Identification of a novel bean α-amylase inhibitor with chitinolytic activity

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Received 9 September 2005; revised 13 September 2005; accepted 14 September 2005

Available online 28 September 2005

Edited by Stuart Ferguson

Abstract ***Zabrotes subfasciatus*** **is a devastating starch-dependent storage bean pest. In this study, we attempted to identify novel α-amylase inhibitors from wild bean seeds, with efficiency toward pest α-amylases. An inhibitor named *Phaseolus vulgaris* chitinolytic α-amylase inhibitor (PvCAI) was purified and mass spectrometry analyses showed a protein with 33 330 Da with the ability to form dimers. Purified PvCAI showed significant inhibitory activity against larval *Z. subfasciatus* α-amylases with no activity against mammalian enzymes. N-terminal sequence analyses showed an unexpected high identity to plant chitinases from the glycoside hydrolase family 18. Furthermore, their chitinolytic activity was also detected. Our data provides compelling evidence that PvCAI also possessed chitinolytic activity, indicating the emergence of a novel α-amylase inhibitor class.**

Keywords: α-Amylase inhibitor; Chitinase; *Zabrotes subfasciatus*; Bruchids; *Phaseolus vulgaris*

1. Introduction

Leguminous plants have become a popular research source, due to the abundance of proteins and peptides involved in plant defense. Amongst these proteins are lectins [1], arcelins [2], chitinases [3], β1,3-glucanases [4], defensins [5] and digestive enzyme inhibitors [6,7]. Such peptides play a key role in defense towards insect-pest predators [8] and pathogens such as bacteria and fungi [9]. A combination of different proteins can be found in unique plant species, which act synergistically against both [10]. Multifunctionality in a single protein can also be observed, contributing to plant defense barriers [11].

In the last two decades, several α-amylase inhibitors have been described in numerous plant species [11,12]. As proposed by Richardson [13], α-amylase inhibitors may be conveniently classified by their tertiary structure into six different classes: lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like. Their specificity has been widely explored, with some capable of acting only against insect α-amylases or against mammalian enzymes [14]. A number of inhibitors have received particular attention as attractive candidates for pest-control due to bifunctional properties. The combination of simultaneous biological activities, inhibiting serine proteinases and α-amylases, is useful and possible for these inhibitors [15].

Although bifunctional proteinase-α-amylase inhibitors are relatively common, only a few reports have described different secondary functions for α-amylase inhibitors. One remarkable example was found in cereal kernels known as Jobs Tear's (*Coix lachryma-jobi*), which showed the presence of a proteinaceous factor with insect α-amylase inhibitory activity and also the capability of hydrolyzing chitin [16]. Chitinase activity is also recognized to play a defense function [17], as an integral part of plant responses to infections and stresses, and as a consequence has great biotechnological potential, especially toward bean bruchids. Storage bean pests, such as the Mexican bean weevil (*Zabrotes subfasciatus*), are extremely starch dependent, feeding on bean seeds and causing severe economical losses. As they possess an external exoskeleton and an internal perithrophic gut membrane covered with chitin, such structural and functional characteristics make bean weevils doubly susceptible to α-amylase inhibitors with additional chitinolytic activity.

Recently, two plant protein classes, designated as XIP (xylanase inhibitor protein) [18] and TAXI (*Triticum aestivum* xylanase inhibitor) [19] have been shown to inhibit xylanases, another type of glycoside hydrolyse (GH). Only XIP proteins were able to inhibit α-amylases [20], showing sequence and structural similarities to chitinases of the family GH18, despite its lack of enzymatic activity [21]. TAXI-I displays a similar structural homology to a pepsin-like family of aspartic proteases, despite their absence of a proteolytic function [22].

Data presented in this report provides compelling evidence that some inhibitors maintained their enzymatic activities, indicating the emergence of a novel α-amylase inhibitor class named "bifunctional chitinolytic α-amylase inhibitor" that could lead to development of novel biotechnological tools with multiple activities toward insect-pests.

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Abbreviations: α-AI2, bean α-amylase inhibitors variant 2; AMY-1, α-amylase 1 from barley; CAZy, carbohydrate active enzymes; GH, glycoside hydrolase; MALDI-ToF, matrix-assisted laser desorption ionization time of flight; PvCAI, *Phaseolus vulgaris* chitinolytic α-amylase inhibitor; PPA, porcine pancreatic α-amylase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAXI, *Triticum aestivum* xylanase inhibitor; XIP, xylanase inhibitor protein; ZSA, *Zabrotes subfasciatus* α-amylase

0014-5793/$30.00
doi:10.1016/j.febslet.2005.09.030

2. Materials and methods

2.1. Isolation of digestive α-amylases from Z. subfasciatus larvae

α-Amylases from the Mexican bean weevil *Z. subfasciatus* larvae were obtained following dissection and collection from the luminal fluid of 18–20 day old larvae reared in common bean (*Phaseolus vulgaris*) seeds. Midguts were dissected in ice-cold 25 mM NaCl. Freshly dissected guts were homogenized and centrifuged at $4000 \times g$ for 20 min at 4 °C to remove the gut walls and cellular debris.

2.2. Purification of P. vulgaris α-amylase inhibitor/chitinase from bean seeds

Wild common bean seeds (*P. vulgaris*) were obtained from Embrapa Rice and Bean (CNPAF/EMBRAPA), Brazil, accession number G12953. Cotyledons were ground into a flour and extracted with 0.15 M NaCl and 0.1% HCl (1:5, w/v, meal to buffer ratio) with continuous stirring for 5 h at 4 °C. The material was then centrifuged at 10000 g, 4 °C for 30 min. The precipitate was discarded and supernatant was submitted to fractionation with ammonium sulfate. Following dialysis, the fraction obtained between 0% and 85% saturation ($F_{0–85\%}$) was applied to an ionic exchange DEAE-Cellulose column equilibrated with 20 mM KPO_4 buffer, pH 6.7, with a flow rate of 30 ml h^{-1}. Retained proteins were displaced with a 0–0.2 M NaCl linear gradient, and applied onto a HPLC reversed-phase analytical column (Vydac 218 TP 1022 C-18) at a flow rate of 1.0 mL min^{-1}. The material in individual peaks was collected, lyophilized and stored at 22 °C. Bean α-amylase inhibitors variant 2 (α-AI2) was purified from the same seeds according Grossi-de-Sa et al. [23].

2.3. α-Amylase and α-amylase inhibitory activity assays

α-Amylase and α-amylase inhibitory activities were measured by the Bernfeld method [24] in 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$. Each assay contained 6.0 U of α-amylase activity (2.5 μM), with inhibitors α-AI2 and *P. vulgaris* chitinolytic α-amylase inhibitor (PvCAI) tested at a standard concentration of 300 μg ml^{-1} or 5.5 and 9.0 μM, respectively, as determined by Bradford method [25]. One α-amylase unit (1 UI) was defined as the amount of enzyme that increased the absorbance at 530 nm by 0.1 OD during 25 min of the assay. Assays were carried out in triplicate. Triplicate inhibition values differed by no more than 10%.

2.4. Chitinolytic activity

Chitinolytic assays were measured using PvCAI and α-AI2 at 300 μg ml^{-1} standard concentration or 5.5 and 9.0 μM, respectively, dissolved in 50 mM acetate sodium buffer, pH 5.0. Chitin azure (2.5 mg ml^{-1}) was used as substrate and enzyme reactions were carried at 37 °C for 24 h. After stopping reactions, mixtures were centrifuged and supernatant optical densities were measured at 575 nm. Each assay was carried out in triplicate, using distilled water as negative control and purified *Trichoderma* sp. chitinase from the GH 18 family at a standard concentration of 300 μg ml^{-1} (9.2 μM) as a positive control.

2.5. Molecular mass analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted as described by Laemmli [26] at room temperature, using low protein molecular weight markers (6.5–66.0 kDa) (GE – Amersham Pharmacia Biosciences) and bromophenol blue as the tracking dye. Freeze-dried samples of HPLC peaks were prepared for matrix-assisted laser desorption time of flight analysis (MALDI-ToF) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA, USA). Samples were dissolved in trifluoroacetic acid 1.0% and the matrix sinapinic acid added (a saturated solution dissolved in acetonitrile per 0.1% TFA 1:1, v:v) (Sigma Co.). Analyses were conduced according Franco et al. [14].

2.6. N-terminal amino acid sequence determination and comparison

Samples were reduced according to Crestfield et al. [27]. Reversed phase HPLC (Vydac C-18), using a linear gradient of 0–100% acetonitrile in 0.1% (v:v) aqueous trifluoroacetic acid, was used to desalt the samples. N-terminal sequences for 25 residues were determined from 2 to 5 pmol of alkylated samples, using an automatic standard Edman degradation in a PPSQ-23 sequencer (Shimadzu Co). The resulting sequence was compared with the SWISSPROT sequence database using the Fasta3 program. The alignment and the calculation of percentage identities were conducted with CLUSTALW.

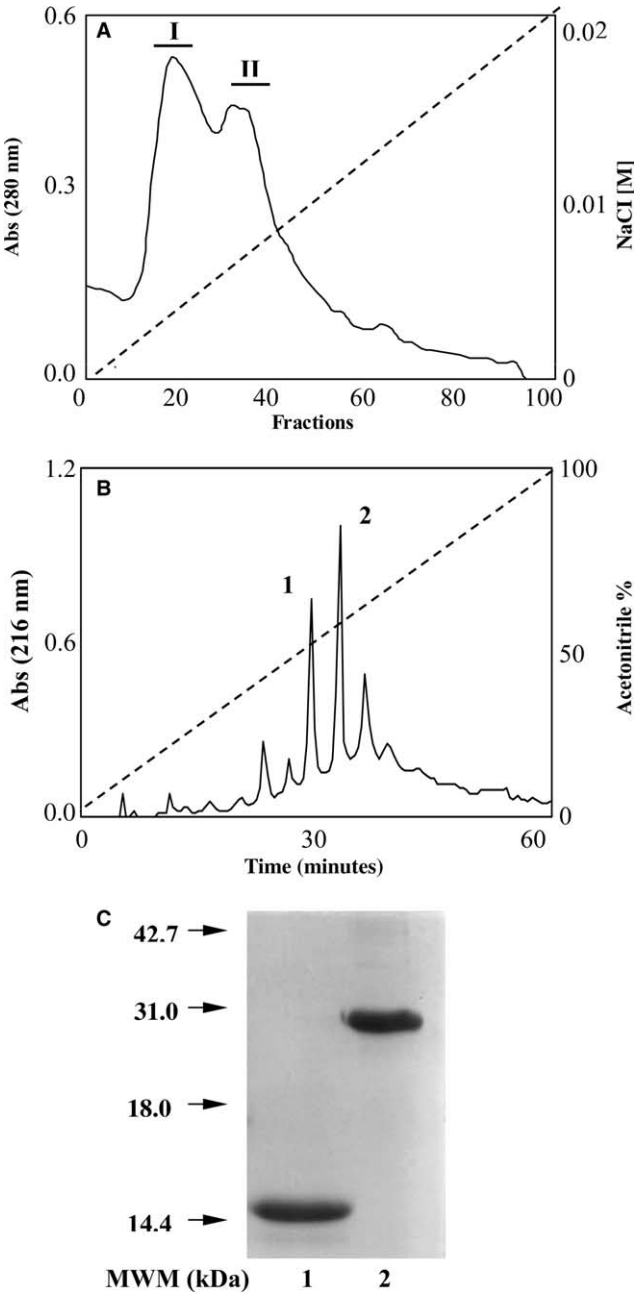

Fig. 1. (A) Retained fraction obtained from ionic exchange DEAE-cellulose chromatography, equilibrated with 20 mM KPO_4 buffer, pH 6.7, with a flow rate of 30 ml h^{-1}. Dashed line represents a 0–0.2 M NaCl linear gradient. (B) Semi-preparative reversed-phase HPLC of fraction II from DEAE-cellulose. The separation was carried out on a Vydac 218TP column using a flow rate of 2.5 ml min^{-1}. TFA (0.1%) was used as ion-pairing agent and the dashed line indicates the acetonitrile gradient. The sample contained 5.0 mg of protein. (C) SDS–PAGE analysis of fractions 1 and 2, stained with Commassie Brilliant Blue R-250.

3. Results and discussion

A crude inhibitor preparation from common bean cotyledons was obtained by aqueous solution extraction and ammonium sulfate precipitation [23]. This fraction was applied on to a DEAE-sepharose ion exchange column from which two principal peaks could be eluted, as shown in Fig. 1A. The absorbance of these peaks was monitored at 280 nm with each identified generically as peaks I and II. Both fractions were tested against porcine pancreatic α-amylase (PPA), showing no inhibitory activity. Peak II was capable of markedly reducing *Z. subfasciatus* α-amylase (ZSA) activity (data not shown). Analytical reversed-phase HPLC of fraction II yielded a number of peaks, shown in Fig. 1B. The absorbance of these peaks was monitored at 216 nm and identified generically as peaks 1 and 2. In both fractions totally purified bands with molecular masses of approximately 14.0 and 31.0 kDa were observed (Fig. 1C). The first fraction was matched by N-terminal sequencing (data not shown) as α-AI2 [23]. This identification was confirmed by mass spectrometry (data not shown), in which the observed masses match perfectly with molecular masses previously reported for α (12 366 Da) and β (15 357 Da) chain sequences. Mass spectrometry results clearly showed the absence of heterotetramers, which have been described previously for a number of lectin-like inhibitors, including α-AI1 and α-AI2 [28]. This fact could be explained by some MALDI-ToF technique properties (ionization treatments and laser strength), which are able to separate highly glycosylated multimeric proteins [28]. Furthermore, mass spectrometry analyses of peak 2 from HPLC analysis showed a majority peak at 33 330 Da and a corresponding homodimer at 66 660 Da, with minor contaminants. This specific molecular mass differs from masses of the six α-amylase inhibitors classes: lectin-like (56 kDa); knottin-like (3 kDa); cereal-type (12 kDa); Kunitz-like (20 kDa); γ-purothionin-like (5 kDa) and thaumatin-like (22 kDa); as proposed by Richardson [13] and reviewed by Franco et al. [11]. The ability of peak II to form dimer is similar to that of another type of α-amylase inhibitor isolated from seeds of Jobs Tear's, which shows a molecular mass of 24 400 Da in the unglycosylated form [16].

Sequence comparisons show that the PvCAI N-terminal amino acid sequence (Table 1) is different to other known α-amylase inhibitors; although it shows clear identity with plant chitinases from carbohydrate active enzymes (CAZy) family 18 [29]. Higher identities of PvCAI were obtained against Q9S8T2, a chitinase from *Cicer arietnum*, with 71% sequence identity [30], as well as with Q9XGB4, a chitinase from *T. repens*, again with 71% identity [31]. The new partial sequence shares cysteines at position 24 that, due to their structural role, are completely conserved in plant chitinases [17]. We also compared the new sequence with the sequences of other common bean chitinases (data not shown), with reduced degrees of sequence similarity in the range of 15–30% identity observed. This suggests that the novel *P. vulgaris* α-amylase inhibitor reported here is the second member of a bifunctional inhibitor class with chitinolytic characteristics.

The inhibitory activities of α-AI2 and PvCAI against PPA and ZSA were determined in vitro as shown in Fig. 2A. α-AI2, as previously described by Grossi-de-Sa et al. [23] inhibited only insect α-amylases, being incapable of inhibiting mammalian enzymes. PvCAI was also assayed toward PPA and ZSA, showing a similar inhibition pattern (Fig. 2A). This is not the first time that such specificity has been observed, with α-amylase inhibitors such as AAI from *Amaranthus* [32], 0.53 from wheat kernels [14] and α-AI2 from wild common bean seeds [23] showing abilities to inhibit only insect enzymes. Furthermore, α-amylase inhibitors with similarities to chitinases-like inhibitors from wheat, such as XIP-I [20], as well as from Jobs Tear's kernels were also isolated [16]. This latter molecule, pertaining to CAZy family GH19 [29], inhibited locust gut α-amylases, but was unable to inhibit enzymes from different sources such as human saliva, porcine pancreas, *Bacillus subtilis*, *Aspergilus oryzae* and barley malt [16]. Furthermore, XIP-I from wheat, which belongs to the CAZy family GH18 [29], was capable of inhibiting xylanases and a barley α-amylase (AMY-1) [20]. The target α-amylases of XIP-I and PvCAI are from similar structural families: both (ZSA and AMY-1) belong to family 13 of GH, having a $(\beta/\alpha)_8$,fold-barrel [33] indicating a possible similarity in mechanism of action. In this case, the presence of substrate is necessary for the enzyme

Table 1
Alignment of the N-terminal sequence of novel common bean α-amylase inhibitor/chitinase (PvCAI) with members of chitinolytical enzymes from Leguminosae seeds family

PIR code	Sequence	Source	Identity	Reference
PvAIC	HEDNAGIAVYWGQDAREGDLVTACN	*P. vulgaris*		
Q9S8T2	AGIAVYWGQNGNEGSLQDACN	*C. arietinum*	71.42	[30]
Q9XGB4	SSSAAGIAVYWGQNGGEGSLEDACN	*T. repens*	71.42	[31]
Q9MBC9	RAENGGIAVYWGQDAREGNLIATCD	*V. unguiculata*	69.56	[34]
P369O8	SSNAAGIAVYWGQNGNEGSLQDACN	*C. arietinum*	65.21	[30]
P29024	PSHAGGISVYWGQNGNEGSLADACN	*P. angularis*	65.00	[35]
P23473	GGIAIYWGQNGNEGTLTQTCN	*P. quinquefolia*	60.00	[36]
Q9S7G9	HSHAAGIAVYWGQNGGEGTLAEACN	*G. max*	64.00	[37]
Q9S8F8	GGIAIYWGQNGGEGTLRDTCN	*P. americana*	60.00	[38]
Q9SXM5	HSHAAGIAIYWGQNGGEGTLAEACN	*G. max*	60.00	[37]
Q43O98	HSNAAGIAVYWGQNGGEGSLADTCN	*P. tetragonolobus*	60.00	[39]
P17541	SSDAAGIAIYWGQNGNEGSLASTCA	*C. sativus*	59.09	[40]
O49876	LSNAAGIVIYWGQNGNEGSLADACN	*L. albus*	56.52	[41]
Q39657	SSDAAGIGIYWGQNGNEGSLASTCA	*C. sativus*	54.54	[42]
Q9M544	SSEAAGIAIYWGQNGNEGSLASTCA	*C. melo*	54.54	[43]
CAA09110	HVDGGGIAIYWGQNGNEGTLTQTCS	*H. brasiliensis*	52.00	[44]
	** **** ** * *			

Identical residues are marked with asterisk.

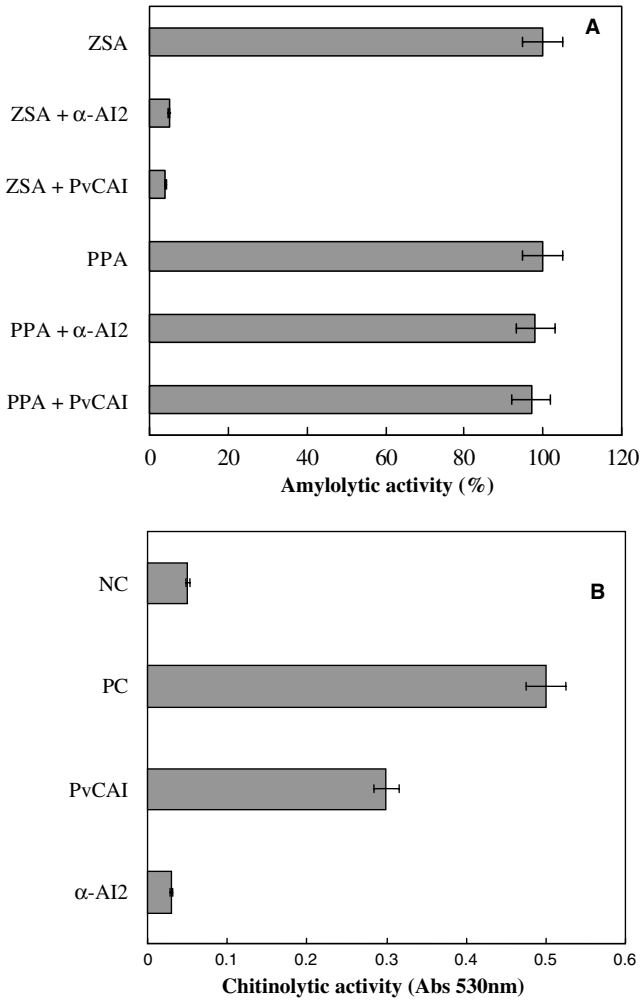

Fig. 2. (A) Inhibitory activities against ZSA and PPA; (B) chitinolytic activities of reversed-phase HPLC fractions 1 (α-AI2) and 2 (PvCAI). PC corresponds to positive control, in which *Trichoderma* sp. chitinase was used, and NC corresponds to negative control, in which distilled water was used. Each assay was carried out in triplicate. Vertical bars correspond to S.D.

inhibitor interaction [20]. In order to be of practical use for the production of transgenic plants, α-amylase inhibitors should ideally be effective against the full range of potential predatory insects and should also lack activity against mammalian enzymes, as observed for PvCAI. However, this may be considered a lesser issue, given that cooking would denature any inhibitors prior to ingestion. Inhibitors such as PvCAI, with greater potential against insect α-amylases than against mammalian α-amylase, are particularly promising candidates for the production of transgenic plants conferring protection against predation.

Purified inhibitors were also tested for their chitinase activity using chitin Azure as substrate and a chitinase from *Trichoderma* sp., a known chitin hydrolytic enzyme, as a positive control expressing enhanced activity. Interestingly, no enzymatic activity could be detected using α-AI2. Furthermore, PvCAI showed significant chitinolytic levels (Fig. 2B). Our data clearly show that the PvCAI chitinase sequence in fact encodes an inhibitor with double function. The presence of a chitinase in bean seeds is not surprising, as capability of degrading chitin is an important attribute for seed defense against bean weevil, a pest with perithrophic membrane and exoskeleton mainly composed of this polysaccharide. The plurifunctionality of this novel family member has major implications for post-genomic studies and predicted gene function. Furthermore, this report provides compelling evidence of the emergence a novel α-amylase inhibitor family, capable of combined functions, and relevant for bean protection from insect feeding and fungal infection.

Acknowledgments: We thank to Paulo Roberto Queiroz e Luzia Helena Lima for technical assistance with chitinase assays and Dr. Robert Miller for critical reading and the correction of English. This work was supported by research grants from the Brazilian Government, EMBRAPA, UCB, CAPES and CNPq.

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