Available online at www.joac.info

ISSN: 2278-1862

Journal of Applicable Chemistry

2014, 3 (4): 1665-1669

(International Peer Reviewed Journal)

Continuous Proteolysis of Casein by the Cell Bound Protease of *Nigella sativa*

M.R.Khan[1*] and H.Bokhari[2]

1. Center of policy and environment, Lahore School of Economics, 19-km Burki Road, Lahore, **PAKISTAN**
2. Department of Chemistry, Government College University, Lahore, **PAKISTAN**

Email: drrafiq@lahoreschool.edu.pk, khanmr1939@yahoo.com, a1hamim@yahoo.com

Accepted on 18th July 2014

ABSTRACT

In this paper, a continuous proteolytic system for preparing protein hydrolysis products for different biological applications is developed. Continuous proteolysis of casein substrate prepared in phosphate buffer (pH 7) by the seeds of Nigella sativa 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 for 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 data indicated that the life of cell bound protease of Nigella sativa was 39 days.

Keywords: Protein, Continuous proteolysis, Casein, *Nigella sativa*.

INTRODUCTION

Nigella sativa (kalongi) carries a great importance in the traditional as well as modern medicine and thus finds extensive applications as treatment of a large number of diseases related to respiratory health, stomach, kidney, circulatory and immune system support, asthma, bronchititis etc, due to presence of different proteases in its seeds[1,2].. The seeds are used to improve digestion and produce warmth, especially in cold climates. They are sometimes scattered in the folds of woolen fabrics to preserve them from insects [3-8].

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 hydrolysis 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 [9,10].
Considerable work has also been carried in Pakistan to hydrolyze casein by proteases immobilized on activated charcoal [11] and on DEAE-cellulose [12] and promising results have been reported. This work has been extended 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 first

experiment was conducted on naturally occurring cell bound proteases of Carum copticum and a continuous proteolytic system with high proteolyic activity on casein substrate was successfully developed [13]. Continuing the same approach work being reported here was carried to develop a successive continuous proteolysis of casein by the seeds of Nigella sativa in a chromatographic column with the hope that 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.

MATERIALS AND METHODS

Fine quality sample of *Nigella sativa* commonly known as kalongi 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 x 1 inch (dia) was used for setting up the cell-bound protease bed in it.

Assay of protease activity: The protease activity assay was done by the Method of McDonald and Chen [14]. The same method was applied to determine the products of proteolysis eluted from the column. An adequate volume of the test sample usually 1mL 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 trichloroacetic acid (5%). The contents were allowed to settle down and then filtered. The protein hydrolyzed was measured by developing a blue color with Folin-Ciocalteu phenol reagent and reading the optical density of the color at 660 nm in a (Cecil 7200) spectrophotometer. 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 *Nigella sativa* 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 °C. The undigested soluble protein was precipitated with 5 mL of trichloroacetic acid (5 %) that also stopped the reaction. The mixture was allowed to stand for 0.5 h and then filtered through Whatman 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 color produced was read at 660 nm in a (Cecil 7200) spectrophotometer. 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 *Nigella sativa:* The dry seeds of *Nigella sativa 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 Nigella sativa. 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 0.5 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 three days 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 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 *Nigella sativa.* 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 color with Folin and Ciocalteu phenol reagent as was done in assay of protease activity. The color was read at 660 nm in Spectrophotometer. The process was continued for 24 d and 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 undergoes 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 2.2 units mL^{-1}. As the total volume of buffer added was 350 mL, the total number of activity units present in 350 mL was 770. In other words, the units of activity of soluble protease per gram were 22. The number of protease units per 100 mg, seeds was 3.5. Thus, unit g^{-1} and unit 35 g^{-1} came out to be 35 and 1225, 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.

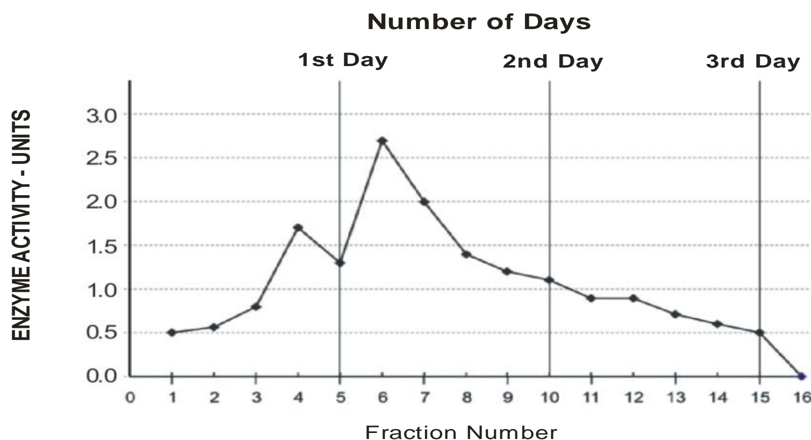

Fig. 1. Change in enzyme activity of the soluble protease of the seeds of *Nigella sativa* packed in the column during elution with 0.2 M citrate phosphate buffer pH 7.0 (Days1-3).

The profile indicates that the enzyme activity of soluble protease decreases exhibiting rise and fall with the addition of fresh 0.2M citrate phosphate buffer pH 7.0. It displays some increase in Fraction 6. After 16 fractions the optical density becomes minimum and finally stays there. Comparision of enzyme activity of soluble proteases of Nigella sativa with that of *Carum copticum* [13] shows that latter exhibits higher activity than Nigella sativa. This may be due to the reason that *Carum copticum* seeds are softer than of Nigella sativa which are quite compact. The progress of proteolysis of casein by the cell bound protease of Nigella sativa is demonstrated in fig.2.

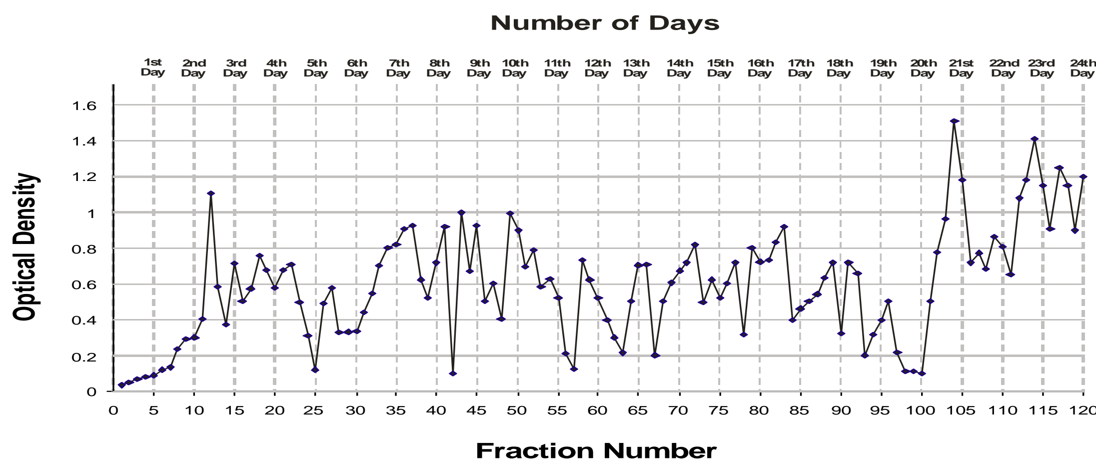

Fig.2: Elution diagram showing the extent of proteolysis by naturally immobilized enzyme after the application of casein substrate in 0.2M citrate phosphate buffer at pH 7.0 to *Nigella sativa* seeds column(Days 1-24).

The results indicate that there is continuous rise and fall of extent of proteolysis and different sharp peaks in different regions are obtained. Starting from fraction 1 with optical density 0, the minimum optical density of around 0.05 is encountered with fractions 25, 42, 52 and 100. The position of different peak enzyme fractions encountered in different regions of graph is as under. i). From Fraction 1 to Fraction 50 : Two peaks of fraction 12, fraction 43, fraction 45 and fraction 49 are notable in this portion. The optical density with fraction 12 is 1.1, fraction 43 is 1.0, fraction 45 is 0.9 and with fraction 49 is 1.0.
ii).From Fraction 51 to Fraction 100 : In this region again occasional rise and fall of peaks is noted. Here optical density falls from 1.0 to 0.9 with fraction 51. Fractions 52, 72 and 78 peaks with optical density 0.8 while fraction 83 peaks with optical density 0.9 and ends at 0.05 of Fraction 100. Many other peaks exhibiting significant proteolysis are also notable in the profile. iii) From Fraction 101 t0 Fraction 120 : The extent of proteolysis suddenly rises to such a height that supersedes all the predecessor peaks and the fraction 104 exhibits an optical density of 1.5; the highest came across throughout the profile. There is then successive decline of extent of proteolysis marked by the optical density with fraction 114 (OD 1.4), fraction 117 (OD 1.25) and fraction 120 (OD 1.2). The progress of proteolysis of casein after seven days stoppage is shown in fig. 3.

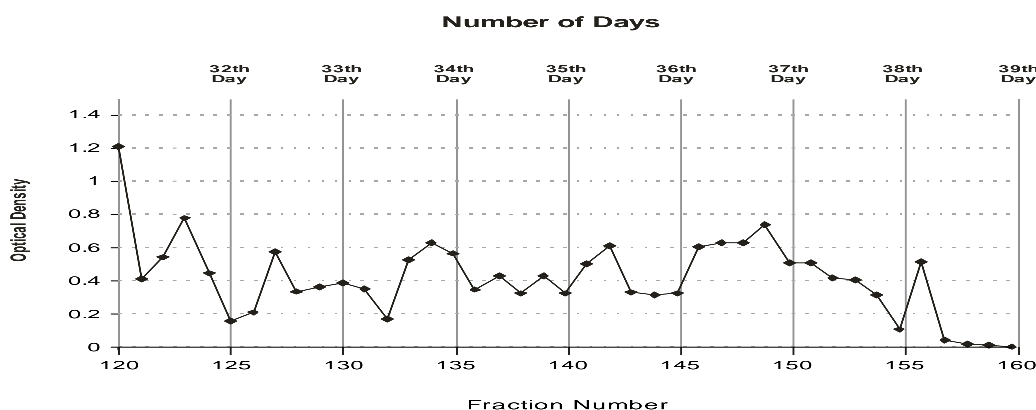

Fig.3: Elution diagram showing the extent of proteolysis by immobilized enzyme after keeping the *Nigella sativa* seeds column closed for one week continuous elution (Days 32-39).

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 at 39 d. The profile also indicates that the seed powder of *Nigella sativa* 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 *Nigella sativa* due to water absorption that exposed more cell-bound protease with the passage of time. During the course of proteolysis for more than a month, no putrefaction was encountered because of the presence of some antibacterial compounds [15] in seeds of *Nigella sativa.*

APPLICATIONS

The extent of proteolysis was significantly high, which went on increasing with the passage of time. The data indicated that the life of cell bound protease of Nigella sativa was 39 days. Thus the developed system, being based on harmless natural seeds of Nigella sativa, can be better applied for the proteolysis of some proteins for making soluble amino acid preparations such as drips for instantaneous supply of nitrogen to the patients needing nutrition for their survival.

CONCLUSIONS

To conclude, the life span of cell bound protease of *Nigella sativa* seeds placed in a column is greater than that of soluble proteases bound to other materials such as activated carbon [11] and DEAE cellulose [12]. This conclusion is quite in agreement with conclusion drawn in case of similarly reported system based on the seeds of Carum copticum packed in a chromatographic column [13].

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