1. Introduction

*Araucaria angustifolia* is the major native conifer with economic importance in Brazil, found mainly in the states of Paraná, Santa Catarina and Rio Grande do Sul. Its seeds, popularly known as pine nuts, are the source of protein for many native species, such as birds and rodents, as well as for human beings (Machado Mello & Peroni 2015). Amylase and protease inhibitors are widely distributed in angiosperm seeds and perform multiple functions such as...
as storage proteins, defence and regulating endogenous enzymes (Oliva & Sampaio 2009). There are many plants, have not already been studied, in which the possibility of finding new types of inhibitors could open new possibilities of biotechnological application (Konarev et al. 2004). Serpins are members of an extensively studied super family of protease inhibitors with peculiar characteristics. Their molecular weight vary between 30 and 50 kDa, they are resistant to drastic changes in pH, are thermolabile with a unique suicide-like substrate mechanism of inhibition (Gettins 2002). Most of the serpins are irreversible inhibitors of serine proteases. Their structures are highly conserved, although individual molecules may assume different conformations in accordance with the mechanism and regulation of its activity (Pearce et al. 2007). Protease inhibitors extracted from plants are an interesting source of new drugs as deacetyl-3-cinnamoyl-azadirachtin, extracted from *Azadirachta indica* leaves which showed NS3/4A inhibitory activity (Ashfaq et al. 2015). Furthermore, proteases itself extracted from plants can play several industrial applications (Khan et al. 2015). The aim of this work was to verify the presence of amylase or protease inhibitors in *A. angustifolia* seeds.

2. Results and discussion

The *A. angustifolia* (AaTI) extract showed no inhibitory activity on amylases, but satisfactory activity on trypsin, being this activity present only in the seed embryo (Figure 1). The inhibitor isolation was carried out by using of the trypsin-sepharose affinity chromatography (Figure S1 – see supplementary material). A reverse-phase high-performance liquid chromatography on a C\textsubscript{18} column of the B fraction (AaTI fraction) revealed two peaks, at 9.9 and 27.6 min retention time (Figure S2 – see supplementary material). However, amino acid analysis of both peaks showed only the peak eluting at 27.6 min had protein content (Table S1 – see supplementary material). SDS-PAGE electrophoresis of the AaTI fraction showed a double band around 35 kDa, and the profile does not change after treatment with a reducing agent (see Figure S3). Their molecular weight similarity was confirmed by mass spectrometry, where the peak 27.6 showed two molecular species (35,450 and 36,955 Da, Figure S4 – see supplementary material). The N-terminal sequence of this peak shows two parallel sequences, confirming the presence of two molecular forms. The sequences are **EIESFVLQNQVNIKQMI** and **QNSNVIRIASPN**. Figure S5 (see supplementary material) shows the similarity between

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**Figure 1.** Determination of crude extracts inhibitory activity of the endosperm and embryo on α-amylase and trypsin. *p* value <0.0001 was considered to indicate significance (****).
the sequences of AaTI and the serpin class of inhibitors (Rawlings et al. 2010). It is clear for us that the 35.450 Da form correspond to the AaTI without reactive centre loop, cleaved by trypsin coupled in the chromatography column, but it is a question for further investigation. AaTI is thermolabile, being virtually inactive at 60 °C (Figure S6 – see supplementary material) and resistant to pH changes, (Figure S7 – see supplementary material). These behaviours are in agreement with the general characteristics of the serpin family (Guo et al. 2015). AaTI is a good inhibitor for trypsin ($K_{\text{app}} = 85$ nM), similar to those described for other serpins (Roberts & Hejgaard 2008), and a less effective for plasmin ($K_{\text{app}} = 7.04$ μM), (Figures S8 and S9 – see supplementary material). Figure S10 (see supplementary material) shows the results of a fibrin plate assay, where AaTI clearly blocked the fibrinolysis. AaTI does not block the action of other proteases like human plasma kallikrein, porcine pancreatic kallikrein and chymotrypsin (data not shown).

The inhibition mechanism of the serpins is mainly irreversible (Gettins 2002); however, the asα-2-antiplasmin (SerpinF2) and TriaeZ2a, a serpin found in wheat (Ostergaard et al. 2000), have a reversible mechanism of inhibition. This fact corroborates with our hypothesis that AaTI is a Serpin, since the purification of the inhibitor by trypsin-Sepharose affinity chromatography indicates a reversible mechanism of inhibition.

The description of this inhibitor is quite relevant, since only a few serpin inhibitors have been characterised in plants. These results lead to an additional motivation for the development of iotech products, once the AaTI could be coupled to a Sepharose resin and used to purify human plasmin, a commercially very expensive enzyme.

4. Conclusions

Our studies of the inhibitory properties of *A. angustifolia* seed showed no inhibitory activity against amylases, but satisfactory activity against two serine proteases, trypsin and human plasmin. The inhibitor was named AaTI and it exhibited same features of the serpin family, such as molecular weight, thermolability, resistance to pH change and the N-terminal sequence similar to those of other members of the serpin super family.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Proc. 2009/53799-5); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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