Kasetsart J. (Nat. Sci.) 40 : 129 - 134 (2006)

Effects of Sucrose Concentration on Crude Bromelain Production of *In Vitro* Culture of Pineapple (*Ananas comosus* var. 'Pattavia')

Budsaraporn Ngampanya* and Siriporn Phongtongpasuk

ABSTRACT

Crude bromelain production of pineapple var. 'Pattavia' shoots induced from crown-derived tissues was conducted under *in vitro* condition. Change in sucrose concentration showed the significant effect on protein yield, total enzymatic activity and specific enzymatic activity. When high concentration (30-45 g/l) of sucrose was applied, protein yield, total enzymatic activity and specific enzymatic activity were higher than those of induced shoots supplemented with low concentration (15 g/l) of sucrose. However, increasing sucrose concentration to 45 g/l resulted to 1.5-2 times lower protein yield, total enzymatic activity and specific enzymatic activity than those derived from natural crowns. It might be suggested that sucrose alone did not entirely affect the production of crude bromelain in pineapple var. 'Pattavia'.

Key words: crude bromelain, *Ananas comosus*, sucrose, *in vitro* culture

INTRODUCTION

Plants are the important source of natural products, the so-called secondary metabolites, which play a major role in the adaptation of plants to their environment. These substances are also used as a source of pharmaceuticals, fragrances, agrochemicals and food additives (Wink, 1990). A variety of these chemicals such as ginseng saponins (Zhang *et al*., 1996; Zhang and Zhong, 1997; Andrijany *et al*., 1999), anthocyanin (Zhong and Yoshida, 1995), canthinone alkaloids (Liu *et al*., 1990), anthraquinones (Nazif *et al*., 2000), taxol (Wang *et al*., 1999; Wu *et al*., 2001), plant cysteine protease (Apte *et al*., 1979; Fernandez and Pomilio, 2003; Perez *et al*., 2003; 2004) and etc. were produced by means of *in vitro* techniques.

Bromelain is an enzyme in the family of cysteine protease which found extensively in the pineapple plant. It was first introduced as a therapeutic compound in 1957 (Kelly, 1996). Additionally, it was also the choice enzymes for food processing (Takagi *et al*., 1992). In pineapple plant, bromelain is accumulated in the entire part with different extent and properties depending on its source (Rowan *et al*., 1990). Few reports emphasized on the production and characterization of this enzyme in this crop by way of plant cell and tissue culture have been recently investigated. The determination of protease derived from pineapple callus was first investigated in 2003 in order to classify the catalytic type of enzyme (Fernandez and Pomilio, 2003). The two additional reports were focused on the culture condition

Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Sanamchandra Palace Campus, Muang, Nakornpathom 73000, Thailand

* Corresponding author, e- mail: nbudsar@su.ac.th

optimization to stimulate the excretion of protease during pineapple micropropagation in temporary immersion bioreactors (Perez *et al*., 2003; 2004). Relatively higher concentration of sucrose was reported to be favorable to enhance a varieties of plant-specific secondary metabolites such as rosmarinic acid in *Coleus blumei* cells (Martinez *et al*., 1993) and anthocyanin in *Perilla frutescens* cells (Zhong and Yoshida, 1995). In addition, Perez *et al*. (2004) was reported the different effects of sucrose concentration on micropropagation rate and excretion of protease in pineapple culture. Therefore, the present research was conducted to study the relation of sucrose concentration with crude bromelain production and accumulation in induced pineapple shoots so as to optimize the culture condition for obtaining the high yielding of bromelain by using typical liquid culture system.

MATERIALS AND METHODS

Plant material

Crowns of pineapple (*Ananus comosus* var. 'Pattavia') collected from the field in Prachuapkhirikhan Province were used as explant sources. The buds were excised from the crown and then surface sterilized in 70% ethanol solution for 3 minutes, followed by 3-time rinse in sterile distilled water, immersed in 15% clorox solution (5.25% w/v sodium hypochlorite) for 15 minutes and additional rinse in sterile distilled water for 3 times, respectively. The explants were placed on solidified MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/l of 6-benzyladenine (BA), 30 g/l of sucrose and 8 g/l of agar. The cultures were incubated at 25±2°C with a 16 h photoperiod of fluorescent light for 4 weeks. The emerged shoots were then transferred from solidified medium to liquid medium in order to increase the multiplication rate of shoot. The cultures were placed on rotary shaker with a constant shaking at 120 rpm and incubated at the same condition as previously described for additional 3 weeks.

Effect of sucrose on crude bromelain production

Liquid MS medium supplemented with 2 mg/l of BA and three different concentrations of sucrose (15, 30 and 45 g/l) were prepared. Three of 3-cm-long shoots were placed into 100-ml-plant container containing 50 ml of medium. The cultures were then placed on rotary shaker with constant shaking at 120 rpm and incubated at 25±2°C with a 16h photoperiod of fluorescent light for 4 weeks. Then protein concentration, total enzymatic activity and specific enzymatic activity were measured.

Determination of protein content and bromelain activity

Crude bromelain was extracted from shoots according to the procedure of Narangajavana (1987). Shoots were fed by distilled water with a ratio of 1: 2 (w/v) and blended by using blender. The homogenates were filtered, and filtrates then were twice precipitated with 41% and 75% acetone, respectively. Pellets were freeze-dried by lyophilizer to obtain crude bromelain powder.

Protein content was determined in accordance with Lowry *et al.* (1951). Absorbency (650 nm) was recorded and a standard curve for bovine serum albumin was used.

Bromelain activity was evaluated according to the modification of Kunitz's method (1947). The reaction mixture contains 1 ml of crude bromelain solution (10 mg of crude bromelain powder suspended in 3 ml of 0.1 M sodiumphosphate buffer pH 7.6), 0.2 ml of 0.2 M 2-mercaptoethanol and 1 ml of 1% casein solution. Reaction was allowed for 20 minutes at 37°C, and then stopped with 3 ml of 5% trichloroacetic acid. Absorbency (280 nm) was measured and a tyrosine standard curve was used. Enzyme activity was

defined as CDU/kg shoot fresh mass (CDU=the amount of enzyme which produces 1 μg of tyrosine per minute in 1% solution of casein at 37°C). Specific activity was calculated as the rate of proteolytic activity relative to protein content.

Statistical analysis

All measurements were carried out in triplicate (n=3), and results were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute, 1990). Differences between means were determined by the least significant different test, and significance was defined at P<0.05.

RESULTS

The analysis of the data showed significant effects of sucrose concentration on protein content, enzymatic and specific activity (Figure 1), which tended to increase with sucrose level. The maximum yield of protein content, enzymatic and specific activity were obtained from induced shoots grown in liquid MS medium supplemented with 45 g/l sucrose. Sucrose concentration of 45 g/l showed the significant different in protein content (Figure 1A) from those at the concentration of 30 and 15 g/l. In addition, enzymatic and specific activities of induced shoots were also affected by the sucrose concentration. In case of enzymatic activity (Figure 1B), there was significant different between concentration of 45 and 30 g/l sucrose as well as the significant different between concentration of 30 and 15 g/l sucrose was also obtained. In contrast to specific activity (Figure 1C), there was no significant different between concentration of 45 g/l and 30 g/l sucrose. However, the concentration of sucrose (30-45 g/l) still revealed significant different in specific activity from the lower concentration (15 g/l sucrose). All data shown here suggests that the high concentration of sucrose seems to effect to yield of crude bromelain from cultured pineapple shoots. Apart from the effects of sucrose on crude bromelain production in induced shoots, the maturity of plant materials used for enzyme extraction was also considered. The protein content (2,067.9±308.62), enzymatic (12,831.8±1,355.86) and specific activity (6.23±0.32) detected in natural crowns were higher than those obtained from 2-month old induced shoots. It might be suggested that sucrose alone did not entirely affect the production of crude bromelain in pineapple.

DISCUSSION

Commonly, sucrose is used in plant cell, tissue, and organ culture as a carbon source for supporting the growth and differentiation under *in vitro* condition. The declining of photosynthesis rate in cultured cells requires the exogenous supply of carbon substrate, otherwise the normal growth of such cells are unable to maintain (Zhong and Yoshida, 1995). It is in consistence with our observation that the growth of induced shoots grown in liquid MS medium without supplying of sucrose was retarded in the first two weeks after subculture (data not shown). The increasing of protein content upon the elevated level of sucrose shown in this experiment was supported by the fact that increasing of sugar concentration in a medium which consists of all nutrients in excess will result in a proportional increase in biomass (Zhong and Yoshida, 1995; Perez *et al*., 2004). Apart from the role of sucrose as carbon source for cultured plant, it also provides the carbon necessary for secondary metabolites production in plant. There are reports indicated the effect of sucrose in both positive and negative on metabolite concentration (Martinez *et al*., 1993; Zhong and Yoshida, 1995; Wang *et al*., 1999; Perez *et al*., 2004). For crude bromelain production from pineapple var. 'Pattavia' reported here (Figure 1B, 1C), the high concentration of sucrose ranged from 30-45 g/l had the significant effect to the value of enzymatic and specific activity when compare to

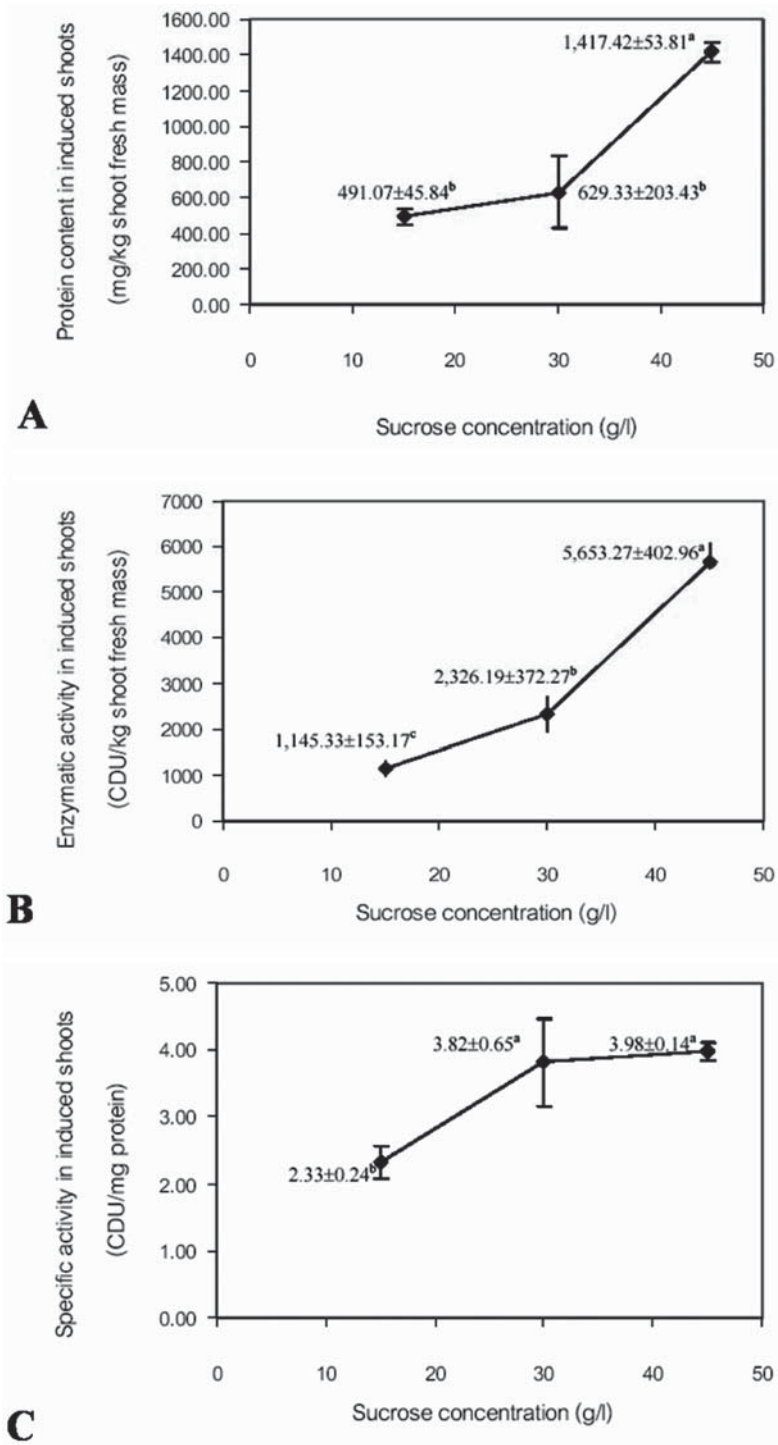

Figure 1 Relationship between sucrose concentration and protein content (A), proteolytic activity (B), and specific activity (C) of crude bromelain in induced shoots of pineapple 'Pattavia'. *Vertical bars* represent average ± SD. $^{a\text{-}c}$ means in the same figure followed by different superscript are significant different (p<0.05).

the value of enzymatic and specific activity derived from induced shoots grown in liquid medium supplemented with 15 g/l sucrose. However, the specific activity derived from induced shoots grown in medium supplemented with 30 g/l sucrose was not significantly different from those grown by supplying 45 g/l sucrose. In this case, it may suggest the positive effect of sucrose on the production and accumulation of crude bromailn when the sucrose concentration was not supplied excess than 45 g/l. At the higher concentration of sucrose than 45 g/l, enzyme may less accumulate in cells. Perez *et al*. (2004) described that protease was excreted from pineapple shoots to culture medium during culture when the shoots were fed by high concentration of sucrose. In this case, pineapple shoots might have been under osmotic stress. In order to enhance the production and accumulation of enzyme in pineapple shoots, therefore, other factor causing stress condition should be considered.

CONCLUSION

Sucrose concentration effect protein content, crude bromelain activity and specific activity of induced shoots of pineapple var. 'Pattavia' grown in typical liquid culture system. In addition, the proper concentration of sucrose supplementing in liquid MS medium to obtain high amount of enzymatic specific activity should not exceed 45 g/l. Factors which may influence the synthesis of bromelain such as age of plant, type of cells and location of bromelain production, other nutrients in medium and culture system should be further investigated.

ACKNOWLEDGEMENTS

This work was supported by Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University.

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