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# Purification and Partial Characterization of Trypsin Inhibitor from Watermelon Seeds

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## ABSTRACT

Several fractions demonstrating trypsin inhibitory activity were isolated from the seeds of watermelon. One of the inhibitors was purified to apparent homogeneity and partially characterized employing ion-exchange chromatography on DEAE-Sephadex A-50, gel filtration on Sephadex G100 and affinity chromatography on Trypsin-Agarose. The inhibitor has relative molecular mass of 10,000 by gel filtration. The inhibitor was resistant to heat up to 70°C for 30 minutes and acidic condition down to pH 4.0. Possibly the inhibitor belongs to the family of low molecular weight heat resistant protease inhibitors.

## INTRODUCTION

Enzyme inhibitors are important tools of nature for regulating the activity of enzymes in cases of emergency, like pathogenic attack or germination. However, detailed functions are not clear. It is generally agreed that the presence of secretory trypsin inhibitors (Kazal) in the pancreas of vertebrates prevents premature activation of trypsinogen and in turn of other pancreatic zymogens. The predisposition to pulmonary emphysema found in individuals with genetically determined low levels of $\alpha_1$ - protease inhibitor strongly suggests protection against excessive proteolysis of lung tissues (Laurell and Eriksson, 1963). Plant seeds are known to produce a variety of proteinase inhibitors that are thought to protect the seed against insects and microbial pathogens (Antcheva *et al*, 1996), by inhibition of insects and microbial proteinases. Ryan (1989) demonstrated that wounding of potato leaves leads to great increase in the level of a polysaccharide hormone proteinases inhibitor-inducing factor, which in turn leads to huge increases in inhibitor levels.

Inhibitors of serine proteinases are the best studied among proteinases inhibitors (Bode and Huber, 1992; Richardson, 1990). The number of well-characterized inhibitors of serine proteinases far exceeds the number of described inhibitors of the three other mechanistic classes of proteinases (Laskonowski and Kato, 1980). It is not clear whether this relative abundance is a true reflection of distribution of inhibitors in nature or only of the preference and convenience of the biochemists that isolated them.

Many other exciting studies on the role of inhibitors are reported but in most cases the results while suggestive are far from unequivocal. In some cases added research complicates rather than support the naive original assignment.

Serine proteinase inhibitors were purified and characterized from many seeds and leguminous plants (Joubert, 1982; Tschesche, 1974), but to the best of our knowledge no attempt to purify and characterize the inhibitor from the seed of watermelon has been reported. In this study we report the purification and partial characterization of low molecular mass serine protease inhibitor from watermelon seeds.

## MATERIALS AND METHODS

### *Materials*

Seeds of watermelon were collected and sun-dried. DEAE-Sephadex A-50, Sephadex G100, bovine trypsin, bovine chymotrypsin, N-benzyl-L-arginine ethyl ester hydrochloride (BAEE) and N-acetyl L-tyrosine-L-arginine ethyl ester (ATEE) were from Sigma Chemical Company USA. All other chemicals used were of analytical grade and obtained from Aldrich, the Netherlands. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) molecular mass markers were purchased from Pharmacia Biotechnology AB Uppsala, Sweden.

### *Methods*

*Enzyme assay:* Protease - inhibitory activities were measured by the decrease in proteolytic activity after incubating with inhibitor for 5 minutes at 25°C. One unit of inhibitory activity is defined as being equivalent to the loss of one unit of trypsin or chymotrypsin activity (1µmole/min at 25°C). Trypsin activity was measured using BAEE as substrate by the method of Schwert and Takenaka (1955). For a

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typical experiment 0.5µg of the enzyme was incubated with 0.5µg of inhibitor fraction for 5 minutes at 25°C. The residual activity' was determined spectrophotometrically at 253nm. Chymotrypsin activity was measured using ATEE as substrate by the method of Schwert and Takenaka (1955). For a typical experiment 0.5µg of the enzyme was incubated with 0.5µg of the inhibitor fraction in a 1ml, quartz cuvette for 5 minutes at 25°C, the residual activity was estimated at 237 urn. Molar inhibitory capacity of the inhibitor towards trypsin and chymotrypsin was determined with BAEE and ATEE respectively.

*Protein determination:* Protein concentration was determined by Warburg and Christian method (1945).

*Polyacrylamide gel electrophoresis:* this was carried out in the presence of sodium dodecyl sulphate and 2-mercaptoethanol and was performed according to the method of Laemmli (1974) using Pharmacia mini electrophoresis system.

*Thermal stability:* Thermal stability of the inhibitor was checked between 30°C to 70°C by incubating the inhibitor at the specified temperature for a period of 5-30 minutes. The residual inhibitory activity was determined as described previously.

*pH stability:* The effect of pH on the inhibitory properties of the inhibitor was checked between pH 4.0-10.0. The control experiment was carried out at these pH values. Buffers used for the assay were 0.05M glycine/HCl (pH 4.0), 0. 05M sodium acetate (pH 5.0-6.0), 0.05 M Tris/HCl (pH 7.0-8.0) and 0.05M glycine/NaOH (pH 9.0-10.0).

*Purification:* 100g of watermelon seeds were finely grounded, dissolved in 0. 05M. Tris/HCl buffer pH 7.0 and left over night. The resultant suspension was macerated for 5 minutes in a Warring blender and then centrifuged at 10,000 rpm for 30 minutes to remove seed debris. The supernatant was subjected to 70% $(NH_4)_2SO_4$ precipitation. The precipitated material was centrifuged at 10.000 rpm for 30 minutes. The resultant precipitate was dissolved in 0.05M Tris/HCl, pH 7.0 and dialyzed with the same buffer in Amicon ultrafiltration unit containing a PM5 membrane. The dialyzed material was applied to a column (5.0 x 20 cm) DEAE-Sephadex A-50 resin equilibrated with 0.05M Tris/HCl, pH7.0 at a flow rate of 40 ml/hr. Several fractions (A1-A4) with trypsin inhibitory activity' were obtained (figure 1). Fractions with highest trypsin-inhibitory activity (AI) were pooled and concentrated in an Amicon ultrafiltration unit containing a PM5 membrane. The concentrated material was applied to a column (1.6 x 90 cm) of Sephadex G100 resin equilibrated with 0.05M Tris/HCl pH 7.0 at 2. flow rate of 8 ml/hr, fractions with highest inhibitory activity were pooled and concentrated. The concentrated material was applied to the column (1.0 x 5.0 cm) of Trypsin -Agarose equilibrated with 0.05 Tris/HCl, pH 7.0 at a flow rate of 4 ml/hr. The column was washed with equilibration buffer until the absorbance of the eluent at 280nm returned to base line. The above material was eluted with 0.05M Glycine/HCl buffer, pH 3.0 (figure2). The pH of the eluted material was immediately neutralized with 0.1M NaOH.

## RESULTS AND DISCUSSION

Summary of the purification results is shown in Table 1. From table 1, it can be seen that the inhibitor is purified 270 times with about 42% yield. Watermelon seeds seem to be a rich source of thermostable trypsin inhibitor. The inhibitor shows strong trypsin inhibitory activity and a moderate inhibitory activity against chymotrypsin. The inhibitory characteristics of the inhibitor against bovine trypsin and chymotrypsin are shown in figure 3a and 3b respectively. The titration curves showed that the inhibitor stoichiometrically inhibited trypsin at 1:1 molar ratio, chymotrypsin was also inhibited but the binding of the enzyme by the inhibitor was very much weaker. This differential inhibition was not surprising, since it has been reported that Bowman-Birk type of proteinase inhibitors from legume seeds have been found to inhibit both trypsin and chymotrypsin at independent reactive sites (Joubert, 1982). The purity of the final preparation was checked on SDS-PAGE. It was found to be homogenous with a single band that was close to the dye front. The relative molecular mass of the inhibitor was found to be 10, 000 by gel filtration on Sephadex G100.

**Table 1. Purification results of trypsin inhibitor from watermelon seeds**

| Step | Total Protein (mg) | CPN | Total Activity | Purification fold | % Yield |
|---|---|---|---|---|---|
| Crude homogenate | 2000 | 0.03 | 60 | 1 | 100 |
| $(NH_4)_2SO_4$ precipitation | 12.00 | 0.04 | 48 | 1.3 | 80 |
| DEAE-Sephadex A50 | 30.8 | 1.2 | 36.4 | 40 | 61.7 |
| SephadexG100 | 7.2 | 3.8 | 27.4 | 126.7 | 45.7 |
| Trypsin-Agarose | 3.1 | 8.1 | 25.11 | 270 | 41.9 |

**Figure 1: Ion exchange Chromatography on DEAE-Sephadex A-50 (pH 7.0)**

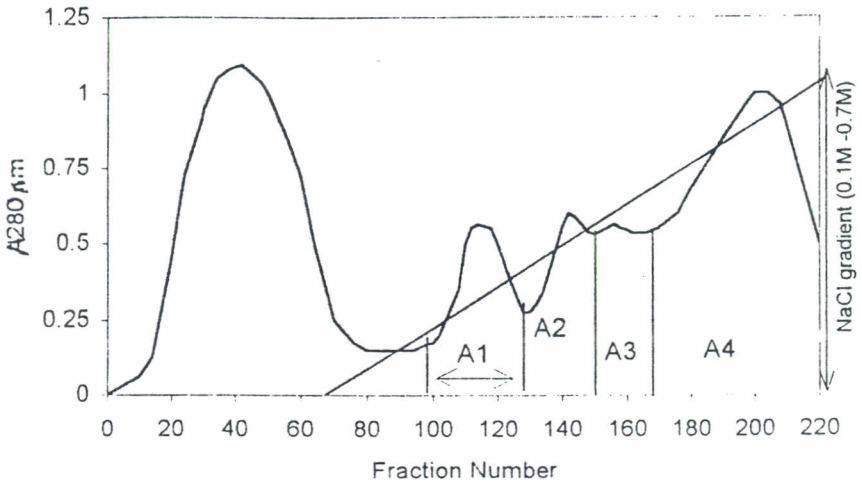
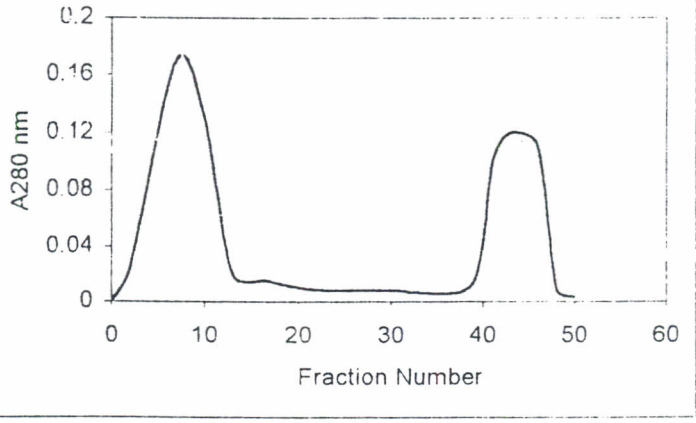



**Figure 2: Affinity Chromatography onTrypsin Agarose**



The inhibitor could be classified as Bowman-Birk type because of its low relative molecular mass. Protease inhibitors from leguminous seeds are divided into two general classes depending on their relative molecular mass and cystine contents. These are, Bowman-Birk types that have relative molecular mass of 8, 000-10, 000 and a high cystine content usually 7 disulphides (Joubert, 1982). The inhibitors from soybeans (Odani and Ikenaka, 1972; 1978), Lima beans (Tan and Stevens, 1971; Stevens et al, 1974), garden beans (Wilson and Lowskowwski, 1975), adzuki beans (Ishikawa *et al*, 1979) and macrotyloma axillare seed (Joubert *et al*, 1979) belong to this class. The other class namely the Kunitz-type proteinase inhibitors, have molecular mass approximately 20, 000 and low cystine content. The Kunitz soybean trypsin inhibitor is a representative of this class (Koide and Ikenaka, 1973).

*Thermal stability:* The inhibitory activity of the inhibitor was found to be unaffected by incubation in solution at temperature up to 70$^0$C for 10 minutes. However, the inhibitor lost 10% and 14.6% of its activity by incubating for 30 minutes at 60$^0$C and 70$^0$C respectively (figure 4). Similar heat resistant ability was reported for trypsin inhibitor from Paprika (*Capsicum annuujm*) seeds (Ryan, 1989). The heat resistance ability of the inhibitor could be due to the presence of many disulphide linkages. Bowman-Birk types of inhibitors were reported to contain many disulphide linkages (Joubert, 1982).

*pH stability:* The inhibitor retained its inhibitory activity at pH 4.0 - 8.0 and only lost 5% of its activity at pH 9.0 and 10.0 (figure *5)*. The loss of inhibitory activity at these pH values could be due to the effect of alkaline solution on the stability of trypsin - inhibitor complex.

This trypsin inhibitor from watermelon may belong to the class of Bowman-Birk type of inhibitors, which are low molecular weight, heat and acid resistance trypsin inhibitors.

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Figure 3a:Molar Inhibitory Activity of the Inhibitor against Bovine trypsin

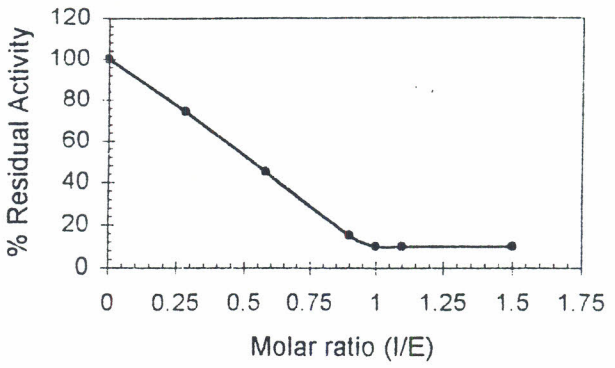


Fig. 3b:Molar Inhibitory Activity of the Inhibitor against chymotrypsin

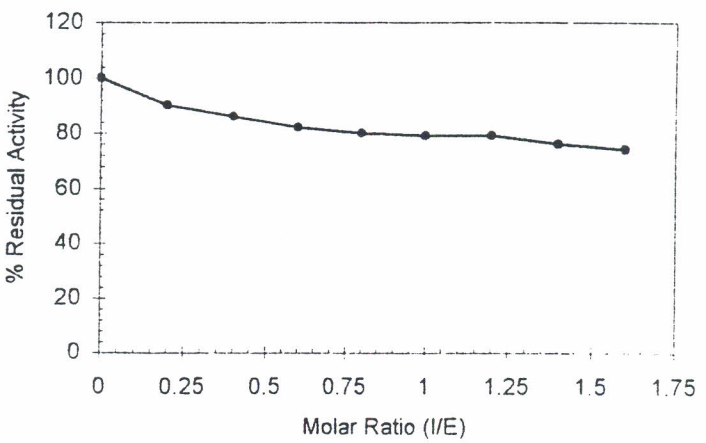

Fig.4: Effect of Temperature on Inhibitory Activity of the Inhibitor

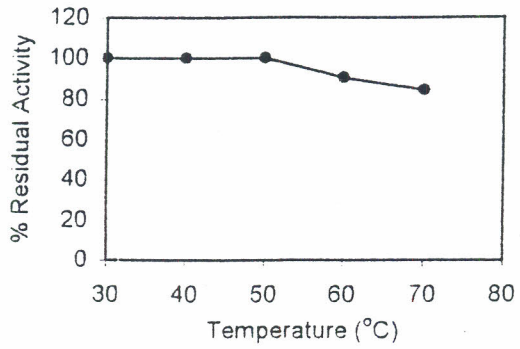


Fig.5: Effect of pH on Inhibitory Activity of the Inhibitor