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Continuous Proteolysis of Casein by the Cell Bound Protease of *Carum copticum*

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In this paper, a continuous proteolytic system to prepare protein hydrolysis products for different biological applications is developed. Continuous proteolysis of casein substrate prepared in phosphate buffer (pH 7) by the seeds of *Carum copticum* packed in a column was attempted. Assuming that the seeds contained a mixture of soluble and cell-bound proteases, the soluble protease was eluted by passing buffer (pH 7) through the packed seeds. The buffered substrate was subsequently run into the column to constitute the system of continuous proteolysis. The products of proteolysis collected in the form of 5 mL fractions were assayed by Lowry's method. The system operated successfully for proteolysis on continuous basis. The extent of proteolysis was significantly high, which went on increasing with the passage of time. The activity of the system did not vanish within the time span of the experiment carried here. The data indicated that the life of cell bound protease of *Carum copticum* is very long.

Key Words: Protein, Continuous proteolysis, Casein, *Carum copticum*.

INTRODUCTION

Carum copticum (Ajwain) carries a great importance in the traditional as well as modern medicine and thus finds extensive applications as treatment of a large number of gastrointestinal diseases[1] such as gastric irritation, ulcers, lack of appetite, *etc.* due to presence of different proteases in its seeds. Thus, the proteases are under investigation in different contexts. The examples are immobilization of the proteases *Eurphorbia royleana*[2], antibacterial activity of some medicinal plants and seeds[3], biochemical and antibacterial studies of *Carum copticum*[4], isolation and characterization of proteases of *Carum copticum*[5], antihypertensive, antispasmodic activities of the *Carum copticum*[6]. Continuous proteolysis systems are also one of different aspects under study.

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Different techniques are under intensive research to set up systems for continuous hydrolysis by the enzymes. One of such techniques is the immobilization of the enzymes by binding them with some matrices filled in chromatographic columns and subsequently passing through them the buffered substrates and collection of hydrolyzed products eluting out of the column. All that is being done is on the assumption that the enzymes immobilized in this way are equipped with enhanced life and increased stability[7-9].

Considerable work has also been carried in Pakistan to hydrolyze casein by proteases immobilized on Amberlite-50[10] and on DEAE A-50[2] and promising results have been reported.

The work being reported here is the extension of the work but with the change that instead of using artificially immobilized enzyme matrices, the naturally occurring proteases bound to plant seed cells reported to exhibit high protease content packed in chromatographic column were used to carry out enzyme hydrolysis on continuous basis. The products of proteolysis may seek commercial applications to manufacture amino acid drips for nitrogen deficient patients and for constructing media for microbial growth for clinical tests or for production of compounds of technical interest in industry and so on.

EXPERIMENTAL

Fine quality sample of *Carum copticum* commonly known as Ajwain in South Asia are easily available in the local market. It was particularly examined for the elimination of any impurity contained in it.

High quality casein supplied by Merck was used. A column of size 18 × 1 inch (dia) was used for setting up the cell-bound protease bed in it.

Assay of protease activity: The protease activity assay was carried out by the Method of McDonald and Chen[11]. The same method was applied to determine the products of proteolysis eluted from the column. In this method, an adequate volume of the test sample usually 1 mL was incubated with buffered substrate. The soluble products formed as a result of protease action were lower proteins, peptides and amino acids in the form of a mixture. Undigested proteins were precipitated with an adequate volume of 5 mL trichloroactetic acid (5 %). The contents were allowed to settle down and then filtered. The protein hydrolyzed was measured by developing a blue colour with Folin-Ciocalteu phenol reagent and reading the optical density of the colour at 660 nm in a (Cecil 7200) spectrophotometer[3].

The unit of protease activity was defined as the amount of the enzyme that caused an increase in optical density of 0.1 under the assay conditions defined.

Protease activity of the soluble enzyme: Before setting a system for continuous proteolysis, the soluble part of the enzyme present in the seeds was removed and assayed. 35 g of *Carum copticum* seeds were stirred with 350 mL of buffer pH 7. The mixture was allowed to settle for three days for maximum dissolution of soluble enzyme in the buffer and subsequent removal. The mixture was then filtered. The filtrate was assayed for protease activity using 1 mL sample. 1 mL of the filtrate was incubated with 4 mL of buffered casein (1 %) pH 7 for 1 h at 30 ºC. The undigested soluble protein was precipitated with 5 mL of trichloroactetic acid (5 %) that also stopped the reaction. The mixture was allowed to stand for 0.5 h and then filtered through Whatmann filter paper no. 41. 1 mL of the filtrate was transferred to 5 mL of alkaline mixture prepared by mixing 100 mL of sodium carbonate (2 %), 1 mL sodium potassium tartrate (2.7 %) and 1 mL copper sulfate (1 %). Then 2 mL of NaOH (1 N) was added to make the mixture alkaline. After at least 10 min, 0.5 mL Folin-Ciocaulteu phenol reagent was added and then contents mixed. The blue colour produced was read at 660 nm in a (Cecil 7200) spectrophotometer after 0.5 h. Blank was prepared by the same procedure except 1 mL sample was substituted by 1 mL of buffer (pH 7).

Protease activity of the powdered *Carum copticum*: The dry seeds of *Carum copticum* were powdered using an electric grinder. The protease activity of the seed powder was determined to have an idea about the total units of protease present in the seed sample. 100 mg of seed powder along with 1 mL buffer (pH 7) constituted the sample and heat denatured 100 mg powder made the blank. The remaining procedure was the same as above.

Packing of the column: Suspension of the seeds was prepared in buffer pH 7 as above and transferred to the column. The column was allowed to stand for 2 h for the proper settling of the cells of *Carum copticum*. The tap was opened to allow the buffer to flow. The flow of the drops was adjusted so that each drop fell after 30 to 40 s. It took 1.5 h to collect a 5 mL fraction. The soluble enzyme was collected eluting with buffer as 5 mL fractions. The elution was carried till the buffer coming out of the column had no protease activity. This also guaranteed that the soluble enzyme had been completely removed. To keep the flow continuous, 20 mL buffer was transferred taking care that the seed bed was not disturbed. It took about one week to remove the soluble enzyme completely. Ten fractions were collected and their protease activity was assayed. The optical density was plotted against fraction number to construct the elution diagram.

Continuous proteolysis of casein: The process of continuous proteolysis of casein was started after the soluble enzyme had washed out of the column. For this purpose, 1 % casein substrate was run through the column and 5 mL of fractions of the enzyme-affected substrate were collected as

above. Thus, casein had reasonable time to be in contact with the cell-bound protease of *Carum copticum*. Every fraction was analyzed for the products of proteolysis. For analysis, 2.5 mL of every alternate fraction was treated with 2.5 mL of trichloroacetic acid (5 %) to precipitate unhydrolyzed protein. The precipitate was filtered off and 1 mL of the filtrate was used to develop colour with Folin and Ciocalteu phenol reagent as was done in assay of protease activity. The colour was read at 660 nm in the spectrophotmeter. The process was continued for 40 d and for first 24 days 2.5 mL of fractions were collected daily. The column was run for 7 h a day and for 6 d a week. After 24 d, the running was stopped for 1 week to seek whether the protease under goes a change without flow of the substrate. This means that determination of extent of proteolysis was resumed on 32nd day.

RESULTS AND DISCUSSION

The protease activity of the bulked soluble enzyme was 3.2 units/mL. As the total volume of buffer added was 350 mL, the total number of activity units present in 350 mL was 1120. In other words, the units of activity of soluble protease per gram were 32. The number of protease units per 100 mg, were 5.2. Thus, unit/g and unit/35 g came out to be 52 and 1820, respectively. This means that the seed powder contained protease more than that solubilized by the buffer pH 7 from the whole seeds and the balance was naturally immobilized.

The change in protease activity per mL in terms of OD of different fractions analyzed with the number of the fractions collected is exhibited in Fig. 1.

The profile indicates that the activity of the fractions collected remains constant in the beginning. After that it goes on decreasing with the increase in fraction number. In other words, it decreases with the increase in the elution volume or increase in the time for which the column is run. After collection of 10th fraction, the activity became zero indicating that the soluble enzyme was completely washed after elution of 50 mL buffer.

The progress of proteolysis of casein by the cell bound protease of *Carum copticum* is demonstrated in Fig. 2 for the first 24 d. The results indicate that the rate of hydrolysis of casein by the cell bound protease went on increasing with the increase in fraction number or elution volume if interpreted in terms of peaks encountered in the pattern. The rate of hydrolysis thus increased with the passage of time.

The progress of proteolysis of casein after seven days stoppage is shown in Fig. 3. The results indicate that immobilized enzyme is not denatured even after 7 d stoppage. The profile shows that the activity of the enzyme in the column exists even after 40 d.

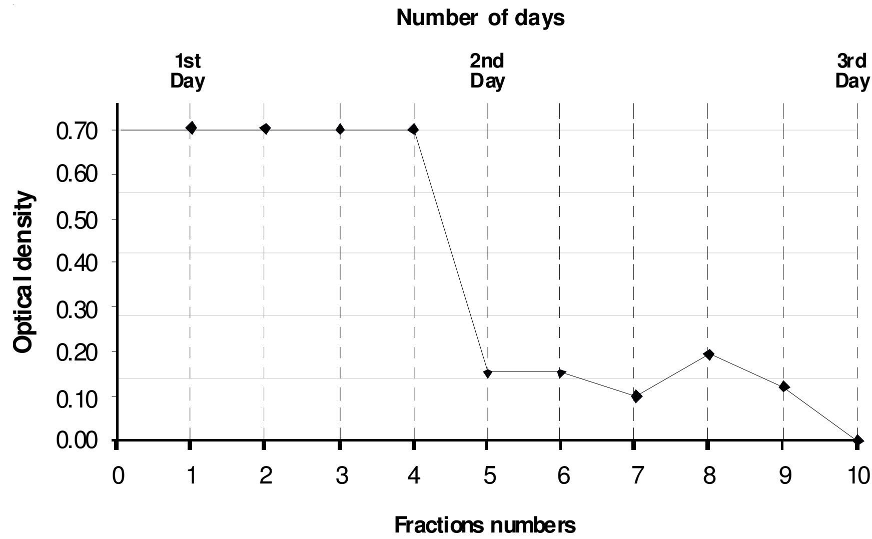

Fig. 1. Change in the optical density of the soluble nitrogenous substances showing to seeds of *Carum copticum* during elution with buffer pH 7 (days 1-3)

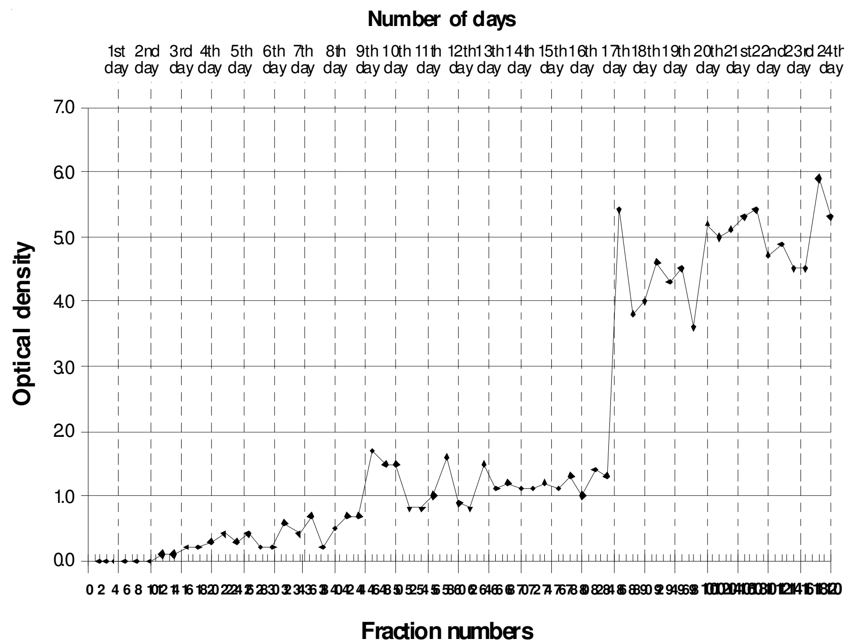

Fig. 2. Elution diagram showing the extent of proteolysis immobilized enzyme at pH 7 after the application of the case in substrate (days 1-24)

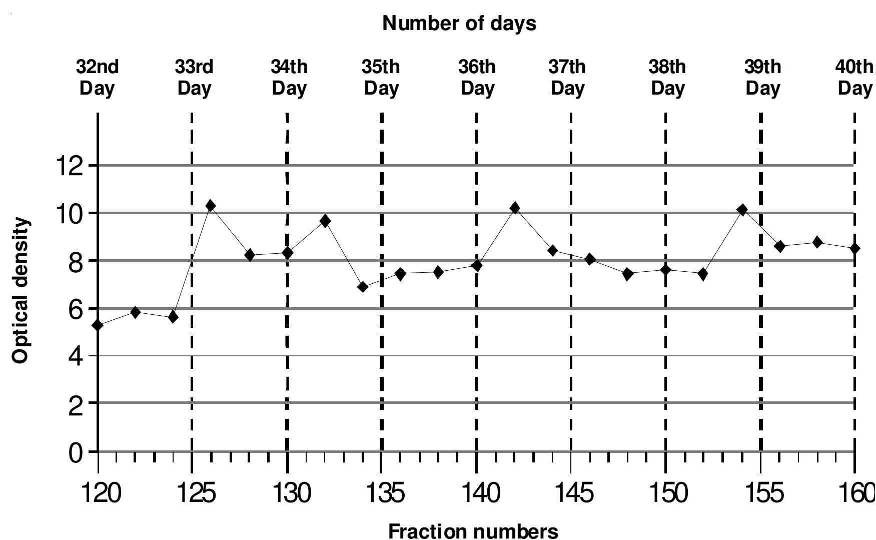

Fig. 3. Elution diagram showing the extent of proteolysis by the immobilized enzyme after keeping the column closed for 1 week continuous elution (days 32-40)

The results indicate that the seed powder of *Carum copticum* contains soluble protease more than that solubilzed by the buffer pH 7 from the whole seeds. The difference of 700 units per 35 g may not be due to cell-bound protease only as, while estimating the soluble protease, the whole seeds were treated with buffer pH 7 and thus, some soluble protease might have remained inside the cells.

The interesting feature of continuous proteolysis system developed here is that the extent of hydrolysis of casein went on increasing with the passage of time for a prolonged period. The increase in activity with increase in running time may be interpreted in terms of loosening of the cells of *Carum copticum* seeds due to water absorption that exposed more cell-bound protease with the passage of time.

The largest extent of proteolysis is indicated by the fractions 108, 120 collected in the last week of the work-in-progress. The column should have been run as long as until no activity encountered.

During the course of proteolysis for more than a month, no putrefaction was encountered because to presence of some antibacterial compounds[3] in seeds of *Carum copticum*.

To conclude, the life span of the cell-bound protease of *Carum copticum* seems infinite. The life span of cell bound protease of *Carum copticum* seeds placed in a column is greater than that of soluble proteases bound to other materials such as Amberlite-50 and DEAE A-50[2,10].

This is an important aspect to work upon, but this claim is not absolute. It is suggested that the work may be repeated and analysis may be carried for at least 6 months to 1 year.

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