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 enzymes 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 Mavitura, 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 achieved 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 preproteinsase composed of 345 amino acids and secreted aszymogène (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.), Papyra (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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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.

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.
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Therefore the present results were in consistent with the earlier reported (Chaurasiya and Hebbare, 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 CaCl₂ concentrations on the proteolytic activities of crude-plant-enzyme extracts**

Table (1) shows the influence of CaCl₂ 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 CaCl₂ percentages. In addition the proteolytic activities of extracts ordered as a follows: bromelain > cichorium > papain at all CaCl₂ 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 CaCl₂ percentages on the proteolytic activities of crude-plant-enzyme extracts**

| CaCl₂ %   | Bromelain       | Cichorium       | Papain        |
|-----------|-----------------|-----------------|---------------|
| 0 (Control) | 31.39±0.37<sup>Aa</sup> | 24.69±0.48<sup>Ab</sup> | 21.96±0.30<sup>Ca</sup> |
| 0.01      | 27.70±0.55<sup>Ab</sup> | 23.52±0.37<sup>Bb</sup> | 20.95±0.24<sup>Cb</sup> |
| 0.02      | 23.12±0.24<sup>Bc</sup> | 22.40±0.55<sup>Cb</sup> | 19.91±0.30<sup>Bc</sup> |
| 0.03      | 21.27±0.18<sup>Bd</sup> | 21.11±0.37<sup>Cd</sup> | 18.70±0.18<sup>Be</sup> |
| 0.04      | 19.63±0.24<sup>Ce</sup> | 19.87±0.48<sup>Ce</sup> | 17.62±0.18<sup>Bc</sup> |
| 0.05      | 18.74±0.50<sup>Cf</sup> | 18.62±0.37<sup>Be</sup> | 16.33±0.18<sup>Bf</sup> |
| 0.06      | 17.86±0.30<sup>Ag</sup> | 17.38±0.37<sup>Bg</sup> | 15.05±0.24<sup>Bg</sup> |
| 0.07      | 16.53±0.37<sup>Ah</sup> | 16.13±0.24<sup>Bh</sup> | 13.92±0.18<sup>Bh</sup> |
| 0.08      | 15.29±0.24<sup>Ai</sup> | 15.01±0.18<sup>Ai</sup> | 12.68±0.25<sup>BJ</sup> |
| 0.09      | 13.48±0.24<sup>Aj</sup> | 12.48±0.18<sup>Bj</sup> | 11.35±0.18<sup>C</sup>  |
| 0.10      | 11.07±0.24<sup>Ak</sup> | 10.91±0.42<sup>Al</sup> | 9.99±0.32<sup>Al</sup>  |

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 crude-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.

![Graph showing proteolytic activity vs volume of crude extracts](image)

**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.

| Treatments | Proteolytic activity (µg tyrosine/1 ml crude-plant-enzyme extract)\(^a\) during the storage at 4°C |
|------------|-----------------------------------------------------------------------------------------------|
|            | Bromelain                                                                                     |
|            | Cichorium                                                                                     |
|            | Papain                                                                                        |
| Fresh (Control) | 32.52±0.55\(^{Ab}\) 26.33±0.57\(^{Bu}\) 23.40±0.37\(^{Ca}\)                                 |
| 7 Days     | 15.97±0.94\(^{Ab}\) 13.72±0.24\(^{Bu}\) 9.75±0.36\(^{Ch}\)                                   |
| 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)\(^a\) during the storage at -18°C |
|------------|---------------------------------------------------------------------------------------------------|
|            | Bromelain                                                                                     |
|            | Cichorium                                                                                     |
|            | Papain                                                                                        |
| Fresh (Control) | 32.52±0.55\(^{Ab}\) 26.33±0.57\(^{Bu}\) 23.40±0.37\(^{Ca}\)                                 |
| 7 Days     | 29.35±0.25\(^{Ab}\) 23.44±0.39\(^{Bu}\) 6.45±0.18\(^{Ch}\)                                   |
| 14 Days    | 27.66±0.18\(^{Ac}\) 22.24±0.37\(^{Bu}\) 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\(^{Ac}\) 20.47±0.32\(^{Be}\) 1.54±0.11\(^{Cc}\)                                   |

See footnote Table (1)
Previous study done by Chaurasiya and Hebbbar (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 for crude 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).

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

| Temperatures (°C) | Bromelain          | Cichorium          | Papain            |
|-------------------|--------------------|--------------------|-------------------|
| 25                | 32.08±0.30bAd      | 31.51±0.42bAd      | 28.06±0.36bBb     |
| 30                | 35.97±0.30Ac       | 35.49±0.07bAb      | 31.19±0.24bBa     |
| 35                | 37.90±0.25Ab       | 38.34±0.49aAb      | 26.86±0.36bAc     |
| 40                | 42.20±0.18bAe      | 31.31±0.59bBe      | 24.04±0.42bCd     |
| 45                | 36.09±0.42bAc      | 25.41±0.36bBd      | 22.48±0.37bCe     |
| 50                | 26.61±0.24bAc      | 23.72±0.43bBe      | 19.91±0.49bCf     |
| 55                | 21.5±0.25bBf       | 22.48±0.30bMc      | 18.50±0.37bGg     |
| 60                | 16.61±0.48bBf      | 18.86±0.37bNg      | 14.00±0.71bCh     |
| 70                | 11.84±0.18bBh      | 12.68±0.18bBh      | 7.58±0.12bCl      |
| 80                | 6.82±0.30aAI       | 6.78±0.25aAI       | 1.55±0.10aBj      |

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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تأثیر بعض العوامل على نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين

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تم دراسة تأثير كل من الأس الهيدروجيني، كلوريد الصوديوم، كلوريد الكالسيوم، تركيز الإيزيم ودرجات حرارة التخزين على نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين. أظهرت زيادة في الأس الهيدروجيني، كلوريد الصوديوم ودرجات الحرارة إلى زيادة ثم انخفاض في نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام. أيضاً أظهرت النتائج أن نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام قد انخفض معنويًا سواء كان بالإزدياد أو التقصص بالتنسق مع تركيزات كلوريد الكالسيوم ودرجات الحرارة على التوالي. دلت النتائج على أن نشاط التحلل البروتيني لمستخلصات البروميلين، الشيكوريا والبابين الخام قد زاد معنويًا مع زيادة تلك المستخلصات.