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Full Length Research Paper

Isolation and partial characterization of a protease enzyme from *Thaumatococcus daniellii* waste

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A protease enzyme was isolated and partially purified from the pulp of *Thaumatococcus daniellii* fruit by gel filtration on sephadex G-75 followed by ion-exchange column chromatography on DEAE-cellulose. The enzyme showed a specific activity of 4.75 × 10^{-1} unit/mg protein and 6.93 × 10^{-1} unit/mg protein, respectively after each purification procedure. The purified enzyme had a K_m and V_{max} of 2.0 × 10^{-4} M and 1.53 mol/min, respectively, using casein as substrate. The enzyme had an optimum temperature of 35°C and functioned best at pH 7.0 with some residual activity at alkaline pH.

Key words: Protease, isolation, characterization, *Thaumatococcus danielii*.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes, accounting for at least a quarter of global enzyme production (Herbert, 1992). Several proteases are used in the brewing, detergent, leather, dairy and food processing industries (Layman, 1986; Kaliz, 1988). These enzymes are a group of enzymes whose catalytic function is to hydrolyze peptide bonds. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds (Barrett, 1994). Proteolytic enzymes are extensively distributed in all types of organism; 2% of all free products are peptidases (Barrett, 1998). Proteases are one of the components of standard ingredient used for household laundering and reagents used for cleaning contact lenses or dentures (Mala et al., 1998). The conventional methods of leather processing involve the use of hazardous chemicals such as sodium sulphide which creates problems of pollution and effluent disposal. The use of enzyme (proteases) as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution (Mala et al., 1998).

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Thaumatococcus daniellii is a species of tropical flowering plant known for being the natural source of thaumatin. The origin of *T. daniellii* has been traced to Ghana (Green, 1999) and surrounding West Africa countries. It is a useful plant, easy to cultivate as the 'katemfe' shrub under tree plantation and it is native to the Africa rain forest. It grows three to four meters in height and has large papery leaves up to 46 cm long. It bears pale purple flowers and a soft fruit containing one to three black seeds surrounded by a gel and copped with a membranous sac; the aril contains the 'sweet protein' thaumatin (Watson and Dallwitz, 1997). Thaumatin is a low-calorie intensely sweet-tasting protein (on a molar basis about 100000 times as sweet as sucrose) (Edens et al., 1982) and flavour modifier (Green, 1999). It is often used primarily for its flavour-modifying properties and not exclusively as a sweetener (Green, 1999). Thaumatin has been approved as a food additive throughout Europe and in many other countries throughout the world. It is currently marketed by an English company that gets the plant material from Ivory Coast but processed in Great Britain (Zemanek and Wasserman, 1995). Besides flavouring, the plant has a number of uses. The sturdy leaf petioles are used as tools and building materials, the leaves are used to wrap food, and the leaves and seeds have a number of traditional medicinal uses.

The increasing need of proteases in biotechnologically

based industries in the world, especially in Africa, led researchers to find alternative ways of producing this important enzyme. In previous works, proteases were isolated and characterized from peels of cocoyam (Raimi and Rokosu, 2001) and yams (Raimi et al., 2005). Tons of *T. daniellii* pulp is generated and thrown into the environment in South-west Nigeria where the fruit is commonly found. This waste has become a nuisance to the environment and hence this study was aimed at finding the possible use for *T. daniellii* pulp as a source of proteases for industries that need them.

MATERIALS AND METHODS

Materials

T. daniellii fruits were purchased from a local market in Ekiti State, Western Nigeria. DEAE-cellulose and sephadex G-75 were from Pharmacia Sweden. All other reagents were of analytical grade.

Isolation

The fruits were washed with distilled water and the pulp was removed using a knife. 300 g of the pulp was blended with 600 ml of ice-cold 150 mM phosphate buffer (pH 7.4). The extract was filtered using a clean white piece of cloth. The filtrate was centrifuged at 7000 ×g for 10 min. The supernatant (460 ml) was then subjected to 75% ammonium sulfate precipitation. This was stored at 4°C overnight and then centrifuged at 7000 ×g for 10 min. The pellet obtained was re-dissolved with small amount of ice cold 150 mM phosphate buffer (pH 7.4), while the supernatant was discarded. The crude extract was then dialyzed against the same buffer for 72 h with continuous stirring.

Protein determination

Protein concentration was quantitatively analyzed using Biuret method and BSA as standard (Layne, 1957).

Enzyme assay

Enzyme assay was performed using a modification of Kunitz caseinolytic assay as described by Janssen et al. (1994). 0.5 ml of the crude enzyme source was added to 2.0 ml of 0.5% casein in 50 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 37 °C and terminated after 30 min by adding 3.0 ml of 5% TCA. The solution was kept for additional 30 min at room temp and then centrifuged. The absorbance of the supernatant was read at 280 nm. One unit of TCA soluble casein hydrolysis product was defined as an increase of 0.1 in absorbance at 280 nm.

Purification procedure

5.0 ml of dialyzed protein sample was carefully layered on pre-swollen sephadex G-75, which was packed carefully in a Pharmacia chromatographic column (1.0 × 38.5 cm). The column was previously equilibrated with 50 mM Tris buffer (pH 7.8) and the protein was eluted using the same buffer. A flow rate of 0.1 ml/min was maintained and 4 ml fraction was collected into fifty test tubes. Protein was monitored at 280 nm. The peak with the highest enzyme activity was pooled together and purified further on DEAE - cellulose, which was carefully packed in a column (2.5 × 25 cm) previously equilibrated using Tris buffer (pH 7.8) and the protein was eluted using Tris buffer in a linear gradient of increasing NaCl concentration from 0 to 0.3 M. A flow rate of 0.4 ml/min was maintained and the absorbance was monitored at 280 nm. Fractions that showed the highest enzyme activity were pooled and characterized.

Determination of K_m and V_{max}

The enzyme activities were assayed at various concentrations of substrate (casein) in a reaction volume of 2.2 ml incubated for 30 min at 37°C and terminated using 3.0 ml of 5% TCA. K_m and V_{max} were determined from the Lineweaver-Burk plot.

Effect of temperature

The effect of temperature on the enzyme activity was carried-out at a temperature range of 15 to 55°C with an interval of 5°C. Modified Kunitz caseinolytic assay method as described for enzyme assay was used.

Effect of pH

This was carried out following the method described earlier but the pH was varied for the reaction mixture between a range of 2.0 and 11.0 with an interval of 1.0 using 50 mM phosphate buffer with the desired pH.

RESULTS AND DISCUSSION

Proteases are unique class of enzymes because they are of immense physiological and commercial impor-tance. Proteases constitute two thirds of total enzymes used in various industries (Gupta et al., 2002) and this dominance in the industrial market is expected to increase yearly. In this study, a protease was isolated from the pulp of *T. daniellii*. The protein yield from 300 g of *T. daniellii* pulp is shown in Table 1. The elution profile of the protein on sephadex G-75 gave two peaks with peak I having a higher protease activity (Figure 1a). Further purification of pooled fractions of peak I on DEAE- cellulose gave a peak with protease activity (Figure 1b).

The enzyme kinetics study revealed a V_{max} of 1.53 mol/min and a K_m of 2.0×10^{-4} M. This indicates that the enzyme had affinity for casein as a substrate. This is similar to the result of Anwar and Saleemudin (2000), in trying to assess further the suitability of the protease from *Spilosoma obliqua* for its use in laundering detergent, carried out an experiment in which various proteins were used as substrates and it was found that the protease was able to cleave all the proteins tested but casein was the most preferred substrate (Anwar and Saleemuddin, 2000).

The enzyme from *T. daniellii* waste was found to have a temperature optimum of 35°C (Figure 2) with pH

Table 1. Enzyme purification.

Purification Step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (units)	Specific activity (unit/mg protein)	Purification fold	Yield
Crude extract	460	18.3	8395	4.50	2.46×10^{-1}	1.00	100
Dialysis	175	4.0	700	1.70	4.25×10^{-1}	4.53	37.8
Gel filtration on Sephadex G-75	16	3.2	51	1.52	4.75×10^{-1}	25.00	33.8
Ion-exchange on DEAE-cellulose	7	1.5	11	1.04	6.93×10^{-1}	176.31	23.1

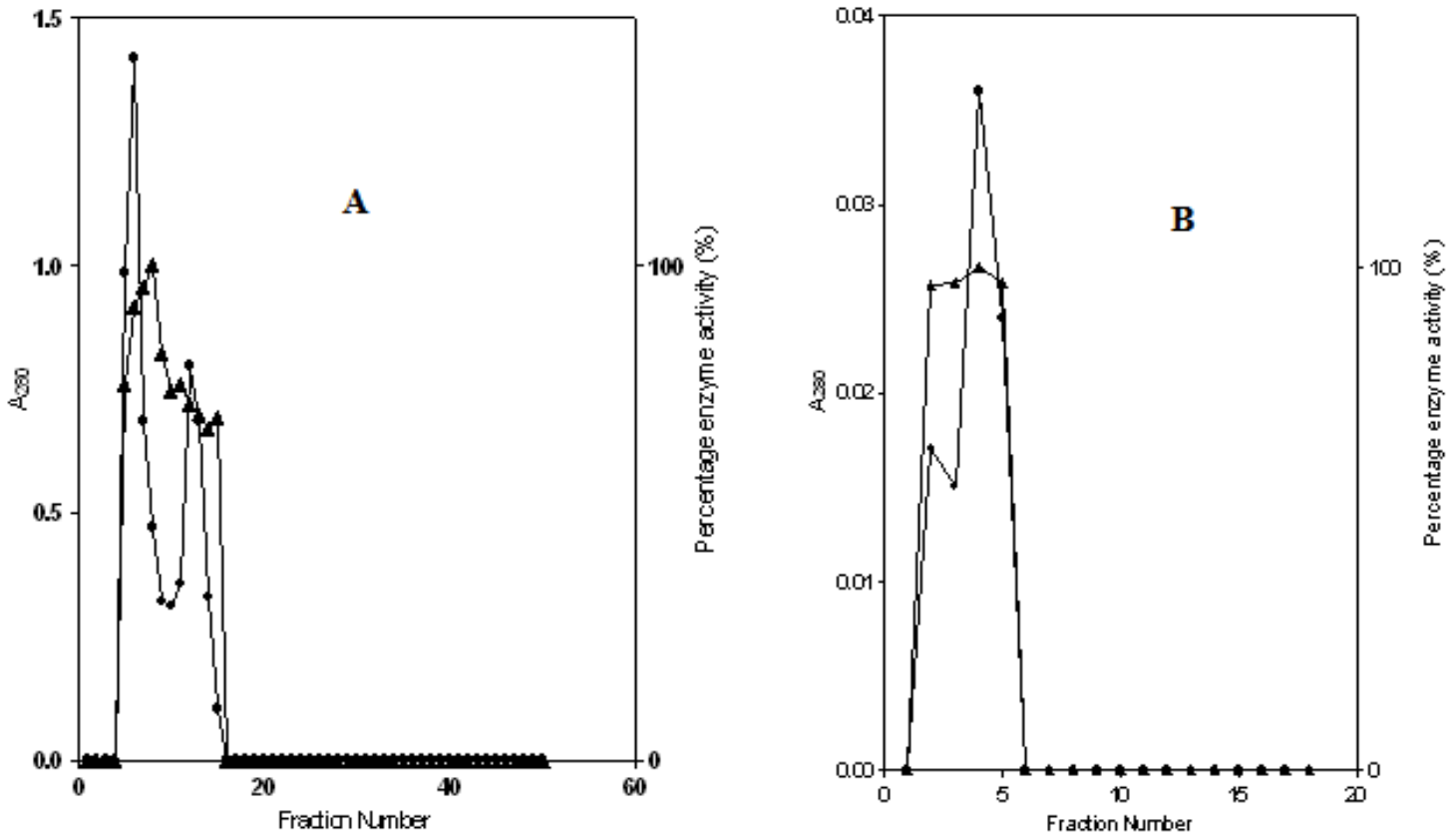

Figure 1. Elution profile and enzyme activity of dialysate on Sephadex G-75 (A) and DEAE-Cellulose (B). ●, absorbance at 280 nm; ▲, percentage enzyme activity (% of maximum).

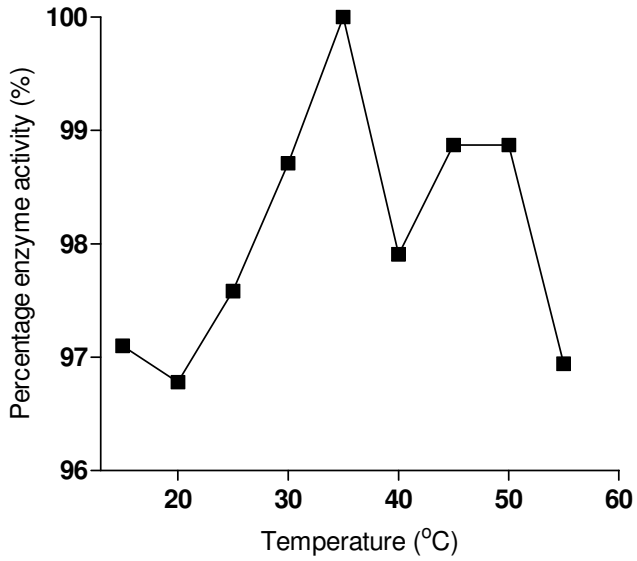

Figure 2. Effect of temperature on enzyme activity.

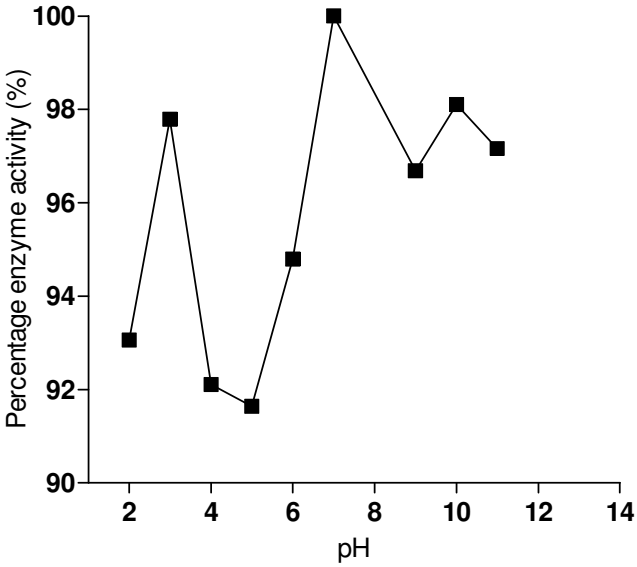

Figure 3. Effect of pH on enzyme activity.

optimum at neutral pH (pH 7.0) but the enzyme also displayed some residual activity at alkaline condition (Figure 3). The largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range (Gupta et al., 2002). The bio-industrial viewpoints of microbial alkaline proteases from sources to cellular role, production, downstream processing, characterization and commercial applications have also been reviewed (Anwar and Saleemuddin, 1998; Kumar and Takagi, 1999). Work on the potential application of alkaline protease in bio-formulations has been intensified (Anwar and Saleemuddin, 2000).

An industrially important alkaline protease, isolated from a selective strain of *Bacillus*, has been shown to be useful as a catalyst for the resolution of N-protected amino acids having unusual side chains (Chen et al., 1991). The major component of this enzyme is subtilisin, which is a serine protease and is widely used as a detergent additive (Chen et al., 1991). The detergent industry has now emerged as the single major consumer of several other hydrolytic enzymes, including proteases acting in the alkaline pH range. The major use of detergent compatible proteases is in the laundry detergent formulation (Vantilburg, 1984; Anstrup and Anderson, 1974). The cost of enzyme production is the major obstacle in the successful application of proteases in industries. Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present, there is considerable interest in the identification of alkaline proteases which are effective over a wide temperature range (Oberoi et al., 2001). In addition, the current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures (Hasan and Tamiya, 1997; Kitayama, 1992; Nielsen et al., 1981). This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures. It is the understanding of this that prompted the research into finding a possible use for *T. daniellii* waste as a source of industrial enzymes in order to reduce the cost of production.

Conclusion

The fact that the protease isolated was active at various temperatures with optimal activity at low temperature of about 35°C is a strong indication that a protease isolated from this waste product could be a suitable source of protease in the laundry detergent industry. This study has therefore been able to demonstrate the presence of a protease enzyme in the pulp of *T. daniellii* which can be exploited commercially.

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