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Purification and characterization of bromelain isolated from pineapple

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Abstract : Bromelains (EC 3.4.4.24) isolated from pineapple fruit and stem have been purified about 18- and 46-folds to homogenity in the same yield of 23%. Molecular weights of fruit- and stem-bromelain were estimated to be 32.5 KDa and 37 KDa by Sephadex G-200, respectively. The enzymes were composed of one subunit. The fruit- and stem-bromelain had their maximum activity at pH 8.0, 70℃ and at pH 7.0, 60℃. Especially the enzymes catalyzed hydrolysis of plant proteins such as ISP (Isolated soybean protein) and wheat gluten with high molecular activity compared to animal proteins. The enzymes were competitively inhibited by sulfhydryl reagent; K_i values of fruit- and stem-bromelain for *p*CMB (*p*-chloromercuribenzoate) were 0.18 mM and 0.10 mM. Activities of the enzymes inhibited by *p*CMB were reversibly restored with increasing concentration of cysteine(Received July 2, 1992, accepted July 19, 1992).

Bromelain (EC 3.4.4.24) is the generic name of the proteolytic enzymes contained in the tissues of plant family *Bromeliaceae* of which pineapple (*Ananas comosus*) was the best known. Bromelain contained in the fruit and stem of pineapple were named as fruit-bromelain and stem-bromelain. Although the fruit-bromelain was discovered much earlier than stem-bromelain,[1,2] in detailed descriptions of the purification and characterization the latter enzyme is better known for those of fruit-bromelain.[3,4] Unlike crude stem-bromelain, which is used widely in industry, fruit-bromelain is not commercially available despite the large quantities of waste pineapple fruit portions at pineapple canneries.[5]

Both bromelains are thiol proteinase,[6,7] but they differ in their physicochemical properties. Stem-bromelain is a basic protein with a molecular weight of approximately 33 KDa and the principal amino-terminal residue is alanine.[6] In spite of such differences in their protein nature, the presence of immunological cross-reaction may be taken as an indicator of structural resemblance. According to the sequential analysis of stem-bromelain, ficin and papain, it had also been shown that partial homology remained in a particular region around the active site of thiol proteinase. Fruit-bromelain seemed to be one of these proteinases in the same category because of its typical cross-reaction with stem-bromelain.[8] It was considered to be a glycoprotein consisting of a single polypeptide and a single carbohydrate side chain.

Recently, application of these enzymes has moved to produce fish protein hydrolysate as a protein supplement for the malnourished groups of the population. Although crude bromelain (a mixture of cysteine proteinases) is of considerable commercial importance, the bromelain have not been fully characterized. In this paper we investigate the physical and chemical characteristics of fruit- and stem-bro-

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melain for the application of these enzymes on the production of fish protein hydrolysate.

Materials and Methods

Materials

CM-Sephadex C-25, Sephadex G and molecular standard kit were purchased from Pharmacia Co. (Sweden). Hydroxyapatite and DEAE-Toyopearl 650 C were obtained from Merck Co. (Germany) and Toso Co. (Japan). All other chemical used were from commercial sources and of reagent grade. Pineapple (*Ananas comonus*) cultivated in Cheju-Do was purchased from local market.

Enzyme extraction and purification

Fruit-bromelain

The fruit enzyme was extracted from pineapple by a procedure based upon the method by Heinicke and Gortner.[9] The fruit was freed of leaves and epithelium, and minced in a Waring blender. Crude extract was prepared by filtration under reduced pressure with the aid of Whatman No. 6 filter paper. The crude extract was cooled to 0~4℃ and one volume of cold acetone was added. The precipitate with low enzymatic activity was discarded. The enzyme was precipitated by the addition of two volumes of cold acetone, and the precipitate was collected by centrifugation and dried under reduced pressure. The dried product was ground in a mortar and yielded about 3.3 g of acetone powder per liter of crude extract. One gram of the acetone powder was suspended in 30 m*l* of 20 mM Tris-HCl buffer (pH 7.4) and dissolved by mild stirring. After centrifuge at 9,200×g for 10 min, the supernatant was put on a DEAE-Toyopearl 650C column (2.6×54 cm) equilibrated with 20 mM Tris-HCl and eluted with an increasing linear gradient of NaCl (20 mM to 700 mM NaCl in Tris-HCl buffer). The active fractions were pooled and concentrated by ultrafiltration through an Amicon PM-10 membrane. The concentrated enzyme was put on a Sephadex G-100 column (1.9×60 cm) equilibrated with 20 mM Tris-HCl (pH 7.4), and eluted with the same buffer. Major fractions were collected and dialyzed against 20 mM phosphate buffer (pH 6.8). The dialyzed enzyme was put on a hydroxyapatite column (2.8×15 cm) equilibrated with the buffer. The column was eluted increasing linear gradient of the buffer (20 mM to 500 mM phosphate buffer).

Stem-bromelain

Stem (1,080 g) was diced into small cubes and minced in a Waring blender with 1000 m*l* of 20 mM phosphate buffer (pH 6.4). The crude extract was prepared in the same procedure described above. The acetone powder of enzyme was prepared by the same method used for fruit-bromelain. One and half gram of the acetone powder was dissolved in 20 m*l* of 20 mM phosphate buffer (pH 6.4), and centrifuged at 9,200×g for 10 min. The supernatant was put a CM-Sephadex C-25 column (3.2×35 cm), equilibrated with the buffer, and active fractions were eluted with an increasing linear gradient of buffer (20 mM to 500 mM phosphate buffer). Enzyme fractions were pooled and concentrated by ultrafiltration. The concentrated enzyme was put on a Sephadex G-100 column (1.9×60 cm) equilibrated with 20 mM phosphate buffer, and eluted with the same buffer. The active fractions were collected and rechromatographed on CM-Sephadex C-25 (1.9×35 cm).

Enzyme and protein assays

Proteolytic activity toward casein was determined as described by Murachi.[10] Proteolytic activity in the acidic pH range was determined by the method of Anson[11] using hemoglobin as a substrate. The standard assay mixture contained 3 m*l* of 1.2% Hammarsten casein (Merck Co.) in 20 mM phosphate buffer, the buffer and the enzyme preparation in a final volume of 3.2 m*l*. The reaction was started by addition of the enzyme to the substrate preincubated at 35℃ for 5 min. After incubation for 10 min, the reaction was stopped by addition of 3.2 m*l* of 0.44 M trichloroacetic acid, and the solution was centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant solution was read at 275 nm. In blank, the enzyme was added after stopping the enzyme reaction which was done in the absence of enzyme. A proteinase unit is defined as the

amount of enzyme that gives an increase in absorbance at 275 nm equivalent to 1 μg of tyrosine per minute at 30℃. Specific activity is expressed as units of enzyme per mg of protein.

Protein concentration was determined by the method of Lowry *et al.*[12] with bovine serum albumin as a standard, and also by measuring the absorbance at 280 nm. The absorption coefficient was calculated to be 1.51 $mg^{-1} \cdot ml \cdot cm^{-1}$ by absorbance at 280 nm.

Molecular weight determination

The molecular weight of the enzyme was determined by gel filtration[13] on Sephadex G-200 with standard proteins (Oriental Yeast, Osaka). The molecular weight of the subunit was determined by the method of King and Lameli[14] with standard proteins (Pharmacia).

Electrophoresis

Electrophoresis on polyacrylamide gel was performed by the methods of Reisfeld[15] and Davie.[16] The gels were stained for protein with 1% Coomassie Brilliant Blue G-250 and destained in ethanol-acetic acid-water (3 : 1 : 6).

Results and Discussion

Purification of fruit- and stem-bromelain from pineapple

Fruit-bromelain was purified with 3 successive column chromatographies : DEAE-Toyopearl 650C, Sephadex G-100 and Hydroxyapatite. Fruit-bromelain was purified about 18-fold with an yield of 23% (Table 1). The most effective purification step proved to be the application of DEAE-Toyopearl 650C. This result was aggreed with the result obtained by Ota *et al.*[17] Through the purification procedure, stem-bromelain was also purified about 46-fold with an yield of 23% (Table 1). The purification fold of stem-bromelain was much higher than other result[15] that stem-bromelain was purified about 7.1-fold by Sephadex G-75 and BioRex 70.

Polyacrylamide electrophoresis were carried out to estimate homogeneity of bromelain. Fruit- and stem-bromelain were found to be homogeneous according to the result of disc gel electrophoresis (Fig. 1). The purified enzyme showed a single protein band upon the disc gel.

Molecular weight and subunit structure of bro-

Table 1. Purification of fruit-bromelain and stem-bromelain from pineapple

〈Fruit-bromelain〉

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	1,240	329,000	266	100	1.0
Acetone fraction	249	184,000	739	56.0	2.8
DEAE-Toyopearl 650C	40.4	133,000	3,300	40.5	12.4
Sephadex G-100	25.4	95,100	3,740	28.9	14.0
Hydroxyapitite	15.5	76,000	4,900	23.1	18.4

〈Stem-bromelain〉

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	2,330	10,100	43.3	100	1.0
Acetone fraction	180.0	51,200	284.7	50.6	6.6
CM-sephadex C-25	26.1	33,500	1,280	33.1	29.6
Sephadex G-100	16.3	25,000	1,530	24.7	35.4
CM-sephadex C-25	11.8	23,500	1,990	23.2	45.9

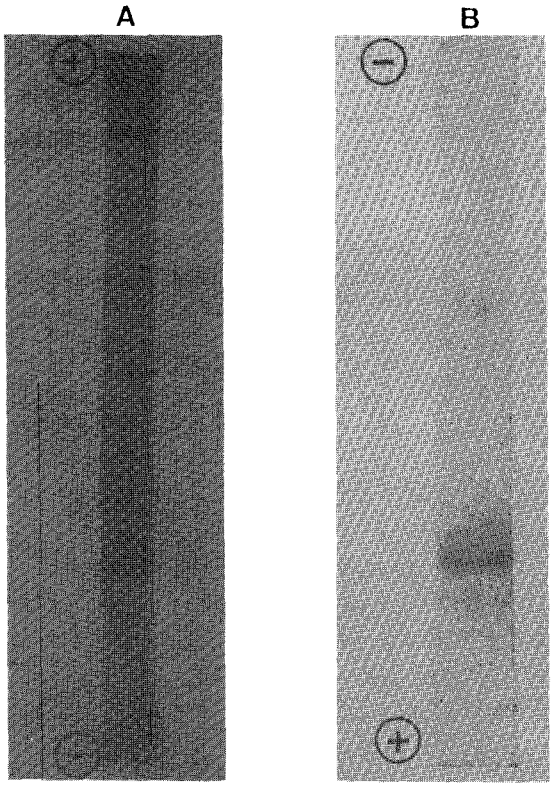

Fig. 1. Polyacrylamide disc gel electrophoresis of the purified fruit-bromelain (A) and stem-bromelain (B). Purified enzyme (20 μg) were subjected to electrophoresis with 1% polyacrylamide gels in Tris-glycine buffer system. After running at current 5 mA, the gel was stained with Coomassie Brilliant Blue R-250.

melain

The fruit- and stem-bromelain had molecular weight of about 32.5 KDa and 32.0 KDa respectively, as determined by Sephadex G-200. The subunit structure was examined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The denatured fruit- and stem-bromelain migrated as a single band of stained protein, and molecular weight of the former and the latter was 32.0 KDa and 33.6 KDa on the basis of their mobility relative to those of standard proteins. These results showed that both enzymes were composed of one subunit. The molecular weight of stem-bromelain agreed with the results of Yamada (M.W 33 KDa)[18] and Ota (M. W 35.7 KDa).[17] The molecular weight of fruit-bromelain was similar with the molecular weight of 31 KDa reported by Yamada,[18] but disagreed with the molecular weight of 18 KDa reported by Ota.[17]

Effect of pH on enzyme activity and stability

To investigate pH effects on the enzyme activity, activity was measured against casein above pH 7.0 and hemoglobin below pH 7.0. The optimum pH of fruit- and stem-bromelain were pH 8.0 and pH 7.0, respectively (Fig. 2). The result agreed with the reports that the pH optima for these enzymes were close to neutral pH.[8] Activities of fruit and stem-

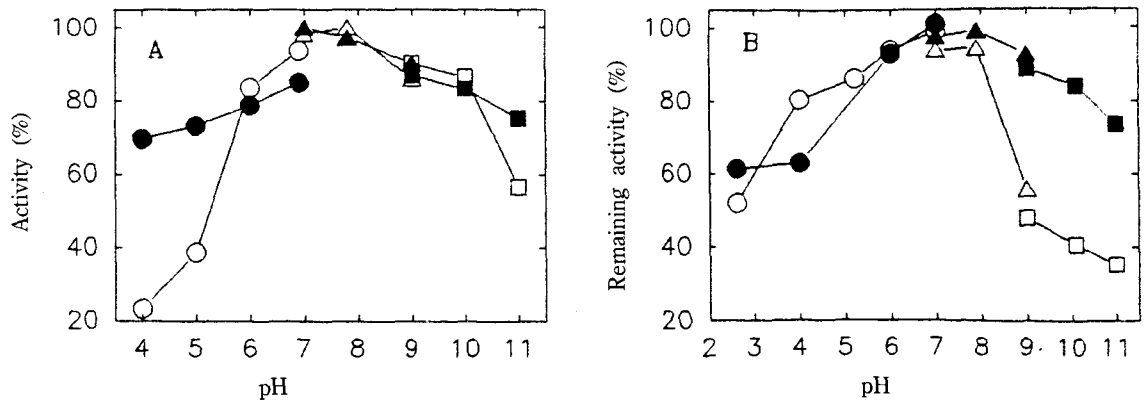

Fig. 2. Effect of pH on the activity (A) and stability (B) of bromelain.
(A) The assay was carried out under the standard reaction condition at various pH with hemoglobin, pH 2.5~7.0 and casein, pH 7.0~11.0, (B) The enzyme solution was incubated at various pH for 24 hr at 25 ℃, and the remaining activity was assayed. The buffers used were citrate-phosphate (○ or ●, pH 2.5~7.0), Tris-HCl (△ or ▲, pH 7.0~9.0), and carbonate-bicarbonate (□ or ■, pH 9.0~11.0). ○, △, □ : Fruit-bromelain, ●, ▲, ■ : Stem-bromelain

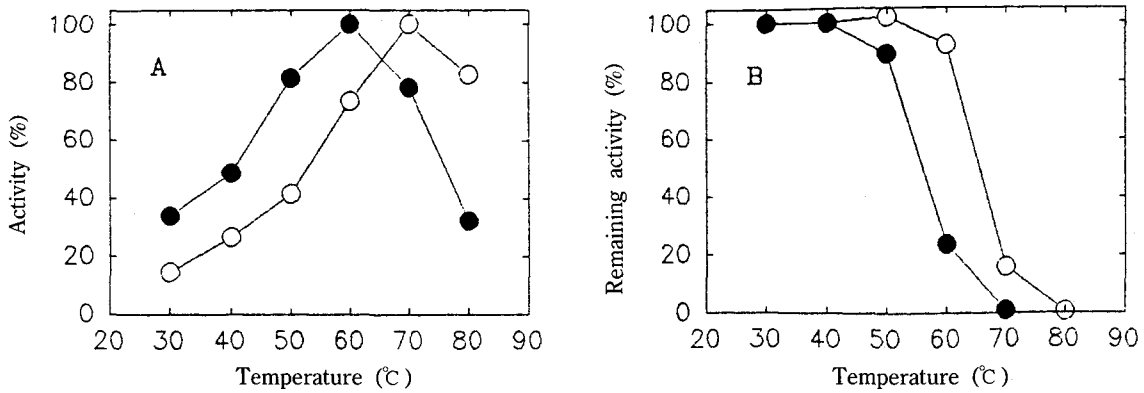

Fig. 3. Effect of temperature on the activity (A) and stability (B) of bromelain.
(A) The assay was carried out under the standard reaction conditions described in "Materials and Methods" at various temperature, (B) The enzyme solution was incubated at various temperature for 30 min, and the remaining activity was assayed. ○—○: Fruit-bromelain, ●—●: Stem-bromelain

bromelain were sensitively affected by pH changes. Fruit-bromelain was found to be less sensitive on pH changes than stem-bromelain. The enzyme was kept at various pH for 24 hr at room temperature and the residual activity was measured for examining the enzyme stability. Fruit-bromelain retained full activity in the range of pH 7~8, while stability of stem-bromelain on pH was showed maximum in pH 6~8, which was the same result of Bull *et al.*[18] Stem-bromelain was more stable on a higher pH than fruit-bromelain (Fig. 2). Both enzymes were fairly stable at the range of pH 6~8. However, fruit-bromelain was more stable at lower pH and less stable at higher pH than stem-bromelain.

Effect of temperature on enzyme activity and stability

For the determination of optimum temperature, reactions were performed at 30, 40, 50, 60 and 70 ℃. These results showed that the stem-bromelain was slightly active at 30 ℃ and exhibited maximum activity at 60 ℃, while fruit-bromelain was fully activated at 70 ℃ (Fig. 3). To test heat stability of bromelain, enzymes were heated at 30, 40, 50, 60, 70 and 80 ℃ for 30 min. The fruit-bromelain showed full activity in temperature below 50 ℃, but fast decrease in activity with an increase in temperature above 60 ℃, with complete inactivation at 80 ℃ (Fig. 3). This result was aggreed with Greeberg's report.[19] Choi *et al.*[20] reported that optimum temperature of fruit-bromelain was 60 ℃ and heat stability of the enzyme was relatively higher than our result. Activity of stem bromelain was stabilized in temperature below 40 ℃, but the activity was decreased fast above 50 ℃, with complete inactivation at 70 ℃. It indicated that stem-bromelain was more heat labile than fruit-bromelain. Bromelain was unsually heat resistant, maintaining their full activity at temperature of 50 or 60 ℃.

Substrate specificity of bromelain

Ability of the enzymes to catalyze the hydrolysis of various proteins from animal and plant sources was investigated, and the velocity of hydrolysis was expressed as percentage of the activity measured with casein. Table 2 showed that the enzyme hydrated all proteins except elastin tested. ISP (Isolated Soybean Protein) was the most active substrate. Expecially proteins from plant sources were comparatively well hydrated in fruit-bromelain. Activities of stem-bromelain on substrates except hemoglobin were lower, as compared to those of fruit-bromelain. Bromelain had broad specificities of substrate. Rowan[1] reported that bromelain had same activity on casein and hemoglobin, but the result of Table 2 showed that activity on casein was higher than that on hemoglobin. Ota reported that

fruit-bromelain was relatively much more active against casein, BAA (benzoyl-L-arginine amide) and BAPA (benzoyl-L-arginine-*p*-nitroanilide).[21] In generally fruit-bromelain hydrolysed various substrates with higher molecular activity than stem-bromelain because fruit-bromelain hydrolysed substrate at many sites compared to those of stem-bromelain.[22]

Effect of compounds on bromelain activity

The effects of compounds on the enzyme activity were shown in Table 3. The enzyme was strongly inhibited by sufihydryl reagent such as *p*CMB and was slightly activated by 2-mercaptoethanol, sulfhydryl protecting reductant. This result indicated that bromelain had sulfhydryl group as active site for catalytic activity.[23] It was reported that stem-bromelain had one reactive sulfhydryl group per molecular.[24] In order to estimate whether cysteine could restore the activity of bromelain inhibited by *p*CMB or not, the effect of cysteine on bromelain inhibited with 1 mM *p*CMB was shown in Table 4. Activities of bromelain were considerably restored with increasing concentration of cysteine. In fruit bromelain, the activity was recovered 1.0 mM concentration of cysteine, and was activated above that concentration. However, 50 mM concentration of cysteine was necessary to restore the activity of stem-bromelain. The addition of cysteine prevented the inhibition of the activity from *p*CMB and activated the bromelain. The difference of restoring concentration of cysteine depended on the degree of enzyme inhibited by *p*CMB and concentration of enzyme.

Fruit- and stem-bromelain was strongly inhibited by sulfhydryl agent. The enzymes were reversibly inhibited by *p*CMB and the inhibited enzyme activity was restored and activated by the addition of cysteine. Consequently, the enzymes were classified

Table 2. Effect of substrates on the activity of bromelain

Substrate	Relative activity (%)	
	Fruit-bromelain	Stem-bromelain
Casein	100	100
Collagen	99.5	69.1
Elastin	11.1	3.8
Hemoglobin	62.2	72.9
ISP	171	120
Wheat gluten	126	53.8

The reaction mixture, consisted of 1.2% substrate and 10 μl of enzyme solution containing 1.65 mg/ml of stem-bromelain or 0.33 mg/ml of fruit-bromelain, was incubated at 30 ℃ for 10 min.

Table 3. Effect of compounds on the bromelain activity

Compounds	Remaining activity (%)			
	Fruit-bromelain		Stem-bromelain	
	0.1 mM	1 mM	0.1 mM	1 mM
None	100	100	100	100
2-Mercaptoethanol	—	101	—	112
EDTA	—	101	—	96.2
*p*CMB	5.9	N.D	12.4	N.D
Cancanavalin A	—	98.7	—	102

10 μl of enzyme solution containing 1.65 mg/ml of stem-bromelain or 0.33 mg/ml of fruit-bromelain was incubated with various compounds at 30 ℃ for 10 min, and the remaining activity was measured under standard reaction condition described in "Materials and Methods".
N.D : Not Detected, *p*CMB : *p*-Chloromercuribenzoic acid, EDTA : Ethylenediamine tetraacetic acid.

Table 4. Effect of cysteine concentrations on the reactivation of bromelain inhibited by *p*CMB

Cysteine concentration (mM)	Relative activity (%)	
	Fruit-bromelain	Stem-bromelain
Control	100	100
0.0	N.D	N.D
0.5	8.0	1.0
1.0	97.3	47.6
1.5	128	50.5
2.5	167	60.1
50.0	182	95.2

After 10 μl of enzyme solution containing 1.65 mg/ml of stem-bromelain or 0.33 mg/ml of fruit-bromelain was incubated with 1 mM *p*CMB at 30 ℃ for 10 min, the reaction mixture, consisted of various concentration of cysteine and substrate, was incubated at the same condition for determining the bromelain activity.
N.D : Not Detected.

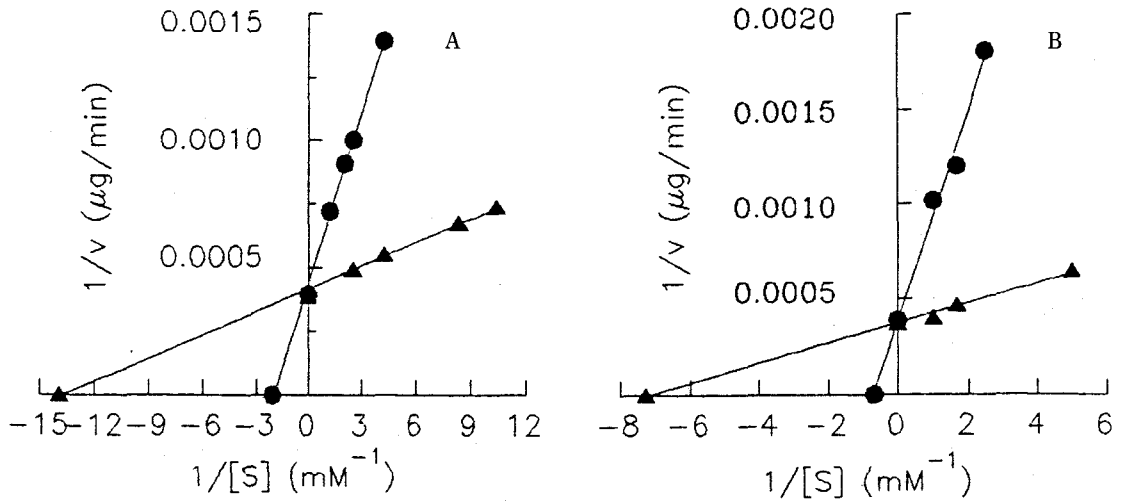

Fig. 4. Lineweaver-Burk plot for fruit-bromelain (A) and stem-bromelain (B) catalyzed hydrolysis of casein
The activity of bromelain was measured on casein with 1 mM *p*CMB and without *p*CMB. Velocity (v) was expressed as μg of product formed per min. ▲—▲: Without *p*CMB, ●—●: With *p*CMB.

into thiol-proteinase category essential to sulfhydryl group for catalytic activity.

Determination of K_m and K_i

The velocity of the enzyme reaction was determined on various concentrations of casein. The reaction rate was determined by measuring the appearance of product versus time. The K_m value of fruit- and stem-bromelain were 0.18 mM and 0.34 mM, respectively. Affinity of fruit-bromalain on casein as substrate was stronger than that of stem-bromelain. Yamada also reported that K_m value of fruit-bromelain was smaller than that of stem-bromealin.[25]

To identify the type of inhibition, the bromelain activities were assayed in the present and absent *p*CMB. Fig. 4 was shown that the bromelain activities were strongly inhibited by *p*CMB. V_{max} was unaffed ($V'_{max}=V_{max}$), but K_m was increased ($K'_m > K_m$). Because of the competition between inhibitor and substrate at the same active site, a greater amount of substrate should be required for half saturation. These results indicated that *p*CMB served as typical competitive inhibitor for the hydrolysis of casein. K_i vlue were determined to be 0.18 mM for fruit-bromelain and 0.10 mM for stem-bromelain from double-reciprocal plots. It has known that *p*CMB was a substance that reversibly bound with the free from of thiol enzyme to produce a binary *p*CMB-enzyme complex incapable of binding casein. Thus in the presence of enzyme, casein and *p*CMB, enzyme could bind with casein to yield enzyme-casein, or enzyme could bind *p*CMB and casein simultaneously to yield a ternary enzyme-*p*CMB-casein complex: The binding of *p*CMB and casein was mutually exclusive.[26]

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파인애플 bromelain의 정제 및 특성

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초록 : 파인애플의 과육과 줄기로부터 fruit-bromelain을 정제도 18배와 수율 23%, stem-bromelain을 정제도 46배와 수율 23%로 각각 전기영동적으로 균일하게 정제하였다. 두 효소의 분자량을 Sephadex G-200에 의해 측정한 결과, fruit-와 stem-bromelain의 분자량은 32.5 KDa와 37 KDa이었으며, 이들은 공히 1개의 subunit로 구성된 효소단백질이었다. Fruit-bromelain은 pH 8.0과 70℃, stem-bromelain은 pH 7.0과 60℃에서 각각 최대 활성을 나타내었다. 동·식물 유래의 천연단백질을 중심으로 기질 특이성을 검토한 결과 동물성 단백질보다는 밀단백 또는 ISP와 같은 식물성 단백질에 높은 기질 특이성을 보였으며, casein에 대한 fruit-와 stem-bromelain의 K_m값은 0.18 mM와 0.34 mM이었다. *p*CMB는 경쟁적 저해제로서 fruit-와 stem-bromelain의 K_i값은 0.18 mM과 0.10 mM이었다. *p*CMB에 의해 저하된 두 효소의 활성은 cysteine에 의해 재활성화 되었으며 또한 과량의 cysteine 첨가에 의해 활성화됨으로써 이 효소들은 전형적인 thiol계 단백분해효소임을 알 수 있었다.