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Purification of a bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana*) by chromatography and its use as an affinity ligand

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ABSTRACT

An ammonium sulphate fraction (20–60%) of bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana*) was purified by affinity chromatography to give 6.59-fold purity with 81.48% yield. The same ammonium sulphate fraction was also subjected to ion exchange chromatography and was purified 4.28-fold with 75.95% yield. The ion exchange fraction was subjected to gel filtration and the inhibitor was purified to 6.67-fold with 67.36% yield. Further sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to check the homogeneity of purified amylase/trypsin inhibitor obtained through affinity, ion exchange and gel chromatography. The molecular weight of the inhibitor was found to be 14 kDa. This purified inhibitor was used as affinity ligand for the purification of a commercial preparation of pancreatic amylase.

1. Introduction

The amylase inhibitors are widely distributed in higher plants and microorganisms. In case of higher plants, many kinds of amylase inhibitors have been purified, mainly from cereals and legume seeds. Research on plant amylase inhibitors for most part has been concerned with cereals plants because of the importance of cereals as a rich source of protein for animal and human consumption. The inhibitors are widely distributed among cereal grains such as ragi, rye, kidney bean, barley, maize and pea [1]. Some of the amylase inhibitors are produced by microorganism such as *Streptomyces diastaticus* [2], *Streptomyces tendae*, *Streptomyces aurofaciens* [3].

Plant proteins that inhibit various types of enzymes have been extensively studied. The most commonly occurring inhibitors are the proteinase and amylase inhibitors. α-Amylase inhibitors, which are proteinaceous in nature, are widely distributed in the plant kingdom [4]. These inhibitors have been isolated and purified from several sources, particularly from cereals.

Cereal amylase inhibitors are mostly double headed in nature. Double-headed inhibitors often show two different binding sites which are specific to different proteases. However, in some cases, especially from cereals, bifunctional protease/α-amylase inhibitors have also been reported [5,6]. These inhibitors have specific action against mammalian and insect amylase and trypsin enzyme. Cereal inhibitors have generally low molecular weight (10,000–50,000). These inhibitors have natural role in the control of endogenous α-amylase activity or in the defense against pathogen and pests [7]. Amylase/trypsin inhibitors are reported to be anti-nutritional factor and have therapeutic application [8]. Apart from this defense mechanism these bifunctional inhibitors are potentially valuable 'two-in-one' affinity ligands for the purification of proteases and α-amylases.

Lately there has been considerable effort in developing affinity-based efficient bioseparation protocols for enzymes/proteins with fewer steps [9,10]. A critical factor is the cost of the affinity ligands. Hence efficient purification procedure for a double-headed inhibitor serves two purposes: (a) reduces the cost of the affinity ligand; (b) twin uses of the same affinity media mean greater convenience as well as further economy.

In the present work, the ammonium sulphate fraction (20–60%) from ragi was taken for further purification by affinity chromatography. Attempts were also carried out to purify amylase/trypsin inhibitor from ragi by ion exchange chromatography on cation exchanger. This was followed by gel filtration [11]. The purified amylase/trypsin inhibitor was further used as affinity ligand for the purification of a commercial preparation of pancreatic amylase [12].

2. Materials and methods

2.1. Materials

Ragi seeds (*Eleusine coracana*) were purchased from local market. Seralose 6B was procured from Sisco Research Lab, Mumbai,

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India. Pancreatic amylase (12.5 U/ml) was procured as a gift sample from Biocon India Ltd., Bangalore, India. Trypsin (450 U/mg) was procured as gift sample from Advanced Biochemical Ltd., Thane, Mumbai. Sulphopropyl cation exchanger (ECONO-PAC High S) pre-packed column was purchased from Bio-Rad Ltd., India. Sephadex G-50 was purchased from Amersham Bioscience Ltd., Chennai, India. Disodium hydrogen phosphate, sodium dihydrogen phosphates and enzyme grade ammonium sulphate, acrylamide, N,N-bisacrylamide, glycerol, glycine, ammonium persulphate and tetra methylene ethyl diamine (TEMED) were purchased from Sisco Research Lab, Mumbai. India. Molecular marker was purchased from Banglore Genei Pvt. Ltd. Tris buffer AR, copper sulphate pentahydrate, sodium potassium tartarate AR, Folin-Ciocalteu phenol reagents were purchased from E Merck (India) Ltd. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Estimation of amylase inhibitor/trypsin inhibitor activity and protein content

The amylase inhibitor activity was calculated using starch as the substrate [13]. The assay was carried out at 37 °C. One unit of amylase inhibitor was defined as that amount, which inhibits the activity of porcine pancreatic amylase by 1 U under the assay conditions [14]. Trypsin inhibitor activity of the crude ragi extract as well as various fractions obtained during the course of purification was estimated using casein as the substrate [15]. One trypsin inhibitor unit was equivalent to decrease in absorbance by 0.01 units at 280 nm.

Protein content of inhibitor rich fractions was determined by the Folin–Lowry method [16] using bovine serum albumin as the standard protein. Fold purity and recovery yield (% yield) were calculated as mentioned below:

$$\text{Fold purity (FP)} = \frac{\text{Specific activity after purification}}{\text{Specific activity before purification}}$$

$$\text{Recovery (\% yield)} = \frac{\text{Total activity after purification}}{\text{Total activity before purification}} \times 100$$

2.2.2. Preparation of crude inhibitor extract of ragi (E. coracana)

Preparation of crude inhibitor extract of ragi was carried out using the method followed by Saxena et al. [17].

2.2.3. Purification of amylase/protease inhibitor

2.2.3.1. Ammonium sulphate precipitation. Ammonium sulphate precipitation was carried out using the method followed by Saxena et al. [17] and the 20–60% fraction was used for further purification studies.

2.2.3.2. Affinity chromatography. Seralose 6B, agarose-based matrix was activated with Cyanogen Bromide (CNBr) using the method followed by Hermanson et al. [18]. The gel was then washed with ice-cold water and ice-cold coupling buffer (0.1 M $NaHCO_3$, pH 8.5). 20 ml of this CNBr activated matrix was suspended in equal volume of coupling buffer (0.1 M $NaHCO_3$, pH 8.5), which contained 2% (w/v) of trypsin ligand. The gel was stirred at 4 °C, 24 h and then washed several times with coupling buffer (0.1 M $NaHCO_3$, pH 8.5), 1 M NaCl and water to remove unreacted ligand. The excess active site on the gel was blocked by suspending it in 1 M ethanolamine (pH 9, 1 h) and then packed in a column (Pharmacia LKB 10 cm, internal diameter 1.0 cm; bed volume 3 ml) with equilibrating buffer (0.02 M phosphate buffer, pH 6.9, 30 ± 2 °C). The column was equilibrated with 5 column volumes of binding buffer (0.02 M phosphate buffer, pH 4.0) at a linear flow rate of 0.693 cm/min. After loading of 20–60% ammonium sulphate fraction, the column was washed using same equilibration buffer (0.02 M phosphate buffer, pH 6.9, linear flow rate of 0.693 cm/min) till the effluent was free from protein. After washing, elution was carried out using a mixture of 50 mM HCl and 20 mM NaCl (pH 3.0). The effluent fractions (0.5 ml, 20 fractions, isocratic elution) were collected using a fraction collector (Bio-Rad Model 2110). Enzyme inhibitor activities (U/ml) of all the fractions were analyzed using the aforementioned methods. The effluent protein concentration was detected at 280 nm and the elution chromatogram was plotted [11].

2.2.3.2.1. Batch study to determine adsorption of amylase/trypsin inhibitor on affinity matrix. Adsorption isotherm, required in order to determine adsorption capacity of adsorbent for amylase/trypsin inhibitor, was determined as follows.

Dilutions of ammonium sulphate fraction (20–60%) containing different concentrations of amylase inhibitor (0.095–11.44 U/ml) and trypsin inhibitor (3.3–400 U/ml) was loaded in stoppered tubes containing 1 ml of pre-equilibrated matrix (0.02 M phosphate buffer, pH 6.9, 30 ± 2 °C). The tubes containing loaded amylase/trypsin inhibitor fraction was kept for equilibration on a rocker shaker, till the supernatant equilibrium concentration of protein and enzyme activity was reached. 0.1 ml sample was removed from supernatant and assayed for enzyme inhibitor activity as described in Section 2.2.1. A graph was plotted between adsorbed enzyme inhibitor activity (q^*) versus corresponding supernatant equilibrium concentration (C^*). The plots indicate the nature of adsorption. The amount of enzyme bound to the adsorbent q^* was calculated as the difference between total amount of enzyme units loaded and units present in the supernatant after 2 h of equilibration. Maximum adsorption capacity q^{max} was determined from the plot of q^* versus C^* and the type of isotherm was found by plotting a graph of $1/q^*$ versus $1/C^*$. Dissociation constant k_d between enzyme and ligand was determined as follows:

Adsorption isotherm equation:

$$q^* = \frac{q_m C^*}{k_d + C^*} \quad (1)$$

where k_d = dissociation constant of the equilibrium reaction.

Rearranging Eq. (1) we get

$$\frac{C^*}{q^*} = \frac{C^*}{q_m} + \frac{k_d}{q_m} \quad (2)$$

Value of k_d can be determined from straight-line plot of C^*/q^* against C^*. The intercept of such plots on the C^* axis is at $-k_d$.

2.2.3.3. Purification of amylase/trypsin inhibitor by ion exchange chromatography. Purification was carried out using pre-packed Bio-Rad sulphopropyl cation exchanger (ECONO-PAC High S) matrix [19] having a column diameter of 10 mm and bed volume of 3 ml. The column was equilibrated using 4 column volumes of 0.02 M phosphate buffer, pH 6.9. 4 ml of ammonium sulphate purified enzyme inhibitor sample was loaded onto this column and linear flow rate was maintained at 0.693 cm/min. This was followed by washing the column with the equilibrating buffer (0.02 M phosphate buffer, pH 6.9) to remove unbound or weakly bound protein. Elution (0.5 ml, 16 fractions, gradient elution) was done by increasing the ionic strength of buffer (0.02 M phosphate buffer, pH 6.9) stepwise from 0.1 to 1 M using NaCl. Protein eluted in each step was checked for amylase/trypsin inhibitor activity [20].

2.2.3.4. Purification of amylase/trypsin inhibitor by gel permeation chromatography on Sephadex G-50. The partially purified ion exchange fraction (fractions 3–15) was further purified by gel chromatography. Sephadex G-50 was selected as the matrix [20,21]. 5 g of Sephadex G-50 was kept for swelling in 80 ml of 0.02 M phosphate buffer, pH 6.9. After 6 h the gel was deaerated by applying