Study on Changes in thiol protease activity during germination and its purification

[1]Dr. Krishan Pal and [2]Ankita Devi

[1]Associate Professor, Department of Botany, OPJS University, Churu, Rajasthan (India)
[2]Research Scholar, Department of Botanu, OPJS University, Churu, Rajasthan (India)
e-mail: ankita39999panwar@gmail.com

Abstract: Thiol protease activity during germination of mungbean seeds was monitored for 5 days using BANA as substrate to determine the optimum stage to harvest seeds for purification of this enzyme. The activity of thiol protease increased markedly after the first day of imbibition, reaching a peak after 2 days and then declining gradually to 15% of the maximum after 5 days. In dry seeds (0 day after imbibition), the enzyme showed 73% of the maximum activity. Thus, 2-day old germinated seeds were used for purification of the enzyme. After ammonium sulphate fractionation the overall fold purification was 12.08 with a recovery of 67 %. The profile of the enzyme elution through DEAE Sephadex A-50 column. The use of anion-exchange chromatography at pH 6.8 separated Leu-βNA hydrolyzing activity (Leucine aminopeptidase activity) and some unwanted proteins from the enzyme. The adsorbed BANA hydrolyzing (thiol protease) activity eluted at 0.3 M NaCl whereas Leu-βNA-hydrolyzing activity was eluted at 0.38 M NaCl. The partially purified fraction obtained from anion exchange column after dialysis, was chromatographed on Sephadex G-100 column. Lots of contaminating proteins were removed at this step and a single peak of enzyme activity was obtained. The enzymatically active fractions were pooled and the pooled fraction when analyzed by native PAGE did not give a single protein band. The next step of purification was affinity column chromatography which was so designed as to separate all the non-thiol proteins from the thiol protease. The affinity column was prepared by immobilizing p-aminophenylmercuric acetate on CNBr-activated Sepharose 4B. The bound enzyme was eluted from the organomercurial column with 50 mM sodium acetate buffer, pH 5.5 containing 0.2M NaCl, 1mM EDTA and 10mM cysteine. The affinity chromatography showed a single peak of enzyme activity. The fractions containing enzyme activity were pooled and immediately dialyzed.
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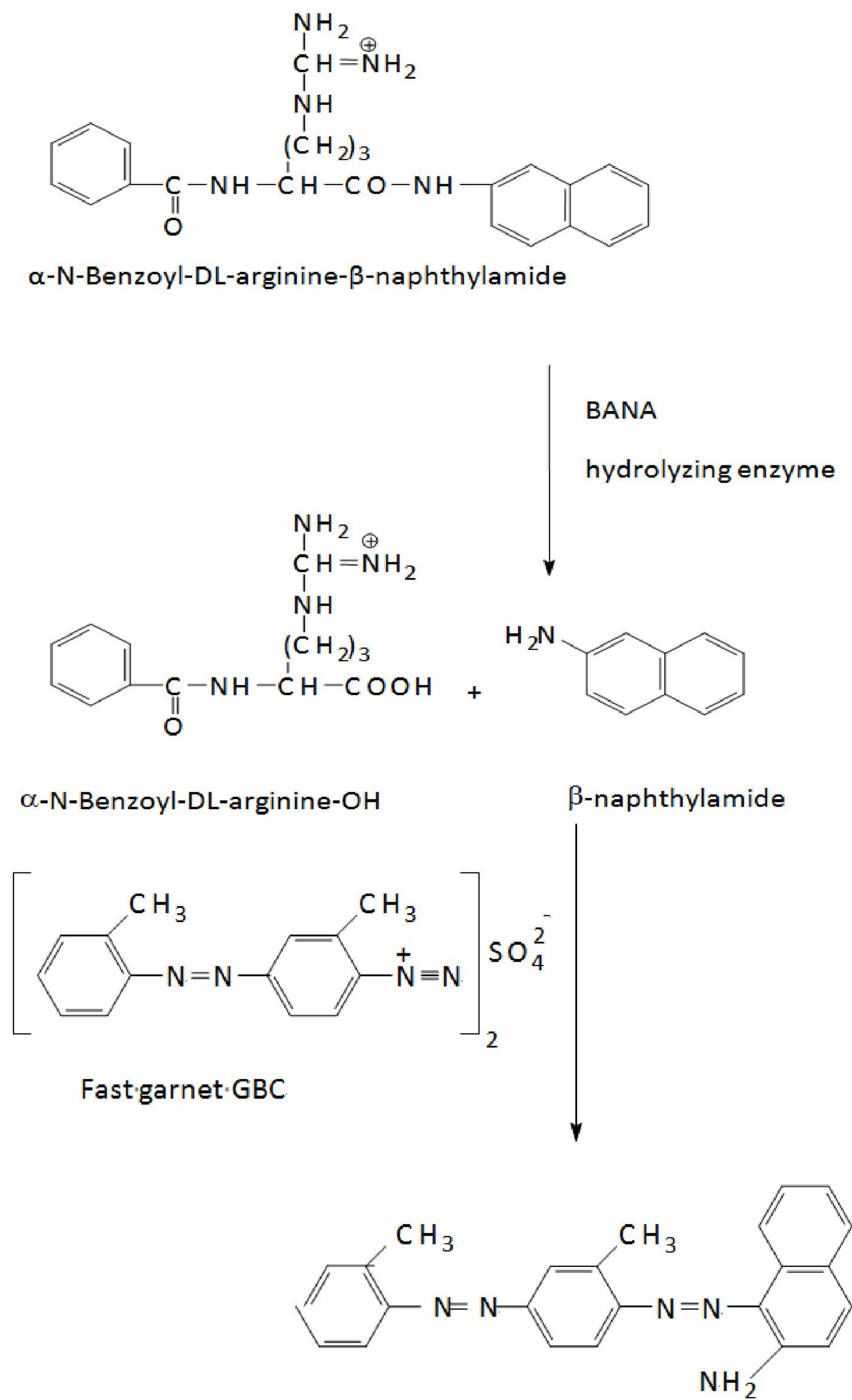
Introduction:

Proteolytic enzymes catalyze the cleavage of peptide bond (s) in proteins. Peptide hydrolases also called peptidases or proteases may be endopeptidases which cleave interior peptide bonds and exopeptidases which cleave the terminal peptide bonds (Barrett, 1994). Exopeptidases may be differentiated according to their substrate specificity as amino peptidases, acting at a free N terminus, and carboxypeptidases, which degrade peptides at the C-terminus. Endopeptidases may be classified as cysteine-, serine-, aspartic-, and metalloproteinases on the basis of their active site residue (Barrett, 1986). However, there are some endopeptidases with unknown mechanism of catalysis. Although all four classes of proteinases have been described in plant cells, most described to date from vegetative organs are cysteine proteinases.

Cysteine proteinases also referred to as thiol proteases are actually endopeptidyl hydrolases with a cysteine residue in their active center are usually identified on the basis of their active site inhibitors (iodoacetate, iodoacetamide and E64) and activation of the enzymes by thiol compounds. Most of these cysteine proteinases have acidic pH optima *in vitro*, suggesting that they are localized in the vacuoles *in vivo*. Cysteine proteinases being labeled with the prefix C and comprise more than 40 families of peptidases grouped into at least six super families or clans. Most plant cysteine proteinases belong to the papain (C1) and legumain (C13) families. The members of cysteine proteinases: caspases (family C14) and calpains, the calcium-dependent proteinases (family C2), have recently been found in plants. Two additional families of cysteine proteinases: ubiquitin C-terminal hydrolases (family C12) and ubiquitin-specific proteinases (C19), the components of the ubiquitin-proteasome-dependent pathway that catalyze deubiquitination of proteins, have also been detected in plants (Vierstra, 2003). Calpains are cytoplasmic, calcium-dependent cysteine proteinases requiring micro- or millimolar concentrations of Ca^{2+} for activity. Legumains belong to the asparaginyl- specific subclass of the cysteine endopeptidase family which cleaves peptide bonds with Asn or Asp. Caspases show a high specificity with absolute requirement for an Asp residue adjacent to the cleavage site (Woltering et al., 2002).

Serine proteases appear to be the largest class of proteases in plants. The MEROPS database lists 248 enzymes for *Arabidopsis* representing 8 of the known 12 clans. Three of them, the chymotrypsin, subtilisin, and carboxypeptidase clans, share a common reaction mechanism based on a well-characterized "catalytic triad" comprising a serine, an aspartic acid, and a histidine residue.



Materials and Methods:

Seed material

Mungbean seeds [Vigna radiata (L.) Wilczek] were procured from the Department of Plant Breeding, CCS Haryana Agricultural University, Hisar, India.

Enzyme assays

Assay of thiol protease

The activity of thiol protease was measured by using the synthetic substrate α-N-Benzoyl-DL-arginine-β-naphthylamide (BANA) by the method of Kamboj et al. (1990). The reaction mixture in a total volume of 1.0 ml contained 100µl enzyme extract and 875ul of 50mM sodium acetate buffer, pH 4.3 containing 2mM cysteine which was pre-incubated for 10 min at 37ºC. The enzymatic reaction was started by adding 25ul of the substrate BANA stock solution (40mg/ml of DMSO). After 20 min incubation, the reaction was stopped by adding 1ml of coupling reagent which was prepared by mixing equal volume of fast garnet GBC dye (1mg/ml of water) with a solution of 10mM $HgCl_2$ and 50mM EDTA in water pH 6.0. After 10min, the red color obtained by coupling of β-naphthylamine with fast garnet GBC was estimated at 520nm by extracting it in 2.0ml n-butanol. Blank experiments were also conducted where the enzyme samples were added after the coupling reagent.

Calculations

Thiol protease activity was calculated in terms of nmoles of β-naphthylamine released per min per ml enzyme by using the formula:

Thiol protease activity (nmoles/min/ml) =

$$\frac{OD520 \times 2.0 \times 10^{-3} \times 10^{9} \times 10}{\varepsilon \times t}$$

Where,

ε is the molar extinction coefficient of β-naphthylamine which is equal to 25,000 under the assay conditions.

t is the reaction time in minutes.

2×10^{-3} is the volume of n-butanol in litres used for extracting the red colour.

10 is the multiplication factor for calculating the enzyme activity per ml, as only 100 µl enzyme was used for the reaction.

10^9 is used for converting moles into nmoles.

Out unit of enzyme activity was expressed as that amount of enzyme which released one nmole of β-naphthylamine from BANA substrate at 37 ºC per min under the assay conditions. Specific activity was expressed as units of enzyme activity per mg enzyme protein.

To determine the molar extinction coefficient, ε, 13.76 mg of β-naphthylamine was dissolved in 1.0 ml of DMSO. This solution was then diluted to 100 ml with water. Then 50, 100 and 200 µl of this solution (equivalent to 20, 40 and 80 nmoles of β-naphthylamine, respectively) were diluted to 2.0 ml with assay buffer. To each of these solutions, 2.0 ml of coupling reagent was added and allowed the color to develop for 10 min which was then extracted in 2.0 ml n-butanol and estimated at 520 nm. The extinction coefficient was 25,000 as determined by the formula given below: **Thiol**

protease catalyzed reaction: liberation and coupling of β-naphthylamine

$$\varepsilon = \frac{OD_{520} \times 2.0 \times 10^{-3}}{\text{concentration of } \beta\text{-naphthylamine in moles}}$$

Similarly, the molar extinction coefficient of 4-methoxy-β-naphthylamine was determined which was found to be 36,000.

Results:

Thiol protease activity during germination of mungbean seeds was monitored for 5 days using BANA as substrate to determine the optimum stage to harvest seeds for purification of this enzyme. The activity of thiol protease increased markedly after the first day of imbibition, reaching a peak after 2 days and then declining gradually to 15% of the maximum after 5 days. In dry seeds (0 day after imbibition), the enzyme showed 73% of the maximum activity (Fig 1). Thus, 2-day old germinated seeds were used for purification of the enzyme.

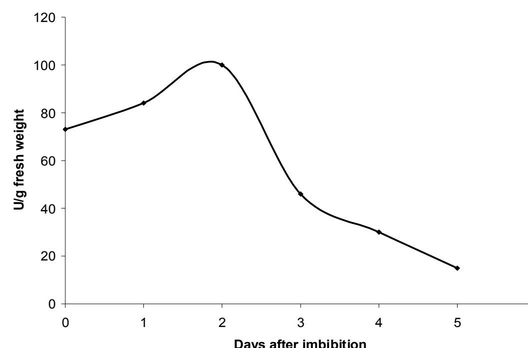


Fig 1 Changes in proteolytic activity (with BANA) during germination.

The thiol protease was isolated and purified to apparent homogeneity from cotyledons of 2 day-old seedlings by following the steps of homogenization, acid fractionation, ammonium sulphate fractionation (20-70%), DEAE-Sephadex A-50 column chromatography at pH 6.8, gel filtration chromatography on Sephadex G-100 column and affinity chromatography on Organomercurial-

Sepharose 4B. The overall purification was 573 fold with a recovery of 3.3% (Table 1).

After ammonium sulphate fractionation the overall fold purification was 12.08 with a recovery of 67 %. The profile of the enzyme elution through DEAE Sephadex A-50 column. The use of anion-exchange chromatography at pH 6.8 separated Leu-βNA hydrolyzing activity (Leucine aminopeptidase activity) and some unwanted proteins from the enzyme. The adsorbed BANA hydrolyzing (thiol protease) activity eluted at 0.3 M NaCl whereas Leu-βNA-hydrolyzing activity was eluted at 0.38 M NaCl. The partially purified fraction obtained from anion exchange column after dialysis, was chromatographed on Sephadex G-100 column (Fig 4.4). Lots of contaminating proteins were removed at this step and a single peak of enzyme activity was obtained. The enzymatically active fractions were pooled and the pooled fraction when analyzed by native PAGE did not give a single protein band. The next step of purification was affinity column chromatography which was so designed as to separate all the non-thiol proteins from the thiol protease. The affinity column was prepared by immobilizing p-aminophenylmercuric acetate on CNBr-activated Sepharose 4B. The bound enzyme was eluted from the organomercurial column with 50 mM sodium acetate buffer, pH 5.5 containing 0.2M NaCl, 1mM EDTA and 10mM cysteine. The affinity chromatography showed a single peak of enzyme activity. The fractions containing enzyme activity were pooled and immediately dialyzed.

Table 1: Purification of mungbean thiol protease

Purification steps	**Total Activity (nmoles/min)**	**Total Protein (mg)**	**Specific Activity (Units/mg)**	**Yield (%)**	**Purification fold**
Crude extract supernatant,S1	1320.0	5420	0.24	100.0	1
Acid inactivated supernatant, S2	1257.0	3160	0.39	95.2	1.60
20-70% $(NH_4)SO_4$ pellet, P_4	882.0	300	2.90	66.81	12.08
DEAE-Sephadex A-50 pool	547.2	32	17.10	41.44	71.25
Sephadex G-100 pool	425.7	5.80	73.40	32.25	305.83
Organomercurial Sepharose- 4B	42.9	0.31	137.50	3.25	572.92

Corresponding Author Address:
Mrs. Ankita Devi
Research Scholar, Department of Botany,
OPJS University, Churu, Rajasthan (India)
e-mail- ankita39999panwar @gmail.com
Phone no. +91-9811471572

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