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Development of a New Type of Fermented Cheese Whey Beverage with Inhibitory Effects against Angiotensin-Converting Enzyme

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Summary

Cheese whey was digested with 7 kinds of proteases for 24hr at 37°C (trypsin, proteinase-K, actinase-E, thermolysin and papain) or 25°C (pepsin and chymotrypsin). Strong inhibitory activity of more than 95% against angiotensin-converting enzyme (ACE, EC 3. 4. 15. 1) of the rabbit lung was generated by proteinase-K and thermolysin digestion. The digested cheese whey was then fermented at 37°C for 24hr with 2% (v/v) inoculum of 2 kinds of lactic acid bacterial culture (each 1% of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*). Through the comparison of the ACE inhibitory activity before and after lactic acid fermentation, proteinase-K was selected as the most suitable enzyme among the 7 proteases tested, because it showed almost no decrease in activity after fermentation (from 89.9 to 89.8%). Based on the results of the preliminary experiments, a new type of fermented cheese whey beverage containing ACE activity was prepared. The IC_{50} value in the fermented cheese whey beverage was 50 ng/ml.
Key words : Angiotensin-converting enzyme (ACE), Cheese whey, Protease digestion, ACE inhibitor, Lactic acid fermentation, Beverage

Recently, milk proteins have been reported to show several biological and physiological activities after digestion with several proteases such as pepsin or thermolysin. Opioid peptides, immunopeptides, mineral-binding peptides and antihypertensive peptides were derived from milk proteins (mainly from casein) after protease digestion (1). Several inhibitors of angiotensin I-converting

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enzyme (ACE), which catalyze both the production of vasoconstrictor angiotensin II and the inactivation of vasodilator bradykinin, have been isolated from human and bovine casein (2-6) such as CEI_{12} (from α_{S1}-casein), $CEI_{7\beta}$ (from β-Casein), casoxin (from κ-casein) and κ-caseinosin (from caseinoglycopeptide (CGP, κ-casein : f106-169)).

However, little is known about the derivation of the ACE inhibitory activity from whey proteins or cheese whey. Sweet cheese whey is a by-product of rennet coagulated cheese manufactured from bovine milk, and it contains many valuable components (7-9) such as lactose, whey proteins, minerals, and also CGP (10).

In our previous study, we found strong inhibitory activity against ACE was detectable after protease digestion of cheese whey and that the inhibitors were mainly came from whey proteins (11). In this report, we describe the development of a new type of fermented cheese whey beverage with antihypertensive and physiological effects.

Materials and Methods

Materials

Cheese whey powder (CWP) was obtained from Snow Brand Milk Products Co. Ltd (Tokyo, Japan). Trypsin (from porcine pancreas), chymotrypsin (from bovine pancreas), thermolysin (from *Bacillus thermoproteolyticus*), papain (from *Carica papaya*) and angiotensin-converting enzyme (ACE, from rabbit lung) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pepsin (from porcine gastric mucosa) and proteinase-K (from *Tritirachium album*) were obtained from Boehringer Mannheim GmbH (Boehringer, Germany). Actinase-E (from *Actinomyces* ssp.) was purchased from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Hippuryl-hystidyl-leucine (HHL, substrate against ACE) was obtained from Sigma Chemical Co. Ltd. (St. Louis, USA). Two strains of lactic acid bacteria (*Lactobacillus* (*L.*) *delbrueckii* ssp. *bulgaricus* NIAI B6 and *Streptococcus* (*St.*) *thermophilus* NIAI 510) were purchased from the National Institute of Agriculture Industry (NIAI, Tsukuba, Japan).

Enzymatic digestion of CWP by seven proteases under laboratory and industrial conditions

Digestion of proteins in CWP by 7 kinds of proteases (pepsin, trypsin, chymotrypsin, proteinase-K, actinase-E, thermolysin and papain) was performed under optimal laboratory conditions of the buffer, pH and temperature as shown in Table 1. CWP (100 mg, protein content : 10.9% (w/w)) was dissolved in the optimum buffer (10 ml) followed by incubation for 24hr with 5 μg (protein : enzyme = 100 : 5, w/w) of pepsin or chymotrypsin at 25°C, or with other proteases at 37°C. For industrial preparation, CWP (100 mg) dissolved in distilled water

(10 ml) was treated with each protease (5 μg) for 24hr at 37°C. After protease digestion, the samples were heated at 98°C for 10 min to inactivate the remaining protease activity, and the ACE inhibitory activity was determined.

Determination of protein

Protein content in the samples was determined by the Folin-Lowry method (12). Absorbance was measured at 750 nm by a Shimazu UV-VIS spectrophotometer Model UV-120 (Kyoto, Japan). Bovine serum albumin (BSA) was used as a standard protein for calibration.

In vitro assay of inhibitory activity against ACE

The inhibitory activity against ACE was measured *in vitro* by the method of Yamamoto *et al.* (13), which was a modification of the original method of Lieberman (14), with a scale-down (1/5) modification in this study. The content of hippuric acids liberated from HHL (substrate) by enzymatic reaction of ACE was photometrically determined at 228 nm after ethyl acetate extraction. The concentration of ACE inhibitors (peptides) reducing 50% of the ACE inhibitory activity was defined as the IC_{50} value.

Evolution of ACE inhibitory activity before and after the fermentation step

CWP (100 mg) was dissolved in distilled water (10 ml), and then each protease (5 μg, protein : enzyme = 100 : 5, w/w) was added. After incubation at 37°C for 24hr, the sample solution was heated at 98°C for 10 min to inactivate the enzymatic activity. After addition of 2% (v/v) of lactic acid bacterial culture (1% each of *L. bulgaricus* NIAI B6 and *St. thermophilus* NIAI 510), fermentation was performed at 37°C for 24 hr. The ACE inhibitory activity and pH value of each sample were measured before and after the lactic acid fermentation.

TABLE 1. *The Digestive Conditions about Buffer, pH and Temperature on 7 Different Proteases Tested*

Enzyme	Buffer	pH	Temperature (°C)
pepsin	0.05 N HCl	2.0	25
trypsin	0.02 M Tris-HCl*	8.0	37
chymotrypsin	0.02 M CH_3COONH_4	8.0	25
proteinase-K	0.02 M Tris-HCl	7.5	37
actinase-E	0.02 M Tris-HCl*	8.0	37
thermolysin	0.02 M Tris-HCl*	8.0	37
papain	0.02 M Sodium phosphate	7.0	37

HCl = hydrochrolic acid, Tris = trishydroxymethyl aminomethane, CH_3COONH_4 = ammonium acetate

*Containing 10 mM of $CaCl_2$

A trial to develop a cheese whey beverage with proteinase-K digestion and lactic acid fermentation

CWP (7g) and sucrose (10g) were dissolved in distilled water (83 ml), and then proteinase-K (14 μg) was added. The 7 (w/w)% CWP solution was incubated at 37°C for 2 or 4 hr. After protease digestion, the sample solution was heated at 75°C for 15 min to inactivate the enzyme. After inoculation of 2% (v/v) of bacterial culture (1% (v/v) each of *L. bulgaricus* NIAI B6 and *St. thermophilus* NIAI 510), the sample was incubated at 37°C for 4, 8 or 24 hr. The numbers of living bacteria cells in the samples were countered by a plate counting method on a plate-count agar containing Bromo Creasol Purple (BCP) (Nissui, Tokyo) according to the official standard assay method in Japan. Each plate was incubated at 37°C for 72±3 hr, and the cells in 30-300 yellow colonies were counted on each plate. During the operation, the ACE inhibitory activity and pH values were measured in some samples at each step before and after the protease digestion

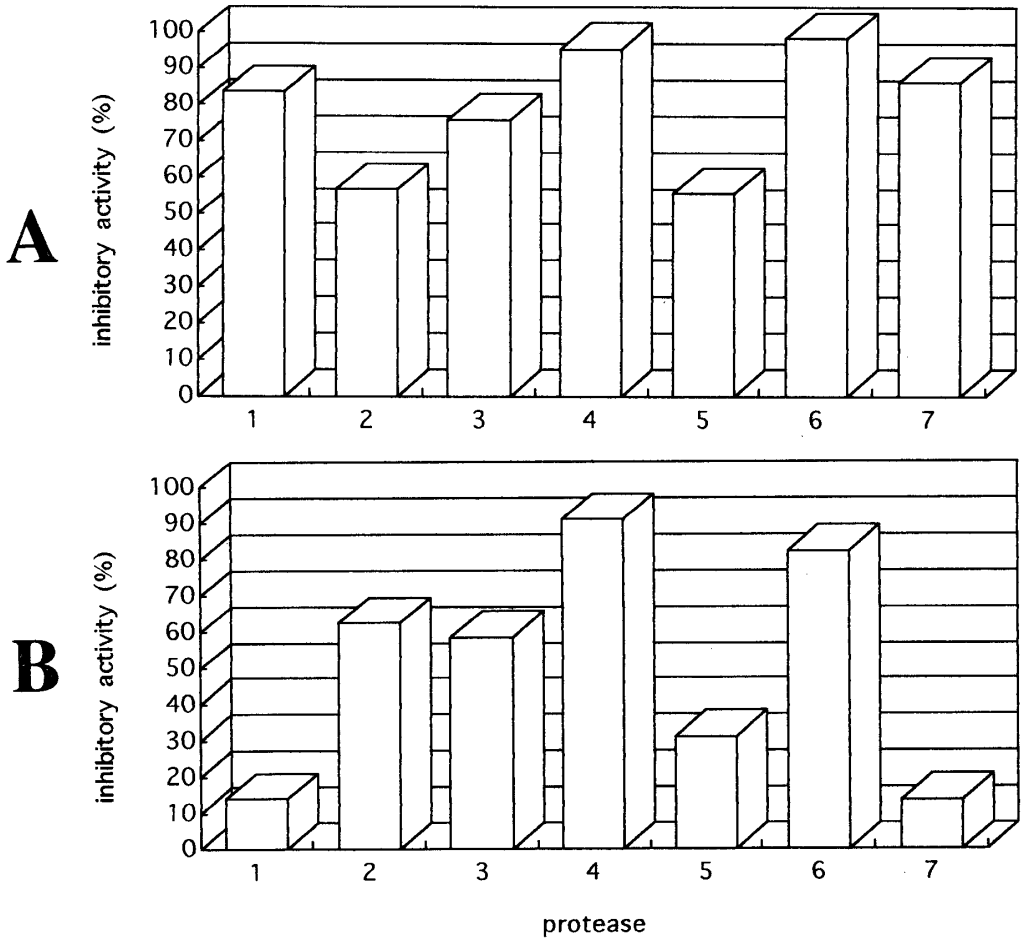

FIG 1. The comparison of ACE inhibitory activity derived from cheese whey by protease digestion in optimum buffer (A) or in distilled water (B).
Proteases: 1; pepsin, 2; trypsin, 3; chymotrypsin, 4; proteinase-K, 5; actinase-E, 6; thermolysin, 7; papain

and fermentation.

Results and Discussion

Whey proteins in cheese whey powder (CWP) were digested for 24 hr with 7 different proteases at 37°C (trypsin, proteinase-K, actinase-E, thermolysin and papain) or 25°C (pepsin or chymotrypsin) in each optimal buffer as shown in Table 1.

Fig. 1-A shows the derived inhibitory activity against angiotensin-converting enzyme (ACE) by 7 proteases under optimal digestive conditions. Before the protease digestion, the starting CWP solution showed no ACE inhibitory activity (data not shown). Strong ACE inhibitory activity of more than 95% was induced by digestion with proteinase-K (No. 4) or thermolysin (No. 6); the highest activity (98.6%) was observed in thermolysin. The activity induced by digestion with trypsin (No. 2) or actinase-E (No. 5) was not so strong (50-60%). The results indicated that thermolysin was the most prominent protease for induction of the highest ACE inhibitory activity from CWP under optimal buffer, pH and temperature conditions.

Fig. 1-B shows the results of the derived ACE inhibitory activity from CWP by the same proteases in distilled water (non-buffered condition) and at constant temperature at 37°C (not optimal temperature for pepsin and chymotrypsin). Strong activity of more than 80% was derived by proteinase-K (No. 4) and thermolysin (No. 6), with the highest activity (89.9%) by the former. Generation of only weak activity ($<15\%$) by pepsin (No. 1) and papain (No. 7) under these conditions was thought to be due to unsuitable pH and a lack of free metal ions,

TABLE 2. *The Changes of pH and ACE Inhibitory Activity of Cheese Whey after Protease Digestion and Lactic Acid Fermentation*

Protease	After protease digestion		After fermentation	
	pH	ACE Inhibitory activity (%)	pH	ACE Inhibitory activity (%)
pepsin	6.82	13.99	4.08	4.24
trypsin	6.92	62.53	4.04	39.20
chymotrypsin	6.82	58.51	4.04	74.28
proteinase-K	6.81	89.90	4.07	89.76
actinase-E	6.94	31.26	3.98	30.83
thermolysin	6.87	82.48	3.76	76.52
papain	6.38	13.74	4.06	6.74

One % (w/v) CWP solution (starting sample, 10 ml) was digested by each protease (5 μg, substrate : enzyme = 100 : 5, w/w) at 37°C for 24hr. Lactic acid fermentation was performed with 2 kinds of bacteria at 37°C for 24hr.

TABLE 3. *The change of pH and ACE inhibitory activity of cheese whey after proteinase-K digestion and fermentation by lactic acid bacteria*

Sample	Before fermentation		After fermentation (hr)					
			4		8		24	
	pH	%*	pH	%*	pH	%*	pH	%*
a-2**	6.21	98.87	5.76	95.50	5.38	96.87	4.22	97.62
a-4***	6.21	99.75	5.75	98.37	4.98	95.63	4.51	97.87

*ACE inhibitory activity
**CWP sample digested for 2 hr with proteinase-K
***CWP sample digested for 4 hr with proteinase-K

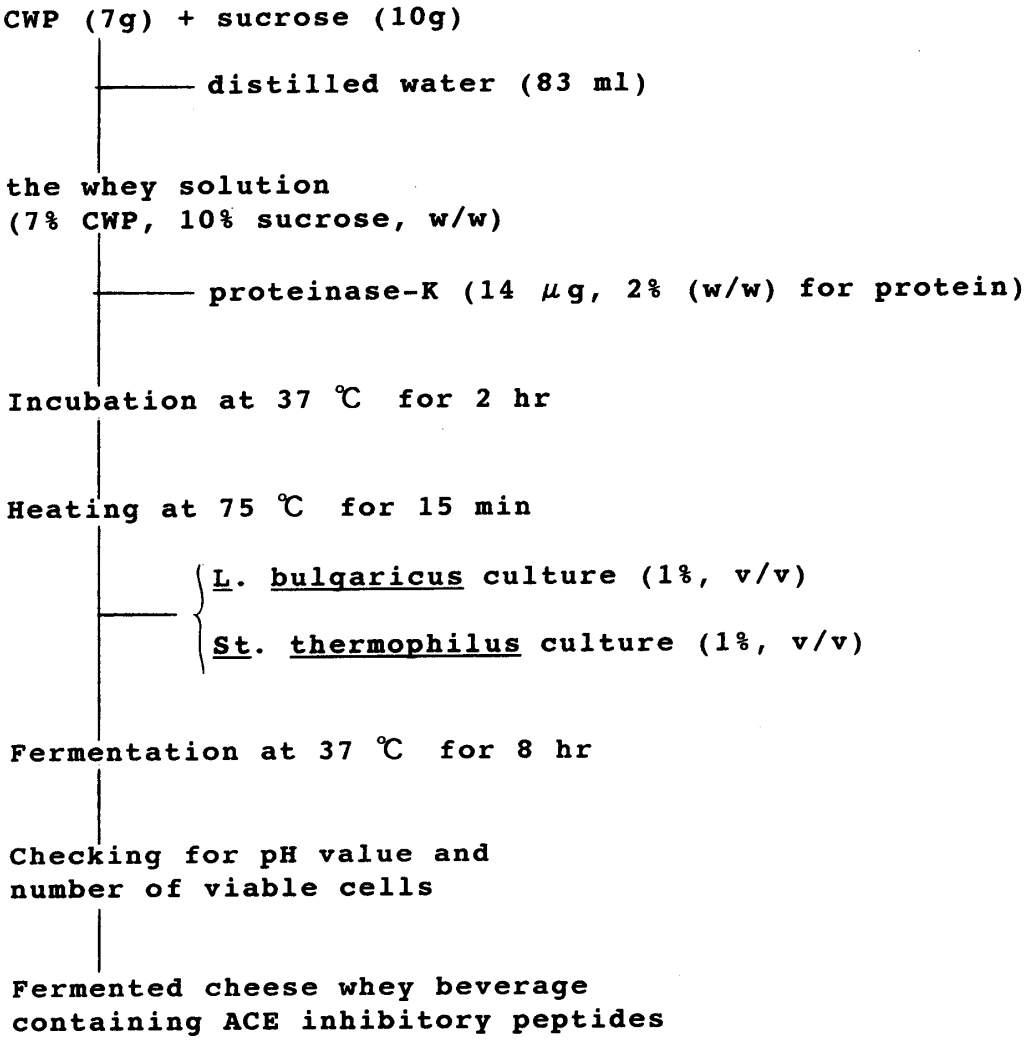

FIG 2. A scheme for the preparation of a fermented cheese whey beverage containing ACE inhibitory peptides.

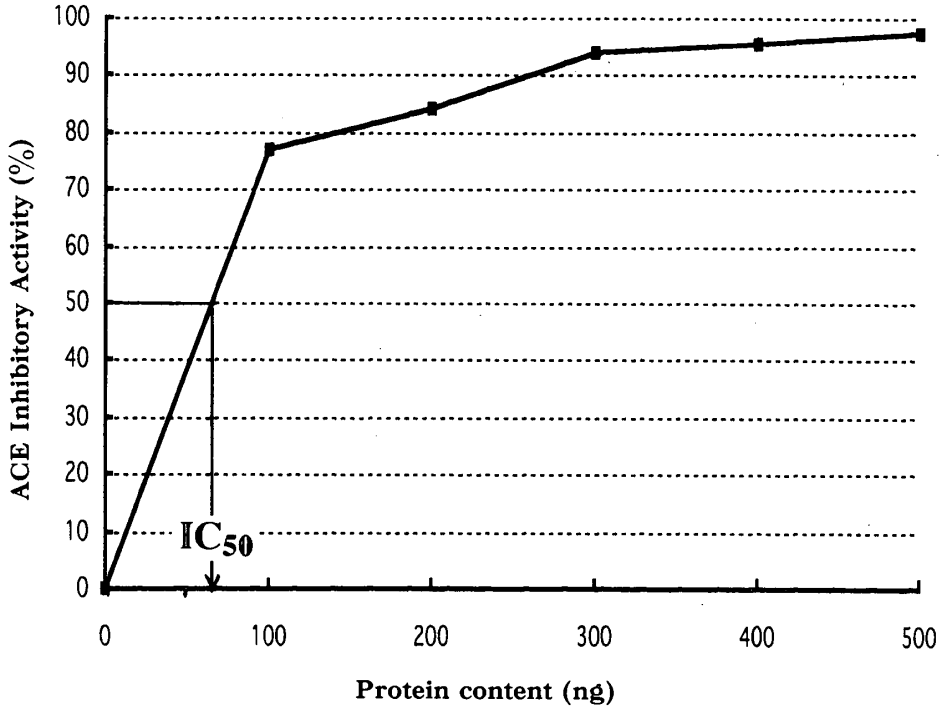

FIG 3. The ACE inhibitory activity (IC_{50}) of a fermented cheese whey beverage.

respectively. Proteinase-K was selected as the most suitable enzyme for attaining the highest ACE inhibitory activity in non-buffered conditions.

Next, CWP was digested by 7 kinds of proteases in distilled water at 37°C followed by fermentation with 2 kinds of lactic acid bacteria by considering the industrial making of a fermented cheese whey beverage.

Table 2 shows changes in the pH value and ACE inhibitory activity during the production process of fermented cheese whey beverage. After protease digestion for 24 hr and before fermentation, pH of the sample solutions was in the range of 6.4 to 7.0. After lactic acid fermentation for 24 hr, pH values decreased to ca. 3.8–4.1. These results indicate that bacteria grew well with the production of a satisfactory amount of lactic acid in any sample.

ACE inhibitory activity decreased after lactic acid fermentation in the samples digested with pepsin, trypsin, thermolysin and papain, but no change was observed in proteinase-K and actinase-E samples. On the other hand, a considerable increase was observed with chymotrypsin treatment. The decrease in ACE inhibitory activity after fermentation was thought to be due to a partial consumption of the active peptides by bacteria as nitrogen sources or further digestion by bacterial peptidases during the fermentation. The highest ACE inhibitory activity derived by proteinase-K (89.9%) was maintained at the same level after lactic acid fermentation, and therefore proteinase-K was considered to be the most prominent protease for the production of a new type of fermented cheese whey

beverage having an antihypertensive effect.

Next, the most appropriate digestion time (2 or 4 hr) and fermentation time (4, 8 or 24 hr) were investigated to determine the best conditions for producing fermented cheese whey beverage. Table 3 shows the ACE inhibitory activity at each step of the production process. ACE inhibitory activity was very high after 2 hr and 4 hr of digestion by proteinase-K. With the progress of fermentation, pH gradually dropped, but there was only a slight decrease in ACE inhibitory activity during fermentation. From the results of these experiments, the optimal conditions for making the new type fermented cheese whey beverage was determined (Fig. 2).

Fig. 3 shows the IC_{50} value of the ACE inhibitory activity of a cheese whey beverage produced according to the method shown in Fig. 2. About 50 ng (protein content per ml) of the beverage inhibited 50% of the activity in an *in vitro* assay.

Recently, two ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, were isolated from sour milk fermented with *L. helveticus* and *Saccharomyces cerevisiae* having antihypertensive activity (15, 16). As several ACE inhibitory peptides usually posses a proline residue in the C-terminus or internal sequence, a specific amino acid residue or sequence may be needed for ACE inhibition activity (15, 17-19).

Isolation of peptides with ACE inhibitory activity from CWP digested by proteinase-K and their sequencing are in progress.

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