99.8	117.3
Na^{+}	97.4	98.9
Fe^{3+}	79.8	85.8
Fe^{2+}	75.4	87.7
Zn^{2+}	85.9	85.5

Control: Enzymes without adding metal ions.

Table 5. Effect of inhibitors on MCA of purified non-mutant and mutant enzyme.

Inhibitor	Concentration (mmol/L)	Relative Activity (%)	
		G186D/E13D	Non-Mutant
PMSF	1	93.4	93.1
1.10-Phenantrolin	1	0	0
Aprotinin	1	73.6	72.6
Leupeptin	0.2	95.2	96.5
Pepstatin A	0.05	15.2	24.5
EDTA	50	77.2	71.1
control	100	100	100

The level of pH4.6-SN increased with the increase in ripening time in each of the three cheeses. The extent of the increase in pH4.6-SN level was greatest ($P\leq0.05$) in the non-mutant cheese at 180 d of ripening (Table **6**), and there were no obvious differences ($P< 0.05$) between mutant cheese and

Table 6. Composition, pH and pH 4.6 SN (soluble nitrogen) of Cheddar cheeses made by non-mutant, mutant rennet (G186D/E13D) and commercial rennet at 180 d of ripening[1].

Enzymes	pH	Moisture%	Protein%	Fat%	pH4.6 SN%
Commercial	5.16(0.03)[a]	38.37(0.82)[a]	26.72(1.42)[a]	31.53(1.17)[a]	5.32(0.09)[a]
Non-mutant	5.16(0.11)[a]	37.54(1.12)[a]	23.25 (0.59)[b]	30.92(0.84)[a]	6.81(0.24)[b]
G186D/E13D	5.21 (0.02)[a]	36.68(0.93)[a]	25.29(1.14)[ab]	29.25(0.63)[a]	5.19(0.18)[a]

[a,b]Means within a row with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).
[1]Values represent means (SD; n = 3).

Table 7. Texture profile analysis parameters hardness, cohesiveness, springiness, and chewiness for Cheddar cheeses made with commercial rennet (control), non-mutant rennet, and mutants rennet (G186D/E13D) at 180 d of ripening[1].

Enzymes	Hardness (N)	Springiness	Cohesiveness	Chewiness (N)
Commercial	1134.44(12.34)[a]	0.39(0.01)[a]	0.25(0.01)[a]	93.44(5.11)[a]
Non-mutant	982.11(14.59)[b]	0.37(0.01)[a]	0.25(0.01)[a]	72.13(5.38)[a]
G186D/E13D	1160.71(16.24)[a]	0.36(0.07)[a]	0.22(0.01)[b]	88.90(6.01)[a]

a, b Means within a row with different superscripts are significantly different (Tukey's HSD; P <0.05).
1 Values represent means (SD; n = 3).

commercial cheese. The level of the increased pH4.6-SN was greatest in non-mutant cheese, which supports the earlier results showing that the protein content was lowest in non-mutant cheese of 180d ripening. Table **7** summarizes the mean values for the parameters obtained in the instrumental texture evaluation. Hardness ($p<0.05$) of non-mutant cheese was marked lower than that of the mutant cheese. The softening of cheeses was related to the hydration of the protein matrix and the effects of proteolysis [38]. This is in agreement with previous observations in which the level of the increased pH4.6-SN resulted in cheese softening. With respect to springiness, cohesiveness and chewiness, there was no significant difference ($p<0.05$) between the three cheeses, except for a slight lower inclination of non-mutant rennet cheese with respect to springiness and chewiness.

CONCLUSION

Site 186 and 13 of MPR are key positions, which relate to temperature sensitivity and substrate specificity. Themostability of MPR decreases remarkably when G186 is substituted by Asp and light reduction of proteolytic activity in MPR happens to be E13, substituted by Glu. Site 186 and 13 belonged to different core regions of MPR. They did not have any influence on each other, which is also proved by mutant G186D/E13D due to the reduction in the thermostability and proteolytic activity simultaneously.

Except for reduction in thermostability and proteolytic activity, the other properties of enzyme of mutant rennet were similar to that of the non-mutated rennet. Most of the textures of cheese made by non-mutated MPR were no different from the other two cheeses, except in protein content, pH4.6-SN, hardness and chewiness. Above all, these properties may assure the practical use of this double mutant as an improved milk coagulant. The effective expression system of Pichia pastoris allowing extracellular secretion used in this study will provide a possibility to produce the improved enzyme on large scale.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The financial support for this work from the Science & Technology Innovation Project of Jilin Academy of Agricultural Science (ZYCX201315), Science & Technology Development Project Agreement in Jilin Province (20150307019NY), Jilin Postdoctoral Science Foundation Funded Project (RB201305) is gratefully acknowledged.

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Received: February 17, 2014 Revised: March 21, 2015 Accepted: June 9, 2015