

Heat Resistant Proteases Isolated from Medicinally Significant Spices: Partial Purification and Biochemical Characterization

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Abstract- Proteases or proteinases are essential constituents for all the existing live forms. They act as important industrial enzymes occupying about 60% of total enzyme market. In the present study proteases extracted from nine medicinally important spices; *Carum copticum*, *Syzygium aromaticum*, *Cuminum cyminum*, *Nigella sativa*, *Cinnamomum verum*, *Foeniculum vulgare*, *Zingiber officinale*, *Cinnamomum tamala* and *Curcuma longa*; used in our food and as household medicines on regular basis, have been investigated. Amongst these spices, the specific activity of the isolated protease enzyme was found to be significantly high in *Nigella sativa* (204 units/mg) and *Curcuma longa* (124 units/mg) and therefore, their extract was further partially purified and biochemically characterized. The crude extract of the two spices on being subjected to salt precipitation using $(\text{NH}_4)_2\text{SO}_4$ as neutral salt, yielded three fractions at 0-30%, 30-60% and 60-90% saturation level. The specific activity of protease enzyme was found to be highest in 0-30% fraction, obtained from both the sources. The values of specific activity and purification-fold of enzyme from *Nigella sativa* and *Curcuma longa* were found to be 409 U/mg, 590 U/mg and 2.0-, 4.8-, respectively. The enzyme showed maximum activity at pH and temperature conditions of 5.0 and 40°C, respectively, at 20 min of incubation time, in both the cases. The pH stability values of protease from *Nigella sativa* ranged from 5.0 to 9.0 whereas in *Curcuma longa* values were from 4.0 to 8.0. The enzyme from *Nigella sativa* and *Curcuma longa* exhibited resistance against heat treatment upto 60°C and 50°C, respectively, increasing the industrial feasibility. The present work hereby, indicates that *Nigella sativa* and *Curcuma longa* may serve as good source of thermostable protease; an enzyme of great relevance in various chemical and bio-industries along with pharma sector.

Keywords- Protease; Heat Resistant; *Nigella sativa*; *Curcuma longa*; *Zingiber officinale*; Therapeutics

I. INTRODUCTION

Proteases are biocatalyst that perform a multitude of chemical reaction and are commercially used in detergents, food, pharma, diagnostic and fine chemical industries [1, 2]. They are group of enzymes which catalyse the cleavage of peptide bonds, generally referred to as proteolytic enzymes or proteinases. Proteases are ubiquitous in nature widely distributed in plants, animals and microorganisms.

The significant role of proteases has made them industrially important. They account for 65% of total worldwide enzyme sale. Several proteases have been

isolated from latex, fruits and seeds. They have major applications in industrial process such as laundry, silk, pharmaceutical, food, degradation of gelatin on X-ray films [3] and bioremediation process. They are widely used in detergents, leather, waste management and silver recovery [4].

A lot of work has been reported on proteases of microbial origin holding industrial potential due to their biochemical diversity. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes [5]. It would be advantageous to utilize a fungal protease as fungal expression systems are capable of producing larger quantities of enzymes than bacterial expression system [6]. Proteases are produced by many species of fungi such as *Aspergillus* [7], *Mucor* [8], *Fusarium* [9], *Cephalosporium* [10] and *Rhizopus* [11]. Filamentous fungi, such as *Aspergillus* have been the organism of choice for large scale production of bulk industrial enzymes [12]. *Actinomycetes* being a protease producing bacteria have the potential for use industrial purpose, pharmaceutical and cytotoxic agent [13]. The sources of protease are enormous. *Bacillus sp.* was found to be predominant and rich source of alkaline proteases. Many of the fungi have also been reported to produce extracellular alkaline protease [14]. *B. subtilis* yield maximum protease production and be used efficiently for industrial purposes [15]. Among various proteases, bacterial proteases are more significant compared to with animal and fungal protease [16]. They are present in *Bacillus subtilis*, *B. amyloliquefaciens*, *Pseudomonas sp.*, *Lysobacter enzymogene*, *E. coli* etc. [17]. *B. subtilis* produce alkaline protease that can be used in textiles, leather and food industries [18]. Plants are also the rich source of protease enzyme [19] but not been worked upon much. In this study, spices such as *Carum copticum*, *Syzygium aromaticum*, *Cuminum cyminum*, *Nigella sativa*, *Cinnamon verum*, *Foeniculum vulgare*, *Zingiber officinale*, *Cinnamomum tamala* and *Curcuma longa* have been explored as source of protease enzyme.

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II. MATERIALS AND METHODS

Spices were procured from Green Earth products Pvt. Ltd., New Delhi. All chemicals were of reagent grade and obtained from standard commercial firms.

A. Screening of various spices for Protease enzyme

Screening of various spices viz; Ajwain (*Carum copticum*), Cloves (*Syzygium aromaticum*), Cumin (*Cuminum cyminum*), Black cumin (*Nigella sativa*), Dal chini (*Cinnamon verum*), Fennel (*Foeniculum vulgare*), Tej patta (*Cinnamomum tamala*), Ginger (*Zingiber officinale*) and Turmeric (*Curcuma longa*) was done for protease activity by using standard protocols.

B. Extraction of Protease enzyme

The pre-weighed samples were crushed in sodium acetate buffer (pH 5.0, 0.05 M), filtered through Whatman filter paper and centrifuged for 20 minutes at 10,000 rpm at 4°C. The pellet was discarded, supernatant collected and subjected to further purification.

C. Determination of specific activity

Specific activity was determined by using the following relationship:

Specific activity= Total enzyme units /Total protein (mg)

- Protein determination: Protein content of the enzyme extract was determined by Lowry method [20] using BSA as standard.
- Protease Assay: Protease activity was assayed using Folin-Ciocalteu method [21]. The reaction mixture containing casein (1%), enzyme, 0.05 M sodium acetate buffer (pH 5.0) was incubated for 30 min at 30°C followed by addition of 0.5 M NaOH and folins reagent. 1 International Unit of protease enzyme is defined as 1µg of tyrosine released per minute per ml under standard assay conditions. The amount of protease produced was measured with the help of a tyrosine standard graph [22]. The activity was reported as mean of three determinations.

D. Partial purification of protease enzyme

- Ammonium sulphate fractionation: The crude extract of the screened spices were subjected to precipitation using salting out process. Ammonium sulphate fractionation (0-30%) was done by adding salt in the extract according to the required saturation level, slowly while keeping on ice [23]. The ice-cold saturated solution of the protein was stirred continuously and kept at 0-4°C for at least one hour followed by centrifugation at 10,000 rpm for 10-15 min. Pellets were collected and dissolved in minimal amount of sodium acetate buffer (0.05 M, pH 5.0) and used as 0-30% fraction, the supernatant being subjected to next fractionation steps and further two fractions (30-60% and 60-90%) were obtained in the similar manner.

E. Biochemical characterization of Protease enzyme

- Time course: To determine the time course, the reaction mixture was incubated at different time intervals ranging from 10 min to 90 min and the protease activity was determined using standard conditions.
- Temperature Optima: The reaction mixture containing enzyme and substrate was incubated at 30°C for time period ranging between 10-90 minutes and the product released estimated by Folin's method.
- pH optima: To determine the pH optima, suitable buffers of different pH values ranging from 3.0 to 9.0 were used. The reaction mixture was incubated for optimum time period and activity determined using standard assay.
- pH stability: The enzyme alone was incubated for 2 hours with suitable buffers of pH ranging from 3.0 to 9.0 and further assayed using optimum assay conditions and residual protease activity, determined.
- Heat Resistance: The enzyme was incubated alone at different temperature ranging from 10°C to 90°C for 2h followed by standard assay.

III. RESULTS AND DISCUSSION

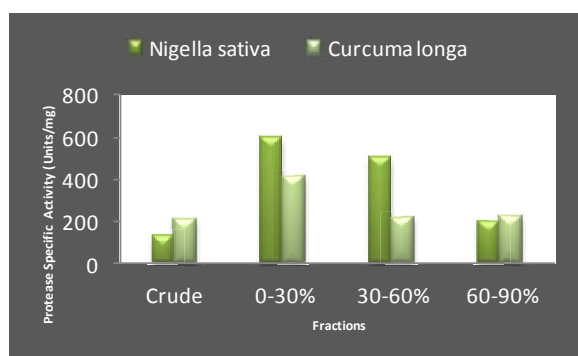
Protease plays an essential role in various pathological processes. Arthritis, tumor invasion and metastasis, infections and number of degenerative disease have been linked with proteolytic enzymes which have widespread applications in bioremediation processes [24]. This research work was undertaken to study the various biochemical aspects of high specific activity protease enzyme isolated from spices. Out of the nine spices studied, the extract of *Nigella sativa* (204 units/mg of protein) and *Curcuma longa* (124 units/mg) was found to contain protease with maximum specific as shown in Table I, and was further characterized. A better understanding of the functions of enzymes could be determined by purification of enzyme [25]. Figure 1 shows the specific activity of fractions of protease isolated from *Nigella sativa* and *Curcuma longa* at three different saturation levels of (NH₄)₂SO₄. It can be seen from Table II that 0-30% fraction has highest specific activity with 2- fold and 4.8- fold purification level in *N. sativa* (409 U/mg) and *C. longa* (590 U/mg), respectively. Therefore, 0-30% enzyme fraction from both the sources was used for further characterization studies. The time course of enzyme catalyzed reaction for protease isolated from *N. sativa* and *C. longa* (figure 2). It shows that the enzyme substrate reaction reached to maximum value in 20 min and 30 min for *N. sativa* and *C. longa*, respectively, later no change in activity was observed. Figure 3 illustrates temperature optima of protease isolated from *N. sativa* and *C. longa*, showing maximum activity at 40°C, in both the cases. Increase in temperature above optimum level affects important factors like protein denaturation, protein

ionization state and solubility of species in solution reducing enzyme activity [26]. Enzymes, being proteinaceous in nature, have properties that are quite pH sensitive. pH can affect activity by changing the charges on an amino acid residue which is functional in substrate binding or catalysis [27]. The pH optima of protease from *N. sativa* and *C. longa* were found to be (Figure 4) 5.0, in both the cases. Similar observation was reported in metalloprotease production in *P. fluorescens* [28] and *Adhatoda vasica* [29] with an optimum activity at pH 5.0. The proteases produced by isolates with enzymatic activity optima at pH 5.0 could be used to coagulate milk proteins for the dairy industry, as debittering agents in cheese and in peptide synthesis [30]. Figure 5 shows the pH stability curve of protease from *N. sativa* and *C. longa*. The protease from *N. sativa* was found to retain its activity in the pH range of 5.0 to 9.0 whereas respective values were from 4.0 to 8.0 in *C. longa*. The most significant result is the heat resistant property of the enzymes obtained from the two species, the temperature values being 60°C and 50°C for *Nigella sativa* and *Curcuma longa* (figure 6), respectively. Proteases from various sources with temperature stability upto 50°C have been reported by various workers [31-36]. Thus, it is imperative to state that spices may serve as good source of thermostable proteases which further have great importance in therapeutics and many other industries.

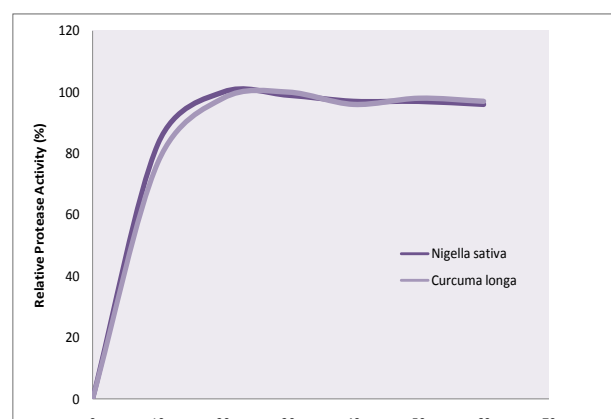
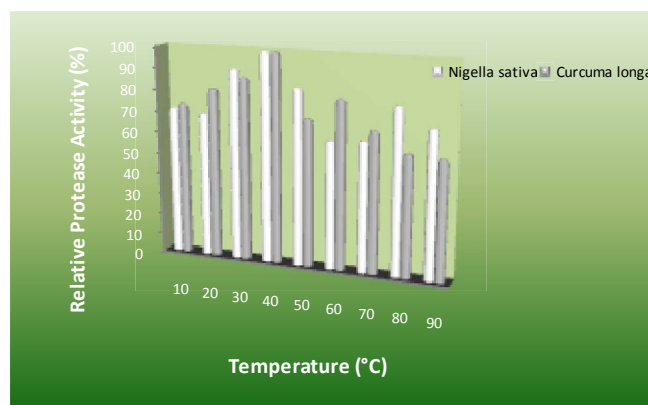
TABLE I. Specific activity of protease isolated from various spices.

S. No.	Spice	Specific Activity* (Units/mg)
1.	Ajwain (<i>Carum copticum</i>)	100.7
2.	Cumin (<i>Cuminum cyminum</i>)	85.3
3.	Black cumin (<i>Nigella sativa</i>)	204.0
4.	Dal chini (<i>Cinnamon</i>)	91.4
5.	Tej patta (<i>Cinnamomum tamala</i>)	71.1
6.	Cloves (<i>Syzygium aromaticum</i>)	67.8
7.	Turmeric (<i>Curcuma longa</i>)	124.0
8.	Fennel (<i>Foeniculum vulgare</i>)	98.2
9.	Ginger (<i>Zingiber officinale</i>)	105.6

*The values are average of triplicate readings

Figure 1. Specific activity of various fractions of protease enzyme isolated from *N. sativa* and *C. longa* after $(\text{NH}_4)_2\text{SO}_4$ fractionation.TABLE II. Specific activity and purification-fold of various fractions of protease isolated from *Nigella sativa* and *Curcuma longa* after ammonium sulphate fractionation.

S. No.	Fractions	Specific activity (units/mg)		Purification-fold	
		<i>Nigella sativa</i>	<i>Curcuma longa</i>	<i>Nigella sativa</i>	<i>Curcuma longa</i>
1.	Crude	204	124	1.0	1.0
2.	0-30% fraction ($(\text{NH}_4)_2\text{SO}_4$ saturation level)	409	590	2.0	4.8
3.	30-60% fraction ($(\text{NH}_4)_2\text{SO}_4$ saturation level)	211	502	1.0	4.1
4.	60-90% fraction ($(\text{NH}_4)_2\text{SO}_4$ saturation level)	217	195	1.1	1.6

Figure 2. Time course of enzyme catalyzed reaction for proteases isolated from *N. sativa* and *C. longa*. The reaction mixture was incubated at different time intervals ranging from 10 min to 90 min and the activity was determined using standard conditions.Figure 3. Temperature optima of proteases isolated from *N. sativa* and *C. longa*. The reaction mixture containing enzyme and substrate was incubated at different temperature ranging from 10°C to 90°C for time period of 20 minutes and further assayed under standard conditions.

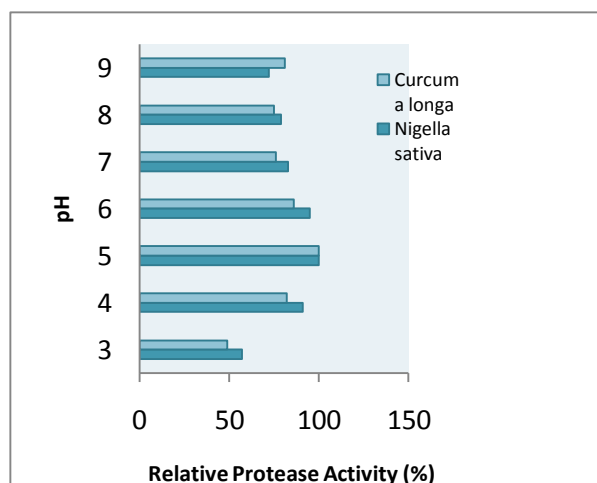


Figure 4. pH optima of proteases isolated from *N. sativa* and *C. longa* using suitable buffers of different pH values ranging from 3.0 to 9.0 at 40°C for 20 minutes.

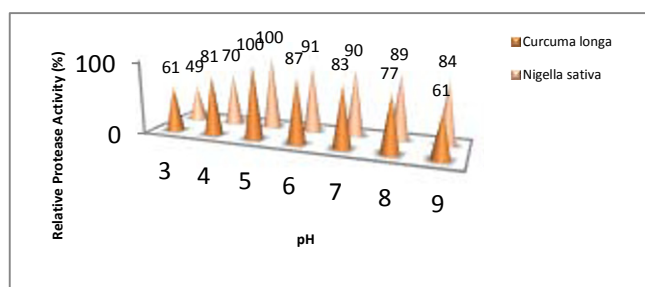


Figure 5. pH stability curve of proteases isolated from *N. sativa* and *C. longa* by incubating enzyme alone for 2 hours with buffers of pH values ranging from 3.0 to 9.0 and further assayed using optimum conditions.

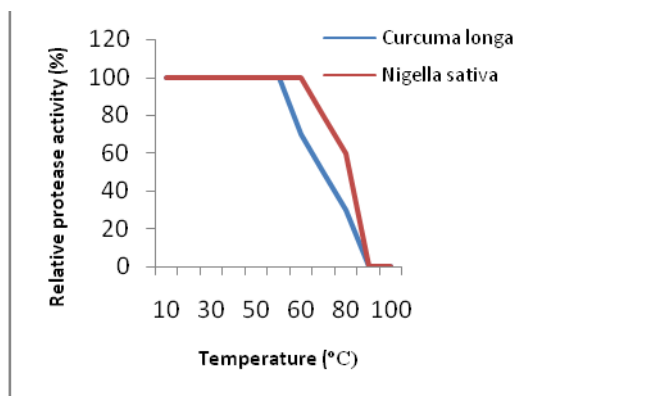


Figure 6. Profile showing heat resistance pattern of proteases isolated from *Nigella sativa* and *Curcuma longa* by incubating the enzyme alone at different temperature ranging from 10°C to 90°C for 2h followed by standard assay.

IV. CONCLUSION

In the present work out of the nine spices studied, *Nigella sativa* and *Curcuma longa* were found to be good source of protease enzyme. Very limited work has been done on enzymes from these spices which call for a more detailed research in future. Proteases obtained from spices may serve as potential candidate for targeting many diseases. From the results obtained, it may be concluded

that spices which are of great medicinal and culinary importance can be explored for heat resistant protease; an enzyme of immense value in therapeutics, chemical, leather, food and other bio-industries.

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