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ORIGINAL PAPER

Application of an acid proteinase from *Monascus purpureus* to reduce antigenicity of bovine milk whey protein

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Abstract An acid proteinase from *Monascus purpureus* No. 3403, MpuAP, was previously purified and some characterized in our laboratory (Agric Biol Chem 48:1637–1639, 1984). However, further information about this enzyme is lacking. In this study, we investigated MpuAP's comprehensive substrate specificity, storage stability, and prospects for reducing antigenicity of whey proteins for application in the food industry. MpuAP hydrolyzed primarily five peptide bonds, Gln^{4}–His^{5}, His^{10}–Leu^{11}, Ala^{14}–Leu^{15}, Gly^{23}–Phe^{24} and Phe^{24}–Phe^{25} in the oxidized insulin B-chain. The lyophilized form of the enzyme was well preserved at 30–40°C for 7 days without stabilizers. To investigate the possibility of reducing the antigenicity of the milk whey protein, enzymatic hydrolysates of the whey protein were evaluated by inhibition ELISA. Out of the three main components of whey protein, casein and α-lactalbumin were efficiently degraded by MpuAP. The sequential reaction of MpuAP and trypsin against the whey protein successfully degraded casein, α-lactalbumin and β-lactoglobulin with the highest degree of hydrolysis. As a result, the hydrolysates obtained by using the MpuAP–trypsin combination showed the lowest antigenicity compared with the single application of pepsin, trypsin or pepsin–trypsin combination. Therefore, the overall result suggested that the storage-stable MpuAP and trypsin combination will be a productive approach for making hypoallergic bovine milk whey protein hydrolysates.

Keywords *Monascus purpureus* · Acid proteinase · Preservation stability · Whey protein · Antigenicity · Tofuyo

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Introduction

Acid proteinase is a well-known group of proteolytic enzymes that have been widely employed in the food industry [33]. Among the reported fungal acid proteinases only the penicillopepsin (EC 3.4.23.20), aspergillopepsin (EC 3.4.23.18), rhizopuspepsin (EC 3.4.23.21) and mucorpepsin (EC 3.4.23.23) have been extensively studied [8]. Genus *Monascus* is one of the emerging sources of enzymes and the species of this genus are frequently utilized in the fermentation industry in East Asia [13, 14]. Yasuda and

co-workers studied the various enzymes produced by the genus *Monascus* that is used for the microbial fermentation of tofuyo which is a cheese-like vegetable protein food in Okinawa, Japan [29, 34–36]. One of the researches in the same research group uncovered a remarkable amount of proteinases produced by *Monascus pilosus* and *Monascus purpureus* [14]. Although a small number of acid proteinases from the genus *Monascus* have been reported [13, 32, 35, 36], the information about these *Monascus* enzymes is very limited.

Yasuda et al. [36] reported a research note on an extracellular acid proteinase produced by *Monascus* sp. No. 3403 that was recently re-identified as *Monascus purpureus* (unpublished). Unfortunately, the preliminary report was insufficient to allow industrial applications. Information about features such as storage stability, specificity, and applications of the enzyme are vital for scientific and industrial perspectives. Even though a substantial number of fungal proteinases have been reported, no one has ever revealed their storage stability which is an essential criterion for selecting an enzyme for industrial applications and also for assuring the enzyme vitality during transportation.

Cow's milk proteins are regarded as good nutrition for all ages because of their exceptional amino acid profiles. Nonetheless, except caseins, the majority of the other protein components in cow's milk are concentrated in whey and they are eventually discarded as by-product in the cheese industry. For this reason, the whey protein is of potential interest in many food industries such as dietary supplements, dairy, and feed industries. However, cow's milk allergy is one of the major causes of food hypersensitivity [17, 31]. Approximately 2.2% of infants exhibit cow's milk protein hypersensitivity in the first year of life [6]. Several studies concerning the relative antigenicities of cow's milk proteins in humans suggest that the main allergenic components are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and α-caseins which are the main protein components in the milk whey and those that are almost absent, or present at much lower amounts, in human breast milk [2, 16, 28].

Enzymatic hydrolysis approaches have been widely utilized in the food industry as one of the possible ways to reduce milk protein antigenicity [4, 19, 20]. The proteinases involved in human digestion have often been experimented on to manufacture milk protein hydrolysates with reduced antigenicity [12, 24, 28]. Enzyme systems of plant proteinases (papain and bromelain), bacterial proteinases (alcalase and neutrase), and combinations of extracellular proteinases with gastric proteinases have also been tested to manufacture low-antigenic whey protein hydrolysates [19, 20]. However, only a limited number of reports can be found on the application of fungal proteinases for the production of hypoallergenic whey protein hydrolysates [4].

The aim of this study was to explore the possibility of using our enzyme (MpuAP) for the production of hypoallergenic whey protein hydrolysates with a comparison of that of pepsin and trypsin, either singly or in combination. To this end, we investigated the relationships between hydrolytic properties of cow's milk proteins and the reduction of antigenicity in the whey protein hydrolysates. In addition, we also investigated the substrate specificity towards natural proteins, peptide bond specificity on the oxidized insulin B-chain, and preservation stability of our enzyme without stabilizers by lyophilization for industrial applications; these factors were not addressed in detail in our previous note.

Materials and method

Chemicals

Hammarsten milk casein was purchased from Wako pure chemical industries, Ltd, (Osaka, Japan). Pepsin (porcine gastric mucosa, activity 0.8–2.5 units/g of protein) and trypsin (bovine pancreas, activity 3.3 Anson units/g of protein) were purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade.

Organism and cultivation

Monascus purpureus No. 3403 was previously isolated by Yasuda et al. [36] and the strain was maintained in our laboratory. The strain was cultivated on steamed rice at 37°C for 7 days in a controlled environment. The resulting red mold rice (red-koji) was used for the enzyme extraction. The acid proteinase (9.1 U/mg of protein) was purified according to the method suggested by Yasuda et al. [36].

Enzyme activity and determination of protein concentration

Enzyme activity was measured in the same way reported by Yasuda et al. [36]. The enzyme was incubated with 2% casein solution made at pH 3.0 as a substrate. The liberated amount of tyrosine was determined by a colorimetric method measuring optical density at 660 nm. The amount of released tyrosine was calculated from a tyrosine standard curve. One unit of the acid proteinase activity was defined as the amount of enzyme that yielded 1 μmol of tyrosine per minute at 37°C at pH 3.0.

Protein concentration was determined by using a DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Absorbance at 280 nm was used to monitor the protein concentrations during the enzyme purification and to assess the protein contents in the experiment of bovine whey protein antigenicity reducing.

Analysis of the cleavage sites on the oxidized insulin B-chain

The oxidized insulin B-chain (100 μg) was dissolved in 0.5 ml of 0.1 M lactate buffer at pH 3.0 and it was incubated with 50 μl of the purified enzyme (1 U for casein) at 55°C. Aliquots were removed from the main reaction mixture at 5, 10, 30 and 60 min incubation, and the remaining enzyme activity was terminated by adding a drop of saturated ammonium hydroxide. The resulting peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) at 30°C on a COSMOSIL $5C_{18}$-AR-300 column (4.6 × 150 mm, Nacalai Tesque Co., Ltd, Kyoto, Japan) with a linear gradient of 0–100% acetonitrile solution containing 0.05% trifluoroacetic acid. Each fraction of the peptide peaks was collected and analyzed by using the ESI–MS esquire 3000plus-R (Bruker Daltonics, Billerica, MA) and identified cleavage sites on the oxidized insulin B-chain.

Preparation of bovine whey protein hydrolysates

The whey from cow's milk was freshly prepared by acid coagulation in the way that was generally practiced in cottage cheese manufacturing. In this process, the fresh milk was heated up to boiling temperature and acidification was done at 80°C in the cooling process. The prepared whey solution was centrifuged at 8,000× *g* for 15 min and the supernatant was dialyzed in distilled water at 4°C overnight. The dialyzed whey solution was concentrated by using rotary evaporation and the partially concentrated whey protein solution was lyophilized and stored at −20°C until use as a whey protein concentrate (WPC). A 2% (w/v) whey protein solution was prepared in deionized water. Whey protein solution was prepared as the substrate at two pH levels: pH 3.0 (with 1 N HCl) and pH 8.0 (with 1 N NaOH).

Enzymes were mixed with substrate (whey protein) at a 0.5% protein equivalent basis. The substrate made at pH 3.0 was incubated individually with acid proteinases (MpuAP and pepsin), and the whey protein solution made at pH 8.0 was incubated with trypsin. All reactions were performed at 40°C. The pH levels of each reaction mixture were maintained stable at pH 3.0 ± 0.05 (for MpuAP and pepsin) and pH 8.0 ± 0.05 (for trypsin) by continuous addition of 0.5 N HCl and 0.5 N NaOH (pH-stat method). During reaction, aliquots were removed at 1, 2, 3, 4 and 5 h and enzyme was inactivated by heating at 95°C in a water bath for 10 min. The supernatant of the hydrolyzed solutions were collected after centrifugation and stored at −20°C for further analysis. In the preliminary experiments conducted, it was observed that the individual application of each enzyme was insufficient for making completely hydrolyzed whey protein. Therefore, we also examined the enzyme combination reactions. In the enzyme combination study, the reactions with individual acid proteinases were terminated after 180 min of incubation and the rest of the reaction was continued with trypsin by maintaining the required pH. Trypsin was introduced at the same proportion (0.5% protein equivalent basis) and aliquots were removed from each reaction tube at 60 and 120 min, and reaction was terminated by heating at 95°C in a water bath for 10 min. The hydrolyzed solutions were centrifuged and the resulting supernatants were collected and stored at −20°C for further analysis.

Degree of hydrolysis (DH)

The degree of hydrolysis (DH) of WPC acted upon by different enzymes was determined according to Adler-Nissen's method [1], which is verified by Spellman et al. [26].

Tricine-SDS–PAGE

The whey protein hydrolysis pattern was visualized on tricine-SDS–PAGE gel in the way described by Schägger and von Jagow [23]. Gels were stained with 0.2% Coomassie brilliant blue R-250 (Sigma) in acetic acid/methanol/H_2O (1:1:5) media and de-stained in an acetic acid/methanol/H_2O (1:3:6) solution.

Inhibition ELISA

The relative antigenicity of the whey protein and the whey protein hydrolysates was determined by inhibition ELISA test. It was performed by measuring the amount of free antibodies that remained in the immune reaction, where the antigen (i.e., the whey protein hydrolysate) and a constant amount of antibodies (rabbit anti-whey antibodies (IgG)) were mixed; the free antibodies react with whey protein-coated ELISA plate and could be measured by an enzyme-coupled immunoassay. The detailed procedure is given below.

The wells of the ELISA plate (Nunc, Roskilde, Denmark) were coated with 100 μl of the whey protein (1 mg/ml) prepared in 0.05 M sodium bicarbonate buffer (pH 9.6). In 2-ml glass tubes, 10^{-3}, 10^{-2}, 10^{-1}, 10^{0}, 10^{1}, 10^{2} and 10^{3} μg/ml of the whey protein hydrolysates prepared in phosphate buffered saline (PBS) (0.15 M NaCl in 0.01 M phosphate buffer at pH 7.4) containing 0.05% Tween 20 (PBST) were mixed with a constant amount of the anti-whey antibodies (100 μl, against bovine whey protein produced in rabbit, 1:1,000 in 0.05 M PBS, Sigma) and kept overnight at 4°C. After removal of the unbound whey protein in the reacted plate, residual free binding sites in each well were blocked with 0.4% fish gelatine in PBST (200 μl/well) at 37°C for 1 h. The unbound blocking solution was removed from the wells and washed gently three times with

PBST. An equal amount of the whey protein hydrolysates (50 µl) which were incubated overnight with constant amount of the anti-whey antibodies was added to each well and incubated at 37°C for 1 h. The unbound anti-whey antibodies in the wells were removed and then washed three times with PBST. The amount of anti-whey which reacted with the plate-bound antigens was determined by adding 100 µl of peroxidase-conjugated goat anti-rabbit IgG (1:1,000 in 0.05 M PBS, Sigma) as the secondary antibody into each well and incubated at 37°C for 1 h. The excess peroxidase-conjugated goat anti-rabbit IgG were removed and wells were washed three times with PBST. FAST-OPD (*o*-phenylenediamine dihydrochloride; FAST™ OPD-Sigma, prepared in deionized water) solution (100 µl) was added to each well as the substrate for the secondary antibody and the reaction plate was incubated at room temperature for 30 min. The reaction was terminated by adding 100 µl of 0.2 M H_2SO_4 and absorbance was measured at 490 nm using a micro-plate reader (Bio-Rad, Benchmark plus, UK). Measurements of 5 replicates were recorded.

Results and discussion

Substrate specificity

The acid proteinase of *M. purpureus* (9.1 U/mg of protein) was purified to homogeneity according to the method described by Yasuda et al. [36]. Although the substrate specificity of the enzyme has been reported briefly [36], in this study we examined further details of substrate specificity towards various natural proteins and the result is summarized in Table 1. Substrates were prepared at 1% concentration in 10 mM lactate buffer at pH 3.0. Standard assay method was performed in the same manner except for different substrates made in the same concentration. These results indicated that the enzyme is capable of acting on a broad range of the substrates and hydrolyses animal proteins more favorably than plant proteins. In particular, the enzyme showed the capability of hydrolyzing bovine whey protein. This outcome led us to experiment on reduction of whey protein antigenicity by hydrolyzing whey protein with the purified enzyme alone and also combined with other digestive proteinases i.e., pepsin and trypsin.

Table 1 Substrate specificity of the acid proteinase of *M. purpureus*

Substrate (1%)	Relative activity (%)
Cytochrome *c*	100
Human hemoglobin	75
Bovine albumin	45
Egg albumin	34
Hammarsten casein	88
Whey protein concentrate	80
Gluten	22
Soybean protein isolate	68

Effect of lyophilization on preservation stability

To investigate preservation stability of MpuAP, we prepared liquid and lyophilized forms of the enzyme. Both forms of MpuAP were individually stored at 5, 30 and 40°C for 7 days. The remaining activities of the two forms of enzyme under three different storage conditions were measured and compared with the activity of freshly prepared samples as shown in Fig. 1. The liquid form of MpuAP was normally preserved in 10 mM McIlvain's buffer at pH 3.0. The lyophilized form of MpuAP could be reactivated with addition of distilled water. Both liquid and lyophilized forms of the purified enzyme showed substantially no loss of activity after 7 days under the refrigerated conditions at 5°C. The enzyme activity of lyophilized form was fully preserved after 7 days of incubation at 30°C but minimal reduction of the enzyme activity was observed in the liquid form under the same condition. At 40°C, the liquid form of MpuAP showed a significant decrease in activity i.e., about 35% loss of activity after 7 days of storage, but it was only 8% in the lyophilized form.

The storage stability of the enzyme is usually affected by the purity level and the composition of the final preparation. In particular, the composition of enzyme preparations for use in research, analytical and diagnostic applications must be more precisely specified. Generally, sugars or inorganic salts are used for stabilizing the final product of the enzyme for storage or distribution [5]. In this experiment, we used only the purified enzymes for storage. It was found that even the pure form of MpuAP in lyophilized form was

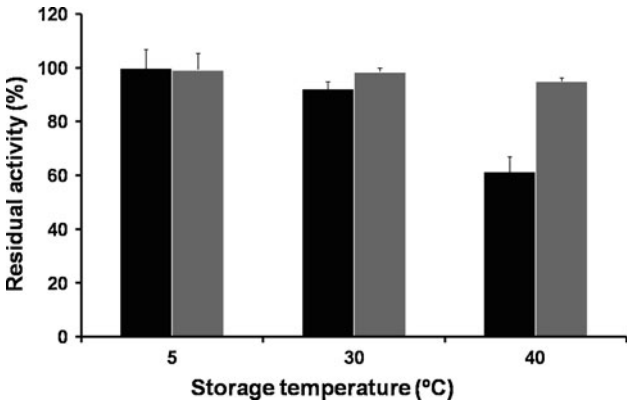

Fig. 1 Preservation stability of free (*black bars*) and lyophilized (*grey bars*) forms of the purified acid proteinase at three different storage temperatures for 7 days. Results are presented as means ± standard deviations ($n = 3$)

unusually more stable than that of the liquid form. The stability of MpuAP at 40°C also suggested that lyophilized MpuAP is more capable of withstanding minor temperature changes than the free form of the enzyme. This characteristic may bring an additional advantage for storage and transportation purposes.

Cleavage analysis on the oxidized insulin B-chain

The oxidized insulin B-chain is extensively used as the model polypeptide in the field of proteinases to investigate the substrate recognition. To evaluate the preference of hydrolysis against the peptide bond, digestion of the oxidized insulin B-chain by MpuAP was performed according to the method described above. Comparison of the cleavage sites of the oxidized insulin B-chain with other acid proteinases is summarized in Fig. 2. MpuAP hydrolyzed primarily five peptide bonds in the oxidized insulin B-chain viz. Gln^4–His^5, His^{10}– Leu^{11}, Ala^{14}–Leu^{15}, Gly^{23}–Phe^{24} and Phe^{24}–Phe^{25}. Of these cleavage sites, the peptide bonds Gln^4–His^5, Gly^{23}–Phe^{24} and Phe^{24}–Phe^{25} are specific to all acid proteinases of the genus *Monascus* [7, 13]. The cleavage sites of Leu^{15}–Tyr^{16} and Tyr^{16}–Leu^{17} appeared to be common to all the fungal acid proteinases except for MpuAP [9, 15, 21, 30]. On the other hand, the enzyme from *M. purpureus* preferentially split off His^{10}–Leu^{11} and Gly^{23}–Phe^{24} in the oxidized insulin B-chain compared with other fungal acid proteinases. In this regard, the substrate specificity of MpuAP was demonstrably different from other fungal acid proteinases including genus *Monascus* and it indicates some deviation of the enzyme from other enzymes.

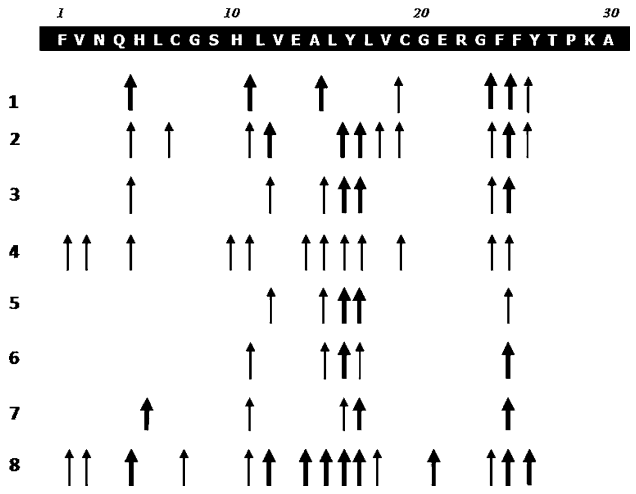

Fig. 2 Comparison of cleavage sites of the purified acid proteinase with various fungal acid proteinases on the oxidized insulin B-chain. *1* Purified acid proteinase of *M. purpureus*, *2* MpiAP1 of *M. pilosus* [13], *3* MpiAP2 of *M. pilosus* [13], *4* acid proteinase from *M. kaoliang* [7], *5* aspartic proteinase from *Rhizopus hangchow* [9], *6 Aspergillus saitoi* aspartic proteinase [30], *7 Aspergillus fumigatus* acid proteinase [21], *8 Penicillium janthinellum* aspartic proteinase [15]

Enzymatic hydrolysis of whey protein

Whey protein solution (2% w/v) prepared at pH 3.0 was individually hydrolyzed with MpuAP and pepsin, while the same substrate prepared at pH 8.0 was also hydrolyzed with trypsin. The enzymes were applied to each substrate at 0.5% protein equivalent basis and the reactions were terminated at 60-min intervals. The DH of whey protein achieved by MpuAP and gastric enzymes (pepsin and trypsin) was examined under in vitro conditions and it is depicted in Fig. 3. Initially MpuAP showed comparatively higher rates of DH than pepsin and trypsin. However, at 5 h of incubation, the highest DH (40%) was observed in trypsin followed by MpuAP (36%), and the lowest one was observed in pepsin (25%).

Electrophoretic patterns during whey protein hydrolysis obtained by several enzymatic actions are presented in Figs. 4 and 5. MpuAP and trypsin completely hydrolyzed intact casein protein within 1 h of incubation, whereas pepsin limitedly hydrolyzed it and its partially digested protein band was retained at 5 h of incubation. Out of the three main proteins in bovine milk whey, MpuAP completely hydrolyzed not only casein but also α-LA within 1 h of incubation. On the other hand, the digestion of α-LA by pepsin and trypsin was slower than that of MpuAP. However, *β*-LG was hard to be degraded by all enzymes; only trypsin could gradually degrade it during 5 h of incubation. These results suggest that the acid proteinases showed incapability of hydrolyzing *β*-LG at their optimal pH levels due to *β*-LG being stable at low pH [11]. In contrast, the action of trypsin on *β*-LG is relatively eased because it is optimally active at higher pH levels. Overall, these outcomes suggest that the enzyme combination reaction using MpuAP and trypsin might allow complete degradation of components of the whey protein.

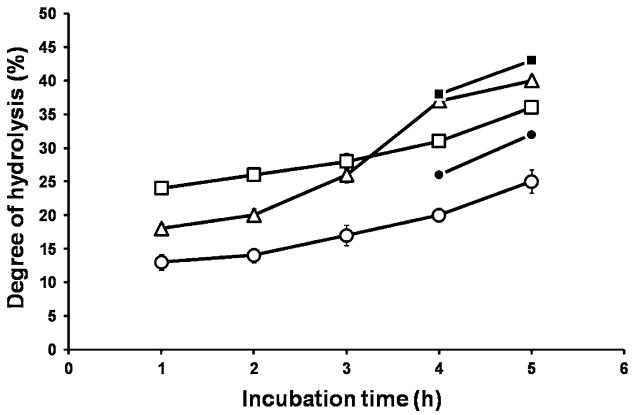

Fig. 3 Degree of hydrolysis of bovine whey protein for different enzymes (*open squares* MpuAP, *open circles* pepsin, *open triangles* trypsin) and combination of trypsin with 3 h partially hydrolyzed whey protein obtained by using pepsin/trypsin (*filled circles*) and MpuAP/trypsin (*filled squares*). Whey protein solution was 2% (w/v) and all enzymes in reaction mixture were 0.5% on protein equivalent basis

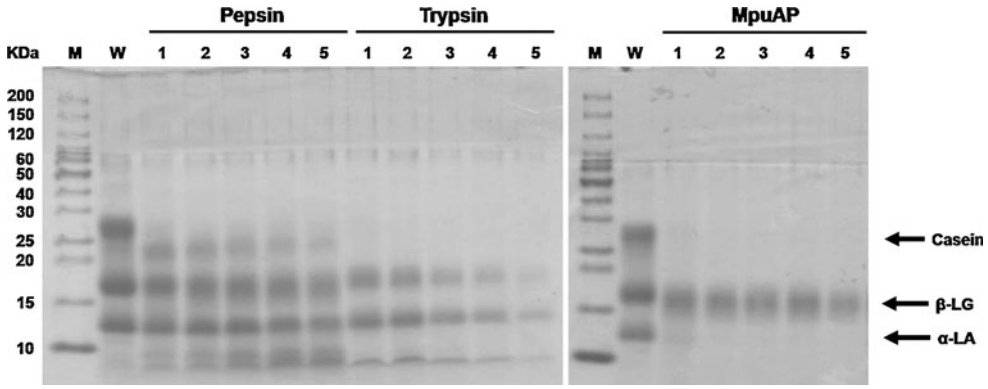

Fig. 4 Tricine-SDS–PAGE analysis of whey protein hydrolysates obtained by single application of MpuAP, pepsin and trypsin at different incubation times. *Lane M* molecular weight markers, *lane W* undigested whey protein, lane numbers *1–5* represent the time of incubation (h). *α-LA* α-lactalbumin, *β-LG* β-lactoglobulin

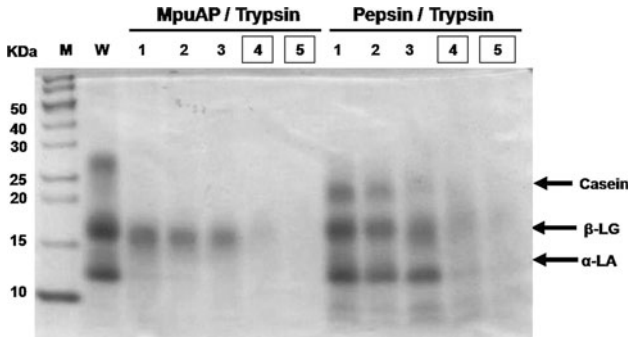

Fig. 5 Tricine-SDS–PAGE analysis of whey protein hydrolysates obtained by MpuAP, pepsin and their individual combinations with trypsin at different incubation times. *Lane M* molecular weight markers, *lane W* undigested whey protein, lane numbers *1–3* represent the time of incubation (h), and lane numbers *4* and *5* in *boxes* represent the overall incubation time after trypsin incorporation at 3 h for partially hydrolyzed whey protein by single application of MpuAP and pepsin. *α-LA* α-lactalbumin, *β-LG* β-lactoglobulin

To achieve the lowest antigenicity via an efficient degradation of the whey protein, we also investigated the enzyme combination study. As we expected, the remaining band corresponding to β-LG (as shown in Fig. 4) faded rapidly after application of trypsin to the reaction mixture of the partially digested whey protein by MpuAP (lanes 4 and 5 in Fig. 5). The structural basis of the resistance of β-LG at acidic pH levels was studied previously [11, 18, 22]. The acid stability of β-LG against enzymatic digestion is explained by increasing of internal hydrogen bonding that arises from the particular folding and occurs either between two titrated carboxyl groups or between one amide and one carboxyl group [3, 11]. Moreover, β-LG becomes less stable at pH 8.0 and trypsinization itself also helps to solubilize the insoluble whey protein denatured by heat treatment [10, 27]. Therefore, the change of pH (from pH 3.0 to 8.0) in the partially hydrolyzed whey protein provided a better environment for trypsin than the remaining proteins. In this experiment, MpuAP showed better removal of casein, α-LA and, to some extent, β-LG from the whey protein than pepsin when trypsin was applied. Thus, it is considered that the combination of MpuAP with trypsin is more beneficial to rapidly degrade whey protein than combination of pepsin.

Antigenicity of whey protein hydrolysates

Antigenicities of the whey protein hydrolysates harvested at different intervals of incubation with MpuAP, pepsin, trypsin and combination of acid proteinases with trypsin were measured by inhibition ELISA with rabbit anti-bovine whey antibodies. The change in antigenicity of the whey protein hydrolysates obtained at different time of incubation is depicted in Fig. 6. In this figure, rise of absorbance levels at 490 nm indicates antigenicity reduction in the whey protein hydrolysates. Hence we confirmed that the DH ratio in Fig. 3 inversely correlates with antigenicity in the whey protein hydrolysates in a time-dependent manner. The highest antigenicity reduction was achieved by the sequential reaction by the combination of MpuAP with trypsin.

Interestingly, the antigenicity reduction by single tryptic hydrolysis was not so high, even though tryptic hydrolysis showed comparatively higher DH ratio in Fig. 3. Enzymatic proteolysis occasionally brings increase of unpredicted protein antigenicity and the specificity for the degradation of epitopic areas on the antigenic substrate has an influence for the reduction of antigenicity [4]. In this experiment, tryptic hydrolysis of the whey protein might produce unpredictable antigenicity from the digested peptides. During the process of partial hydrolysis of the whey protein by MpuAP, some β-LG could have been exposed the epitopic areas to the action of trypsin. In fact, after incorporation of trypsin into the partially hydrolyzed whey protein (after complete removal of casein and α-LA in 3 h of incubation) by MpuAP, the reduction of antigenicity was obviously accelerated (Fig. 6). On the other hand, digestion of α-LA and β-LG by pepsin is always a slower process [4, 12]. These results suggest that the incorporation of trypsin into the partial hydrolyzed whey protein by acid proteinases may accelerate the antigenicity reduction and further prevent generation of unpredicted antigenicity.

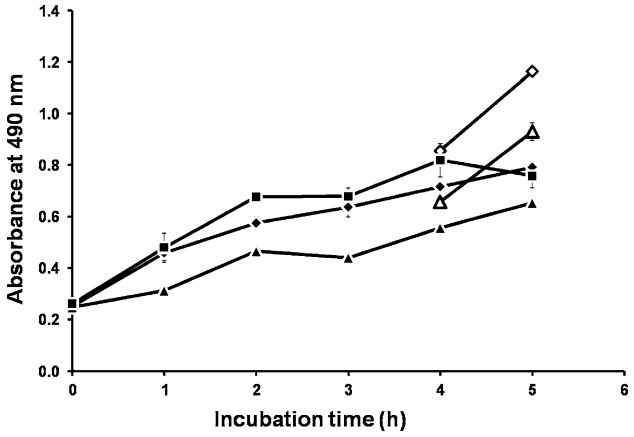

Fig. 6 Inhibition ELISA for bovine whey protein. Inhibition of rabbit IgG binding to whey protein concentrates by whey protein hydrolysates at different incubation times. Shorter lines represent the hydrolysates that were obtained by incorporation of trypsin into the individual partially hydrolyzed whey protein after 3 h of incubation with MpuAP and pepsin (*filled diamonds* MpuAP, *filled triangles* pepsin, *filled squares* trypsin, *open triangles* Pepsin/trypsin and *open diamonds* MpuAP/trypsin). Whey protein solution was 2% (w/v) and all enzymes in reaction mixture were 0.5% on protein equivalent basis

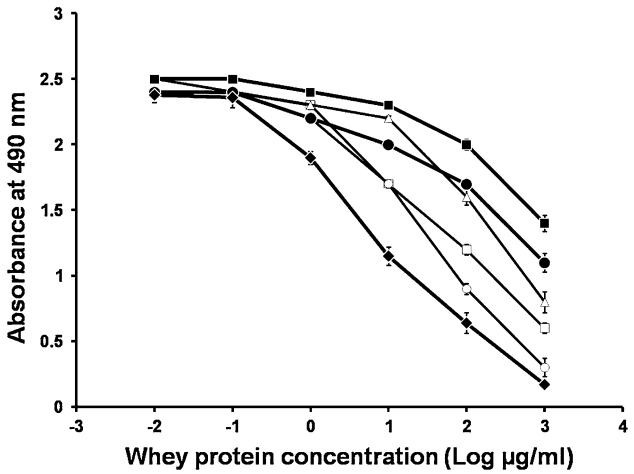

Fig. 7 Inhibition of rabbit IgG binding to whey protein concentrates by whey protein hydrolysates obtained by using different enzymes and their combinations with trypsin. Hydrolysates were obtained after 5 h of overall incubation. Trypsin was incorporated into the individual partially hydrolyzed whey protein after 3 h of incubation with MpuAP and pepsin. Single enzymes are indicated in *thin lines* (*open squares* MpuAP, *open circles* pepsin and *open triangles* trypsin). Enzyme combinations are indicated in *thick lines* (*filled circles* pepsin/trypsin, *filled squares* MpuAP/trypsin), and undigested whey protein is indicated in a *thick line* with the marker of *filled diamonds*

Inhibition of rabbit IgG binding to whey protein by the whey protein hydrolysates obtained by using different enzymes and combination systems is shown in Fig. 7. In the inhibition ELISA, the absorbance at 490 nm is inversely correlated with the antigenicity of the whey protein hydrolysates. Antigenicity reduction of the whey protein hydrolysates was enhanced by lowering concentration of the whey protein, and further its level was dominantly higher than other reaction systems under high concentration conditions of the whey protein. Almost complete loss of the antigenicity was observed at below 10 µg/ml of the whey protein in the combination reaction of MpuAP with trypsin. In a similar experiment, some non-hydrolyzable antigenic portions of α-LA and β-LG are identified in the hydrolysates of pepsin–trypsin combination [25]. The difference in the absorbance at 490 nm between unhydrolyzed whey protein and hydrolyzed whey protein indicates the degree of antigenicity reduction. Therefore, it clearly demonstrates that only the whey protein hydrolysates obtained by the combination of MpuAP with trypsin had the highest absorbance difference between the control (i.e., unhydrolyzed whey) and the hydrolysates compared with any other hydrolysates obtained by using only single enzyme or its combination.

Conclusion

The findings that the acid proteinase from *M. purpureus* No. 3403 has broad substrate specificity towards natural proteins, unique cleavage sites on the oxidized insulin B-chain, and long-term preservability in the lyophilized form are interesting. The results of the study on reducing antigenicity of whey protein demonstrate that the MpuAP–trypsin combination satisfied the complete digestion of antigenic proteins within a short time of incubation. Furthermore, the whey protein hydrolysates made with the MpuAP–trypsin combination system showed the lowest antigenicity and there were no detectable small peptide residues in tricine-SDS–PAGE analysis. Therefore, it might be possible to construct an infant formulas manufacturing process without ultrafiltration. Taken all together, the acid proteinase of *M. purpureus* has several prominent properties and might be applicable to produce hypoallergenic whey protein hydrolysates as a nutritional supplement, production of hypoallergenic infant formulas and cheeses such as ricotta and brown cheeses, and to use in bakery industries where whey protein is used as an additive.

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