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Simple Purification of Bromelain from Pineapple

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Abstract

Bromelain(EC 3.4.22.4), the collective name for the proteolytic enzymes found in tissues of the plant family *Bromeliaceae*(pineapple), has been used as a tenderizing agent in food processing, and as an antiinflammatory agent in pharmaceuticals. In this paper, we describe the simple purification method of bromelain using Korean pineapple fruit. After removing contaminants at 30% saturation of ammonium sulfate, the supernatant obtained was treated again with ammonium sulfate to 80% saturation. With the above salt fractionation, partially purified bromelain could be obtained. To get highly purified bromelain, the previous 30% to 80% ammonium sulfate treated precipitate was dialyzed against 25mM sodium acetate buffer(pH 5.0) followed by passing through a CM-cellulose cation exchange column. Fruit bromelain was eluted as a major peak at 0.5~0.8M NaCl gradient. The present method is simpler with high yield than the traditional purification method-acetone treatment and several consecutive chromatographic processes.

Key words: bromelain, Korean pineapple, purification

INTRODUCTION

Bromelain(EC 3.4.22.4) is the generic name of the proteolytic enzymes found in the stem and fruit of the pineapple(*Ananas comosus*). The name of bromelain was coined quite recently, originally to apply to "any protease from any member of the *Bromeliaceae*". Bromelain is a glycoprotein, and its active site is represented by the reactive sulfhydryl group of cysteine(1). The sequence of amino acids near the active site is similar to that of papain. Following a limited number of application of pineapple in folk medicine by the natives in some tropical countries, bromelain has been widely used in food processing as a meat-tenderizing enzyme and as a cleaning enzyme(2).

The history and several characteristics of bromelain have been reported in several reviews(3-6). Bromelain also has widespread applications in pharmaceutical therapy. It is often used in cases of inflammation associated with traumatic injuries, cellulitis, furunculosis and ulcerations as well as digestive aids. The mechanism of bromelain affecting these varied biological effects relates in part, to its modulation of the arachidonate cascade (7,8). In Korea, bromelain is imported every year(9) and the amount imported has been increasing. Pineapple, the source of bromelain, found by Coumbus in 15th century in Gouadeloupe, is widely grown today around the globe in tropical and subtropical regions, including southern part of Korean peninsula.

Traditionally, bromelain was purified by using acetone precipitation followed with several chromatographic methods(10). Because bromelain is used for various purposes, many different purification methods have been developed(11-13). In the present paper, we developed a simple purification procedure of bromelain from Korean pineapple using salting out and only one step of cation exchange chromatography.

MATERIALS AND METHODS

Pineapple and chemicals

Pineapple grown at Keoje Island was used as bromelain resources in the present study. Commercial bromelain for standard marker and its artificial substrate, Nα-CBZ-$_L$-lysine-ρ-nitrophenyl ester(CLN), were purchased from Sigma Co.(St. Louis, USA). Electrophoretic reagents were purchased from Bio-Rad(Richmond, USA), and CM-cellulose was from Pharmacia Co. (Uppsala, Sweden). All other reagents used were of standard reagent grade.

Preparation of pineapple fruit juice

The peel of the pineapple was removed and cut into

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cubes of $2\times2\times2cm^3$. Pineapple cubes were homogenized and the juice was pressed out using hemp cloth. The extracted juice was centrifuged in GSA rotor at 2,800×g for 10min. at 4°C. Large particles, including some contaminants, were removed as precipitates, and supernatants were used as samples for purification of bromelain.

Salt fractionation

Ammonium sulfate was used to fractionate proteins of pineapple. After adding ammonium sulfate to the juice of pineapple fruit to a given concentration, the solution was kept at 4°C overnight. A SS34 centrifuge rotor was used to obtain precipitated proteins by centrifugation at 12,000×g for 10min. In the case of fractionation, the same steps were repeated after the second percentage of ammonium sulfate was added to the previous supernatant of previously treated ammonium sulfate.

CM-cellulose ion exchange chromatography

Ammonium sulfate fractionated precipitates were dialyzed against acetate buffer(25mM sodium acetate, 1mM EDTA, 0.01% sodium azide, pH 5.0) with dialysis membrane(Specman Co., Molecular cut-off=10,000). After removing unrefolded precipitates by centrifugation at 12,000×g for 5min. supernatants were applied to CM-cellulose column. CM-cellulose column(2.5×15cm) was preequilibrated with 3 volumes of the acetate buffer. Dialysate of 10ml was loaded to column, and washed out unabsorbed materials with 200ml of acetate buffer. NaCl gradient with acetate buffer containing 0M NaCl and 2M NaCl(each 250ml) were employed to separate absorbed bromelain. Flow rate and fraction volume were 13 drops/min and 5ml, respectively. Fig. 1 shows the flow chart for the purification process.

Enzymatic activity assay

Proteolytic activity of bromelain was determined using an artificial substrate, 15mM Nα-CBZ-L-lysine-ρ-nitrophenyl ester(CLN)(14) in acetonitrile containing 20%(v/v) water was used. First, 10μl of sample containing bromelain was mixed with 3ml of acetate buffer (10mM, pH 4.6) containing 0.1M KCl and 1.0mM L-cysteine, and incubated for 1min at 25°C. After incubation, 50μl of substrate was immediately added by stirring and then the increase in absorbance at 340nm was

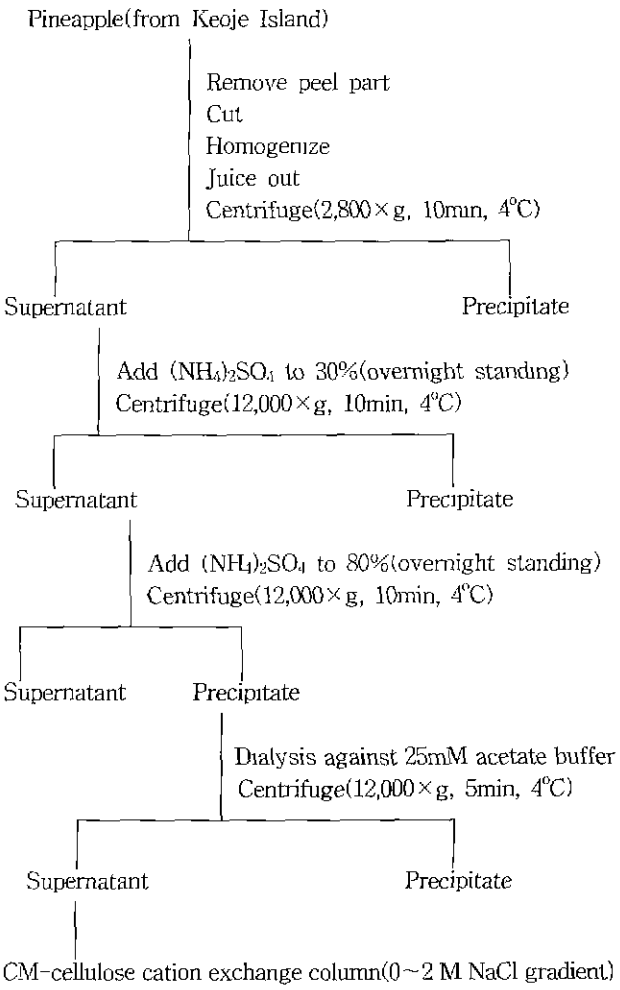

Fig. 1. The net flow chart of the process for purification of bromelain.

monitored for several min. in Shimatdzu spectrophotometer. Enzyme unit was calculated as the amount of enzyme which increases the absorbance unit of 0.1 per min. in the linear range.

Protein determination

Protein concentration of samples in the purification step was determined by the method of Lowry(15) using bovine serum albumin as a standard. Protein concentration of eluents from the chromatography was shown as its optical density values at 280nm.

SDS-polyacrylamide gel electrophoresis and determination of molecular weight

Electrophoresis was carried out at 12% SDS-polyacrylamide gel by the Laemmli method(16). Ammonium sulfate treated samples were treated with trichloro-

acetic acid(TCA) for removing salts present in the samples. After TCA was added to the samples to give final concentration of 10%, protein precipitated was collected by centrifuging at 12,000×g for 10min. Then, residual acid was washed with cold acetone(-20°C).

After electrophoresis the gel was stained using 0.05% Coomassie Brilliant blue R-250 in 10% methanol and 10% acetic acid, and destained with destaining solution(5% methanol, 7% acetic acid), changing several times till the bands showed distinctively. A low molecular weight standard marker of Pharmacia Co. (Uppsala, Sweden) was used as a standard which contains phosphorylase b(M.W. 94,000), bovine serum albumin(M.W. 67,000), ovalbumin(M.W. 43,000), carbonic anhydrase(M.W. 30,000), soybean trypsin inhibitor (M.W. 20,100) and α-lactalbumin(M.W. 14,000).

RESULTS AND DISCUSSION

Partial purification of bromelain with salt fractionation

Bromelain was purified from the fruit of pineapple grown in Keoje Island, Korea. After removing large particles of pineapple juice by centrifugation, ammonium sulfate was treated for salt fractionation to the supernatant to the given percentage. After centrifugation, the attached salt within precipitate was washed out before electrophoresis by TCA treatment and acetone washing. Fig. 2 shows the electrophoretic patterns of the ammonium sulfate fractionated proteins from pineapple juice. The band of bromelain(noted by an arrow) was hardly noticeable in 10, 20, 30% concentrations of ammonium sulfate, while contaminants of low molecular weight were predominant. However, the intensity of bromelain band was increased with further addition of ammonium sulfate from 40% concentration. This result indicates that major impurities other than bromelain can be easily excluded with 30% treatment of ammonium sulfate.

For further purification of pineapple proteins, ammonium sulfate fractionation was carried out again with the supernatant of 30% ammonium sulfate treated pineapple juice. The same procedures described above were followed again and the collected precipitated sample was applied on 12% SDS-polyacrylamide gel electrophoresis(SDS-PAGE). Fig. 3 shows the results of electrophoretic patterns of fractionated proteins. In this figure, bromelain is shown to increase with increasing concentration of secondly treated ammonium sulfate. To achieve the maximum yield of bromelain by salt fractionation, 30% to 80% saturation of ammonium sulfate to the pineapple juice can be recommended. When compared to the result of Choi et al. (13) using ammonium sulfate during purification of bromelain, we could obtain bromelain with high yield. It may arise from that we treated salt with two times, while they did not.

CM-cellulose chromatography

To obtain highly purified bromelain, we used a cation

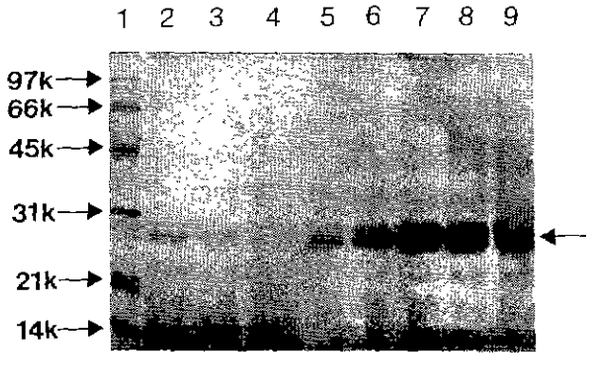

Fig. 2. 12% SDS-PAGE patterns of $(NH_4)_2SO_4$ treated pineapple fluid.
Lane 1, size marker; lane 2, $(NH_4)_2SO_4$ was added to final 10%; lane 3, 20%; lane 4, 30%; lane 5, 40%; lane 6, 50%; lane 7, 60%; lane 8, 70%; lane 9, 80%. Arrow indicates the band of bromelain.

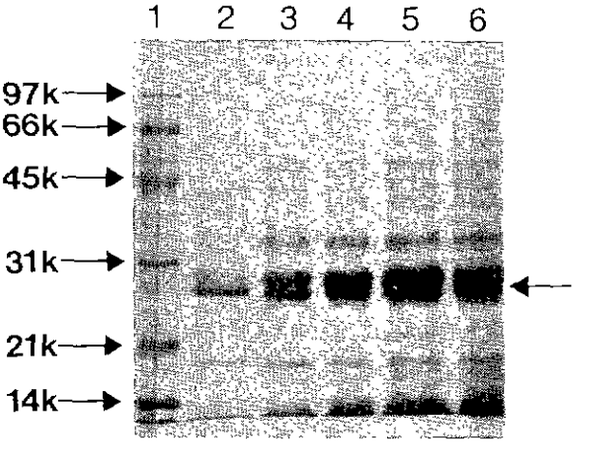

Fig. 3. 12% SDS-PAGE patterns of salt fractionated pineapple fluid.
Lane 1, size marker; lane 2, secondly treated $(NH_4)_2SO_4$ was final 40%; lane 3, 50%; lane 4, 60%; lane 5, 70%; lane 6, 80%. Arrow indicates the band of bromelain.

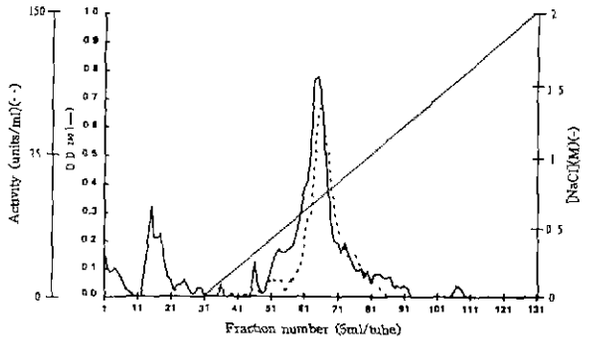

Fig. 4. CM-cellulose cation-exchange chromatography of 30~80% $(NH_4)_2SO_4$ fractionated pineapple fluid.

exchange chromatography. Partially purified bromelain with 30% to 80% ammonium sulfate fractionation was dialyzed against an acetate buffer(25mM sodium acetate, 1mM EDTA, 0.01% sodium azide, pH 5.0) for renaturation. Unrenaturated aggregates were removed with centrifugation, and supernatants were applied to a CM-cellulose column, preequilibrated with acetate buffer. Fig. 4 shows the elution profile of chromatography. Protein was monitored at 280nm, and bromelain was determined on the basis of its hydrolytic activity to artificial substrate, Nα-CBZ-L-lysine-ρ-nitrophenyl ester(CLN). Many contaminants were eluted out at the washing stage, while bromelain was absorbed to CM-cellulose resin in 25mM acetate buffer. To isolate bromelain, continuous gradient of acetate buffer containing 0 to 2M NaCl was applied. Between 0.5 and 0.8M NaCl concentration, the elution of bromelain reached its peak. Some groups(8,9,11) also used ion exchange chromatography for purification of bromelain. However, they could obtain purified bromelain only after a second gel filtration chromatography step. In the present paper, we could purify bromelain with only one step of CM-cellulose chromatography because we had already removed small molecular contaminants in the step of salt fractionation.

Fig. 5 shows the result of SDS-PAGE pattern of each purification step. Compared to the purchased bromelain, which was purified with acetone precipitation (10), our partially purified bromelain with salt fractionation was proved to be pure. Because ammonium sulfate is cheaper than acetone, the procedure developed in this study is highly recommended for the purification of bromelain in Korean pineapple. The molecular weight of purified bromelain from Korean pineapple, grown at

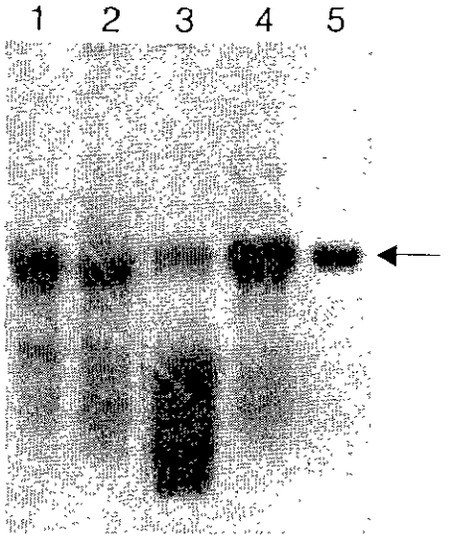

Fig. 5. 12% SDS-PAGE patterns of each purification steps.
Lane 1, pineapple juice ; lane 2, acetone extracted bromelain with Murachi method (10); lane 3, commercial bromelain purchased from Sigma Co.; lane 4, 30~80% $(NH_4)_2SO_4$ fractionated bromelain, lane 5, CM-cellulose fraction of bromelain.

Table 1. Summary of the purification yields

Step	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Yield (%)
Pineapple juice	23,800	17,000	0.71	100
$(NH_4)_2SO_4$ (30~80%)	9,570	12,410	1.30	73
CM-cellulose chromatography	5,800	9,800	1.69	58

Keoje Island, is about 30,000 daltons supporting the result of Murachi group(6,10), while different from other group (11). These differences may be caused by the differences of varieties(13). The quantitative results of these purification steps were summarized in Table 1.

In this study, we purified bromelain from Korean pineapple with salt fractionation and cation exchange chromatography. Partially purified bromelain could be obtained in 30% to 80% ammonium sulfate concentration. CM-cellulose column chromatography was used for further purification. Bromelain was eluted as a major peak at 0.5~0.8M NaCl gradient. As compared to the purchased bromelain, partially purified and highly purified bromelain in this research shows high purity. Even though partially purified bromelain contains some

contaminants, it is acceptable for use in food processing. As compared to the traditional purification method(10), the present method is simple with high yield. Because bromelain used in Korea was usually imported from abroad, this study may attribute to the effective use of Korean pineapple and substitution of imported bromelain.

ACKNOWLEDGEMENTS

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