Isolation and Characterization of Proteases of *Carum copticum*

M. R. KHAN, M. AZAM AND S. A. NAWAZ
Department of Chemistry
Government College of Science, Allama Iqbal Town
Wahdat Road, Lahore, Pakistan

(Received 10th May, 2004, revised 13th May, 2006)

Summary: The present work was carried out to separate and characterize different proteases of *Carum copticum*. The enzymes were extracted with 0.1 M phosphate buffer of pH 7.0. Crude extract was subjected to fractionation by ammonium sulphate precipitation. Fraction-1 at 68 % and Fraction-2 at 80% concentration were obtained. The resulting fractions were subjected to gel filtration chromatography and subsequent characterization of isolated proteases. The results indicated that Fraction-1 contained three major proteases A, B, C, while Fraction-2 also contained three designated as D, E, and F. The overall picture showed that the proteases of *Carum copticum* were mixture of acidic, neutral and alkaline proteases. The fraction A contained a major component with pH optimum at 3.0 and minor component with pH optimum at 6.0. The fraction E on the other hand, contained acidic protease with pH optimum 3.0 as major component.

Introduction

Proteases are the enzymes, which belong to the class hydrolases and catalyze the degradation of proteins, peptones and polypeptides to simpler components. Purification and partial characterization of Q-Enzyme from potato by DEAE cellulose as well as polyethylene glycol and $(NH_4)_2SO_4$ precipitations has been reported [1]. An endopeptidase has been purified from sprouts of bamboo *(Pleioblastus hindsi* Nakai) to electrophoretic homogeneity by four purification steps [2]. A new protease was purified to mass spectroscopic homogeneity from the latex of *Araujia hortorum* Fourn fruits by ultracentrifugation and ion exchange chromatography The enzyme has a molecular mass of 24,031 and an isoelectric point higher than 9.3. The optimum pH range for casein hydrolysis was 8.0–9.5. The enzyme showed remarkable caseinolytic activity at high temperatures, although its thermal stability decayed rapidly [3]. Aspartic proteinases were purified from sunflower seed extracts by affinity chromatography on a pepstatin A-EAH sepharose column and by Mono Q column chromatography. The final preparation contained three purified fractions. These purified enzymes showed optimum pH for hemoglobinolytic activity at pH 3.0 and it was completely inhibited by pepstatin [4]. Latex of the medicinal plant *Ervatamia coronaria* was found to contain at least three cystein proteases with high proteolytic activity, called ervitamins. One of these proteases named ervitamin B has been purified to homogeneity using ion exchange chromatography and crystallization. The enzyme hydrolyzed denatured natural substrates such as casein and azoalbumin with a high specific activity. The temperature optimum was around 50-55^{0}C. The enzyme was basic with an isoelectric point of 9.3 and had no carbohydrate content [5].

An endopeptidase, balansain I, was isolated from unripe fruit extract of *Bromelia balansae Mez* (Bromeliaceae) by anion exchange chromatography. It had pI=5.45 and mol. wt=23192. It exhibited pH profile with a maximum activity around 9.0 and inhibited only by cysteine peptidase inhibitor. Enzymatic hydrolysis of milk and soy protein yield characteristic pattern at pH 9.0 [6]. Five aspartic proteinases have been purified and characterized from sterile pitcher fluid of several species of *Nepenthes* [7]. A proteinase was isolated from jack fruit (*Artocarpus integrifolis*), for using as dairy enzyme, by subjecting jack fruit phosphate buffer extract to ammonium sulfate fractionation followed by gel chromatography. Enzyme activity increased up to 55°C for 60 minute reaction time and still constant thereafter. The optimum pH was pH 7.5.Michaelis constant (K_m) values of 2.0 mg ml^{-1} and a maximum initial velocity (V_{max}) of of 0.75 mole mg^{-1} when casein used as a substrate. Cu^{+2}, K^{+2}, Fe^{+2} and Zn^{+2} strongly inhibited the enzyme [8]. Two proteases were purified and characterized from *Avena sativa*, involving in Rubisco proteolytic cascade. They exhibit caspase specifity and display amino acid sequence homologous to plant subtilisin like Ser proteases [9]. An acidic protease from the cotyledons of 4 day old germinating Indian bean (*Dolichos*

lablab L. *var lignosus*) seedlings was purified by a five steps procedure. This protease involve in degradation of proteins of germinating Indian bean. The mol. mass of this acidic protease was found 32 KDa [10]. A renin like milk-clotting protease from twigs of *Streblus asper* was purified by a factor of 65 times with 36% recovery using ethanol precipitation, ion exchange and size exclusion chromatography. It was an acid protease with an optimum pH of 5.5 and retained 96% of its residual activity between pH 5.0 and 6.0. It had an aspartic acid residue at the active site which declared it a milk clotting aspartic protease [11].

The work reported in this article was conducted to determine the protease activity of the seeds of *Carum copticum* to investigate the nature of its proteases in crude state. This was followed by their subsequent separation with ammonium sulphate precipitation and gel filtration chromatography. The characterization of isolated protease fractions by determining of their characteristics such as effect of temperature on reaction velocity, thermostability, effect of pH on enzyme activity and determination of pH stability was carried out and the comparison of their characteristics was made.

Results and Discussion

The effect of buffer pH on extraction of proteases is demonstrated in Fig. 1, while that of soaking time is shown in Fig. 2.

From Fig.1, it is evident that the maximum protease extraction occurs with 0.1 M phosphate buffer of pH 7.0. Fig.2 shows that the protease activity goes on increasing with the gradual increase in time of soaking up to 24 hours.

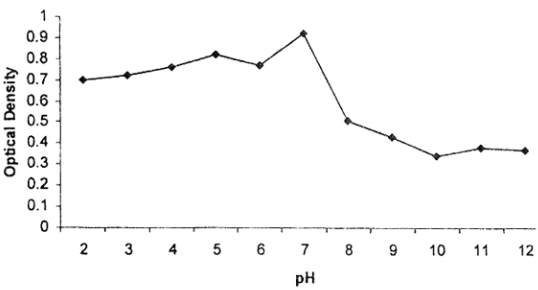

Fig.1: Effect of buffer pH on protease extraction.

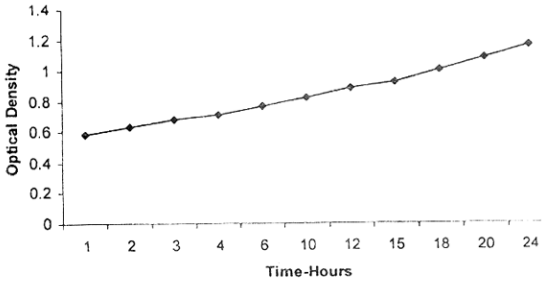

Fig.2: Effect of soaking time on protease extraction with buffer pH 7.0.

The variation of extracted protease activity in successive operations is shown in Fig. 3, which indicates that the amount of enzyme extracted decreases slowly after successive batch operations. . It is even highly significant after sixth batch operation. It means that *Carum copticum* seed powder is very rich in cell bound proteases.

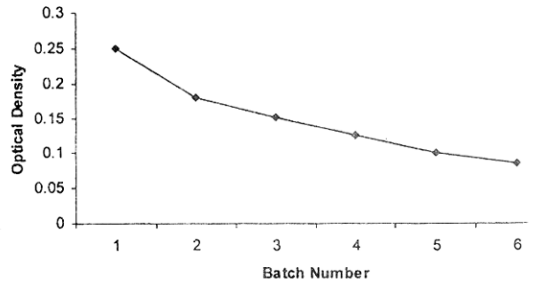

Fig. 3: Effect of grinding with sand on protease extraction.

The variation of the protease activity with the concentration of powdered seeds is shown in Fig. 4. The profile shows that protease activity increases with the amount of seed powder.

The pH profile of crude proteases of *Carum copticum* extracted with 0.1 M phosphate buffer of pH 7.0 is exhibited in Fig. 5.

The profile exhibits two pH optima, one at pH 3.0 and other at 5.0. Thus, the enzyme extracted is a mixture of acid proteases.

The pH profile of crude proteases of *Carum copticum* extracted with 0.1 M phosphate buffer of pH 5.0 is exhibited in Fig. 6.

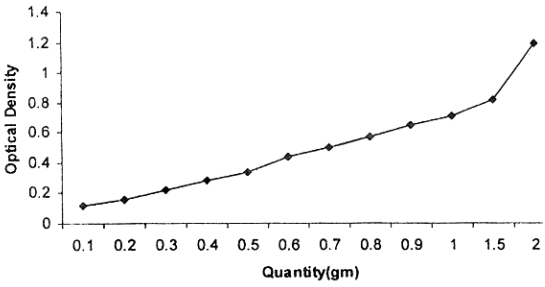

Fig.4: Variation of protease activity with concentration of powdered seed.

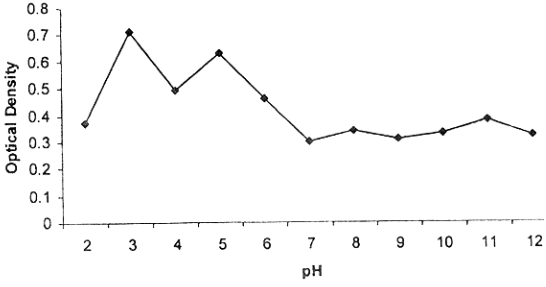

Fig. 5: pH profile of protease of crude *Carum copticum* extracted with buffer pH 7.0.

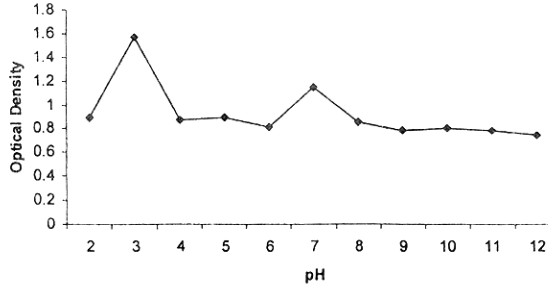

Fig.6: The pH profile of crude protease of f *Carum copticum* extracted with buffer pH 5.0.

The pH profile indicates two optima one at pH 3.0 and other at pH 7.0. This means that the enzyme is a mixture of acid and neutral proteases.

The elution diagram of the gel filtration chromatography of Fraction-1 is exhibited in Fig 7

The elution diagram of the gel filtration chromatography of Fraction 2 is exhibited in Fig. 8

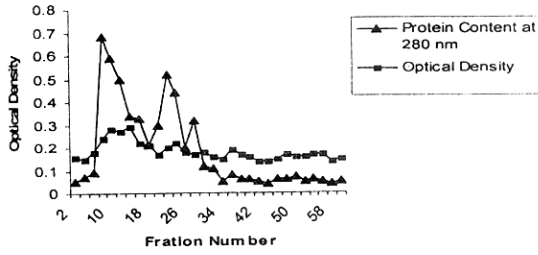

Fig 7: The gel filtration chromatogram of Fraction-1.Eluant:0.1 M phosphate buffer of pH 7.0.

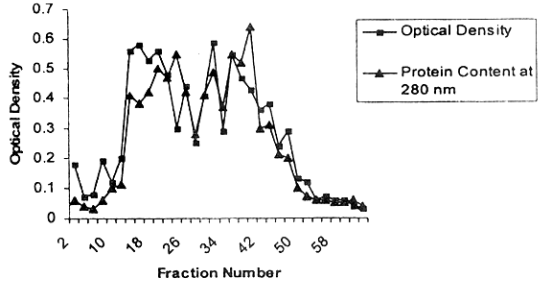

Fig.8: The gel filtration chromatogram of Fraction-2. Eluant:0.1 M phosphate buffer of pH 7.0.

Table-1: Progress of purification by ammonium sulphate fractionation and gel filtration

Material	Total Protein mg	Protease Activity-Unit Total	Specific	% Yield	
Crude Extract	1952	3320	1.70	100	
Fraction 1	150	748	5.0	22.5	
Fraction 2	46.0	230	5.0	6.5	29%
1-A	10.5	131	12.5	4.0	
1-B	6.0	7.5	12.7	2.3	
1-C	5.6	71	13.0	2.1	8.4
2-D	6.5	84.0	12.4	2.5	18.4%
2-E	6.1	76.0	12.5	2.3	
2-F	6.0	75	12.5	2.2	10.0

Table-1 indicates that only two fractions designated as Fraction-1 and Fraction-2 respectively were obtained at 68% and 80% ammonium sulphate saturation. The specific activity of both fractions was quite high as compared to that of the crude extract as many unwanted protein were eliminated in this step. Much of the activity was left in the supernatant even after 80% saturation and subsequent centrifugation. The data indicate that ammonium sulphate fractionation is quite helpful in separation of proteases of *Carum copticum*.

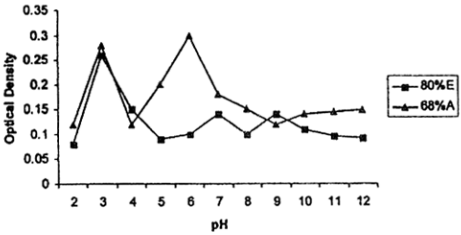

Fig.9: Effect of pH on Enzyme Activity.

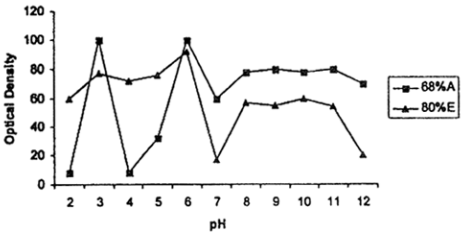

Fig.10: The pH stability of proteases of *Carum copticum.*

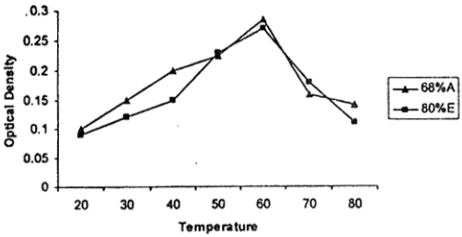

Fig.11: Effect of Temperature on reaction velocity.

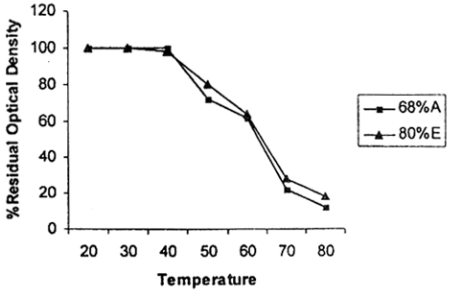

Fig.12: Thermostability of proteases of *Carum copticum*

Here the characterization of isolated fractions only A and E could be studied due to relatively high protease activity (Fig 9-12).

The pH optimum of isolated fractions A and E (Fig. 9) differ significantly. There are two pH optima in the profile of the Fraction A at pH 3.0 and 6.0 respectively means that it contains more than one component. The peak height of the protease corresponding to pH 3.0 is nearly same as of protease corresponding to pH 6.0 indicating that former is an acidic protease, while latter is a neutral protease and both seem to be major components of Fraction A. The Fraction E, on the other hand, exhibits one optimum and that is at pH 3.0 (Fig. 9). Other peaks including a small peak corresponding to pH 7.0 are negligible. This means that Fraction E has one major component and that is an acidic protease. This corresponds to the major component of Fraction A with pH optimum 3.0, If these are the same as being indicated by the pH profiles, this means, this component is dominantly present in Fraction E and this seems to be better purified as compared to Fraction A which also contains the neutral protease component as a major component.

The Fig. 11 shows that both fractions are stable in the range of 8.0 to 11.0 at the alkaline side.

The results of Fig. 11 show that the reaction velocity of both fractions increases with increase in temperature till 60^0 C after which protease activity decreases due to enzyme denaturation. The temperature optimum of both is $60\ ^0$C.

It is evident (Fig. 12) that isolated fractions A and E are stable up to 40 ^{0}C. It seems that both proteases start denaturing quickly near 50 ^{0}C.

The pH profile of the fraction A and E are comparable with those of Ali *et al* [12], who reported that the protease of crude *Carum copticum* powder was a mixture of acid and alkaline proteases (pH optima 3 & 7). Although, the protease with optima at or near pH 7 is classified as neutral protease, those workers considered appropriate to use the term alkaline, as the same could be a weighted average of neutral and alkaline proteases. Here the specific and distinguished presence of one acidic and other neutral protease has been proved.

The project can be extended further to study the advance characteristics of the isolated fractions such as amino acid composition and their sequence, substrate specificity, molecular weight determination, electrophoretic patterns, etc., after further purification

by ion-exchange chromatography, isoelectric focusing, etc.

Experimental

Assay of Protease Activity

The protease activity was assayed by the method of McDonald and Chen [13]. In this method, an adequate amount of the test sample was incubated with buffered substrate. The soluble products formed as a result of proteases action were lower proteins, peptides and amino acids. Undigested protein was precipitated with an adequate volume of 5% TCA. The contents were allowed to settle down and then filtered. The protein hydrolyzed was measured by developing a blue color with Folin-Ciocalteau phenol reagent and the optical density of the color was read at 660nm in a colorimeter. Protein content of each fraction was also determined by method of Anson [14].

Extraction of Protease with Buffer of Different pH

0.1 M phosphate buffers of different pH from 2-12 were tested to achieve the maximum enzyme extraction. 100 mg powdered sample was taken in each of eleven test tubes was added 10ml buffer at room temperature. The contents of the tubes were shaken and filtered. The activity of each filtrate was assayed taking 1 ml of sample from each tube. Activity was plotted as a function of pH.

Study of Effect of Cell Breaking on Protease Extraction

100 mg of sample was subjected to grinding in pestle mortar with 10 ml of 0.1 M phosphate buffer of pH 7.0 and silica sand. The filtrate was tested for protease activity taking 1 ml sample. To the residue was added again 10 ml buffer, the mixture was ground again and filtered. The activity of the filtrate was assayed as above. In this way, six batch operations were carried out. Batch vs. activity profile was constructed.

Study of Effect of Soaking Time on Protease Extraction

To check the effect of soaking time on proteases extraction, 100 mg *Carum copticum* seed powder was taken in eleven test tubes and to each was added 10 ml 0.1 M phosphate buffer of pH 7.0 and kept at room temperature. The contents of the tubes were occasionally shaken and filtered successively after one hour, two hour, three hour, etc, up to 24 hours to vary the soaking time. The contents of each tube were filtered and each filtrate protease assayed taking 1 ml of the sample. The activity per ml was plotted as function of time.

Study of Effect of Quantity of Powder Sample on Protease Activity

To determine the effect of quantity of powder sample on protease activity, different quantities of powdered samples ranging from 0.1 to 2.0 gm were taken in test tubes. To each was added 4ml substrate and protease activity was assayed as described above. The protease activity was plotted against quantity of the sample.

Fractional Precipitation with Ammonium Sulphate

50 gm of powdered sample was transferred into a conical flask and to it was added 200 ml 0.1M-phosphate buffer of pH 7.0 and mixture was stirred occasionally. It was kept for 24 hours at room temperature and then filtered. To the filtrate was added, a calculated amount of solid ammonium sulphate to constitute after dissolution 10% ammonium sulphate concentration. As there was no precipitation, it was further added to constitute 20% and process was repeated up to 68% concentration. The contents of the flask were kept in refrigerator at 4°C for 24 hours and material was centrifuged to separate the precipitates as Fraction-1. To the supernatant was added more ammonium sulphate to raise the concentration up to 80%. The precipitate was separated as above to constitute Fraction-2. Supernatant was preserved for enzyme assay as it contained some residual protease activity. The protease activity of both the fractions was assayed and also their protein content was determined.

Gel Filtration Chromatography

The fractions obtained as a result of fractional precipitation were subjected to gel filtration chromatography. A column of size 2.6x30 cm was evenly packed to obtain maximum efficiency. A plug of cotton was placed on the bore of stopcock to support the gel. To equilibrate the gel, 12gram of Sephadex G-75 (Pharmacia Fine Chemicals Ltd) was soaked in excess quantity of 0.1M-phosphate buffer of pH 7.0 .The mixture was kept in a refrigerator for 24 hours as to swell and equilibrate the Sephadex with buffer. Degassing of the gel was carried with the help of vacuum pump. Homogeneous packing was achieved,

a little buffer was added to the top of the column and the column was closed and left overnight to equilibrate with buffer and stabilize.

The sample of Fraction-1 was dissolved in 0.1 M phosphate buffer of pH 7.0.and applied to the top of the column using a syringe to transfer the sample on the gel. A used and subsequently cleaned drip bottle equipped with drop controller was used as reservoir of 0.1 M phosphate buffer of pH 7.0. The flow rate was equal to number of drops oozing out of the column stopcock per minute. 5 ml fractions were collected manually and every fraction was checked for assay of protease activity and protein content.

Study of Effect of Temperature on Reaction Velocity

The variation of reaction velocity with temperature was studied in the range of 20 to 80^{0}C by incubating 1ml sample with 4ml of casein substrate in a test tube for one hour. The assay was carried out as usual .The change in optical density was plotted as a function of temperature and the optimum temperature for protease activity was determined.

Determination of Thermostability

To determine thermostability, 1 ml of enzyme sample was incubated at different temperatures ranging from 20 to 80^{0}C in a thermostat for 15 minutes with occasional shaking. The residual activity samples, was assayed at 30^{0}C using casein as a substrate. The percentage of residual activity was plotted as a function of temperature and thermo-stability was judged from the curve.

Effect of pH on Enzyme Activity

The effect of pH on enzyme activity towards casein was studied within the pH range 2.0 to 12.0 using 0.1 M phosphate buffer of pH 7.0. The activity could not be tested with casein within the whole range as this precipitates down in pH range 4.0-6.0. The optical density was plotted as a function of pH and pH optimum for the protease activity was noted.

Determination of pH stability

10 ml of enzyme sample was taken in each of eleven test tubes and pH of the tubes was adjusted in the range of 2.0 to 12.0. The contents of tubes were allowed to stand for about 4 hours and then pH was pulled back to 7.0. One ml from each sample was incubated with casein substrate of pH 7.0 at 30^{0}C for 1 hour and protease activity was determined as above. The percentage residual protease activity was plotted against pH.

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