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Purification and biochemical characterisation of a novel protease streblin

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ABSTRACT

A serine protease, streblin, was purified 4.6-fold with 75% recovery from the latex of a plant, *Streblus asper*. This is the first report of identification and purification of a serine protease from the genus *Streblus* of the Moraceae family. Streblin, has a molecular mass of 64 kDa and the extinction coefficient ($\epsilon^{1\%}_{280}$) is 5.29. Streblin is a basic protein with pI value of 9.2 that acts optimally at pH 9.0; such optimum activity at high pH has not been reported for most of the isolated plant serine proteases and the enzyme is stable over a wide range of pH (3.0–12.5). The enzyme is also thermostable, retaining complete activity at 15–85 °C and acts optimally at 65 °C. Furthermore, it is highly stable in the presence of various denaturants in which SDS resistance is the most striking property of the purified enzyme. Streblin strongly coagulated skimmed milk. Easy availability of the latex, simple purification procedures, high stability of streblin against pH, or autodigestion, and under various conditions make the enzyme a good system for exploring the biophysical chemistry of proteases. In addition to its high milk-clotting ability, it could be used in the cheese industry, as well as other food and biotechnological industries.

1. Introduction

Proteolytic enzymes account for approximately 60% of all enzyme sales, because of their broad substrate specificities and activities over a wide range of pH and temperature. Additionally they have been widely applied in pharmaceutical, medicinal, food, detergent, leather and biotechnological industries (Nallamestty, Kundu, & Jagannadham, 2003).

As cardosins are probably the plant proteases that have met with most success in cheese making, Macedo, Malcata, and Oliveira (1993) comprehensively reviewed fundamental and applied aspects of the manufacture of Serra cheese. In the food industry, proteases are used for coagulation of milk in the cheese industry, as well as for improvement of functional and nutritional properties of proteins, hydrolysis of gelatin, soy protein, casein and whey protein (Sumantha, Larroche, & Pandey, 2006). They are also used in brewing, cheese elaboration, and bread manufacturing (Pande, Dubey, Yadav, & Jagannadham, 2006). Similarly, extracts of *Centaurea calcitrapa* degrade caseins from milk from different species, suggesting that such plant extracts can be used as an alternative to commercial animal rennets, especially in the manufacture of caprine and ovine milk cheeses (Tavaria et al., 1997). The fruit extract of *Opuntia ficus-indica* is apparently a good substitute for animal rennet, as it exhibits both clotting and caseinolytic activities (Pintado et al., 2001). Proteolytic enzymes from plant sources are well suited to the pharmaceutical and food industries, as they are active over a wide range of temperature and pH, and possess broad substrate specificity and high stability under extreme conditions (Patel, Singh, & Jagannadham, 2007).

Apparently, most of the isolated and characterised plant proteases have been classified as cysteine proteases, which are widely used in several processes in the food industry (Uchikoba, Yonezawa, & Kaneda, 1998). The major drawback in the use of cysteine proteases is that their activity is readily reduced by air oxidation and metal ions. Therefore, application of these enzymes requires reductants and chelating agents and, thus, are not economical or convenient (Tomar, Kumar, & Jagannadham, 2008). By contrast, plant serine proteases are both stable and active under harsh conditions of raised temperatures and high pH, as well as in the presence of either surfactants or oxidising agents. Thus, they are more useful and economical for industrial applications (Sharma, Kumari, & Jagannadham, 2009). Therefore, the search for new potential plant serine proteases still continues, in order to make these industrially applicable and cost-effective, as well as to understand their physiological role in plants.

Abbreviations: BSA, bovine serum albumin; CBB, coomassie brilliant blue; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; DTNB, 5,5μ-dithiobis (2-nitrobenzoic acid); DTT, di-thiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(raminoethyl ether) tetraacetic acid; GuHCl, guanidine hydrochloride; IAA, iodoacetic acid; IEF, iso electric focusing; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; TEMED, *N,N,N,N*-tetramethylethylenediamine; TFA, trifluoroacetic acid; β-ME, β-mercaptoethanol.

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Thiol proteases from plant latex, such as bromelain, calotropins, ficin and papain, are commonly used in several processes in the food and dairy industries, but their proteolytic activity is inhibited by air oxidation or metal ions. Thus, these proteases require reducing and chelating agents for their activity, which restricts their commercial application. By contrast, different serine proteases, including plant proteases, do not need such co-factors (Singh, Kumar, Rao, & Jagannadham, 2010).

Serine proteases are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes. In plants, they are widely spread among different taxonomic groups and are found to be involved in a number of physiological processes, such as protein degradation and processing, microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, and senescence (Antao & Malcata, 2005). Despite being the largest class of proteases in plants, the functions and regulatory roles of plant serine proteases are poorly understood, probably due to a lack of identification of their physiological substrates. Once thought to be rare in plants, in recent years, several serine proteases have been isolated and purified from different plant parts of various plant species, including seeds, latex and fruits (Mohamed Ahmed, Morishima, Babiker, & Mori, 2009).

Studies in this direction are herein carried out, utilising a novel protease, streblin, from the latex of *Streblus asper*, a medicinally important plant. *S. asper* Lour (Family: Moraceae) is a small tree. Various parts of this plant are used in Ayurveda and other folk medicines for the treatment of different ailments, such as filariasis, leprosy, toothache, diarrhea, dysentery and cancer. Research carried out using different *in vitro* and *in vivo* techniques of biological evaluation support most of these claims (Rastogi, Dinesh, Kulshreshtha, & Rawat, 2006).

2. Materials and methods

2.1. Materials

Latex was collected in 0.01 M Tris–HCl buffer, pH 8.0, by superficial incisions on stems of *S. asper* found in Varanasi, India, and frozen at −20 °C for 24 h. The latex was thawed and centrifuged at 20,000*g* for 40 min to remove the gum and other insoluble materials. The supernatant was subjected to anion-exchange chromatography, for which a DEAE column was purchased from Pharmacia. BSA, hen egg white lysozyme, azocasein, azoalbumin, haemoglobin, DTNB, DTT, GuHCl, urea, *o*-phenanthroline, EDTA, EGTA, SBTI, $HgCl_2$, PCMB, NEM, PMSF, acrylamide, *N*,*N*-methylene bisacrylamide and CBB R 250, were obtained from Sigma Chemical Co. (United States). CBB G 250 was from Eastman Kodak. TFA was obtained from Applied Biosystems. Ampholine carrier ampholytes were from LKB. All other chemicals were of the highest purity available.

2.2. Safety

Acrylamide is a potent neurotoxin and carcinogen, and it was handled with safety gloves. The handling of phenol and TCA was done carefully because of their highly corrosive nature to skin. All other experiments were carried out with the utmost precaution and care.

2.3. Purification of the protease, streblin

Chromatography was performed at room temperature. Supernatant, obtained in the above step, was subjected to anion-exchange chromatography on a DEAE-Sepharose fast flow column pre-equilibrated with 0.01 M Tris buffer, pH 8.0. The column was washed with the same buffer until no protein was detected in the eluant. The bound proteins were eluted with a linear gradient of 0–0.8 M NaCl in the same buffer at a flow rate of 2.5 ml/min, and fractions of 2.5–3 ml were collected. The absorbances at 280 nm, as well as caseinolytic activity of the proteins in all the fractions, were determined. Bound proteins were eluted in two peaks with caseinolytic activity. The active and homogeneous fractions of peak one were pooled, concentrated and stored at 4 °C for further experiments. The pure enzyme thus obtained was named as streblin and assayed for protein content as well as protease activity (Fig. 1).

2.4. Protein concentration

Protein concentration, at different stages of purification, was determined spectrophotometrically by absorbance at 280 nm, as well as by the method of Bradford (1976), using BSA as a standard.

2.5. Assay for protease activity

The hydrolysing activity of the protease was determined using denatured natural substrates such as casein and azoalbumin (chromogenic substrate). Enzyme, at a concentration of 15 μg in a total volume of 0.5 ml of 0.05 M Tris–HCl buffer, pH 7.5, was incubated at 37 °C for 10 min. An equal volume of 1% casein solution (w/v), prepared at the same pH, was added to the enzyme solution, making the final volume 1 ml, and the reaction mixture was incubated further for 30 min at 37 °C. The reaction was stopped by addition of 0.5 ml of 10% TCA and the mixture allowed to stand for 10 min at room temperature. Soluble peptides were separated by centrifugation for 10 min, using a tabletop centrifuge. The absorbance of the TCA-soluble peptides in the supernatant was measured at 280 nm. In the case of azoalbumin (0.6%, w/v) as substrate, 0.5 ml of supernatant, after TCA precipitation, was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The development of colour was measured spectrophotometrically by absorbance at 440 nm. With haemoglobin (1% w/v) as a substrate, activity is determined in the same manner as for casein. A control assay, without the enzyme, was done and used as blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions that gave rise to an increase of 1 unit of absorbance at 280 or 440 nm per minute of digestion. The number of units of activity per milligramme of protein was taken as the specific activity of the enzyme.

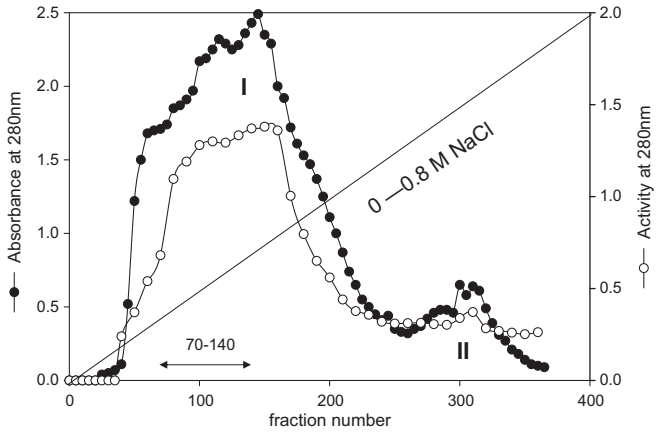

Fig. 1. Elution profile of streblin on anion-exchanger: DEAE-Sepharose column was pre-equilibrated with 10 mM tris buffer, pH 8.0. The unbound proteins were washed out with the equilibration buffer and the column was eluted with a linear salt gradient of 0.00–0.80 M NaCl, same pH. Fractions of 3 ml were collected at a flow rate of 4 ml/min and assayed for protein content (●) and proteolytic activity (**O**). The fractions of pool I (70–140) were pooled as indicated by a horizontal arrow.