Effect of some Factors on the Proteolytic Activities of Bromelain, Cichorium and Papain Extracts

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Abstract: Effects of pH values, sodium chloride, calcium chloride, enzyme concentrations and storage temperatures on the proteolytic activities of crude bromelain, cichorium and papain extracts were studied. The increase of each pH values, sodium chloride percentages and temperatures showed an increase and then decrease in the proteolytic activities of crude-plant-enzymes. The experimental results suggests that the proteolytic activities of crude-plant-enzymes extracts significantly ($p<0.05$) decreased with the increase of calcium chloride, while decreased during the storage with cooling and freezing conditions. Ultimately, the proteolytic activities of crude bromelain, cichorium and papain extracts were increased significantly ($p<0.05$) with increase of enzyme concentrations.

Keywords: Bromelain; Cichorium; Crude-plant-enzymes extracts; Papain; Proteolytic activities

INTRODUCTION

Many plants were employed as a source of proteolytic enzymes such as proteases. The protease indicates to proteases and peptidases (Gonzalez-Rabade *et al*., 2011). Rawlings *et al*. (2010) showed that the families of proteases were as a follows: aspartic, asparagine, cysteine, glutamic, metallo, serine and threonine. In addition the plants have five classes from endoproteases which included aspartic, cysteine, metallo, serine and threonine. Most of plant proteases classified as cysteine proteases and seldom as aspartic proteases, and retained active over a range of each pH values and temperature. The proteases enzymes were used in different applications, *e.g.* food, pharmaceutical, detergent, preparation of leather and wool, tenderization of meat and dairy processing (Doran, 2002; Gonzalez-Rabade *et al*., 2011). Also, the proteases of plants can be used as substitute for rennet (Tamer and Mavituna, 1997; Uhlig, 1998). The plant proteases include of bromelain, ficin and papain, which extracted from *Ananas comosus*, *Ficus carica* and *Carica papaya,* respectively (Gonzalez-Rabade *et al*., 2011).

Papain proteases were isolated from latex of papaya fruit, and applied in cheese, flavoured protein hydrolysates and food complements (La Valle *et al*., 2000; Losada, 1999), emulsifiers' production (Pardo *et al*., 2000), pharmaceutical industry, cancer treatment (Targoni *et al*., 1999) and digestion disorders (Mello *et al*., 2008). Papain enzyme is stable and active at the pH values from 4 to 10 at high temperatures (Cstorer and Ménard, 1994). The preproteinase composed of 345 amino acids and secreted as zymogene (Mitchel and Chaiken, 1970). A single-chain was the shape of mature papain and contains 212 amino acids after the cleavage of an activation peptide (Kamphuis and Kalk, 1984), in addition the enzyme contains three disulfide bonds with isoelectric point (IEP) of 8.75 (Storer and Ménard, 2013).

Bromelain is a type of proteolytic enzyme, derived from pineapple, contains four cysteine endopeptidases and the range of IEP values from 4.6 to 10. The bromelain activity was ranged over pH values from of 5.5-8.0. The high temperature was accompanied with inactivate of bromelain, therefore the denaturation has been resulted (de Lencastre Novaes *et al*., 2016). Stem and fruit bromelains have several applications in food industry, pharmaceutical and used as a digestive aid (Rowan *et al*., 1990).

Cichorium intybus L. distributed in Asia and Europe, usually known as chicory, and belongs to family *Asteraceae* (Bais and Ravishankar, 2001). Cichorium enzymes considered a cheap source; therefore it can be replace of microbial enzymes in acceleration of each Domiati and Ras cheese ripening by adding enzyme to cheese curd (Abou-zeid and El Sisi, 2014; Abou-zeid, 2015).

The purpose of the present study was aimed to know the effects of pH, sodium chloride, calcium chloride, enzyme concentrations and storage temperatures on the proteolytic activities of crude extracts of bromelain, cichorium and papain.

MATERIALS AND METHODS

Materials

Chicory (*Cichorium intybus* L.), Papya (*Carica papaya*) and Pineapple (*Ananas comosus* L.) were purchased from local market (Ismailia, Egypt). Calcium carbonate, casein, Folin-ciocalteu reagent, L-tyrosine, sodium carbonate and trichloroacetic acid were obtained from El-Nasr-pharmaceutical chemical Co. (Cairo, Egypt). All chemicals were of analytical grade in the present study.

Methods

Extraction of crude enzymes

The plant extraction was done according to (Hale *et al*., 2005) with some modifications. Bromelains, cichorium and papain were obtained from pineapple, chicory and papaya respectively. The fresh plants were milled with a mortar and pestle, and then centrifuging (7000 rpm/10 min., at 4°C). The supernatant was used without further fractionation.

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Proteolytic activity determination

The proteolytic activity of plant extracts was measured using method of Chopra and Mathur (1983). Aliquot 1 ml from crude plant extracts was added to 1% casein in 0.05 M phosphate buffer (pH 7). The mixture was incubated at 37°C/20 min after mixing. The reaction was stopped by adding 2 ml from 0.4 M trichloroacetic acid (TCA) followed filtrating. With regard to the blank, the substrate was precipitated by TCA before adding enzyme extracts and treated similar to describe above. Add 5.0 ml of 0.4 M sodium carbonate to 1 ml of filtrate obtained after TCA precipitation, 1 ml of Folin-Ciocalteu reagent was added to the filtrate, and then incubated at 37°C/20 min for colour development and the absorbance was performed at 750 nm.

Statistical analysis

The obtained results were analyzed statistically in one way analysis of variance using computer program software SPSS 16 (SPSS Inc., Chicago, USA). To determine the changes between means, Duncan analysis was used at $p<0.05$.

RESULTS AND DISCUSSION

Effect of pH values on the proteolytic activities of crude bromelain, cichorium and papain extracts

As depicted in Fig. (1), the increase of pH values from 6-7 and 6-6.50 for (bromelain and cichorium) and papain extracts respectively has been resulted increases in the proteolytic activities, and then the proteolytic activities of their extracts decreased due to occurrence of greater electrostatic interaction (Chaurasiya and Hebbar, 2013). The optimum proteolytic activities for bromelain, cichorium and papain extracts were 31.71, 26.41 and 26.21 µg tyrosine/1 mL at pH 7, 7 and 6.5, respectively.

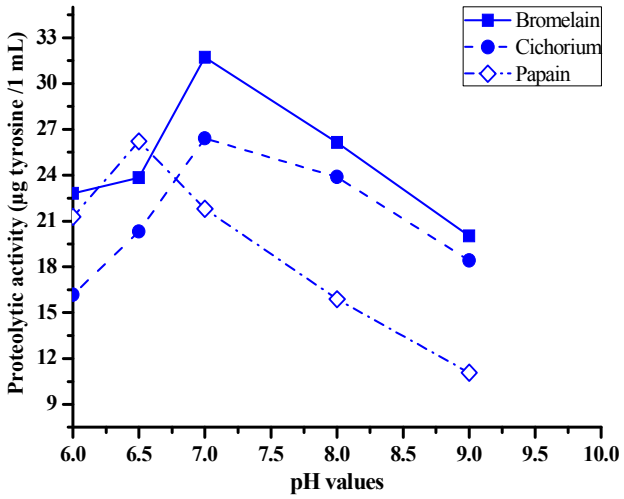

Fig. (1): Effect of pH values on the proteolytic activities of crude-plant-enzymes extracts

Results of the present study was in agreement with that observed by Kang and Warner (1974), who found that the slight reduction in papain activity was noticed at pH 5.0, then the activity begin to loss at pH 7, while the higher activity reduction observed at pH 9. Also, the optimum activity for crude papain enzyme was noticed at pH 6.4, followed the enzyme activity was decreased (Foda *et al*., 2016). On the other hand, the obtained results were in harmony with that obtained by de Lencastre Novaes *et al*. (2016), they showed that activity of bromelain was increased up to the pH 7, while the activity noticeably decreased with the advancing of pH values. Also, the similar observations were obtained by Chaurasiya and Hebbar (2013), they reported that the conformation of bromelain didn't change at the pH range from 4.5-9.8, moreover the optimum activity were observed at pH 7, while the activity was decreased at pH 5.7 due to acidic of pineapple fruit. In this work, the density of positive charge and electrostatic repulsion showed a higher at low pH values, which in turn led to reduction in the positively charged bromelain (Omotoyinbo and Sanni, 2017).

Effect of NaCl concentrations on the proteolytic activities of crude plant extracts

Fig. (2) shows the effect of NaCl levels on the proteolytic activities of crude bromelain, cichorium and papain extracts. The increase of NaCl percentages from 0 to 2% increased the proteolytic activities of all crude-plant-enzyme extracts, while the proteolytic activities of these extracts decreased at range of 2-6% NaCl.

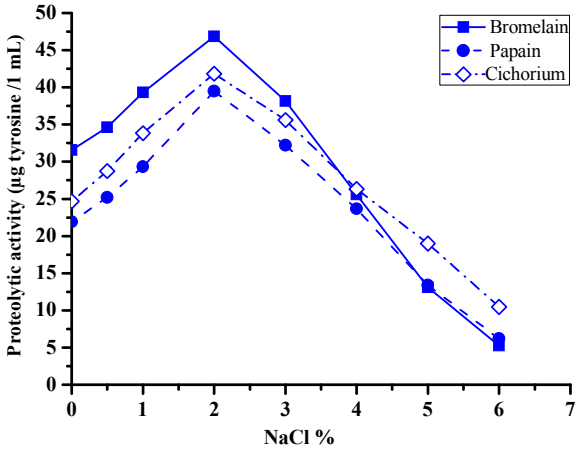

Fig. (2): Effect of NaCl percentages on the proteolytic activities of crude bromelain, cichorium and papain extracts

Therefore the present results were in consistent with the earlier reported (Chaurasiya and Hebbar, 2013), those found the proteolytic activity of enzyme has been increased with coinciding of the reduction of salt percentages, while a higher salt levels led to decrease of the proteolytic activity. Foda *et al*. (2016) showed that the optimum activity of papaya pectin esterase was obtained with 0.3 M NaCl, and then the enzyme activity was decreased. The highest proteolytic activities at 2% NaCl was for bromelain, cichorium and papain respectively. Increases of NaCl concentrations up to 6% caused sharp decrease in the proteolytic activity. At large concentration of 6% NaCl, the cichorium extract showed less sensitively than bromelain or papain extracts. The increase of proteolytic activities of crude-plant-enzyme extracts can be due to the salting in, whereas the salting out led to reduction of the proteolytic activities (Polacsek-Racz and Pozsar-Hajnal, 1976).

Effect of $CaCl_2$ concentrations on the proteolytic activities of crude-plant-enzyme extracts

Table (1) shows the influence of $CaCl_2$ concentrations on the proteolytic activities of different plant extracts. Generally, the proteolytic activities of all crude-plant-enzyme extracts significantly ($p<0.05$) decreased with the increase of $CaCl_2$ percentages. In addition the proteolytic activities of extracts ordered as a follows: bromelain > cichorium > papain at all $CaCl_2$ treatments. Kaur *et al.* (2015) showed that the bromelain enzyme has been inhibited at pH 3.5 and with 0.5 mM calcium. Haq *et al*. (2005) showed that the relationship of structure activity for bromelain enzyme with calcium ions has been influenced by salt effects, electrostatic shielding of charge and nonspecific binding of protein molecule.

Table (1): The influence of $CaCl_2$ percentages on the proteolytic activities of crude-plant-enzyme extracts

$CaCl_2$ %	Proteolytic activity (µg tyrosine / 1 ml crude-plant-enzyme extract)▼		
	Bromelain	**Cichorium**	**Papain**
0 (Control)	31.39 ± 0.37^{Aa}	24.69 ± 0.48^{Ba}	21.96 ± 0.30^{Ca}
0.01	27.70 ± 0.55^{Ab}	23.52 ± 0.37^{Bb}	20.95 ± 0.24^{Cb}
0.02	23.12 ± 0.24^{Ac}	22.40 ± 0.55^{Ac}	19.91 ± 0.30^{Bc}
0.03	21.27 ± 0.18^{Ad}	21.11 ± 0.37^{Ad}	18.70 ± 0.18^{Bd}
0.04	19.63 ± 0.24^{Ae}	19.87 ± 0.48^{Ae}	17.62 ± 0.18^{Be}
0.05	18.74 ± 0.50^{Af}	18.62 ± 0.37^{Af}	16.33 ± 0.18^{Bf}
0.06	17.86 ± 0.30^{Ag}	17.38 ± 0.37^{Ag}	15.05 ± 0.24^{Bg}
0.07	16.53 ± 0.37^{Ah}	16.13 ± 0.24^{Ah}	13.92 ± 0.18^{Bh}
0.08	15.29 ± 0.24^{Ai}	15.01 ± 0.18^{Ai}	12.68 ± 0.25^{Bi}
0.09	13.48 ± 0.24^{Aj}	12.48 ± 0.18^{Bj}	11.35 ± 0.18^{Cj}
0.10	11.07 ± 0.24^{Ak}	10.91 ± 0.42^{Ak}	9.99 ± 0.32^{Ak}

Capital letters, values of averages are significant ($p<0.05$) with the different letters between each row; Small letters, values of averages are significant ($p<0.05$) with the different letters within each column; ▼, Mean ± S.D.

Effect of enzymes concentrations on the proteolytic activities of crude-plant-enzyme extracts

The increase of concentrations of bromelain, cichorium and papain extracts resulted increases in the proteolytic activities (Fig. 3). The highest proteolytic activity of different extracts was found in bromelain extract, followed by cichorium and papain extracts. Furthermore, the relationship between concentrations of crud-plant-enzyme extracts and the proteolytic activities were positive. Foda *et al*. (2016) reported that the reaction activity of crude papain enzyme was increased with the increase of enzyme concentration until limit level, then the activity has been decreased the inhibition effects / or the reverse reactions which can be resulting the direction and steric retardation for the excess amount of enzyme.

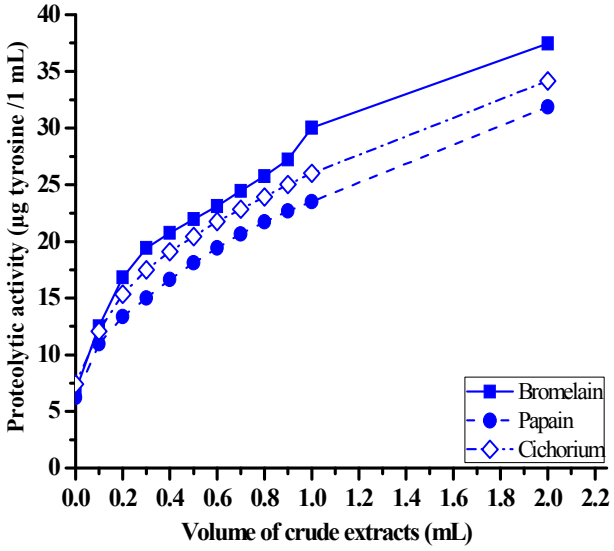

Fig. (3): Effect of enzymes concentrations on the proteolytic activities of crude bromelain, cichorium and papain extracts

Effect of storage time and temperature on the proteolytic activities of crude-plant-enzyme extracts

Table (2) represents the proteolytic activities of the enzyme extracts during the storage at 4°C and -18°C. It was remarkable that the proteolytic activities of crude bromelain, cichorium and papain extracts statistically ($p<0.05$) decreased during the storage either at 4°C or -18°C due to the cold denaturation and the interactions between both water and protein molecules (Tantos *et al*., 2009). Also, the storage under freezing conditions presumably results the denaturation process, therefore the changes in solute concentration has been occurred due to formation of ice (Bhatnagar *et al*., 2007). Similar observations were obtained by Dias *et al*. (2010), they found that the low temperature of proteins was caused to unfolding, and therefore the denaturation.

Table (2): Effect of the storage stability on the proteolytic activities of crude bromelain, cichorium and papain extracts at different temperatures

Treatments	Proteolytic activity (µg tyrosine/1 ml crude-plant-enzyme extract)▼ during the storage at 4°C		
	Bromelain	**Cichorium**	**Papain**
Fresh (Control)	32.52 ± 0.55^{Aa}	26.33 ± 0.57^{Ba}	23.40 ± 0.37^{Ca}
7 Days	15.97 ± 0.94^{Ab}	13.72 ± 0.24^{Bb}	9.75 ± 0.36^{Cb}
14 Days	8.02 ± 0.50^{Ac}	7.34 ± 0.43^{Ac}	6.25 ± 0.24^{Bc}
21 Days	1.55 ± 0.09^{Ad}	1.53 ± 0.08^{Ad}	1.41 ± 0.10^{Bd}
Treatments	**Proteolytic activity (µg tyrosine/1 ml crude-plant-enzyme extract)▼ during the storage at -18°C**		
Fresh (Control)	32.52 ± 0.55^{Aa}	26.33 ± 0.57^{Ba}	23.40 ± 0.37^{Ca}
7 Days	29.35 ± 0.25^{Ab}	23.44 ± 0.39^{Bb}	6.45 ± 0.18^{Cb}
14 Days	27.66 ± 0.18^{Ac}	22.24 ± 0.37^{Bc}	1.55 ± 0.10^{Cc}
21 Days	26.17 ± 0.30^{Ad}	21.35 ± 0.30^{Bd}	1.55 ± 0.12^{Cc}
30 Days	25.13 ± 0.30^{Ae}	20.47 ± 0.32^{Be}	1.54 ± 0.11^{Cc}

See footnote Table (1)

Previous study done by Chaurasiya and Hebbar (2013) reported that the proteolytic activity for bromelain was decreased as the storage period proceeding due to the lower of specific activity. Storage of all crude-plant-enzyme extracts at 4°C was resulted a sharp decrease in the proteolytic activities. Within 14 days the proteolytic activities decreased nearly 75% of the original activities and the end of storage (21 days), the extracts lost most of their activities. Also, storage of crude-plant-enzyme extracts at -18°C has a pronounce decrease in the proteolytic activities of bromelain and cichorium, while papain was greatly affected and lost most of its activity. Results of the present work were in harmony with that described by Storer and Ménard, (2013), who found that the native papain has been loosed 50% from its activity in 7 days due to oxidation of active site thiol group.

Effect of the incubation temperatures on the proteolytic activities of crude bromelain, cichorium and papain extracts

The obtained results of the proteolytic activities of crude-plant-enzyme extracts are showed in Table (3). The optimum temperatures for the proteolytic activities of crude bromelain, cichorium and papain extracts were 40, 35 and 30°C, respectively. The proteolytic activities of all extracts were significantly ($p<0.05$) decreased with elevation of temperatures due to the denaturation process. Similar results were obtained by Xue *et al*. (2010), they found the increase of temperature has been decreased the thermal stability of enzymes. It is remarkable that, the denaturation of bromelain enzyme via high temperature was attributed to the molten globule state of bromelain (Ahmad and Khan, 2006). Also, Khan *et al*. (2003) showed that the activity of bromelain was decrease 17% at range of 40-60°C. The proteolytic activity of bromelain was higher compared crude papain and cichorium especially at range of 25–50°C, while from 55–80°C, the proteolytic activity of crude bromelain was occupied the low trend compared crude papain and cichorium extracts. Omotoyinbo and Sanni, (2017) found that the gradual increase in the bromelain activity was observed from 30–35°C, while the optimum activity was noticed at 40°C.

Table (3): Effect of temperatures on the proteolytic activities of extracts

Temperatures (°C)	Proteolytic activity (µg tyrosine/1 ml crude-plant-enzyme extract)▼		
	Bromelain	**Cichorium**	**Papain**
25	32.08 ± 0.30^{Ad}	31.51 ± 0.42^{Ac}	28.06 ± 0.36^{Bb}
30	35.97 ± 0.30^{Ac}	35.49 ± 0.07^{Ab}	31.19 ± 0.24^{Ba}
35	37.90 ± 0.25^{Ab}	38.34 ± 0.49^{Aa}	26.86 ± 0.36^{Bc}
40	42.20 ± 0.18^{Aa}	31.31 ± 0.59^{Bc}	24.04 ± 0.42^{Cd}
45	36.09 ± 0.42^{Ac}	25.41 ± 0.36^{Bd}	22.48 ± 0.37^{Ce}
50	26.61 ± 0.24^{Ae}	23.72 ± 0.43^{Be}	19.91 ± 0.49^{Cf}
55	21.15 ± 0.25^{Bf}	22.48 ± 0.30^{Af}	18.50 ± 0.37^{Cg}
60	16.61 ± 0.48^{Bg}	18.86 ± 0.37^{Ag}	14.00 ± 0.71^{Ch}
70	11.84 ± 0.18^{Bh}	12.68 ± 0.18^{Ah}	7.58 ± 0.12^{Ci}
80	6.82 ± 0.30^{Ai}	6.78 ± 0.25^{Ai}	1.55 ± 0.10^{Bj}

See footnote Table (1)

CONCLUSION

All parameters were appeared different changes in the proteolytic activities for crude-plant-enzyme extracts. It was remarkable that, the trends or rates of changes in the proteolytic activities of crude-plant-enzyme extracts was differed with pH values, sodium chloride, calcium chloride, enzyme concentrations, storage conditions and temperatures.

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تأثير بعض العوامل علي نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين

أحمد محمد عبد الدايم ، إكرام حسن الباجوري ، فوزي محمد عباس ، محسن علي فيصل
قسم الألبان - كلية الزراعة - جامعة قناة السويس – الإسماعيلية ٤١٥٢٢ - مصر

تم دراسة تأثير كل من الأس الهيدروجيني، كلوريد الصوديوم، كلوريد الكالسيوم، تركيز الإنزيم ودرجات حرارة التخزين علي نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين. أظهرت زيادة قيم الأس الهيدروجيني، كلوريد الصوديوم ودرجات الحرارة إلي زيادة ثم انخفاض في نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام. أيضا أظهرت النتائج أن نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام قد انخفض معنويا سواء كان بالزيادة أو النقصان بالتوازي مع تركيزات كلوريد الكالسيوم أو درجات الحرارة علي التوالي. دلت النتائج علي أن نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام قد زاد معنويا مع زيادة تلك المستخلصات.