Study on Characterization of thiol protease

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Abstract: The Sepharose 6B column was packed as detailed under 'Materials and Methods'. After proper equilibration with buffer A (0.1M sodium acetate buffer pH 5.5 containing 0.2 M NaCl and 1 mM EDTA), the void volume (V_0) and the total volume (V_t) of the Sepharose 6B column (90 x 0.8 cm) were determined as 36.0 ml and 115.0 ml, respectively. Various molecular weight marker proteins [2.0 mg each of carbonic anhydrase (29 k Da), bovine serum albumin (66 k Da), alcohol dehydrogenase (150 k Da) and β-amylase (200 k Da)] were dissolved in 2.0 ml of eluent buffer A. This mixture was loaded on the column which was eluted at a speed of 15 ml/h and fractions of 3.0 ml each were collected. The elution volume for each marker protein was calculated by estimating protein through absorbance at 280 nm. The purified thiol protease sample was eluted separately from the same column under identical conditions and the elution volume of the enzyme protein was calculated. The molecular weight of thiol protease was calculated from the standard graph plotted between log molecular weight against Ve/Vo which was found to be ~157 kDa.

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Introduction:

Thiol proteases play an essential role not only in plant growth and development but also in senescence, programmed cell death, and in deposition and mobilization of seed storage proteins. In addition, they are also involved in signaling pathways and in response to biotic and abiotic stresses (Grudkowska et al., 2004). Though endopeptidases and carboxypeptidases are present in protein bodies of dry quiescent seeds yet the function of these stored proteases during germination is still a matter of debate. Stored proteinases might be responsible for degrading small amounts of storage proteins in limited regions during germination. Numerous reports reveal that the initiation of reserve protein mobilization is attributed to *de novo* formed endopeptidases which together with stored carboxypeptidases degrade the bulk of proteins in storage organs and tissues after seeds have germinated (Muntz et al., 2001). The purification, characterization and the role of cysteine proteinases in mobilization of storage proteins has been reported primarily from legumes (Baumgartner et al., 1977; Nielsen et al., 1984; Mistushashi et al., 1986; Boylan et al., 1987; Yamoka et al., 1990; Yu et al., 1994) and cereals (Miller et al., 1981; Koehler et al., 1988; Barros et al., 1990; Holwerda et al., 1992; Kato et al.,1996; Sutoh et al., 1999). Most researchers have used azocasein or casein as the substrate for thiol proteases and have suggested its role in mobilization of seed storage proteins. No attempt has been made to purify and characterize these thiol protease using α-N-benzoyl-D,L,Arginine-β-napthylamide (BANA) as the substrate from mungbean cotyledons. Further, there is no report on immobilization of this enzyme. Immobilization is known to enhance the stability of enzymes which would be important for their industrial applications.

Proteolytic enzymes are multifunctional enzymes that have many physiological functions in plants and animals including germination, senescence, apoptosis, complement activation, inflammation process etc. and also having commercial importance in food, leather and textile industry. Commercially they are extremely important as more than 60% of the total enzyme market is made up of proteases; they are isolated from plants, animals, bacteria and fungi. Proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity as well active in wide range of pH, temperature, and in presence of organic compounds as well as other additives. Search for valuable proteases with distinct specificity is always a continuous challenge for varied industrial applications. The purified enzyme was characterized for its molecular weight, pH optimum and stability, temperature optimum and stability, substrate specificity, effect of inhibitors, metal ions, activators, urea, DMSO, ethanol, NaCl and storage stability.

Determination of molecular weight: Molecular weight of the purified enzyme was determined by molecular sieve chromatography on Sepharose 6B column and SDS-PAGE under reducing conditions.

Determination of molecular weight by Sepharose 6B column: The Sepharose 6B column

was packed as detailed under *'Materials and Methods'*. After proper equilibration with buffer A (0.1M sodium acetate buffer pH 5.5 containing 0.2 M NaCl and 1 mM EDTA), the void volume (V_0) and the total volume (V_t) of the Sepharose 6B column (90 x 0.8 cm) were determined as 36.0 ml and 115.0 ml, respectively. Various molecular weight marker proteins [2.0 mg each of carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa)] were dissolved in 2.0 ml of eluent buffer A. This mixture was loaded on the column which was eluted at a speed of 15 ml/h and fractions of 3.0 ml each were collected. The elution volume for each marker protein was calculated by estimating protein through absorbance at 280 nm. The purified thiol protease sample was eluted separately from the same column under identical conditions and the elution volume of the enzyme protein was calculated. The molecular weight of thiol protease was calculated from the standard graph plotted between log molecular weight against Ve/Vo which was found to be ~157 kDa (Fig. 1).

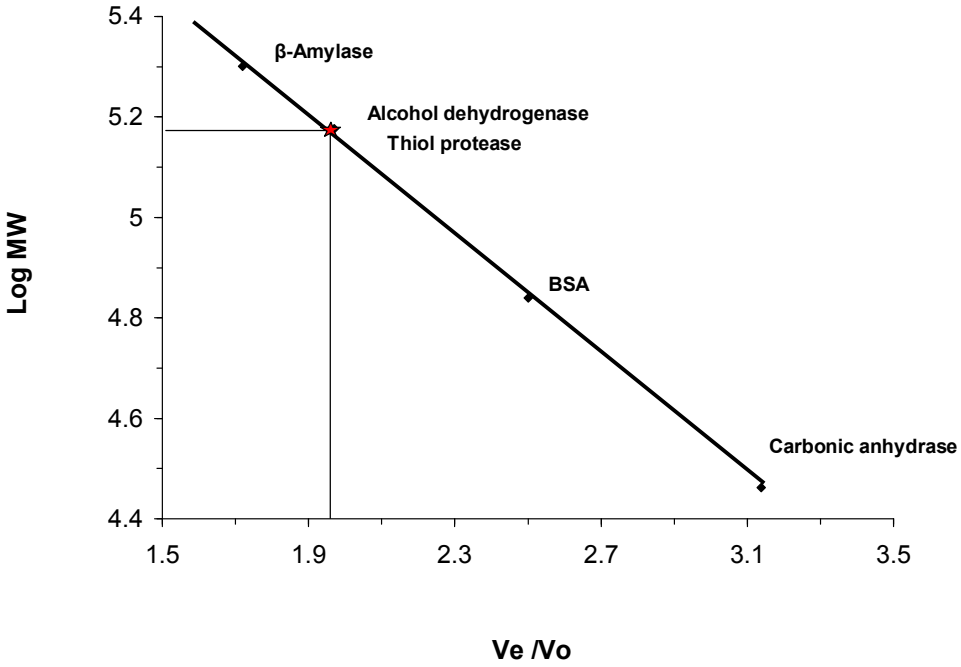

Fig. 1. Determination of molecular weight by Gel filtration through Sepharose 6B

Determination of molecular weight by SDS-PAGE: The molecular weight of thiol protease was also determined by SDS-PAGE under reducing conditions. The sample of enzyme protein and marker proteins were processed. The molecular weight of thiol protease as calculated from the standard graph plotted between log molecular weight against the relative mobility of each standard protein was found to be ~77.6 kDa.

The molecular weight of the purified native enzyme was estimated to be ~157 kDa on analytical Sepharose 6B column. On SDS-PAGE under reducing conditions the enzyme appeared as a single protein band corresponding to a molecular mass of ~ 77.6 kDa indicating that the native enzyme probably consisted of two identical subunits. The molecular weight of thiol protease has been reported as 20-35 kDa from mungbean seeds (Baumgartner and Chrispeels, 1977; Mitsuhashi et al., 1986; Yamaoka et al., 1990) and 71 kDa for buckwheat seeds (Dunaevsky and Belozersky, 1989). An acid protease has been isolated from sorghum seeds having a molecular weight of 80 kDa from gel filtration chromatography (Garg and Virupaksha, 1970). Leucine aminopeptidase from barley has a molecular weight of 260 kDa (Sopanen and Milkola, 1975). An aminopeptidase from mungbean cotyledons was found to have a molecular weight 75 kDa as determined by gel filtration (Yamaoka et al., 1990). An arginine specific peptidase has been isolated from Ragweed pollen having a molecular weight of ~ 80 kDa (Bagarozzi et al., 1998).

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