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New Derivation of the Inhibitory Activity against Angiotensin Converting Enzyme (ACE) from Sweet Cheese Whey

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Summary

Three kinds of samples [whey protein (WP) containing caseinoglycopeptide (CGP+), WP-removed CGP (CGP−), and cheese whey powder (CWP)] were digested with 7 kinds of proteases at 37°C for 24 hr (trypsin, proteinase-K, actinase-E, thermolysin and papain) or 25°C (pepsin and chymotrypsin). Strong inhibitory activity against the angiotensin-converting enzyme (ACE, EC. 3.4.15.1) was generated in all samples by 5 proteases digestion (pepsin, chymotrypsin, proteinase-K, thermolysin and papain). In WP (CGP+), the most potent inhibitors (91.91%) were derived by papain digestion, and in WP (CGP−), digestion by thermolysin induced the highest activity (95.23%). In CWP, the highest activity was derived by thermolysin (98.56%). On the other hand, weak ACE inhibitory activity was derived by trypsin and actinase-E digestion. As no remarkable differences in inhibitory activity were observed between WP (CGP+) and WP (CGP−) samples, the bioactive peptides are considered to come mainly not from CGP but from WP components, such as β-lactoglobulin, α-lactalbumin, serum albumin and/or immunoglobulins. A similar development pattern in the activity between WP (CGP+) and CWP suggested that lactose and minerals do not contribute to the activity in CWP.

The major milk protein is classified into two groups: namely caseins and whey proteins. Whey proteins are less surface-active than caseins, mainly due to their globular structure. Although they have good solubility characteristics, their ability to stabilize emulsions and foam is poor, and therefore, their use in food ingredients is limited (1, 2).

Different types of whey are available as whey protein sources. Acid whey is

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produced in casein or cottage cheese manufacture through direct acidification of skim milk. Sweet cheese whey is a by-product of cheese production through rennet coagulation (3, 4). Though sweet cheese whey contains many valuable components such as lactose, whey proteins minerals and caseinoglycopeptide (CGP, κ-casein : f106-169) (5-7), they are often discarded in many countries, and cause serious water pollution.

Recently, many biologically active peptides have been isolated from human and bovine casein such as opioid peptides, angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides, and immunomodulatory peptides (8). ACE is one of the dipeptidylcarboxy-peptidases which cleave a C-terminal dipeptide portion of angiotensin I and convert it to the potent vasoconstrictor I, angiotensin II, with a strong hypertensive ability (9, 10). A number of ACE inhibitory peptides have been found/identified in different food proteins, but little is known about the peptides which are formed by the protease digestion of proteinacious constituents in cheese whey, such as whey protein (WP) and/or CGP.

In this study, we describe a derivation of inhibitory activity against ACE from proteinacious components in sweet cheese whey.

Materials and Methods

Materials

Cheese whey powder (CWP) was a product of Snow Brand Milk Products Co. Ltd., (Tokyo, Japan). Pepsin (from porcine gastric mucosa) and proteinase-K (from *Tritirachium album*) were purchased from Boehringer Mannheim GmbH (Germany). Five proteases trypsin (from porcine pancreas), chymotrypsin (from bovine pancreas), thermolysin (from *Bacillus thermoproteolyticus*), papain (from *carica papaya*) and ACE (from the rabbit lung) were obtained from Wako Pure Chemical Co. Ltd., (Osaka, Japan). Actinase-E (from *Actinomyces* ssp.) was obtained from Kaken pharmaceutical Co. Ltd. (Tokyo, Japan). Hippuryl-histidyl-leucine (HHL, substrate against ACE) was purchased from Sigma Chemical Co. Ltd., (St. Louis, USA).

Preparation of Whey Protein Samples from CWP

Caseinoglycopeptide (CGP, κ-Cn : f106-169) in CWP (100 g) was removed by extraction with 12 (w/v)% trichloroacetic acid solution (TCA, 1L) with gentle stirring for 30 min. In the preliminary experiment, it was confirmed that CGP was soluble in 12% TCA, and could be removed from CWP by direct extraction of the powder. After centrifugation (9,000$\times$g, 4°C, 15 min), the insoluble portion was dissolved with distilled water (300 ml), followed by neutralization with 1N NaOH solution to pH 7.0. The sample was dialyzed against distilled water at 4°C for 2 days, followed by lyophilization (WP without CGP ; WP (CGP$-$)).

CWP (50 g) was dissolved in distilled water (200 ml). The solution was dialyzed with distilled water at 4°C for 2 days to remove lactose and minerals, followed by lyophilization. (WP with CGP ; WP (CGP+)).

Enzymatic Digestion of Whey Protein Samples

Seven kinds of proteases (pepsin, trypsin, chymotrypsin, proteinase-K, actinase-E, thermolysin and papain) were used for the digestion of WP (CGP+), WP (CGP−) and CWP. Table 1 shows the optimal digestive conditions (buffer, pH, temperature) for the seven proteases used in this experiment. WP (10 mg) or CWP (100 mg) was dissolved in an optimal buffer (10 ml) and digested with pepsin or chymotrypsin at 25°C for 24 hr, or other proteases at 37°C (protein substrate : enzyme = 100 : 5, w/w). After digestion, each sample was heated to 96°C for 10 min to inactivate the protease.

Determination of Protein Content

Protein content in the samples was determined by the Folin-Lowry method (11). A sample solution (1 ml), which was appropriately diluted with distilled water, was mixed with the alkaline-copper reagent (5 ml) and the phenol reagent (0.5 ml, two-fold dilution with distilled water) purchased from Wako Pure Chemicals. After allowing the solution to stand for more than 30 min, the absorbance was measured at 750 nm (blue color), using a Shimadzu UV-VIS spectrophotometer Model UV-1200 (Kyoto, Japan).

Assay of Inhibitory Activity against ACE

The inhibitory activity against ACE was measured *in vitro* by the method of

TABLE 1. *The Optimal Conditions of Buffer, pH and Temperature on 7 Different Protease Treatment*

Enzyme	Buffer	pH	Temperature (°C)
Pepsin	0.05N HCl	2.0	25
Trypsin	0.02M Tris-HCl*	8.0	37
Chymotrypsin	0.02M CH_3COONH_4	8.0	25
Proteinase-K	0.02M Tris-HCl	7.5	37
Actinase-E	0.02M Tris-HCl*	8.0	37
Thermolysin	0.02M Tris-HCl*	8.0	37
Papain	0.02M Sodiumphosphate	7.0	37

HCl : hydrochrolic acid
Tris : Trishydroxymethyl amino methane
CH_3COONH_4 : ammonium acetate
*containing 10 mM of $CaCl_2$

Yamamoto *et al.* (12), which was a modification of the original Lieberman's method (13), with a scale-down (1/5) modification (Table 2). The content of hippuric acids liberated from HHL by the enzymatic reaction of ACE was photometrically determined after ethyl acetate extraction at 228 nm. The formula used for calculation was: Inhibitory activity (%) = [(Ac − As)/(Ac − Ab)] × 100, where A, c, s and b represent absorbance, control, sample and blank, respectively. The concentration of peptides which reduce of the ACE activity by 50% was defined as the IC_{50} value.

Results and Discussion

We first tested whether ACE inhibitory activity can be derived from proteinacious components in sweet cheese whey as well as in casein. Three kinds of whey samples (WP (CGP+), WP (CGP−) and CWP) were digested by 7 proteases, which originated from digestive organs, bacteria, fungi or plants. Each sample was digested for 24 hr under optimal conditions (see Table 1), and then checked for the occurrence of ACE inhibitory activity.

TABLE 2. *The Modified Assay for the Measurement of ACE Inhibitory Activity*

Reagent	As[1]	Ac[2]	Ab[3]
Sample solution[4]	30	—	—
Distilled water	—	30	30
Substrate solution[5]	20	20	20
ACE solution[6]	2	2	2
Stopping solution[7]	—	—	50
	↓	↓	
	incubation (at 37°C, for 1 hr)		—
Stopping solution	50	50	—
Total volume	102	102	102

(μl)

1 : absorbance of sample
2 : absorbance of control
3 : absorbance of blank
4 : samples were appropriately dilluted with distilled water after determination of protein content.
5 : HHL (53.69 mg) was dissolved in 10 ml of borate buffer containing 1 M NaCl (pH 7.8), and then abjusted to pH 8.3 with dilute NaOH solution.
6 : ACE (1 unit) was dissolved in 2 ml of 50(v/v)% glycerol (0.5 mU/μl) and stored at −20°C.
7 : 0.5 N HCl.

Fig. 1 shows the differences in ACE inhibitory activity after protease digestion of WP (CGP+), WP (CGP−) and CWP samples. Table 3 shows the summarized results of the ACE inhibitory activity derived from 3 samples. In WP (CGP+) in Fig. 1-A, four proteases (pepsin, proteinase-K, actinase-E and papain), especially papain, induced a strong inhibitory activity of more than 80%. The highest and lowest ACE inhibitory activities were obtained by papain (91.9%) and trypsin (45.3%) digestion, respectively. In WP (CGP−) in Fig.

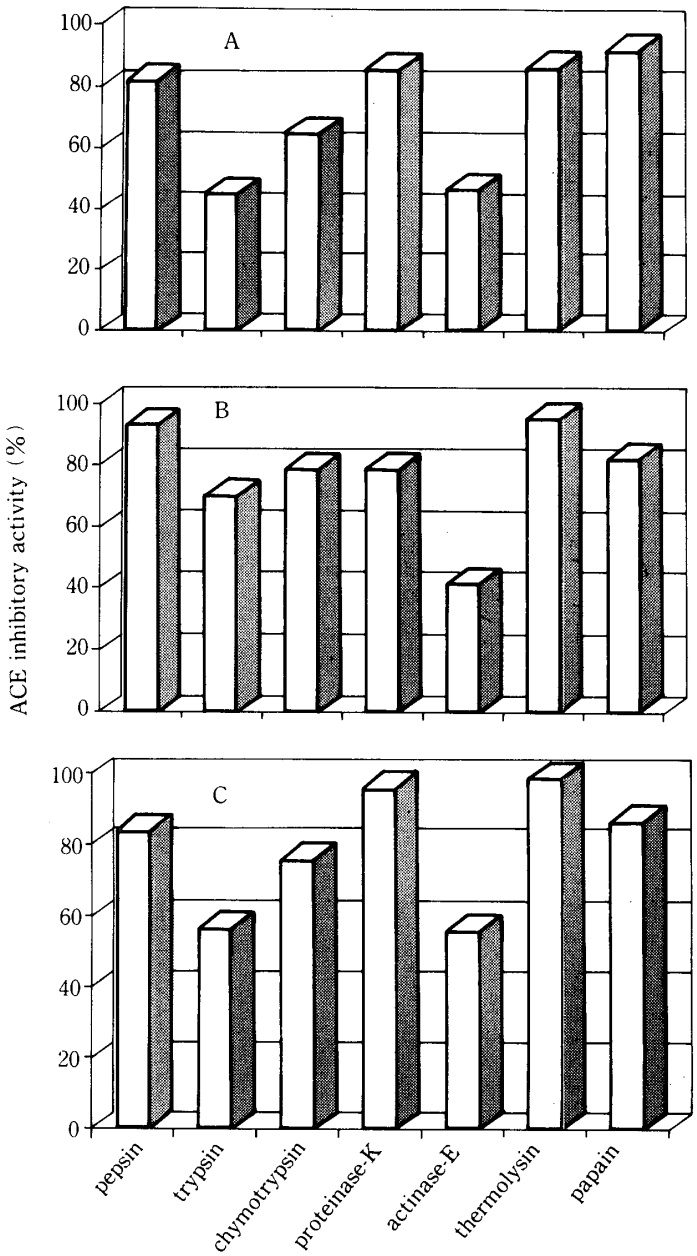

FIG. 1. ACE inhibitory activity derived from A: WP (CGP+), B: WP (CGP−) and C: CWP by protease digestion.

TABLE 3. *The Derivation of ACE Inhibitory Activity from 3 Whey Samples after Digestion with 7 Different Proteases*

Enzyme	ACE inhibitory activity (%)		
	WP (CGP+)	WP (CGP−)	CWP
Pepsin	82.27	93.12	83.74
Trypsin	45.25	69.98	56.69
Chymotrypsin	64.68	78.82	75.97
Proteinase-K	85.69	78.96	95.68
Actinase-E	46.81	41.37	55.68
Thermolysin	86.52	95.23	98.56
Papain	91.91	82.18	86.47

WP (Whey protein); CGP (caseinoglycopeptide, κ-Cn: f106-169); CWP (Cheese whey powder)

1-B, the pepsin and thermolysin digests generated a strong ACE inhibitory activity of more than 90%. Thermolysin and actinase-E induced the highest (95.2%) and lowest (41.4%) activity, respectively. As almost the same derivative pattern developed in WP (CGP+) and WP (CGP−) by 7 different protease treatments, the bioactive peptides which have an ACE inhibitory activity are considered to come mainly not from CGP but from WP components (β-lactoglobulin, α-lactalbumin, bovine serum albumin and/or immunoglobulins).

In CWP in Fig. 1-C, the developing pattern of ACE inhibitory activity was very similar to that in WP (CGP+) (Fig. 1-A), and a strong activity of more than 90% was obtained by proteinase-K and thermolysin digestion. Thermolysin and actinase-E induced the highest (98.6%) and lowest (55.7%) activity, respectively. This result also indicated that lactose and minerals do not contribute to the development of ACE inhibitory activity. Finally, it is concluded that the participation of CGP, lactose and minerals in ACE inhibitory activity in CWP is minimal. The IC_{50} value of the ACE inhibitory activity of CWP after proteinase-K digestion was 50 ng (protein content) per ml of the hydrolysate, as shown in Fig 2.

In the past ten years, a number of ACE inhibitory peptides have been isolated from bovine and human casein (14-18). Maruyama *et al.* (14-17) isolated CEI_{12} (dodecanopeptide) and $CEI_{\beta 7}$ (heptapeptide) as ACE inhibitors from bovine α_{s1}- and β-casein, respectively. Kohmura *et al.* (18) investigated the epitope portion of ACE inhibitors by using synthetic peptides which have the same structure as that found in human κ-casein. Chiba *et al.* (19) also isolated "casoxin C (decapeptide)" from bovine κ-casein. This peptide has many functions as an opioid antagonist, as well as controlling smoth muscle contruction, and as an ACE inhibitor. However, the only ACE inhibitor derived from bovine

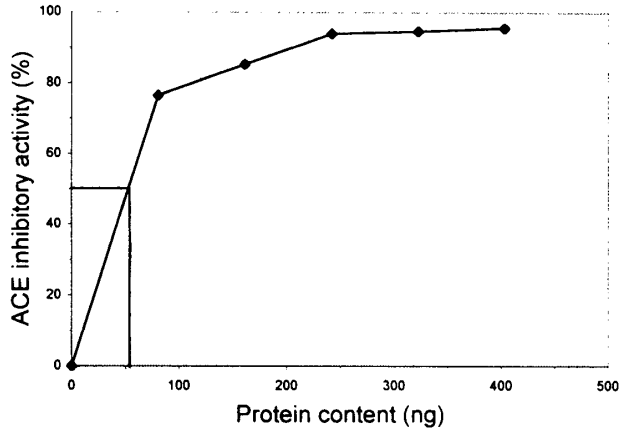

FIG. 2. IC_{50} value of the ACE inhibitory activity on CWP after proteinase-K digestion.

serum albumin (BSA) is "albutensin A (nonapeptide)".

Isolation of ACE inhibitors from CWP digested by thermolysin, and analysis of their structures are now in progress.

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