Isolation and Partial Characterization of Proteolytic Enzymes from Stems of Pineapples Cultivated in Cheju Island

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濟州産 파인애플 줄기로부터 蛋白分解酵素의 分離 및 그 部分的 特性

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Summary

A pineapple is one of the major sources of proteolytic enzymes of plant origin which have been widely used in industry. A bromelain was isolated from stems of pineapples cultivated in Cheju island and its characteristics were partially determined. The optimum temperature and pH for its catalytic activity were 62.5℃ and 7.5 , respectively. Crude enzyme preparations lost catalytic activity rapidly above 40℃ and were stable at pH range 5.6-9.0. Coexistence of substrate casein increased heat stability of the bromelain about 25%, thus the enzyme was stable up to 45℃. Its value of Km for casein was 0.1%.

INTRODUCTION

Poverty of agricultural policy and unstability of prices for agricultural products are enforcing farmers out of crop fields in this country. The same situation is happening in Cheju island. Subtropical climate in Cheju island enables Chejuians to cultivate a pineapple, *Ananas comosus* (L) Merr, for mainly tourists and domestic markets. Recent trends toward freedom on international trading is going to lower market prices of agricultural products including pineapples, which is one of major threats to farmers.

Pineapples are mainly cultivated for harvest of fruit which is used as food and medicine. Leaves can be processed into fabric, paper, and net. But they are usually discarded and found to be fertilizers (Jung, 1988). Pine-

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apples are rich with proteolytic enzymes, a stem bromelain (EC 3.4.22.4) and a fruit bromelain (EC 3.4.22.5) (Takashi, 1976). A bromelain is one of the sulfhydryl proteases and has similar activities to a papain (Yasuda, *et al.*, 1970). The enzyme is presently used in meat tenderizing, clarification of beer, fish processing, and as pharmaceuticals (Jung, 1988; Redd, 1975; Schwimmer, 1981). Use of a bromelain as meat tenderizers has been made possible since proteases of microbial origin act only on actomyosin muscle fiber, whereas a bromelain hydrolyses actomyosin, collagen, elastin, and connective tissue fiber (Lawrie, 1979). The development of domestic meat tenderizer using bromelains has never been attempted in this country.

Proteases from *Streptomyces* (Kim, *et al.*, 1989; Yun, *et al.*, 1989) or *Bacillus* (Bae, *et al.*, 1989; Kim, *et al.*, 1989) species have been isolated and characterized in respect to the signifiance of their industrial application. A ficin was also isolated from the latex of *ficus sp.* and characterized (Seo, 1984). Since Heinicke (1953) identified proteolytic enzymes in pineapple juice and stem in 1953, bromelains have been purified from pineapple stems and their basic characteristics determined (Takashi, 1976; Wang, 1958; Ward, 1985). This study is the begining of our future goal leading to the development of domestic meat tenderizer. We report here partial characteristics of the bromelain isolated from stems of pineapples cultivated in Cheju island.

MATERIALS and METHODS

Isolation of bromelain

Pineapples, *Ananas comosus* (L) Merr, at mature stage were obtained from Seoguipo area of Cheju island. Stems were recovered from pineapple trees and marcerated in 10mM phosphate buffer (pH 7.0) with homogenizer. Proteins were extracted at 4℃ in the refrigerator. Debrises of pineapples were removed by filtering with cheese cloth and fines were discarded after centrifuging the filtrate. Proteins were precipitated by the addition of two volumes of ice-cold acetone to the supernatant and recovered by centrifugation. The protein precipitates were dissolved in minimal amout of water, dialyzed against water, and finally freeze-dried. The resulting powder was used as crude bromelain. Crude bromelain powder was dissolved in 50mM phosphate-80mM mercaptoethanol-40mM EDTA buffer (pH 6.0) or in water appropriately for use in these experiments.

Preparation of casein substrate solution

One gram of hammarsten casein was dissolved in 50㎖ of 0.5M sodium phosphate solution and heated for 30min in boiling water bath, with occasional agitation. Citric acid (0.05M) was added to adjust pH to 6.0 after cooling to room temperature and the mixture was finally diluted to 100㎖ with water.

Proteolytic activity assay

The assay is base on proteolytic hydrolysis of a casein. One ㎖ of bromelain solution was mixed with 2.5㎖ of casein substrate in test tubes and the tubes were incubated at 37-40℃ for adequate time. After incubation, 1.5㎖ of 30% trichloroacetic acid was added to precipitate unhydrolyzed casein and the tubes were further incubated for 30 min to allow the precipitated protein to coagulate completely.

The reaction mixtures were filtered through Whatman No.2 filter paper. The absorbance of the resulting filtrate was determined at 280 nm. This standard assay method was used with slight modification if necessary and the modifications were specified elsewhere.

RESULTS and DISCUSSION

Dependence of the activity on pH

Bromelains are glycoproteins (Ward, 1985) whose functional groups may change depending upon the concentration of hydrogen ions surrounding. Overall or partial structure of the enzyme also may change affecting enzyme activity. Casein hydrolytic activities were determined at pH ranges from 5.3 to 11.0. The pH's of substrate casein were adjusted to desired values prior to the addition of enzyme solution. Optimum activity was obtained at pH 7.5(Fig.1), which coincided

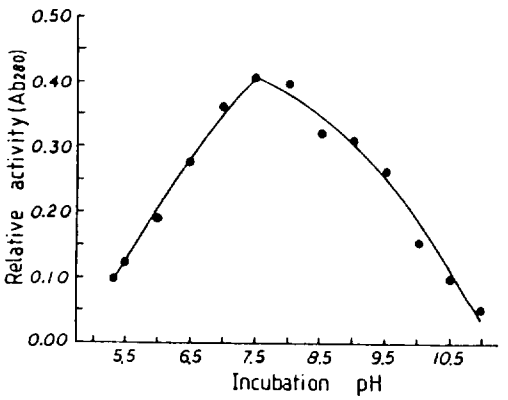

Fig. 1. Dependence of catalytic activity of a bromelain on incubation pH. Catalytic activity for initial 30 min was assayed at each pH.

well with other reported pH optimum 6.0-8.0 (Reed, 1975; Ward, 1985). Thus our bromelain is a neutral protease.

Stability at each pH of the enzyme was determined by incubating the enzyme for 60 min in buffers with different pH's before mixing with substrate casein. The enzyme was stable at least for 60 min without any loss of catalytic activity at pH ranges from 5.6 to 9.0 (Fig.2) but may be less stable than a ficin (Seo, 1984).

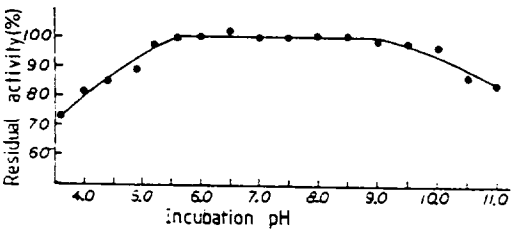

Fig. 2. Effects of pH on bromelain stability. The enzyme was incubated at each pH for 60 min prior to activity assay.

Dependence of the activity on temperature

Proteins are usually labile to heat. Upon heating some forces that support the protein

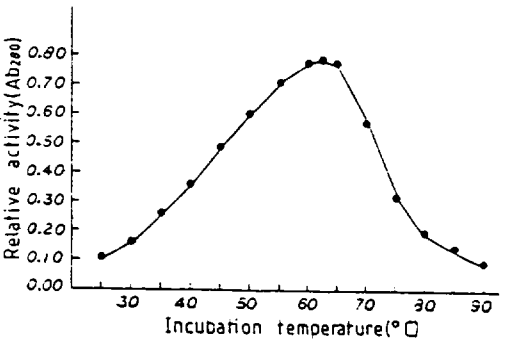

Fig. 3. Dependence of catalytic activity of a bromelain on incubation temperature. Catalytic activity for initial 30 min was assayed at each temperature.

structure are disrupted, which will be reflected in the change of activity. On the other hand, enzyme catalyzed reaction rate increases two to three fold as temperature goes up by 10℃. Optimum temperature for catalytic activtiy was determined to be 62.5℃ after incubation of the enzyme and substrate for initial 30 min at different temperatures (Fig.3). The optimum temperature may get lower as the incubation time gets longer because bromelains lose activity above 50℃ (Fig.4). Accordingly, the optimum temperature will get closer to 50℃

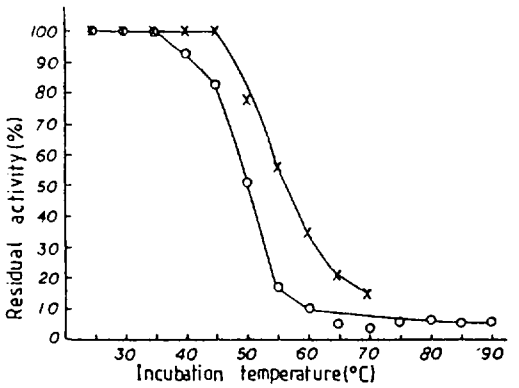

Fig. 4. Effects of temperature on bromelain stability. The enzyme was incubated without substrate casein (O-O) or with it (X-X) at each temperature for 30 min prior to activity assay.

that is known to be optimum one for stem bromelains (Ward, 1985) if the reaction mixtures are incubated further longer.

When the enzyme was incubated at different temperatures for initial 30 min before being mixed with substrate, it lost activity rapidly above 40℃ (Fig.4). However, enzyme-substrate complex was expected to be more tolerable than enzyme alone at high temperatures. When enzymes were mixed with excess amount of casein and incubated for 30 min at each temperature, and the residual activities were assayed under standard conditions, the resulting mixture retained original catalytic activity even at 45℃ and showed about 25% higher residual activity than the enzyme treated with heating alone (Fig.4). Casein forms complex with the enzyme before being hydrolysed and stabilizes the enzyme even for a short time. Supposing the concentration of casein is high enough to saturate the enzyme, it can be regarded to exist as a complex with the substrate. Artificial substrate that can not be hydrolysed may enable us to study the stability of enzyme-substrate complexs in more detail.

Km value for casein substrate

One of the characteristics of a enzyme is the substrate concentration (Km) which gives half maximum reaction velocity. Each substrate has a unique Km value. The Km value

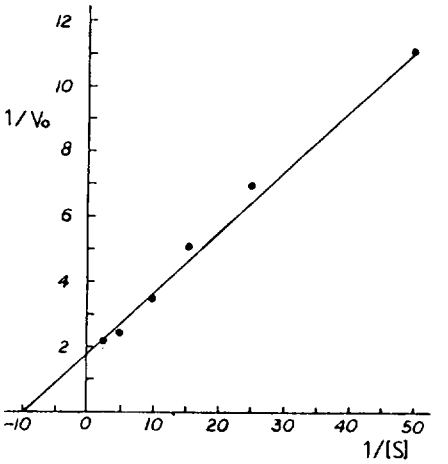

Fig. 5. Lineweaver-Burk plot for hydrolysis of casein by a bromelain. [S], concentration of casein (%); Vo, hydrolysis velosity (Increase of Ab_{284}/min).

tells us how good the substrate is. The lower the value is, the better the substrate is.

Hydrolysis velocities for initial 30 min were determined at different substrate concentrations of casein under standard assay condition. Km value for casein of the stem bromelain appeared to be 0.1% on Lineweaver-burk plot (Fig.5). But it should be stressed that 30 minutes' incubation might be too long for measurement of initial reaction rate even though incubation time depends on enzyme activity.

Perspectives.

Several enzymatic methods for tenderization of meat have been developed and patented (Schwimmer. 1981). However, our further goal is to make a ready-to-use meat tenderizer instantly on the table. The bromelain mentioned in this paper need to be investigated further about the effects that possible seasonings may have on it since the expected tenderizer is desired to be applicable to the recipe for cooking meat.

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摘　　要

즉석 軟肉劑를 제조하기 위한 基礎 調査로 濟州産 파인애플의 줄기로부터 蛋白分解酵素 bromelain을 抽出, 分離하여 部分的으로 그 特性을 규명하였다. 最高 觸媒活性을 나타내는 反應温度는 62.5℃였고, pH는 7.5였다. 이 酵素는 温度에 鋭敏하여 40℃ 以上에서는 活性이 급격히 減少하였으나, 基質인 casein의 共存下에서는 熱安定性이 약 25% 增加하여 45℃에서도 活性損失이 없었다. 또한 pH 5.6-9.0에서 酵素의 活性減少가 없이 安定하였고 casein에 대한 Km値는 0.1%였다.