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Short communication

Glycosylation, amino acid analysis and kinetic properties of a major Kunitz-type trypsin inhibitor from *Acacia victoriae* Bentham seeds

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ABSTRACT

An *Acacia victoriae* trypsin inhibitor (AvTI) was purified from the seeds of prickly wattle (*A. victoriae* Bentham) by salt precipitation, ion-exchange and gel filtration chromatography, and its degree of glycosylation, amino acid composition, and kinetic properties were determined. Gel electrophoresis revealed at least four glycoprotein bands in the crude extract, salt-precipitated and ion-exchange protein fractions, while the purified AvTI showed only one band and a degree of glycosylation of 2.06%. Glutamate (13.3%), aspartate (10.3%), leucine (7.62%) and lysine (7.01%) were the major amino acids in AvTI while the contents of sulphur-containing amino acids, cysteine (1.38%) and methionine (0.75%), as well as of tryptophan (1.17%) were low. Its dissociation constant (K_i) for the inhibition of bovine trypsin was found to be 1.06×10^{-8} M, indicating a high affinity between AvTI and this enzyme, and its role as a competitive inhibitor was confirmed by a double reciprocal plot. These results complement our earlier studies which indicated the presence of three isoforms of this Kunitz-type trypsin inhibitor in prickly wattle seed.

1. Introduction

Acacia victoriae Bentham (prickly wattle) is one of the most economically viable native leguminous plants in Australia (Maslin & McDonald, 2004). Wattle seed meal is recognised as having a significant commercial potential due to its high levels of proteins and carbohydrates, which can be used as ingredients for the manufacture of many food products (Forbes-Smith & Paton, 2002; Seigler, 2002). Several studies have investigated processing-related functionality of wattle seed extracts, including their protein profile, emulsifying properties (Agboola, Ee, Mallon, & Zhao, 2007), and foam and gel formation characteristics (Ee, Rehman, Agboola, & Zhao, 2009a). The nutritional qualities, especially amino acid profile, as well as the influence of environmental factors on the structural properties of the major protein fraction of *A. victoriae*, were also reported (Agboola & Aluko, 2009). These studies have mainly focused on the beneficial properties of wattle seed components as innovative forms of food ingredient, in an attempt to enhance their commercial uptake by the food industry.

Plant seeds contain proteins in amounts varying from ~10% in cereals to ~40% (dry weight) in certain legumes and oilseeds. They also generally have a high concentration (5–15% of total protein) of protease inhibitors (Bhattacharyya, Mazumdar, Leighton, & Babu, 2006; Liener, 1980; Richardson, 1991). From a nutritional perspective, inhibitors of serine proteases, trypsin and chymotrypsin, which are commonly found in plant foods, are the most important and have been studied extensively due to their detrimental role in human and animal nutrition (Belitz & Weder, 1990). On the other hand, the important physiological roles and potential beneficial functions of these inhibitors are also increasingly being recognised. These include roles of protease inhibitors as stores of sulphur-containing amino acids during seed development, as regulators of endogenous enzymes, and as defensive agents against proteolytic action of the digestive enzymes of pathogens and pests (Lawrence & Koundal, 2002; Richardson, 1991; Shewry, 2000).

Plant protease inhibitors are classified, based on the active amino acid at their reactive site, into serine-, cysteine-, aspartic-, and metallo-protease inhibitors, with serine-protease inhibitors being the most widely studied. Kunitz and Bowman Birk families of serine-protease inhibitors are found abundantly in various leguminous plants (Liener, 1980; Richardson, 1991). The Kunitz-type trypsin inhibitors are proteins of molecular mass ~20 kDa that typically contain four cysteine residues, forming two disulphide

Abbreviations: AvTI, *Acacia victoriae* trypsin inhibitor; FPLC, fast-performance liquid chromatography; kDa, kiloDaltons; K_i, inhibition constant; K_m, Michaelis–Menten constant; K_{mapp}, apparent Michaelis–Menten constant; ME, β-mercaptoethanol; MW, molecular weight; PAS, periodic acid-Schiff; RP-HPLC, reversed phase-high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TAME, p-toluenesulfonyl-L-arginine methyl ester; UPLC, ultra-performance liquid chromatography.

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bonds and possessing a single reactive site (an arginine residue) located in one of the protein loops (Richardson, 1991). Two polypeptide chains (one major and one minor), linked by disulphide bonds, have been reported for Kunitz-type trypsin inhibitors found in the Mimosoidae subfamily of the Leguminosae, such as seeds of *Acacia elata* (Kortt & Jermyn, 1981), *Acacia confusa* (Lin, Chu, Wu, & Hsieh, 1991) and *Dimorphandra mollis* (Mello et al., 2001).

We have previously reported the presence of trypsin and α-chymotrypsin activities in the extracts of prickly wattle, which were identified as several bands by gel electrophoresis (Ee, Zhao, Rehman, & Agboola, 2008). We also documented the isolation and partial characterisation of the major Kunitz-type trypsin inhibitor, which was reported to consist of two polypeptide chains with a molecular mass of ~18.3 kDa (Ee, Zhao, Rehman, & Agboola, 2009b). We observed that the *A. victoriae* trypsin inhibitor (AvTI) was of the Kunitz type, existing in at least three isoforms with a narrow range of molecular masses, which suggested the possibility of glycosylation in its native form although the archetypical Kunitz-type inhibitor, soybean trypsin inhibitor, is not a glycoprotein (Kunitz, 1947). Furthermore, since only partial (N-terminal) sequencing of AvTI was carried out, a complete amino acid composition is warranted for a better comparison with other Kunitz-type inhibitors. Significantly, it is not clear what type of inhibitor class AvTI belongs to in terms of its kinetic properties against the enzyme trypsin. The objective of the present study, therefore, was to further characterise AvTI by determining its degree of glycosylation, amino acid composition and inhibition kinetics against trypsin.

2. Materials and methods

2.1. Materials

Whole wattle seeds (*A. victoriae* Bentham) were supplied by Outback Bushfoods, Alice Springs, Australia. GelCode® Glycoprotein Staining Kit (product No. 24562) and Glycoprotein Detection Reagent (product No. 23260) were purchased from Pierce Biotechnology, Rockford, Illinois, USA. All other chemicals used were of analytical grade and purchased from Sigma–Aldrich, Castle Hill, NSW, Australia, or E. Merck, Darmstadt, Germany, unless stated otherwise.

2.2. Isolation and purification of wattle seed protease inhibitors

Wattle soluble proteins were extracted and purified using AKTA fast protein liquid chromatography (FPLC) according to the procedures described in a previous study with the purification stages being monitored by continuous assay of trypsin activity (Ee et al., 2009b). The fraction precipitated with 25–50% (w/v) ammonium sulphate, which had the highest trypsin inhibition activity of the salt fractions, was designated as AS-2. The three active anion-exchange fractions were pooled and designated as 3-I. The active gel permeation fraction was designated as G-90m and the purified anion-exchange fraction, obtained from G-90m, was designated as *A. victoriae* trypsin inhibitor (AvTI).

2.3. Glycoprotein carbohydrate analysis

SDS–PAGE (PhastGel gradient 10–15) of crude extract and protease inhibitors, at all stages of purification, was carried out using the Pharmacia Phast System (GE Healthcare Life Sciences, Uppsala, Sweden). The protein samples (crude extract, AS-2, 3-I, G-90m and AvTI) with final concentrations of 2–5 mg/ml, plus a positive (horseradish peroxidase) and negative control (soybean trypsin inhibitor) were treated with Laemmli's buffer (Laemmli, 1970), and incubated for 10 min at 100 °C before loading onto the gels. One of the gels was stained by PhastGel blue R and the other gel was stained using the GelCode® Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, Illinois, USA) according to the manufacturer's instruction.

Glycoprotein carbohydrate estimation was carried out by using the Glycoprotein Carbohydrate Estimation Kit (Pierce Biotechnology, Rockford, Illinois, USA) and the degree of glycosylation (%) was calculated according to the manufacturer's instruction, in comparison with the provided standards. The optical density of diluted protein sample (1 mg/ml) and standards was obtained using a FLUOstar Omega UV–vis spectrophotometer (BMG Labtech, Offenburg, Germany) at 550 nm. Reported results were based on triplicate measurements.

2.4. Amino acid analysis

For high sensitivity amino acid analysis, purified samples of (AvTI) underwent 24 h gas phase hydrolysis in 6 M HCl at 110 °C. Cysteine analysis was performed using performic acid oxidation, followed by 24 h acid hydrolysis with 6 M HCl at 110 °C. The samples for tryptophan analysis were subjected to 24 h liquid hydrolysis in 5 M NaOH at 110 °C. After hydrolysis, amino acid composition was determined, using an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation, Massachusetts, USA) equipped with a Waters AccQ-Tag™ Ultra column.

2.5. Determination of inhibition kinetics

A Lineweaver–Burk double reciprocal plot (Greco & Hakala, 1979; Lineweaver & Burk, 1934) analysis was employed to determine the constants of inhibition for bovine trypsin (EC 3.4.21.4) by pre-incubating the enzyme (0.1 ml of 0.84 mM bovine trypsin in 1 mM HCl) with increasing concentrations of AvTI in 2.6 ml of assay buffer (41.4 mM Tris–HCl, pH 8.1, with 10.3 mM $CaCl_2$). Enzyme inhibition was carried out using 0.3 ml of at least five different *p*-toluenesulfonyl-L-arginine methyl ester (TAME) concentrations (0.05–10.0 mM). AvTI (13 kDa) was prepared to achieve final concentrations of: 0.00, 0.02, 0.04 and 0.06 μM. The molecular mass of AvTI was calculated by SDS–PAGE analysis under reducing conditions (Ee et al., 2009b). A mixture of the assay buffer (2.6 ml), TAME (0.3 ml) and 0.1 ml of 1 mM HCl, without enzyme, was used as a reference blank. The absorbance at 247 nm (A_{247}) was measured immediately and continuously for 3 min, using a Cary 50 UV–vis spectrophotometer (Varian, Inc., California, USA). The inhibition constant (K_i) was estimated by subjecting the Michaelis–Menten constant (K_m) and apparent Michaelis–Menten constant (K_{mapp}), which were determined by Lineweaver–Burk double reciprocal plot, to the Michaelis–Menten competitive inhibition equation (Dixon, 1953): $K_i = [K_m]\,[\text{Inhibitor}]/[K_{mapp} - K_m]$. Each analysis was carried out in triplicate.

3. Results and discussion

3.1. Glycoprotein carbohydrate analysis

Fig. 1A shows the SDS–PAGE gel electrophretogram obtained for the crude extract of wattle seed, AS-2, 3-I, G-90m and AvTI from ion-exchange chromatography under reducing conditions with glycoprotein staining, while Fig. 1B shows its Coomassie-stained counterpart. Electrophoresis was performed on the two gels, simultaneously, to ensure that the protein bands on the two gels aligned with each other. The glycoprotein staining gel (Fig. 1A) revealed a positive periodic acid-Schiff (PAS) reaction in all