Production and partial purification of proteases from *Aspergillus oryzae* grown in a medium based on whey protein as an exclusive nitrogen source

H. Kumura,¹ T. Ishido, and K. Shimazaki

Laboratory of Dairy Food Science, Division of Bioresources and Product Science, Hokkaido University, Kita 9, Nishi 9, 060-8589, Sapporo, Japan

ABSTRACT

Several attempts have been made to incorporate whey proteins into curd to increase cheese yield. For some types of cheese, degradation of whey proteins that have been incorporated into the curd would be required to obtain acceptable flavor and texture. On the basis of the high potential for protease synthesis in *Aspergillus oryzae*, sodium nitrate as a nitrogen source in a minimal medium for fungi, known as Czapek-Dox medium, was replaced with whey protein isolate to induce the protease to hydrolyze whey protein using *A. oryzae* AHU7146. A solid-phase medium adjusted to pH 6 was suitable for this purpose when incubation was carried out at 25°C for 2 wk. The application of column chromatography enabled the resolution of 3 proteolytic components (1, 2, and 3). With respect to optimal temperature and zymographic analysis, component 1 was similar to component 3. In contrast, component 2 was less abundant than the other components and exhibited activity in the alkaline pH region. The degradation of β -lactoglobulin and α -lactalbumin in whey protein isolate solution by the crude enzyme was primarily attributed to the action of components 1 and 3, based on HPLC analysis and the N-terminal amino acid sequences; however, zymography demonstrated evident proteolysis due to component 2. Because heat-denatured whey protein aggregates were digestible by the crude enzyme, the proteolytic system from *A. oryzae* has the potential as an additive to stimulate the ripening of cheese enriched with whey protein.

Key words: whey protein, *Aspergillus oryzae*, protease

INTRODUCTION

Whey, as a byproduct from the manufacture of cheese, comprises 85 to 90% of the total volume of the original milk and retains about 50% of the nutrients. To increase cheese yields, several attempts have been made

to incorporate whey proteins into curd. The technologies associated with this objective are high heat treatment, ultrafiltration, particulation, and the addition of transglutaminase after the rennet coagulation of milk (Hinrichs, 2001; Cozzolino et al., 2003). A high preheating temperature or ultrafiltration could improve the yield and texture of fresh cheese due to increased water binding to the denatured whey proteins; however, the type of cheese should be considered if these technologies are applied because the heating of concentrated milk to high temperatures could influence the composition and ripening of the cheese, which would result in a slightly poor quality with respect to flavor and texture for some kinds of cheese (Hinrichs, 2001).

Bovine milk protein consists of approximately 20% whey protein that includes β -LG and α -LA as the predominant proteins. Whey proteins are well known for their high nutritional value (Hambræus and Lönnerdal, 2003) and their functional characteristics of gelation and foaming properties (Mangino, 1992; Dickinson, 2003; Singh and Havea, 2003). Therefore, whey proteins have been incorporated into food products, including dairy products, infant formula, and bakery products, among others (Hoppe and Higgins, 1992; Mulvihill and Ennis, 2003). Moreover, numerous studies have shown that peptides from whey protein exhibit various physiological activities such as opioid-like properties, antihypertensive activity, and immune modulation (FitzGerald and Meisel, 2003; Madureira et al., 2010). The extensive hydrolysis of whey protein, in particular of β -LG, would be advantageous to reduce allergenicity of β -LG (Peñas et al., 2006; Morisawa et al., 2009). Because β -LG and α -LA tend to be more resistant to bacterial protease than casein, which is possibly due to their globular structure (Fairbairn and Law, 1986; Madureira et al., 2010), it would be ideal to incorporate whey protein to add intensively proteolytic microorganisms to the starter, including microorganisms used for mold-type cheeses or smear-ripened cheese, to improve cheese flavor and texture. The maturation of the smear-ripened cheese and white-mold cheese proceeds gradually from the surface to the inner part of the curd, whereas the ripening of blue-veined cheese occurs rather uniformly. Therefore, if the proteolytic system of the starter mi-

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¹Corresponding author: kumura@anim.agr.hokudai.ac.jp

croorganism associated with blue-veined cheese could act on whey protein that had been incorporated into the cheese curd, the liberation of the peptides from whey protein, leading to the development of novel types of flavors, could be expected. However, our preliminary study confirmed the low digestibility of protease from *Penicillium roqueforti*, isolated from commercial cheese, on whey protein.

In contrast, *Aspergillus oryzae*, the so-called koji molds, have been used extensively for indigenous Japanese fermentation products such as sake (rice wine), shochu (spirits), shoyu (soy sauce), and miso (soybean paste) for more than 1,000 years (Machida, 2002; Yu et al., 2004). The long history of extensive use in food industries prompted the US FDA to place *A. oryzae* on the list of generally regarded as safe organisms. The safety of *A. oryzae* is also supported by the World Health Organization (Taylor and Richardson, 1979; Barbesgaard et al., 1992; Machida, 2002). The genomics research of *A. oryzae* is complete, and 135 genes encoding secreted proteinases have been found (Machida et al., 2005). Recently, Izumi et al. (2009) studied the effect of α -LA and its hydrolysates on the concentration of glucagon-like peptide-2 (GLP-2) in serum using suckling rats. They observed that the oral administration of a hydrolysate of α -LA by a commercial protease preparation from *A. oryzae* led to almost twice the GLP-2 release in serum compared with that from intact α -LA and other α -LA hydrolysates. Moreover, the length of the small intestine and the number of epithelial cells per crypt were increased when the hydrolysate of α -LA by commercial protease from *A. oryzae* was given compared with the results from giving intact α -LA, which indicated that the hydrolysate promoted GLP-2 secretion along with a contribution to growth and maturation of the small intestine. Therefore, whey protein digested by proteases, in particular derived from *A. oryzae*, could be used not solely as foodstuff (such as seasoning, sauce, confectionary, and an additive to dairy and meat products) to add nutritional value, but as a functional food.

In this study, the nitrogen source in a minimal medium for fungi was replaced with whey protein to induce proteinases responsible for whey protein digestion from *A. oryzae*. Furthermore, the fractionation and partial characterization of the proteinase were performed to evaluate its potential for use in the cheese industry.

MATERIALS AND METHODS

Strain and Growth Medium

Aspergillus oryzae AHU7146, originally isolated from miso and maintained in the culture collection of Hokkaido University, was grown in potato dextrose agar

(Merck KGaA, Darmstadt, Germany) at 25°C for 2 wk. The conidia were isolated and dispersed in 0.9% NaCl solution.

The medium for protease production was based on the minimum medium for fungi, Czapek-Dox. Sodium nitrate was replaced as the nitrogen source with whey protein isolate (WPI). The final medium content was as follows: 0.05% of KCl, 0.1% of KH_2PO_4 , 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002% of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3.6% of glucose, and 5% of WPI; this medium was prepared and adjusted to a final pH of 4.0, 5.0, or 6.0 with 1 M HCl. The solution was aliquoted (15 mL) in an Erlenmeyer flask (100 mL) and autoclaved to prepare the solid-phase medium. Alternatively, the liquid-phase medium was prepared as follows: 15 mL of the solution, whose pH value was adjusted to 8.4, was filter-sterilized and recovered in an Erlenmeyer flask (100 mL); filtration was followed by the addition of 0.7 mL of 1 M HCl to adjust the pH value to 6.0. Each medium-containing Erlenmeyer flask (100 mL) received 3.5×10^4 conidia and was maintained at 15, 20, 25, or 30°C for 1, 2, or 4 wk. Unless otherwise stated, the pH value of the medium was adjusted to 6.0 before autoclaving, and incubation was carried out at 25°C for 2 wk.

Preparation and Purification of the Enzyme

The extraction of the enzyme was performed as follows: 10 mL of distilled water was added to the Erlenmeyer flask containing solid-phase medium previously inoculated with *A. oryzae* AHU7146, and the medium and sample were pounded to clots. The material was transferred to a polyethylene bag. The sample was treated by a stomacher and transferred to a centrifugal tube. Following centrifugation at $10,000 \times g$ and 4°C for 15 min, the supernatant was recovered and solid ammonium sulfate was added to yield 90% saturation. The precipitate was collected by centrifugation at $10,000 \times g$ and 4°C for 15 min and dissolved in 1 mL of 0.02 M sodium phosphate buffer at pH 6.0. The resulting solution was used as a crude enzyme.

The crude enzyme was extensively dialyzed against 0.02 M sodium phosphate buffer at pH 6.0 and applied to a column (10 $\times$ 100 mm) of Butyl-Toyopearl 650S (Tosoh, Tokyo, Japan) that was pre-equilibrated with the same buffer. The column was washed thoroughly with the same buffer to remove unadsorbed materials. The adsorbed materials were then eluted by a linear gradient of (0–50%) ethanol in the same buffer (30 mL), and the elution was continued using 50% ethanol in the same buffer (10 mL) at a flow rate of 1 mL/min. Proteinase-rich fractions were pooled and applied to a column (10 $\times$ 100 mm) of DEAE-Toyopearl 650M (Tosoh) that was pre-equilibrated with 0.02 M sodium

Table 1. Production of protease¹ of *Aspergillus oryzae* AHU7146 under various pH and temperature conditions for defined incubation periods²

pH and temperature	Incubation period		
	1 wk	2 wk	4 wk
pH 4.0			
15°C	3.2 ± 0.4	7.5 ± 0.6	3.1 ± 0.0
20°C	4.8 ± 0.2	1.5 ± 0.2	2.0 ± 0.3
25°C	3.9 ± 1.9	0.6 ± 0.2	5.3 ± 2.7
30°C	1.3 ± 0.2	6.9 ± 1.2	1.7 ± 1.0
pH 5.0			
15°C	4.0 ± 0.3	6.3 ± 0.7	4.3 ± 0.2
20°C	4.3 ± 0.5	5.0 ± 0.0	4.7 ± 0.4
25°C	1.9 ± 0.1	2.5 ± 0.5	1.9 ± 0.5
30°C	1.4 ± 0.0	3.1 ± 0.3	5.6 ± 0.0
pH 6.0			
15°C	4.3 ± 0.3	5.9 ± 0.7	3.4 ± 0.2
20°C	5.6 ± 0.2	4.8 ± 0.4	19.2 ± 3.6
25°C	4.0 ± 0.2	15.9 ± 1.4	15.2 ± 0.3
30°C	1.9 ± 0.5	10.6 ± 0.4	4.4 ± 1.8

¹Protease activity in the crude enzyme was expressed in protease units (PU)/mL; see details in text.

²Average value ± standard error is shown from 2 determinations per experiment from 2 experiments.

phosphate buffer at pH 6.0. The column was washed thoroughly with the same buffer to remove unadsorbed materials, and the adsorbed materials were then eluted by a linear gradient of (0 to 1 M) NaCl in the same buffer (30 mL). The elution was then continued using 2 M NaCl in the same buffer (10 mL) at a flow rate of 1 mL/min.

The materials unadsorbed to Butyl-Toyopearl 650S were directly applied to a column (10 × 440 mm) of DEAE-Toyopearl 650M (Tosoh) that was pre-equilibrated with 0.02 M sodium phosphate buffer at pH 6.0. The column was washed thoroughly with the same buffer to remove unadsorbed materials and then the adsorbed materials were eluted by a linear gradient of 0.15 to 0.55 M NaCl in the same buffer (180 mL), and the elution was continued using 2 M NaCl in the same buffer (40 mL) at a flow rate of 1 mL/min.

Measurement of Protease Activity

The proteolytic activity was determined as described previously with some modifications (Kumura et al., 1991). The substrate, 0.5% casein dissolved in 0.05 M sodium phosphate buffer at pH 6.0 (700 µL), was incubated with an appropriately diluted enzyme solution (50 µL) at a defined temperature for 1 h. The reaction was terminated by the addition of 15% trichloroacetic acid solution (750 µL), and further incubation was carried out for 15 min. The resulting preparation was centrifuged at 10,000 × g and 25°C for 15 min to obtain the supernatant. The optical density at 280 nm was measured to calculate the protease activity. One unit of protease (PU) was expressed as the amount of the

protease that allows an increase in the optical density of the substrate at 280 nm of 0.001 per 1 min. Unless otherwise mentioned, incubation was carried out at 30°C. Alternatively, casein was dissolved in 0.05 M sodium citrate or 0.05 M sodium borate to adjust to the desired pH value.

Analysis of Whey Protein Hydrolysis

The degradation profile of whey protein by fungal protease was investigated by HPLC. One milliliter of 0.5% WPI dissolved in 0.05 M sodium phosphate at pH 6.0 was mixed with 2 U of crude enzyme or individual component, filter-sterilized and incubated at 30°C for 24 or 72 h. The resulting solution was injected into the HPLC.

Furthermore, heat-aggregated whey protein was used as a substrate; cheese whey obtained from an experimental factory at Hokkaido University was adjusted to pH 6.0 using lactic acid and heated above 95°C for 10 min in boiling water. After cooling to room temperature, the precipitate was collected by centrifugation at 10,000 × g and 25°C for 10 min. The precipitates of 20-mg wet weight were transferred to a test tube and dispersed in 1 mL of 0.05 M sodium phosphate buffer at pH 6.0, containing 0.05% NaN₃ and 2 U of proteolytic activity in the form of the crude enzyme equivalent. The test tube was incubated at 30°C for 24 h and centrifuged at 10,000 × g and 4°C for 10 min to recover the supernatant as the sample for HPLC.

The samples described above were loaded on a TSK gel ODS-100S column (4.6 × 150 mm; Tosoh, Tokyo, Japan). As the starting solvent, 0.1% trifluoroacetic acid in water was used. Elution was performed by a

Figure 1. Liquid chromatography of the whey protein isolate (WPI) hydrolysate. The digestion was carried out using an amount equivalent to 2 U of protease at 30°C. (A) Unhydrolyzed WPI; (B) digested by crude enzyme from *Aspergillus oryzae* AHU7146 for 72 h; (C) digested by component 1 for 24 h; (D) digested by component 2 for 72 h; (E) digested by component 3 for 24 h. The constituents of the numbered fractions were subjected to N-terminal amino acid sequence analysis, and the results are shown in Table 2.

linear gradient of acetonitrile, 0 to 100% in 35 min, at a flow rate of 0.4 mL/min, and fractions were collected. The N-terminal amino acid sequence of fragments in selected fractions was determined by means of a protein sequencer (Procise 491 cLC, Applied Biosystems, Austin, TX). The cleavage sites were identified on the basis of the probable amino acid sequence of the released peptide fractions that corresponded to at least 4 serial amino acids that correspond to β -LG, α -LA, and glycomacropeptide.

Protein Determination and Zymography

The protein concentration was determined using a modified Lowry method (Peterson, 1977) with BSA as a standard.

Zymography was carried out according to the method of Raser et al. (1995) with slight modification. In brief, a separating gel containing 0.2% of WPI was used. After electrophoresis, the gels were incubated in a 0.02 M sodium phosphate buffer at pH 6.0 with slow shaking

Figure 2. Ion-exchange chromatography profile of the proteolytic active fractions obtained from the Butyl-Toyopearl 650S adsorptive (A) and nonadsorptive (B) component. The DEAE-Toyopearl 650M was used for separation with a 0.02 M sodium phosphate buffer at pH 6.0 as the starting solvent, followed by a gradient of NaCl. The amount of protein was measured at an absorbance of 280 nm ($\square$), and the enzyme activity ($\bullet$) is also shown. Indicated fractions were pooled and used as components 1, 2, and 3 in the subsequent experiments. OD = optical density. Color version available in the online PDF.

for 30 min. The gels were further incubated overnight at ambient temperature in the same buffer. Finally, the gels were stained with Coomassie blue R-250 (Nacalai Tesque, Kyoto, Japan).

RESULTS

Effect of Temperature and pH on Protease Synthesis

Aspergillus oryzae AHU7146 was grown under various pH and temperature conditions to induce protease activity. As shown in Table 1, at the 2-wk time point, large amounts of protease activity were detected when the pH value and temperature were adjusted to 6.0 and 25°C, respectively. Even if the incubation at 25°C was continued up to 4 wk, no accumulation of the activity

Figure 3. Effect of pH on the proteolytic activity of the components 1 (A), 2 (B), and 3 (C) at 30°C using 0.5% casein substrate dissolved in 0.05 M sodium citrate ($\bullet$), 0.05 M sodium phosphate ($\blacktriangle$), or 0.05 M sodium borate ($\blacksquare$) buffer.

Table 2. Probable sequence of the peptide obtained by HPLC

Probable origin of the fragment	Possible sequence of AA	AA position	Substrate	Enzyme ¹	HPLC peak
GMP ²	AIPPK	M106-A107	WPI ³	1	Figure 1C-4
			Aggregates	CE	Figure 6B-1-3
	QDKTE	N113-Q114	WPI	3	Figure 1E-1
	EIPT	T117-E118	Aggregates	CE	Figure 6B-1, 2
	TINT	P120-T121	WPI	1	Figure 1C-4
	GEPTS	S127-G128	WPI	1	Figure 1C-1-3
				3	Figure 1E-3, 4
	EVIE	P150-E151	WPI	CE	Figure 1B-2-4
	SPPEI	E154-S155	WPI	2	Figure 1D-1, 2
				3	Figure 1E-1, 2, 4
α -LA	VQVT	T161-V162	Aggregates	CE	Figure 6B-1
	LKDLK	E11-L12	Aggregates	CE	Figure 6B-3
	SLPE	V21-S22	WPI	2	Figure 1D-1
	AIVQ	Q39-A40	Aggregates	CE	Figure 6B-1
	NDSTE	N44-N45	Aggregates	CE	Figure 6B-3
	FQIN	L52-F53	Aggregates	CE	Figure 6B-3
	FQINN		WPI	CE	Figure 1B-2, 3
	NKIW	N56-N57	WPI	CE	Figure 1B-3, 4
	KDDQN	C61-K62	WPI	1	Figure 1C-1, 2
			WPI	3	Figure 1E-4
β -LG	DQNP	D63-D64	WPI	1	Figure 1C-4
	CDKF	S76-C77	WPI	3	Figure 1E-1, 2
	DDLTD	D82-D83	WPI	3	Figure 1E-4, 5
	TDDI	L85-T86	WPI	3	Figure 1E-5
	KKIL	V92-K93	Aggregates	CE	Figure 6B-3
	VGIN	K98-V99	Aggregates	CE	Figure 6B-3
	GINY	V99-G100	WPI	1	Figure 1C-4
	KLDQ	E113-K114	Aggregates	CE	Figure 6B-4
	TMKGL	Q3-4T	WPI	1	Figure 1C-3, 4
	LDIQK	G9-L10	WPI	1	Figure 1C-1, 2
			WPI	3	Figure 1E-3
	LKPT	E45-L46	WPI	CE	Figure 1B-1
	EGDLE	P50-E51	Aggregates	CE	Figure 6B-3
	GDLE	E51-G52	WPI	3	Figure 1E-4
	LQKW	L57-L58	WPI	1	Figure 1C-1, 3, 4
	WENG	K60-W61	WPI	1	Figure 1C-1, 2
	AEKTK	I72-A73	WPI	1	Figure 1C-3
			WPI	3	Figure 1E-3, 4
	TKIPA	K75-T76	Aggregates	CE	Figure 6B-3
	VLDT	L93-V94	Aggregates	CE	Figure 6B-3, 4
	AEPEQ	S110-A111	WPI	3	Figure 1E-4
	EQSLA	P113-E114	Aggregates	CE	Figure 6B-3
	VRTPE	L122-V123	WPI	1	Figure 1C-3, 4
	KALP	L140-K141	WPI	1	Figure 1C-2
	LPMHI	A142-L143	WPI	CE	Figure 1B-2
	SFNP	L149-S150	WPI	1	Figure 1C-1, 4
	FNPT	S150-F151	WPI	3	Figure 1E-1-5
	TQLEE	P153-T154	WPI	3	Figure 1E-4

¹1: Component 1; 2: component 2; 3: component 3; CE: crude enzyme. Component 1 was the adsorptive component of Butyl-Toyopearl 650S (details are shown in Figure 2A). Components 2 and 3 were nonadsorptive components of Butyl-Toyopearl 650S (details are shown in Figure 2B).

²Glycomacropeptide.

³Whey protein isolate.

was monitored. In contrast, incubation at 20°C for 4 wk resulted in activity comparable to that obtained at 25°C for 2 wk.

Confirmation of Whey Protein Degradation Using Crude Enzyme by HPLC

The WPI solution was treated with the crude enzyme prepared from *A. oryzae* AHU7146 grown on the solid-phase medium at 25°C for 2 wk. Due to the

hydrolysis, several peaks appeared upon HPLC elution (Figure 1B), some of which were collected to analyze N-terminal amino acids. Some cleavage sites were successfully proposed and are listed in Table 2.

Fractionation of Protease in Crude Enzyme

Because multiple proteolytic components were predicted to be involved in the activity of the crude enzyme, fractionation using column chromatography

Table 3. Purification of protease from *Aspergillus oryzae* AHU7146

Sample ¹	Total volume (mL)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
After extraction	360	4,770	76	1.0	100
Dialyzed crude enzyme	23	3,900	229	3.0	81.8
Ethanol-eluted fraction	11	1,470	2,568	33.7	30.8
Component 1	5	550	3,602	47.2	11.5
Component 2	8	220	98	1.3	4.6
Component 3	12	590	118	1.6	12.4

¹Component 1 was the adsorptive component of Butyl-Toyopearl 650S (details are shown in Figure 2A). Components 2 and 3 were nonadsorptive components of Butyl-Toyopearl 650S (details are shown in Figure 2B).

was performed. Even under a low salt concentration, some of the proteolytic components were adsorbed to the Butyl-Toyopearl ligand, and these components were successfully eluted by a gradient of buffered ethanol solution (data not shown). Figure 2 shows the elution profile of the fractions that were adsorptive or nonadsorptive to the hydrophobic ligand, which were then applied to a DEAE-Toyopearl column. The hydrophobic ligand adsorptive fraction showed a homogeneous chromatographic profile and was recovered as component 1. The hydrophobic ligand nonadsorptive fraction resolved into 2 peaks on the DEAE column, and these peaks were recovered as components 2 and 3. A representative result of purification is summarized in Table 3.

Partial Characterization of Individual Components

Components 1 and 3 were acidic proteases, whereas component 2 retained its activity in the alkaline pH region (Figure 3). The maximum proteolytic activity was obtained at 50°C for components 1 and 3, and at 40°C for component 2 (Figure 4).

The incubation of the WPI solution in the presence of each component led to the release of peptides, and several peaks appeared with HPLC elution (Figure 1 C–E). Some of them were collected to analyze N-terminal amino acids, and proposed cleavage sites are shown in Table 2. Although attempts to identify the substrate specificity toward β -LG of component 2 by HPLC were unsuccessful, the susceptibility of whey protein to this component was more evident than the others from the zymography (Figure 5). The electrophoretic mobility of component 1 was similar to that of component 3, whereas component 2 migrated more slowly.

Digestion of Whey Protein Aggregates by Crude Enzyme

To observe the accessibility of the crude enzyme to the aggregate form of whey protein, whey protein aggregates dispersed in a buffer solution were treated with the crude enzyme. Due to hydrolysis, several peaks

appeared by HPLC elution (Figure 6), some of which were collected to analyze N-terminal amino acids. Some cleavage sites were successfully proposed and listed in Table 2. At the end of the incubation, the sample was recovered to monitor bacterial contamination using standard plate count agar. Because the colony formation was undetectable, the effect of the bacterial protease was neglected (data not shown).

DISCUSSION

Our initial attempt was to induce proteases responsible for the degradation of whey protein with fungi used in cheese production, such as *P. roqueforti* and *P. camemberti*, in a modified Czapek-Dox medium whose nitrogen source was replaced by WPI, as was in this study. Although the growth of these fungi was possible, the secretion of protease was so poor that we selected *A. oryzae* as an alternative enzyme source.

In this study, the secreted protease activity was the highest if the pH value and temperature were adjusted to 6.0 and 25°C, respectively. Under the condition of the liquid-phase medium comprising the same constituents at 25°C for 2 wk, the secreted proteolytic activity from *A. oryzae* AHU7146 was negligible (data not shown). It is well known that solid-phase media are preferable to submerged culture for enzyme production by *A. oryzae* (Kitano et al., 2002; Te Biesebeke et al., 2005). Although this study was intended to induce proteases able to hydrolyze whey protein, casein was found to be preferable as the substrate to enhance the sensitivity of the assay. Therefore, in addition to the observation concerning the effect of temperature and pH on the protease synthesis of *A. oryzae* AHU7146, the purification and characterization of individual components were carried out using casein as the substrate.

By means of column chromatography, the crude enzyme was fractionated into 3 proteolytic components. Components 1 and 3 exhibited similar properties with respect to optimal temperature, electrophoretic mobility during zymographic analysis, and the ability to digest β -LG and α -LA in WPI solution, although component

Figure 4. Effect of temperature on the proteolytic activity of components 1 (●), 2 (■), and 3 (▲) at pH 6.0.

1 was more hydrophobic and still active in the neutral pH region. Component 2 was less abundant than the other components and retained activity in the alkaline pH region.

Because of the small quantities obtained, information such as molecular weight, the amino acid sequence and isoelectric point of the individual components remain unclear. Vishwanatha et al. (2010) adapted a response-surface methodology to induce the acid protease from *A. oryzae* MTCC 5341 and concluded that its optimum factor levels were pH 5.4, incubation at a temperature of 31°C, and 123 h as the fermentation time. It might be possible to modify the resulting ratio of the 3 components obtained in our study if these factors are

Figure 6. Liquid chromatography of the hydrolysate from whey protein aggregates dispersed in a buffer system: (A) indicates that no enzyme was added; (B) indicates digestion by the crude enzyme equivalent to 2 U of protease from *Aspergillus oryzae* AHU7146 at 30°C for 24 h. The chromatography was performed using a TSK gel ODS-100S column (4.6 × 150 mm; Tosoh, Tokyo, Japan) with 0.1% trifluoroacetic acid as the starting solvent, followed by a gradient of acetonitrile. The constituents of the numbered fractions were subjected to N-terminal amino acid sequence analysis, and results are shown in Table 2.

Figure 5. Zymograms using whey protein isolate as the substrate. An amount equivalent to 2 U of protease as a crude enzyme (lane A), component 1 (lane B), component 2 (lane C), and component 3 (lane D) were subjected to the gel. Arrows indicate the transparent band. Color version available in the online PDF.

carefully considered. Furthermore, a genomic database is advantageous to develop microarray analysis, which will help in the identification of molecular species for the corresponding proteinases.

The degradation of β -LG and α -LA in the WPI solution induced by the crude enzyme was primarily due to the action of components 1 and 3; however, the proposed cleavage sites identified using a crude enzyme preparation were inconsistent with those of the individual components, which was attributed to the concomitant peptidases in the preparation of crude enzyme. Indeed, the identification of cleavage sites using the crude enzyme was more difficult, which was interpreted as the result of extensive degradation by the synergic action of proteinases and peptidases. Mizuno et al. (2004) compared the hydrolysis of casein using 9 different commercially available proteases and found that the preparation using protease from *A. oryzae*

Figure 7. Process modification in the manufacturing of soft, semi-hard, and hard cheeses when whey protein particles (WPP) are added. Scheme (A) is from Hinrichs (2001); (B) is an alternative strategy to incorporate WPP into cheese curd. *A. oryzae* = *Aspergillus oryzae*.

showed the shortest average peptide length of 1.4 AA in the sequence and the highest angiotensin-I converting enzyme inhibitory activity. In contrast, Izumi et al. (2009) demonstrated that the oral administration of hydrolysates of α -LA by commercial protease from *A. oryzae* significantly promoted small intestinal elongation and increased the number of crypt epithelial cells compared with intact α -LA. They treated α -LA with the commercial protease at a pH of 7.0, and at the end of the incubation at 50°C for 18 h, the average molecular weight of the resulting peptides was 545, which was longer than that obtained from casein digestion. In this study, we identified the cleavage sites on the basis of the probable AA sequence of the released peptide consistent with at least 4 serial AA residues; however, it was possible to involve peptides composed of fewer than 3 amino acids.

Britten et al. (1994) incubated WPI with a commercial protease from *A. oryzae* under alkaline conditions (pH 8) and observed the preferential hydrolysis of β -LG and resistance of α -LA and BSA to the protease. The results suggest that the protease involved in the prepa-

ration was different from the 3 components used in the current study with respect to optimal pH region and substrate specificity. Rao et al. (1998) reviewed microbial proteases and described that the fungal acid protease had its optimum pH between 4 and 4.5. Components 1 and 3 were acid proteases; however, the proteolytic activity could not be determined using casein as the substrate at this pH because of the poor solubility of the casein. The application of other substrates such as hemoglobin and cytochrome c will clarify the effect of acidic pH on the activity and facilitate its comparison with other acidic proteases from *A. oryzae*. Vishwanatha et al. (2009) found that the acid protease from *A. oryzae* MTCC 5341 preferred hydrophobic amino acids as the cleavage site; however, 3 components in this study showed broad specificity.

Although no cleavage site of component 2 was found in β -LG, zymography demonstrated that proteolysis induced by component 2 was evident. It should be considered that zymography was carried out in the presence of surfactant, which could influence both the substrate structure and the catalytic properties. Tujita and Endo

(1978) reported a membrane acid protease in *A. oryzae* that was activated in the presence of Triton X-100. Moreover, β -LG exhibits oligomeric form between pH 3.5 and 6.5 (Sawyer, 2003). These factors would affect the conformation of the substrate and lead to the alteration of enzyme accessibility. Therefore, the substrate specificity of these components should be carefully interpreted, with the properties of the substrate and hydrolytic environment taken into account. The HPLC elution profile of heat-denatured whey protein digested by the crude enzyme for 24 h (Figure 6B) was similar to that for 72 h (data not shown). In contrast, accumulation of hydrolysate from WPI digested by the crude enzyme was monitored when the HPLC profile after incubation for 72 h (Figure 1B) was compared with that after 24 h (data not shown). The result was, in part, due to the limitation of accessibility of the enzyme to the aggregated form of the denatured protein, and fine particulation of the heat-denatured whey protein would stimulate liberation of the peptide. Thus, even if the whey protein had been heat denatured, that is, aggregate form prepared by heating cheese whey, it can be concluded that the aggregate form would be digested by the crude enzyme from *A. oryzae*.

In conclusion, the 3 components associated with the degradation of whey protein were isolated when a solid-phase medium containing WPI as the nitrogen source was used for growth of *A. oryzae* AHU7146. Figure 7A shows the schema of cheese manufacture attempted by the addition of whey protein particles (Hinrichs, 2001), and it is of interest to postulate an alternative process as indicated in Figure 7B: the application of the proteolytic system of *A. oryzae* to heat-denatured whey protein particles for pre-ripening. Following maturation under optimal conditions, the material will be mixed with curd before pressing so that the curd will receive the pre-ripened whey protein as not only a nutritional additive but also as an adjunct to stimulate the ripening of the cheese matrix. For this purpose, further studies are needed, including the formulation of a protease mixture from *A. oryzae* and the adjustment of the amount of the enzyme and ripening circumstances such as temperature, pH, and incubation period to develop an acceptable flavor.

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