Mutants of common bean alpha-amylase inhibitor-2 as an approach to investigate binding specificity to alpha-amylases

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Abstract – Despite the presence of a family of defense proteins, *Phaseolus vulgaris* can be attacked by bruchid insects resulting in serious damage to stored grains. The two distinct active forms of α-amylase inhibitors, α-AI1 and α-AI2, in *P. vulgaris* show different specificity toward α-amylases. *Zabrotes subfasciatus* α-amylase is inhibited by α-AI2 but not by α-AI1. In contrast, porcine α-amylase is inhibited by α-AI1 but not by α-AI2. The objective of this work was to understand the molecular basis of the specificity of two inhibitors in *P. vulgaris* (α-AI1 and α-AI2) in relation to α-amylases. Mutants of α-AI2 were made and expressed in tobacco plants. The results showed that all the α-AI2 mutant inhibitors lost their activity against the insect α-amylases but none exhibited activity toward the mammalian α-amylase. The replacement of His33 of α-AI2 with the α-AI1-like sequence Ser-Tyr-Asn abolished inhibition of *Z. subfasciatus* α-amylase. From structural modeling, the conclusion is that the size and complexity of the amylase-inhibitor interface explain why mutation of the N-terminal loop and resultant abolition of *Z. subfasciatus* α-amylase inhibition are not accompanied by gain of inhibitory activity against porcine α-amylase.

Index terms: *Phaseolus vulgaris*, α-amylase inhibitors, inhibitor specificity, site directed mutagenesis, structural modeling.

Mutantes do inibidor-2 de alfa-amilase do feijão-comum para investigação da especificidade de ligação a alfa-amilases

Resumo – Apesar de possuir uma família de proteínas de defesa, o feijão-comum (*Phaseolus vulgaris* L.) pode ser atacado por insetos bruquídeos causando sérios danos aos grãos armazenados. O *P. vulgaris* possui duas formas ativas de inibidores de α-amilases, denominadas α-AI1 e α-AI2, que apresentam diferentes especificidades em relação às α-amilases. A α-amilase de *Zabrotes subfasciatus* é inibida por α-AI2 mas não por α-AI1. Em contraste, a α-amilase pancreática de porco é inibida por α-AI1 mas não é por α-AI2. O objetivo deste trabalho foi entender as bases moleculares da especificidade desses inibidores em relação às α-amilases. Para tanto, foram construídos mutantes do α-AI2, os quais foram expressados em plantas de fumo. Todos os inibidores mutantes deixaram de inibir a α-amilase de inseto sem, contudo, passar a exibir atividade contra a α-amilase de mamífero. Os modelos estruturais explicam por que a substituição de His33 do α-AI2 pela seqüência correspondente do α-AI1 (Ser-Tyr-Asn) aboliu a inibição da α-amilase de *Z. subfasciatus*. Dos estudos de modelagem molecular pode-se concluir que o tamanho e a complexidade da interface α-amilase-inibidor explicam por que a mutação da alça N-terminal e a quebra da atividade inibitória para α-amilase de *Z. subfasciatus* não levam ao ganho de atividade inibitória do mutante em relação à α-amilase de porco.

Termos para indexação: *Phaseolus vulgaris*, inibidores de α-amilases, especificidade de interação, mutagênese sítio-dirigida, modelagem molecular.

Introduction

Alfa-amylase inhibitors (α-AI) occur naturally in many food plants and are particularly abundant in cereals and legumes (Franco et al., 2002). Insects gain access to food sources when they evolve amylases that are not affected by inhibitors present in the food source, and plants become resistant when they evolve inhibitors effective against these insect enzymes. Genes encoding these inhibitors have been used to make transgenic crops by gene transfer technology because their expression in plants is harmful to target insects and pests, interfering with their digestion, absorption or reproduction, whereas no antinutritional or toxic effects were observed in rats fed on transgenic pea expressing the insecticidal bean α-amylase inhibitor (Pusztai et al., 1999).

In the common bean (*Phaseolus vulgaris*), α-AI exist as at least two allelic variants that differ in their specificity towards α-amylases, despite their high degree of similarity (78% amino acid sequence identity). Alfa-amylase inhibitor-1 (α-AI1) is the isoform found in cultivated beans (Moreno & Chrispeels, 1989) and inhibits porcine pancreatic α-amylase (PPA), as well as the α-amylases of the cowpea weevil *Callosobruchus maculatus* (CMA) and the azuki bean weevil (*Callosobruchus chinensis*) (Kasahara et al., 1996), but not the amylase of bruchid (*Zabrotes subfasciatus*) (ZSA) (Ishimoto & Kitamura, 1989). Alpha-amylase inhibitor-2 (α-AI2) is found in some wild bean accessions (Suzuki et al., 1993) and, in contrast, only inhibits ZSA.

The growth of larvae of two seed feeding beetles, *C. maculates* and *C. chinensis*, is inhibited when the diet of the larvae contains relatively low levels of common bean α-AI (Ishimoto & Kitamura, 1989). Pea transgenic seeds containing up to 0.1%–1.2% of α-AI1 were resistant to cowpea weevils and azuki bean weevils (Shade et al., 1994). Furthermore, in field trials, peas expressing α-AI1 were protected against damage by the pea weevil (Pusztai et al., 1999), suggesting that gene transfer mechanism can be used to make legumes resistant to bruchids. Common beans resistant to *Z. subfasciatus* have already been produced by traditional breeding techniques but presently there are no inhibitors in beans that are effective against *Acanthoscelides obtectus*, another important storage bean insect pest. A better understanding of the molecular basis of specificity of α-AI and their target amylases therefore has potential practical benefits.

In the last ten years, several studies of the interaction between α-amylase inhibitors and their target enzymes have been carried out (Pueyo et al., 1993; Mirkov et al., 1995; Grossi-de-Sá et al., 1997; Takahashi et al., 1999). Based on the pea lectin structure (Einspahar et al., 1986) and their own sequence alignments, Mirkov et al. (1995) constructed an α-AI1 mutant by replacement of the $_{188}$WSY$_{190}$ triplet with GNV producing an inactive inhibitor. The same triplet was considered later by Grossi-de-Sá et al. (1997) in substitutions using the corresponding α-AI2 sequence. The α-AI2- triplet was also changed to the α-AI1-like sequence. In both cases the specificity of the inhibitor remained unchanged.

Research was further stimulated after the crystal structures of α-AI1 complexed with PPA (Bompard-Gilles et al., 1996), *Tenebrio molitor* α-amylase (TMA) alone (Strobl et al., 1998), and in complex with α-AI1 (Nahoum et al., 1999), were determined. The PPA/α-AI1 structure confirmed that interactions occur primarily by contacts with two inhibitor loops and revealed an extensive network of direct hydrogen bonds. Based on these structures, ZSA and the four pairwise α-AI1 or α-AI2 complexes with PPA or ZSA were modeled (Silva et al., 2000). Although none of the interface parameters analyzed were, alone, able to explain the specificity of inhibitors for their cognate enzymes, lists of interface forming residues were defined as targets for experimental efforts toward specificity alteration via site-directed mutagenesis.

The main goal of this work was to understand the molecular basis of the specificity of α-AI1 and α-AI2 inhibitors in *P. vulgaris* in relation to α-amylases.

Material and Methods

Construction of the mutants by PCR mutagenesis

The PCR (polymerization chain reaction) techniques, as described in Mole et al. (1989) and Landt et al. (1990), were used to construct the mutants (M1, M2, M3 e M4) of the α-AI2 gene. In each case, the substitution assumed the same correspondent amino acid sequence like in α-AI1 (Table 1). For construction of the first mutant (M1), the cDNA (complementary deoxyribonucleic acid) of α-AI2 on the pUC8 plasmid (Grossi-de-Sá et al., 1997) was used as template; the amino acids serine (Ser) and tyrosine (Tyr) were inserted between positions 32 and 33, and His33 was replaced by asparagine (Asn) (SYH33N). These residues are involved in the N-ter-

minal loop and this mutation was repeated in all other mutants.The M1 mutant was used as template to construct the M2 mutant where Gln71 was replaced by histidine (Q71H); this residue is involved in binding to α-amylase but is outside of the two main interacting loops. The M1 mutant was also used to construct the M3 mutant, but in this case the additional mutation involved the C-terminal loop, where the His175, Glu176, Tyr177 and Phe179 were replaced by tyrosine, glutamine, tryptophan and tyrosine, respectively. The M4 triple mutant was constructed to contain all the mutations introduced in the other mutants. The primers used to perform the mutations were α-AI2PR1: 5'CATAGAGT CCACTGT**ATTATAGGA**TAGTTGTAAGTAGCC3'; α-AI2PR2: 5'TGCTTCGCG**GTG**AGTGGTGATATT CA3' and α-AI2PR3: 5'GCCATCTCAGGGGTT**TATC AATGG**AGC**TAT**GAAACGAGAGACGTG3', with the mutated nucleotides in bold letters. The primers of the 3' and 5' α-AI2 sites, in a sense (5'CCGAATTCAT GGCTTCCTCCAACTTA3') or in antisense (5'CTAGAGGATATTGTTGAGGAGGAT3') orientation, were combined in each case to obtain the megaprimer (intermediary step) or the whole gene.

The PCR products were digested by *Eco*RI, gel purified and ligated into pGEMT-easy (Promega Corporation, Madison, Wis., USA). In each cloning step, several clones of each construction were sequenced to confirm that no errors had been produced. To guarantee maximum fidelity, the *Pfu* enzyme (Promega) was used in the reactions. The *Bsm*I and *Pst*I sites were introduced (by PCR) into the ends of both the wild-type and mutants versions of the α-AI2 gene to facilitate the fusion of this gel purified fragment (750 bp) into plasmid pTA-2 (Grossi-de-Sá et al., 1997), containing the PHA-L (phytohemaglutinin) (536 bp) seed-specific promoter. Finally, the *Hind*III-*Eco*RI chimeric (PHA/wild-type or PHA/α-AI2 mutants) cDNA fragments were cloned in respective sites into the plant transformation vector pCAMBIA 1390 (Cambia GPO, Camberra, Australia) containing the nopaline synthase transcriptional (NOS) terminator.

Tobacco transformation

The above mentioned vectors were introduced into tobacco (*Nicotiana tabacum* cv. Xanthi) to express the protein in the seeds. The binary vectors were transferred to *Agrobacterium tumefaciens* GV3101PMP90 (Koncz & Schell, 1986) using heat-shock method (Brasileiro & Carneiro, 1998). Sterilized leaf explants were transformed by a simplified version of the leaf-disc method (Horsh et al., 1985). Five different cultures of *Agrobacterium* harboring each construction were grown in 5 mL YEB medium (Vervliet et al., 1975) supplemented with rifampicin (100 μg/mL), gentamicin (50 μg/mL) and kanamycin (100 μg/mL), for 16 hours, 28±2ºC. For co-transformation experiments, 1 mL of the *Agrobacterium* culture (A600nm = 0.1) was added to 20 mL MS liquid medium (Murashige & Skoog, 1962). Twenty leaf explants were incubated with the *Agrobacterium* culture in a Petri dish for 5 min, at room temperature. Explants were then immediately placed on MS solid medium (0.7% agar) for two days in darkness at 28±2ºC. For regeneration and selection, the explants were transferred to MS solid medium (0.65% agar) containing 1 mg/mL benzylaminopurine (BAP), 500 μg/L cefotaxime and 30 μg/mL hygromycin and maintained under a 16 hours photoperiod at 25±2ºC. Untransformed explants were placed onto the same medium with or without hygromicin as the negative and positive controls, respectively. Shoots regenerated on selection medium were excised at the base and placed in Magenta GA7 boxes containing rooting medium (the same medium as the regeneration medium but without BAP). The hygromycin-resistant plants tested by PCR using both 3' and 5' α-AI2 and hygromycin primers were transferred to soil and grown in a greenhouse at 25±10ºC and 50% humidity. The mature seeds were collected after 4 months.

Protein extraction

The soluble wild type and recombinant proteins expressed in tobacco seeds, were extracted as previously described (Grossi-de-Sá et al., 1997) by grinding 150 mg of dry seeds, in an ice-cold mortar with 1.0 mL 50 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100, 30 mM NaCl and 1% 2-mercaptoethanol. The homogenates were twice centrifuged for 10 minutes at the top speed of a bench centrifuge and the supernatants stored at -20ºC or immediately used to measure the inhibitory activity. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Electrophoresis and immunobloting

Tricine polyacrylamide gel electrophoresis (Tricine/PAGE), 12% polyacrylamide, was performed according to Schagger & Jagow (1987). After separation by Tricine/PAGE, polypeptides were transferred onto a

nitrocellulose membrane and incubated with a 1:1000 dilution of a polyclonal antibody rabbit serum raised against α-AI1 (Grossi-de-Sá et al., 1997). Immunoglobulin G coupled to horseradish peroxidase HRP was used as a secondary antibody and the reaction was visualized using 0.1% (v/v) H_2O_2 in TBS buffer (Koncz & Schell, 1986) mixed with 3% (w/v) HRP color development reagent in methanol.

Alpha-amylase inhibition assay

The α-amylase activity was measured using the dinitrosalicylic acid (DNS) method adapted from Bernfeld (1955) and Ishimoto & Chrispeels (1996) using 1% soluble starch as substrate. To measure inhibitory activity of tobacco seed extracts against either PPA or ZSA expressed in *Sf*9 insect cells, the solutions containing 200 μg of the total extract (previously analyzed in immunoblots) and one unit α-amylase activity (defined as the amount of this enzyme that increased the absorbance at 546 nm by 0.1 absorbance units during 25 minutes) were pre-incubated at room temperature for 20 minutes prior to the addition of 250 μL of the substrate solution followed by incubation at 37°C for 10 minutes. The reactions were stopped by the addition of 500 μL of DNS reagent followed by color development, placing the tubes in boiling water for 10 minutes. After addition of 5 mL distilled water, the absorbance was read at 546 nm. Alfa-amylase inhibitor-1 purified from *P. vulgaris* was used as positive and negative controls in assays with PPA and ZSA, respectively. Assays were carried out in duplicate. Duplicate inhibition values differed by no more than 10%.

Computational methods

In order to study the behavior of mutant M1 in structural terms, four model complexes, PPA-α-AI2, PPA-M1, ZSA-α-AI2 and ZSA-M1, using MODELLER (Sali & Blundell, 1993) were constructed. Experimental results show that the M1 complexes are merely hypothetical, *i.e.*, do not actually form. Since the patterns of inhibition of M2-4 were identical to that of M1, these other mutants were not studied further. The structure of PPA in complex with α-AI1, PDB code 1DHK (Bompard-Gilles et al., 1996) was used to construct the PPA-α-AI2 and PPA-M1 model complexes while the structure of TMA in complex with α-AI1, PDB code 1VIW (Nahoum et al., 1999) was used for modeling ZSA-α-AI2 and ZSA-M1. Alignments were adjusted so that insertions and deletions in the model proteins relative to the templates could be smoothly accommodated. In each case, ten variant models were constructed and refined against MODELLER's objective function which measures the models agreement with template-derived restraints, in combination with the standard CHARM energy function.

The best performing model (that with the lowest objective function) for each of the complexes was taken as the respective final model. Each final model complex was analysed, and compared with the crystal structure of PPA in complex with wild-type α-AI1, using the protein-protein interaction server (Jones & Thornton, 2001). This server determines which residues in a protein complex contribute with the surface areas of the interface and measures geometrical characteristics of the interface which can then be compared with typical values (Jones & Thornton, 1996). Visualization of protein structures was carried out using O (Jones et al., 1991) and Pymol (Delano, 2001) programs.

Results and Discussion

The major interaction between α-AI1 and PPA, as revealed by the crystal structure of the complex, is via two loops of α-AI1 that make numerous hydrogen bonds with the amino acids in the substrate cleft of PPA (Bompard-Gilles et al., 1996; Silva et al., 2000). To assess the role of the two loops for the specificity of α-AI-amylase interaction, chimeric amylase inhibitor was made containing specific changes based on modeling experiments (Silva et al., 2000). Analyzing the full set of previously identified interface-forming residues, four mutants were designed (Table 1). The mutated amino acids were chosen for their putative importance in electrostatic and hydrophobic aspects of the enzyme-inhibitor interaction. One option would have been the simple mutation of putatively important residues to amino

Table 1. Aminoacid sequence involved on the mutations[(1)].

Aminoacid sequences	Interface forming residues (IFR)		
	N-terminal loop	Outside of the loops	C-terminal loop
Wild type inhibitors			
α-AI1	$_{33}$SYN$_{35}$	H$_{73}$	$_{177}$YQWSY$_{181}$
α-AI2	--H$_{33}$[(2)]	Q$_{71}$	$_{175}$HEYSF$_{179}$
α-AI2 mutant inhibitors			
M1	$_{33}$SYN$_{35}$	Q$_{73}$	$_{177}$HEYSF$_{181}$
M2	$_{33}$SYN$_{35}$	H$_{73}$	$_{177}$HEYSF$_{181}$
M3	$_{33}$SYN$_{35}$	Q$_{73}$	$_{177}$YQWSY$_{181}$
M4	$_{33}$SYN$_{35}$	H$_{73}$	$_{177}$YQWSY$_{181}$

[(1)]Numbering as processed in Silva et al. (2000).[(2)]-- Indicating absence of two residues in this loop.

acids of different physicochemical characteristics, or simply to alanine, and the monitoring of the consequent loss of ZSA inhibition. Instead, the chosen residues were mutated to their α-AI1 counterparts so that, in addition to loss of ZSA inhibition, possible acquisition of PPA inhibition could be monitored. Through this dual assay approach, it was expected that an enhanced understanding of the detailed importance of the mutated residues would be achieved.

The PCR products of wild-type and mutant genes were ligated to 5' regulatory sequence of PHA-L, because of known success of this seed-specific promoter in expressing the α-AI activity in plants (Altabella & Chrispeels, 1990). Therefore, all chimeric construction as well as wild-type form of α-AI2 were expressed in tobacco seeds. Since α-AI1 needs to be proteolytically processed to be active, plant expression, leading to proper glycosylation and proteolytic processing, was essential (Altabella & Chrispeels, 1990; Pueyo et al., 1993). Approximately 15–20 independent transgenic plants (tested by PCR) were obtained for each construction.

Four transgenic lines for each construction were used to perform immunoblots with serum raised against α-AI1. The level of transgene expression was approximately equal to all mutants. The similar polypeptide profiles observed in the 12–15 kDa range to tobacco seeds expressing the recombinant proteins and wild-type inhibitor demonstrate that mutations did not destabilize the proteins to a point where they were degraded by endogenous proteases (Figure 1).

To investigate the specificity of the recombinant proteins accumulated in tobacco seeds in relation to α-amylases, total extracts were tested for inhibitory activity against PPA and ZSA. To circumvent possible stability problems, the proteins expressed in tobacco seeds were tested immediately after the preceding extraction. In agreement with previous reports (Suzuki et al., 1993; Grossi-de-Sá et al., 1997, Grossi-de-Sá & Chrispeels, 1997) α-AI1 inhibited PPA but not ZSA, and α-AI2 inhibited ZSA, but not PPA. However, all the mutants lost inhibitory activity against ZSA and none of the mutants acquired specificity toward PPA (Figure 2). Because the level of the expression varied between mutant and wild-type proteins (Figure 1), different concentrations were used in the assays to assess the activity of the mutated proteins.

The high sequence identity between modeled proteins and templates (78% between α-AI1 and α-AI2; 61% for the comparison of ZSA and TMA) along with careful manual positioning of insertions and deletions, ensure reliable models. In the cases of the ZSA complexes, the present models should be significantly more accurate than those previously analysed (Silva et al., 2000) since they were based on the TMA-α-AI1 (Nahoum et al., 1999)

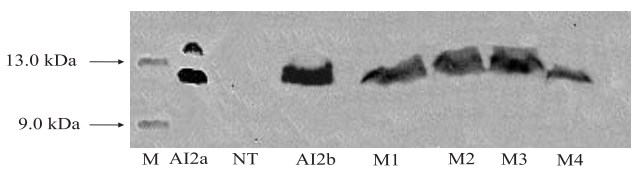

Figure 1. Immunoblot of the α-AI2 and mutants expressed in tobacco seeds. 87.5 μg of the total protein was loaded in each lane. α-AI2a refers to an inhibitor purified from *Phaseolus vulgaris* and α-AI2b to an inhibitor expressed in tobacco seeds while M1, M2, M3 and M4 refer to α-AI2 mutants. NT refer to untransformed plant (negative control). Molecular weight standard are shown on the left.

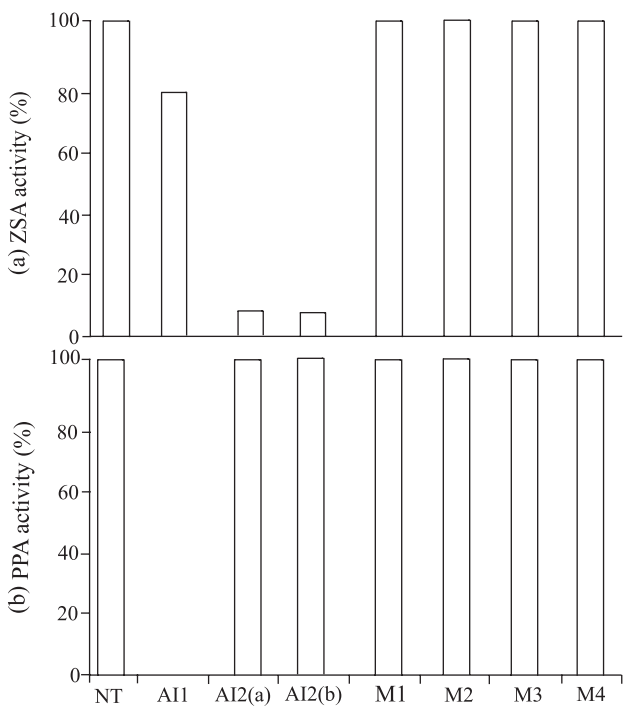

Figure 2. Activities of α-AI1and α-AI2(a), extracted from *Phaseolus vulgaris*, α-AI2(b) and mutants, extracted from transformed tobacco seeds, in relation to ZSA and PPA. NT refers to seed extract from untransformed tobacco. The enzyme activity values were calculated for four transformed lines for each mutant. In each of (a) and (b) the highest activity obtained was defined as 100% and the remaining results expressed as percentages.

complex rather than on the structure of α-AI1 alone. Comparison of the structures of complexed and uncomplexed α-AI1 models reveals significant differences in the conformations of loops that interact with α-amylase.

Attempts to model a ZSA-M1 complex revealed unnaturally close atomic contacts between ZSA and M1 in the region of the latter's α-AI1-like two residues insertion. Comparison with the TMA-α-AI1 crystal structure shows that these clashes arise mainly from the substitution of Thr291 (TMA numbering) with the larger Asp and from different backbone structure of the loop around residue 293 which is one residue shorter in ZSA than in TMA (Figure 3). As well as the steric aspect, it is notable that Asp 291 in ZSA is largely buried in the hypothetical interface with M1, distant from any positive charge with which to interact. In contrast, in the modeled complex with α-AI2, charge compensation is provided in the form of His33 (Figure 3). These results are consistent with the inhibition of TMA (but not ZSA) by α-AI1 and ZSA (but not TMA) by α-AI2 (Suzuki et al., 1993; Nahoum et al., 1999).

The inability of α-AI2 or M1 to inhibit PPA is consistent with the geometric analyses of the hypothetical complexes obtained from the protein-protein interaction server. The server measures surface complementarity as a gap volume index (Jones & Thornton, 1996). Lower values indicate better complementarity and typical values for experimentally determined enzyme-inhibitor complexes are 2.2±0.5. The values obtained for the two crystal structures of α-amylase-inhibitor complexes are 2.2 and 2.3. In sharp contrast, the hypothetical PPA-M1 and PPA-α-AI2 complexes score 2.7 and 3.0, respectively, at the limit or outside the typical range. These poorer complementarity values presumably contribute to the lack of PPA inhibition by α-AI2 or M1, although other factors may also be involved.

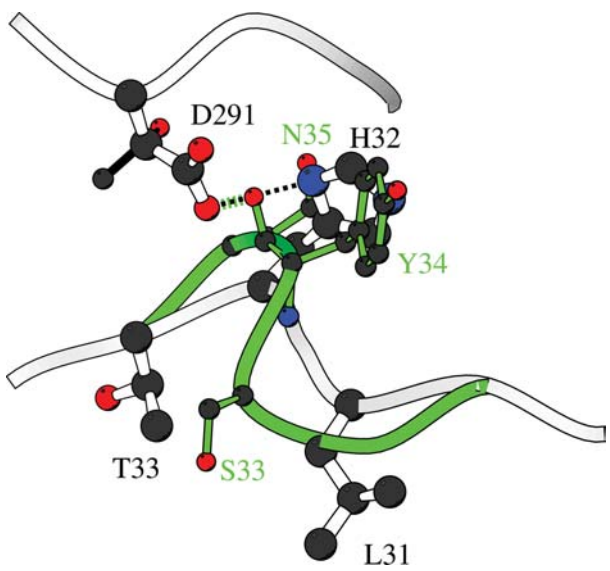

Figure 3. Structural comparison of the ZSA-α-AI2 (white) and ZSA-M1 (green, thinner bonds and smaller atoms) complexes near the side of mutation. The additional two residues present in M1 compared to α-AI2 cause steric clashes with Asp 291 of ZSA as marked by the broad green dotted line. With α-AI2, in contrast, a favorable ionic interaction with His 32 is present. The extra residues do not clash with the Thr (black) that replaces Asp 291 in TMA.

The most striking finding reported is the absence of inhibition of ZSA by M1 which differs from wild-type α-AI2 only in the replacement of His33 with Ser-Tyr-Asn. The explanation obtained through modeling involved combined steric and electrostatic effects. Hence, the alteration in α-AI2 structure produced a drastic change in inhibitory behavior through two distinct mechanisms. This result follows other examples of apparently fine-tuned α-amylase inhibitor specificity (Franco et al., 2000). The multiple factors underlying amylase inhibitor specificity have been reviewed recently (Franco et al., 2002).

The lack of appearance of PPA inhibition even in M4 is explained by consideration of the complexity of the PPA-α-AI1 interface. Eight inhibitor segments containing one to ten residues each contribute to the interface with PPA. This is more than double the number of inhibitor segments typically involved in enzyme-inhibitor interfaces (3±0.9 segments). The size of the interface area between PPA-α-AI1 (1,430 $Å^2$) is much larger than the average for enzyme-inhibitor complexes (785±75 $Å^2$). These analyses show that the interface between PPA and α-AI1 is larger and more complex than typical enzyme-inhibitor interfaces. This naturally makes more difficult the task of precisely determining all the factors involved in conferring specificity for α-AI1 or α-AI2 on a given α-amylase (Silva et al., 2000). Even in the most-changed mutant M4, only 41% of the inhibitor surface area in the PPA-α-AI1 complex has been grafted into the α-AI2 environment.

Conclusions

1. The first interacting loop of the inhibitors is an important contributor to specificity but other factors also exist.

2. Computational analyses are useful for highlighting important interface residues and analyzing the results of their mutation.

3. The interaction between α-AI and α-amylases is atypically complex so that additional work will be required to fully understand all specificity determinants.

Acknowledgements

To Empresa Brasileira de Pesquisa Agropecuária (Embrapa) and to Conselho Nacional de Desenvolvimento Científico (CNPq), for the financial support; to Ana Carolina S. Monteiro from Universidade Federal do Rio de Janeiro and Cristiano Lacorte from Embrapa Recursos Genéticos e Biotecnologia, for discussions in molecular biology strategies and assistance in tobacco transformations; to the Universidade de Brasília for the use of cell Biology Department facilities during the development of the first author's Ph.D. thesis.

References

ALTABELLA, T.; CHRISPEELS, M.J. Tobacco transformation with the bean aAI gene express an inhibitor of insect α-amylase in their seeds. **Plant Physiology**, v.93, p.805-810, 1990.

BERNFELD, P. Amylase, α and β. **Methods in Enzymology**, v.1, p.149-158, 1955.

BOMPARD-GILLES, C.; ROUSSEAU, P.; ROUGÉ, P.; PAYAN, F. Substrate mimicry in the active centre of a mammalian α-amylase: structural analysis of an enzyme inhibitor complex. **Structure**, v.4, p.1441-1452, 1996.

BRADFORD, M.M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of dye binding. **Analytical Biochemistry**, v.72, p.248-254, 1976.

BRASILEIRO, A.C.M.; CARNEIRO, V.T.C. **Manual de transformação de plantas**. Brasília: Embrapa-SPI, 1998. 309p.

DELANO, W.L. **The PyMOL molecular graphics system on world wide web**. <Available in http://www.pymol.org>. Access on: October 2001.

EINSPAHAR, H.; PARKS, E.H.; SUGUNA, K.; SUBRAMANIAN, E.; SUDDATH, F.L. The crystal structure of pea lectin at 3,0 Å resolution. **Journal of Biological Chemistry**, v.241, p.16518-16527, 1986.

FRANCO, O.L.; RIDGEN, D.J.; MELO, F.R.; BLOCH JUNIOR, C.B.; SILVA, C.P.; GROSSI-DE-SÁ, M.F. Activity of wheat alpha-amylase towards bruchid alpha-amylases and structural explanation of observed specificities. **European Journal of Biochemistry**, v.267, p.2166-2173, 2000.

FRANCO, O.L.; RIDGEN, D.J.; MELO, F.R.; GROSSI-DE-SÁ, M.F. Plant α-amylase inhibitors and their interaction with insect α-amylases. **European Journal of Biochemistry**, v.269, p.397-412, 2002.

GROSSI-DE-SÁ, M.F.; CHRISPEELS, M.J. Molecular cloning of bruchid (*Zabrotes subfasciatus*) α-amylase cDNA and interactions of the expressed enzyme with bean amylase inhibitors. **Insect Biochemistry and Molecular Biology**, v.27, p.271-281, 1997.

GROSSI-DE-SÁ, M.F.; MIRKOV, T.E.; ISHIMOTO, M.; COLUCCI, G.; BATEMAN, K.S.; CHRISPEELS, M.J. Molecular characterization of a bean α-amylase inhibitor that inhibits the α-amylase of the Mexican bean weevil *Zabrotes subfasciatus* . **Planta**, v.203, p.295-303, 1997.

HORSH, R.B.; FRY, J.E.; HOFFMAN, N.L.; EICHOLTZ, D.; ROGERS, S.G.; FRALEY, R.T. A simple and general method for transferring genes into plants. **Science**, v.227, p.1229-1231, 1985.

ISHIMOTO, M.; CHRISPEELS, M.J. Protective mechanism of the Mexican bean weevil against high levels of α-amylase inhibitor in the common bean. **Plant Physiology**, v.111, p.393-401, 1996.

ISHIMOTO, M.; KITAMURA, K. Growth inhibitory effects of an α-amylase inhibitor from kidney bean, *Phaseolus vulgaris* (L.) on three species of bruchids (Coleoptera: Bruchidae). **Applied Entomology and Zoology**, v.24, p.281-286, 1989.

JONES, T.A.; THORNTON, J.M. Principles of protein-protein interaction derived from structural studies. **Proceeding of the National Academy of Science**, v.93, p.13-20, 1996.

JONES, T.A.; THORNTON, J.M. **Protein Protein Interaction on world wide web**. Available in: <http://www.biochem.ucl.ac.uk/bsm/PP/server>. Access on: October 2001.

JONES, T.A.; ZOU, J.Y.; COWAN, S.W.; KJELDGAARD, M. Improved methods for building protein models in electron density maps and the locations of errors in these models. **Acta Crystallographica**, v.A47, p.110-119, 1991.

KASAHARA, K.; HAYASHI, K.; ARAKAWA, T.; PHILO, J.S.; WEN, J.; HARA, S.; YAMAGUCHI, H. Complete sequence, subunit structure and complexes with pancreatic α-amylase of an α-amylase inhibitor from *Phaseolus vulgaris* white kidney beans. **Journal of Biochemistry**, v.120, p.177-183, 1996.

KONCZ, C.; SCHELL, J. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. **Molecular and General Genetics**, v.204, p.383-396, 1986.

LANDT, O.; GRUNERT, H.P.; HAHN, V. A general method for rapid site directed mutagenesis using the polymerase chain reaction. **Gene**, v.96, p.125-128, 1990.

MIRKOV, T.E.; EVANS, S.V.; WAHLSTROM, J.; GOMEZ, L.; YOUNG, N.M.; CHRISPEELS, M.J. Location of the active site of the bean α-amylase inhibitor and involvement of a Trp, Arg, Tyr triad. **Glycobiology**, v.5, p.45-50, 1995.

MOLE, S.E.; IGGO, R.D.; LANE, D.P. Using the polymerase chain reaction to modify expression plasmid for epitope mapping. **Nucleic Acids Research**, v.17, p.3319, 1989.

MORENO, J.; CHRISPEELS, M.J. A lectin gene encodes the α-amylase inhibitor of the common bean. **Proceeding of the National Academy of Science**, v.86, p.7885-7889, 1989.

MURASHIGE, T.; SKOOG, F.A. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v.15, p.473-497, 1962.

NAHOUM, V.; FARISEI, F.; LE BERRE-ANTON, V.; EGLOFF, M.P.; ROUGE, P.; POERIO, E.; PAYAN, F. A plant-seed inhibitor

of two classes of alpha-amylases: X-ray analysis of *Tenebrio molitor* larvae alpha-amylase in complex with the bean *Phaseolus vulgaris* inhibitor. **Acta Crystallographica D**: Biology Crystallographica, v.55, p.360-362, 1999.

PUEYO, J.J.; HUNT, D.C.; CHRISPEELS, M.J. Activation of bean (*Phaseolus vulgaris*) α-amylase inhibitor requires proteolytic processing of the pro-protein. **Plant Physiology**, v.101, p.1341-1348, 1993.

PUSZTAI, A.; BARDOCZ, G.G.; ALONSO, R.; CHRISPEELS, M.J.; SCHROEDER, L.M.; TABE, T.J.; HIGGINS, T.J. Expression of the insecticidal bean alpha-amylase inhibitor transgene has minimal detrimental effect on the nutritional value of peas fed to rats at 30% of the diet. **Journal of Nutrition**, v.129, p.1597-1603, 1999.

SALI, A.; BLUNDELL, T.L. Comparative protein modelling by satisfaction of spatial restrints. **Journal of Molecular Biology**, v.234, p.779-815, 1993.

SCHAGGER, H.; JAGOW, G. von. Tricine-sodium dodecyl sulfate-polyacrylamide gel eletrophoresis for the separation of proteins in the range from 1 to 100 kDa. **Analytical Biochemistry**, v.166, p.368-379, 1987.

SHADE, R.E.; SCHROEDER, H.E.; PUEYO, J.J.; TADE, L.M.; MURDOCK, L.L.; HIGGINS, T.J.V.; CHRISPEELS, M.J. Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. **Biotechnology**, v.12, p.793-796, 1994.

SILVA, M.C.M. da; GROSSI-DE-SÁ, M.F.; CHRISPEELS, M.J.; TOGAWA, R.C.; NESHICH, G. Analysis of structural and physico-chemical parameters involved in the specificity of binding between α-amylase and their inhibitors. **Protein Engineering**, v.13, p.167-177, 2000.

STROBL, S.; MASKOS, K.; BETZ, M.; WIEGAND, G.; HUBER, R.; GOMIS-RÜTH, F.X.; GLOCKSHUBER, R. Crystal structure of yellow mealworm α-amylase at 1.64 Å resolution. **Journal of Molecular Biology**, v.278, p.617-628, 1998.

SUZUKI, K.; ISHIMOTO, M.; KIKUCHI, F.; KITAMURA, K. Growth inhibitory effect of an α-amylase inhibitor from wild common bean resistant of the Mexican bean weevil (*Zabrotes subfasciatus*). **Japanese Journal of Breeding**, v.43, p.257-265, 1993.

TAKAHASHI, T.; HIRAMOTO, S.; WATO, S.; NISHIMOTO, T.; WADA, Y.; NAGAI, K.; YAMAGUCHI, H. Identification of essential amino acid residues of an α-amylase inhibitor from *Phaseolus vulgaris* white kidney beans. **Journal of Biochemistry**, v.126, p.838-844, 1999.

VERVLIET, G.; HOLSTERS, M.; TEUCHY, H.; MONTAGU, M. van; SCHELL, J. Characterization of different plaque-forming and defective temperature phages in *Agrobacterium* strains. **Journal of General Virology**, v.26, p.33-48, 1975.

Received on September 2, 2003 and accepted on December 15, 2003