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Kinetic and Biochemical Studies of Thermostable Protease Partially Purified from *Cinnamomum zeylanicum*: A Medicinally Important Culinary Spice

Sawetaji[1*], Aparna[2] and Nidhee Chaudhary[3]

[1,2,3]Amity Institute of Biotechnology, Amity University Uttar Pradesh Sec-125 Noida-201313
E-mail: [1]shwetaji2@gmail.com, [2]aparnakumar.bsc.micro@gmail.com, [3]nchaudhary@amity.edu*

Abstract—*Proteases play key role in various industries like food, pharmaceutical, leather and tanning. They are the largest groups of industrial enzymes which helps in hydrolysis of specific peptide bonds which links the amino acids together forming a protein. Cinnamomum zeylanicum (Cinnamon) commonly named as Dalchini is widely used as sweet and flavored spice with significant medicinal value. Its leaves, fruits, roots, bark and flowers are widely used as household cure for cold, cough, acne, weight loss, digestive problems, controlling sugar level and arthritis pain. In the present study, the crude enzyme extracted from bark sticks of C. zeylanicum has been studied. The specific activity of the crude enzyme was found to be 45.30 U/mg. The protease enzyme isolated from C. zeylanicum was partially purified by salting out method into three different fractions; 0-30%, 30-60% and 60-90% according to ammonium sulfate saturation level; out of the three fractions, 0-30% (94.80 U/mg) fraction) with 2.08-fold of purification level showed the maximum specific activity was dialyzed and on further characterization showed maximum enzyme activity at temperature 30^0Cin 30 minutes of incubation time period at pH 5.0. The temperature and pH stability values for the enzyme were determined upto 40^0Cand 5.0-6.0 respectively. The values of kinetic parameters; Michaelis-Menten constant (K_m) and Maximum Velocity (V_{max}) were found to be 0.3 mg/ml and 34.4 units/min/ml, respectively. Out of various metal ions tested; $Co^{2+}>Na^{+}>Mn^{2+}>Zn^{2+}>Cu^{2+}>K^{+}$were found to inhibit the protease activity in the mentioned descending order. The results obtained suggest that Cinnamomum zeylanicum, a traditional valued spice and medicinal source in India, may also act as good source for thermostable protease enzyme which in addition to various industries have immense therapeuticimportance.*

Keywords: *Cinnamomum zeylanicum, Protease, Specific activity, Thermostable, Medicinal.*

1. INTRODUCTION

Proteases are the largest groups of industrial enzymes which helps in hydrolysis of specific peptide bonds which links the amino acids together forming a protein [1]. Proteases plays an important key role in maintenance of microorganisms as well as applied in food, pharmaceutical. Leather and tanning industries. Proteases plays an important physiological role in different biological processes [2]. Earlier plant proteases was extracted from different sources like papain from papaya, ficin from ficus latex, keratinases and bromelain from pineapple [3]. These plant proteases are widely used in cheese making, as antihelmentic, in milk clotting etc [4]. According to industrial purposes the plant source is generally substituted by microbial sources because of low cost of enzyme in their large scale production [5-6]. Therefore plants proteases availability is limited and nowadays researchers puts more attention to extract more plant proteases for commercial purposes because plant proteases are more active at wide range of pH and temperature[7]. Various sources have been found to contain substantial amount of proteases viz; Ayurveda plants, cereals, spices and dry fruits etc [8].

Cinnamomum zeylanicum (Cinnamon) is widely used as sweet and flavored spice with having its own medicinal value. *C. zeylanicum* is commonly named as Dalchini. Cinnamon is known as one of the most useful spice which was earlier be usedby the prehistoric Egypt communities as a remedy in treating various infections [9]. Cinnamon is basically known for its sweet and flavored smell coming from cooking foods but it's too have its numerous medicinal value in nature. In India plant is found mostly in Western Ghats and hilly areas belongs to Lauraceae family [10]. Cinnamon as a spice is presently available in market in two different forms that are – in powdery and sticks form. This spice is scrapped from the innermost bark of the cinnamon tree by stripped off the bark and get exposed in front of sunlight to dry. Cinnamon isknown for its multi ingrediental properties like asflavouring agent, antioxidant source, anti-spasmodic, curing digestive related problems, high protein content and as a stimulantin treatment of diabetes [11]. According to biochemical study, Cinnamon shows good quantity of protease enzyme in it with having health benefits which includes cold, cough, acne, weight loss, digestive problems, controlling sugar level, anti-inflammatory,

anti-cancerous, anti-microbial and in curing arthritis pain [12]. Keeping these benefits of this plant in account the work has been undertaken to extract and estimate the potential for protease enzyme.

2. MATERIALS AND METHODS

Cinnamomum zeylanicum was purchased from the certified shop. All chemicals were of reagent grade and obtained from standard commercial firms.

2.1 Extraction of protease enzyme

The pre-weighed *C.zeylanicum* bark sticks was crushed in sodium acetate buffer pH 5.0, and further centrifuged at 10,000 rpm for 15 minutes at 4^0C. Thefiltrate was treated as crude extract.

2.2 Protein determination

The protein was estimated spectrophotometrically by Lowry method (1951) using Bovine serum albumin (BSA) as standard [13].

2.3 Protease Assay

Protease activity was assayed using Folin-Ciocalteau reagent. The 6ml reaction mixture contained casein (1%), enzyme, 0.05 M sodium acetate buffer (pH 5.0) incubated for 30 min at 30^0C. After that 0.1N NaOH and folins reagent was added. One International Unit of enzyme is defined as 1µg of tyrosine released per minute per ml under standard assay conditions. The activity was reported as mean of three determinations.Specific activity was determined by using thefollowing relationship:

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

2.4 Partial purification of protease isolated from *C.zeylanicum* by ammonium sulphate precipitation

The crude extract of *C.zeylanium* was subjected to precipitation using salting out process. Ammonium sulphate fractionation of the crude extractwas performed at three saturation levels viz; 0-30%, 30-60% and 60-90%. Ammonium sulphate was added to the extract accordingto required saturation level slowly, while keeping on ice. Theice cold solution of the protein was stirred continuously and precipitation at 0-4°C for an hour. Then the precipitate was centrifuged at 10,000 rpm for 30 min. The pellet was separated and the process was repeated for next saturation levels (30-60% and 60-90%) with the supernatant. The separated pellets were dissolved in minimum amount of sodium acetate buffer (pH 5.0, 0.05 M) and specific activity for each fraction was determined.

2.5 Kinetic and Biochemical characteristics ofprotease

The crude protease enzyme was characterized with respect to the following parameters:

2.5.1 Time course

The reaction mixture containing enzyme was incubated at different time intervals ranging from 10 - 100 minutes to study the effect of time course on enzyme activity.

2.5.2 Temperature and pH optima

Suitable buffers of various pH values ranging from 3.0 to 10.0 were used to study the effect of pH on the enzyme activity. The optimum temperature for the enzyme activity was determined by incubating the reaction mixture in 0.05M buffer (appropriate pH) up to 100°C).

2.5.3 Temperature and pH stability

Suitable buffers of various pH values ranging from 3.0 to 10.0 were used to give shock to the enzyme for 2 hours under suitable temperature to study the effect of pH stability on the enzyme activity. The thermal stability for the enzyme activity was determined by incubating the enzyme at different temperatures for 2 hours (10-100°C).

2.5.4 Effect of metal ions

The effect of various metal ions like Cu^{2+}, Co^{2+} , Mn^{2+} , Ca^{2+} , Zn^{2+},Mg^{2+} and Na^{+} in the form of their respective salts i.e. $CuSO_4$, $CoCl_2$, $MnCl_2$, $CaCl_2$, $ZnSO_4$, $MgSO_4$ and NaCl was studied at 0.25 mM concentration.

2.5.5 Effect of varying substrate concentration

The varying substrate concentration in the range of 0.02 mM and 0.5 mM was used to study the effect on enzyme activity. The Michaelis-Menten constant (Km) and maximum velocity were calculated by using Lineweaver Burk plot (1/v vs. 1/s).

3. RESULTS AND DISCUSSION

The main objective of work analyzed here was to screen different therapeutically important spices for accounting highest specific activity of protease enzymes:

S. No.	Spices	Specific Activity (U/mg)
1.	Cinnamomum zeylanium	52.30
2.	Trigonella foenum graecum	46.90
3.	Piper nigrum	41.20
4.	Myristica fragrans	38.30
5.	Syzygium aromaticum	25.22
6.	Vetiveria zizanioides	16.64

Out of six spices screened, *Cinnamomum zeylanicum* bark sticks accounted protease enzyme with having highest specific activity about 52.30 units/mg as shown in above table. This selected plant source was characterized with respect to different parameters: temperature optima, temperature stability, pH optima, pH stability, time course, effect of metal ions, enzyme substrate concentration variation (Km).

3.1 Time course

Protease shows its maximum activity under the incubation period of 30 minutes (fig.1).

3.2 Effect of metal ions

Protease enzyme shows its highest activity in this order (fig.2) Fe^{2+}>K^{+}>Cu^{2+}>Zn^{2+}>Mn^{2+}>Na^{+}>Co^{2+}, where Fe^{2+} ion act as activator and Co ion as inhibitor.

3.3 Temperature and pH optima

Maximum activity of protease enzyme was observed at temperature 30^0C (fig.3) and pH at 5.0 (fig.5).

3.4 Temperature and pH stability

Maximum stability of protease enzyme was observed at temperature ranging from 30-40^0C (fig.4) and pH ranging from (5.0-6.0) (fig.6).

3.5 Enzyme substrate variation (Michaelis Menten constant)

Cinnamomum zeylanicum, K_m value was about 1.20 µg/ml/min and V_{max} was about 2.4 mg/ml (fig.7).

3.6 Ammonium sulfate precipitation

Out of three fractions 0-30%fraction has specific activity about 74.80 U/mg.

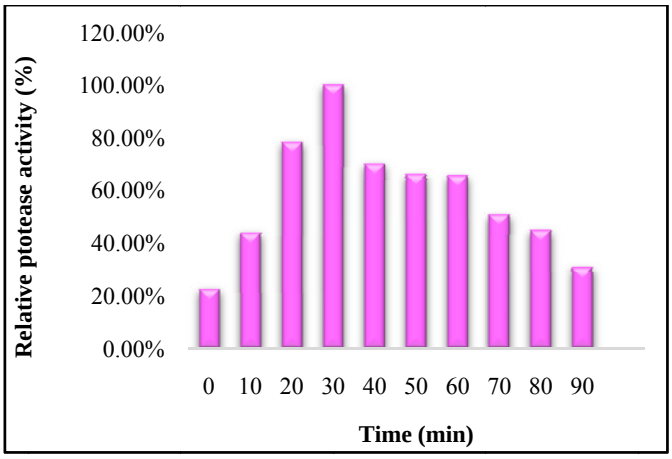

Fig. 1: Time course of protease catalyzed reaction isolated from C.zeylanicum bark sticks.

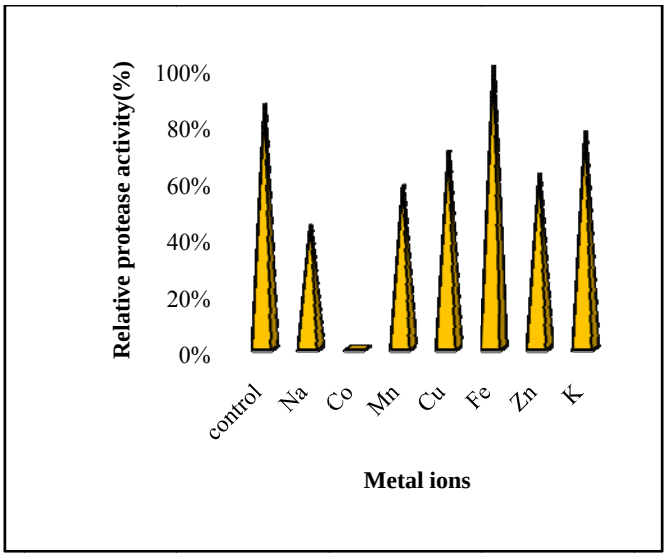

Fig. 2: Effect of metal ions on protease enzyme isolated from C.zeylanicum bark sticks'

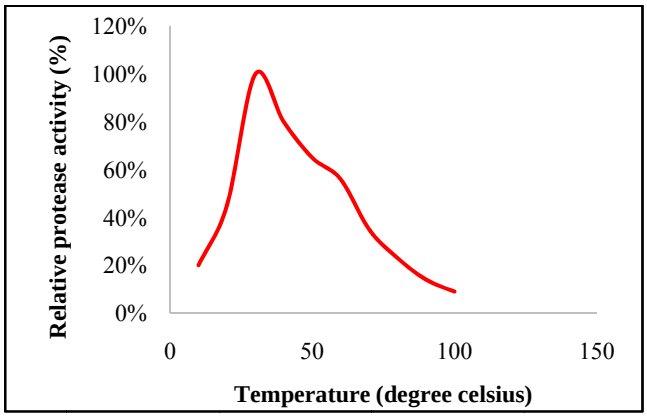

Fig. 3: Temperature optima for protease enzyme isolated from C. zeylanicum bark sticks.

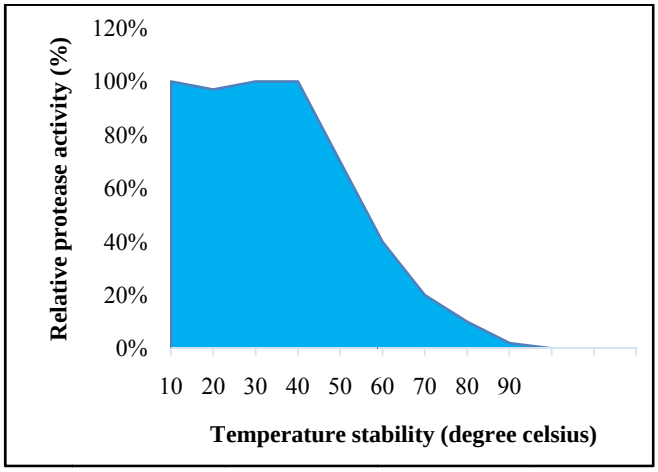

Fig. 4: Temperature stability for protease enzyme isolated from C.zeylanicum bark sticks

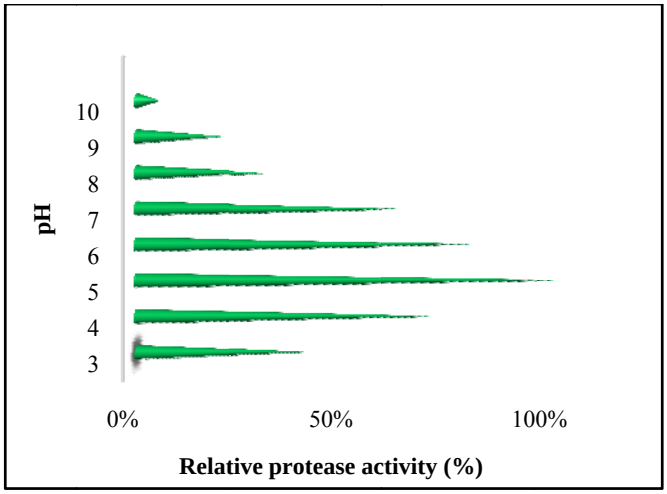

Fig. 5: pH optima for protease enzyme isolated from C. zeylanicum bark sticks.

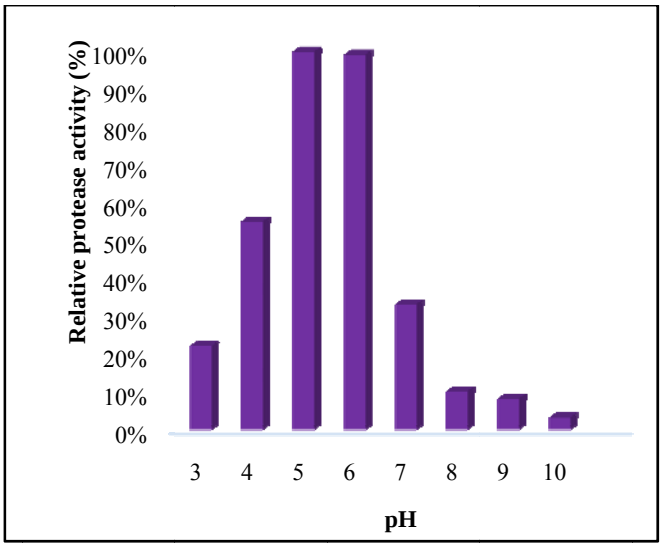

Fig. 6: pH stability for protease enzyme isolated from C. zeylanicum bark sticks.

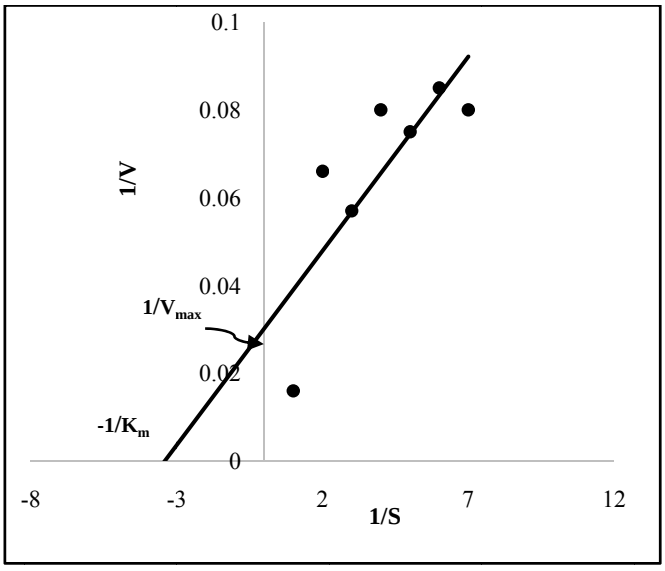

Fig. 7: Lineweaver plot showing Km and Vmax value of protease isolated from C. zeylanicum bark sticks.

4. CONCLUSION

According to results it has been concluded that *C. zeylanicum*, a therapeutically potential source of protease enzyme used traditionally in Ayurvedic medicines for curing many diseases, act as multi-ingredentially flavored spice in different cuisines which have its medicinal value which is very much beneficial for health. In this field further studies are very much require keen attention to develop and make safe supplements to cure many more infectious diseases.

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