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Molecular Cloning and Expression of an α-Amylase Inhibitor from Rye with Potential for Controlling Insect Pests

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Alpha-amylase inhibitors have important roles in plant defense mechanisms, particularly against insects, and several of these inhibitors have been expressed in different crops to increase their resistance to particular insects. In this work, we report the cloning and expression of a gene encoding for a new α-amylase inhibitor (BIII) from rye (*Secale cereale*) seeds. The *BIII* gene contains 354 nucleotides that encode for 118 amino acids sequence. A 313 bp fragment of the gene was expressed in *Escherichia coli* and resulted in a functional inhibitor that reduced the activity of α-amylases of larvae of the coleopteran pests *Acanthoscelides obtectus*, *Zabrotess subfasciatus* and *Anthonomus grandis*. In contrast, the inhibitor did not inhibit the activity of porcine pancreatic α-amylase. Although the amino acid sequence of BIII showed high identity with those of bifunctional inhibitors, the recombinant protein was unable to inhibit trypsin-like serine proteinases. The effects of recombinant BIII were evaluated *in vivo* against *A. grandis*. When first instar larvae were reared on an artificial diet containing four different concentrations of BIII, a reduction in larval weight and a mortality of 83% were observed at the highest concentration.

KEY WORDS: α-amylase inhibitor, CM protein, insect pest, molecular cloning, plant defense.

1. INTRODUCTION

Insect pests and plant pathogens are responsible for severe crop losses worldwide. Attack by pests account for approximately 37% of the losses in agricultural production, with small-scale farmers being hardest hit (Gatehouse *et al.*, 1992) since the crop plants are frequently exposed to insect herbivores that attack roots, leaves, stems and flowers (Bezemer *et al.*, 2003). However, plants have evolved sophisticated defense mechanisms, most of which are constitutively active or may be induced, and involve the accumulation of a wide range of compounds that confer resistance to phytophagous predators (Carlini and Grossi-de-Sá, 2002; Mello and Silva-Filho, 2002). These defensive compounds include proteins and peptides such as lectins (Hartley and Lord, 2004), arcelins (Carlini and Grossi-de-Sá, 2002), RIPs (Stirpe, 2004), defensins (Melo *et al.*, 2002; Thevissen *et al.*, 2001) and enzyme inhibi-

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Abbreviations: AgA, *Anthonomus grandis* α-amylase; AoA, *Acanthoscelides obtectus* α-amylase; BIII, α-amylase inhibitor from rye seeds; Bt, *Bacillus thuringiensis*; CM, proteins chloroform–methanol proteins; HSA, human salivar α-amylase; pBIII, partial nucleotide sequence of BIII; PPA,porcine pancreatic α-amylase; RBI, Ragi bifunctional inhibitor; RIPs, ribosome inactivating proteins; TMA, *Tenebrio molitor* α-amylase; ZSA, *Zabrotes subfasciatus* α-amylase.

tors of insect digestive proteinases and α-amylase that occur in large quantities in cereal and legume seeds (Franco *et al*., 2002; Svensson et al., 2004).

A large number of proteinaceous α-amylase inhibitors have been isolated from plant seeds (Franco *et al*., 2002; Svensson *et al*., 2004), particularly from members of the Leguminosae such as the pigeonpea, *Cajanus cajan* (Giri and Kachole, 1998), cowpea, *Vigna unguiculata* (Melo *et al*., 1999) and common bean, *Phaseolus vulgaris* (Grossi-de-Sá *et al*., 1997), and from cereals such as wheat, *Triticum aestivum* (Franco *et al*., 2000), sorghum, *Sorghum bicolor* (Bloch and Richardson, 1991) and rye, *Secale cereale* (Iulek *et al*., 2000). A large range of α-amylase inhibitors that vary in molecular mass, degree of multimerization and specificity occurs in these species.

Alpha-amylase inhibitors may be classified into six families (lectin-like, knotting-like, Kunitz-like, γ-purothionin-like, thaumatin-like and cereal-type) based on their primary and tertiary structures, disulphide bonds positions and reactive site localization (Richardson, 1991). A prototype group of such inhibitors are CM (chloroform-methanol) proteins, classified as bifunctional inhibitors because of their ability to interact with α-amylases from insects and mammals and also with trypsin-like proteinases (Halford *et al*., 1988). CM (chloroform-methanol) proteins show a typical double-headed α-amylase/trypsin domain (Campos and Richardson, 1983) that can inhibit α-amylases (Barber *et al*., 1986a) and trypsin-like enzymes (Barber *et al*., 1986b; De Leo *et al*., 2002) separately or at the same time (Garcia-Maroto *et al*., 1991). These hydrophobic proteins are soluble in high salt solutions and can be extracted with chloroform/methanol mixtures, and are present in aqueous extracts of developing and mature seeds (Finnie *et al*., 2002). The CM protein family includes lipid transfer proteins (Lerche and Poulsen, 1998; Svensson *et al*., 1986) and proteins related to cold tolerance (Hincha, 2002).

Several CM protein α-amylase inhibitors have been isolated from cereals (Halford *et al*., 1988) and a new inhibitor belonging to this family, known as BIII, was purified from rye (*S. cereale*) seeds (Iulek *et al*., 2000). This protein had a monomer molecular mass of 13,756 Da, was capable of dimerization and had potential glycosylation sites. Despite its homology with bifunctional inhibitors, BIII was not active against mammalian or insect trypsins, although low activity against porcine pancreatic and human salivary α-amylase and high inhibitory activity against *Acanthoscelides obtectus*, *Zabrotes subfasciatus* and *Anthonomus grandis* α-amylases was observed (Iulek *et al*., 2000; Oliveira-Neto *et al*., 2003). These findings suggested that BIII could be potentially useful in producing transgenic crop plants resistant to attack by insects.

In this report, we describe the molecular cloning and nucleotide sequence of the *S. cereale BIII* gene and the heterologous expression of a truncated form of this protein with 103 amino acids sequence containing the motifs responsible for the inhibitory activity. Recombinant BIII protein was purified and its activity against several insect pests was evaluated *in vitro* and *in vivo*.

2. MATERIAL AND METHODS

2.1. Isolation of Midgut Fluid

Anthonomus grandis larvae were obtained from the Biological Control Department of Embrapa (Brasília, Brazil). The larvae were reared at $27 \pm 1°$ C in a relative humidity of $70 \pm 10\%$ and a 14-hr photoperiod. The insects were routinely maintained on a standard diet as described by Monnerat *et al*. (1999). When required, the guts were excised from the larvae and adult insects and placed in isotonic saline solution (0.15 *M* NaCl). Midgut tissues were homogenized in saline solution and centrifuged for 10 min at $10,000\,g$ at 4°C and the supernatant then removed and used for the enzyme assays.

2.2. Cloning of the BIII Gene

To clone a nested fragment of the *BIII* gene, two oligonucleotide primers BIIIFOR2 (5′-GAAGATTGCACCCCATGG-3′) and BIIIREV (5′-GTGTTGTGAACGGTCGTC-3′) were designed and used in a PCR reaction. The amplifications were done in a PTC-100 programmable thermal cycler (MJ Research) under the following conditions:2 min at 94°C then 30 cycles of 30 s at 94°C, 1 min at 50°C and 1 min at 72°C plus a final extension step for 5 min at 72°C, using 2 μg of total DNA from rye leaves (cultivar Br-1 EST 96-66061, obtained from IAPAR-Paraná, Brazil). The resulting 311 bp fragment encoding the bifunctional α-amylase/trypsin inhibitor (referred to here as partial *BIII* or *pBIII*) was amplified. To obtain the 3′ region of the gene, two specific primers BIII3'FOR1

(5′-GTACCTCGCCAAACAGCAGTG-3′) and BIII3'-FOR2 (5′-GGATGCCCTAGGGAGGTCC-3′) were designed using the *pBIII* sequence and a novel degenerate primer RIIINEWREV (5′-CGCAACATGCT-TITTTATTC-3′) was designed using consensus data from the 3′ regions of other α-amylase/trypsin inhibitor genes. Amplification by PCR was done using two steps, as described earlier, except that the annealing temperature was 45°C. In the first step, the primers BIII3'FOR1 and RIIINEWREV were used. To increase the specificity, a second PCR reaction was done using 1 μl of the product of the first PCR reaction as the template and the primers BIII3'FOR2 and RIIINEWREV. The resulting 187 bp product was cloned as described above. The amplified DNAs were cloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI), and the clones were sequenced in both strands in an automated DNA sequencer. Computer analysis of the DNA and deduced amino acid sequences was done using the GCG package (Genetics Computer Group, Inc.).

2.3. Heterologous Expression in *Escherichia coli*

The 311 bp *pBIII* fragment was cloned into the pET102D TOPO® (Invitrogen) vector to produce an N-terminal fusion of pBIII to thioredoxin and a 6× His tag at the C-terminal. This construct was used to transform *E. coli* strain BL21 (DE3) pRYL competent cells. Expression of recombinant protein was induced in cells grown at 28°C using 0.5 m*M* isopropyl-1-thiol-β-d-galactopyranoside (IPTG), added to Luria-Bertani media (LB) containing 100 μg of ampicillin ml^{-1}, 12.5 μg of chloramphenicol ml^{-1} and 12 μg of tetracycline ml^{-1}. After 1–3 hr of induction, the cells were harvested by centrifugation and stored at −20°C prior use. The solubility of the recombinant protein was analyzed at different temperatures and salt concentrations. SDS-PAGE was performed using 12% polyacrylamide gels, as described by Laemmli (1970).

2.4. Purification, Digestion and Refolding of Recombinant pBIII

The cells pellet containing recombinant protein was suspended in lysis buffer (50 m*M* $NaHPO_4$ pH 7.8, 300 m*M* NaCl, 50 m*M* K_2PO_4, 10% glycerol, 0.5% Triton X-100 and 10 m*M* imidazol) and sonicated using 10 pulses at 10 s. The lysate was centrifuged at 10,000 *g* for 20 min and the supernatant then loaded onto a Ni-NTA (Qiagen®) affinity column. Unbound proteins were removed with five washes of buffer (50 m*M* $NaHPO_4$ pH 7.8, 300 m*M* NaCl and 20 m*M* imidazol) and the recombinant proteins were eluted with the same buffer containing 250 m*M* imidazol. After purification, extensive dialysis against 0.1 *M* Tris–HCl buffer (pH 8.0) was performed to optimize the protein digestion by enterokinase in Tris buffer (500 m*M* Tris–HCl pH 8.0 containing 25 m*M* $CaCl_2$ and 1% Tween-20). Enzymatic digestions were done for 40 hr and monitored by SDS-PAGE (data not shown). Renaturation of recombinant pBIII was carried out according to Guay *et al.* (2000). The purified protein was refolded by a slow drop-wise dilution (<20-fold dilution, 25 ml in 450 ml) in stirred refolding buffer (50 m*M* Tris–HCl pH 8.0 containing 0.5 m*M* EDTA, 10 m*M* reduced glutathione and 1 m*M* oxidized glutathione). After stirring overnight at 4°C, the solution was concentrated using an Amicon ultrafiltration system with a nominal cut-off of 5 kDa. The concentrated solution was dialyzed against water and clarified by centrifugation.

2.5. Inhibitory Activity Assays

The inhibitory activity of pBIII was analyzed against porcine pancreatic α-amylase (PPA) and three insect gut α-amylases from larvae of *A. obtectus* (AoA), *Z. subfasciatus* (ZSA) and *A. grandis* (AgA). α-Amylase activity was analyzed by the iodometric method (Figueira *et al.*, 2003). Inhibitory activity was measured by pre-incubating the α-amylase inhibitor with the desired enzyme (0.2 U) for 40 min at 25°C. After the addition of substrate (1% starch), the reaction was left to proceed for 60 min at 25°C and activity was measured by the change in absorbance at 660 nm. The inhibition of proteolytic activity was measured according to Kakade *et al.* (1969) against *Spodoptera frugiperda* and *A. grandis* trypsin-like enzymes, bovine pancreatic chymotrypsin and trypsin, and bacterial proteinases.

2.6. Activity of Recombinant pBIII Towards Cotton Boll Weevils

The bioassays were carried out in six-well plates containing 5.0 ml of artificial diet sterilized in each well (Dias *et al.*, 2000). The recombinant protein was incorporated into the diet at three different concentrations: 0.05%, 0.2%, 0.48% and 0.89%.

Protein concentrations were determined according to Bradford (1976) and the percentage of recombinant α-amylase inhibitor was estimated by optical densitometry (Scan Zero Program) in 12% SDS-PAGE stained with Coomassie blue. Ten first instar larvae were fed one of each of the artificial diets. Two negative controls were used:(i) distilled water and (ii) non-IPTG induced *E. coli* strain BL21 (DE3) pRYL added to the artificial diet at a greater concentration than for the recombinant protein. The mortality was calculated as the percentage of neonate larvae that completed their development through to emergence. Possible sub-lethal effects in adult insects were evaluated by measuring the weight, body size, longevity and fertility. Each treatment was done in triplicate. The results were analyzed using ANOVA, or the non-parametric Kruskal–Wallis test when the data were not normally distributed. Pair wise multiple comparison procedures (Student–Newman–Keuls test) were used to assess the effects of the different treatments (Sokal and Rohlf, 1981).

2.7. Computer Models of the Amylase-Inhibitor Complex

A model of α-amylase from *Tribolium molitor* guts (TMA) complexed with BIII was constructed based on the crystal structure of TMA and the *Ragi* bifunctional inhibitor RBI (Strobl *et al.*, 1998) and the known 3D similarity between RBI and the CM protein family of α-amylase inhibitors. The SwissProt Data Bank (SPDB)-Viewer was used to fit the BIII sequence onto the RBI part of the TMA–RBI complex (1TMQ.pdb). One hundred and three Cα atoms could be superimposed with an rms fit of 2.1 Å. Visual inspection of the initial TMA–BIII model showed several steric clashes. In many cases, these were resolved by manipulating the BIII side chain so that it adopted the conformation seen for the corresponding RBI side chain. Since large conformational changes were beyond the scope of this modeling, the three C-terminal residues were deleted from our model.

For additional model refinement, a scheme of energy minimization was applied, using the program Gromos96 (Stocker and Van Gunsteren, 2000). Since the original model probably contained errors both of overall inhibitor orientation relative to the enzyme and of side chain conformations, a combination of rigid body and positional energy minimization was used. Four rounds of positional refinement (50 cycles) followed by rigid body refinement (100 cycles) were done. Alternate cycles of side chain and rigid body refinement were designed to improve the quality of modeled complex structures.

2.8. Sequence and Model Analyses

The program SPDB-viewer 3.7 was used to visually inspect the models. An alignment of CM proteins was done with CLUSTALW (Pearson and Lipman, 1988). Where crystal structure information was available, this was modified in the light of DALI structural alignments. The Bio-Info Meta server was also used to align the inhibitors. X-PLOR was used to define residues at the interfaces of our models. To be defined as an interface-forming residue, any atom of the inhibitor residue was required to be within 4 Å of an enzyme atom. This same rule applied to enzyme interface-forming residues.

3. RESULTS

3.1. Cloning and Expression of BIII

The *BIII* gene contained a 354 bp open reading frame that encoded a predicted protein of 118 amino acids sequence. The molecular mass of the predicted translated *BIII* gene was 11.5 kDa, with a theoretical pI of 5.3. The YinOYang program (Blom *et al.*, 1999) predicted one possible O-glycosylation site at Thr49 and one putative N-glycosylation site (NLT) at Asn100 (Figure 1).

The deduced amino acid sequence of BIII showed homology to several α-amylase/trypsin inhibitors from barley, wheat, finger millet and rye. BIIII showed 91% identity to CM16, a bifunctional α-amylase/trypsin inhibitor from barley (Lullien *et al.*, 1991) and all 10 cysteine residues were conserved (boxed in Figure 2). In addition, 88% identity was observed between BIII and two other CM proteins, CM17 (Lulien *et al.*, 1994) and CMb (Medina *et al.*, 1993).

pBIII was cloned into the pET102D TOPO® vector and expressed in *E. coli* strain BL21 (DE3) pRYL. After refolding, the recombinant protein was assayed against α-amylase and proteinases of important agricultural pests. A high level of recombinant protein expression was obtained within 3 hr after the addition of 0.5 m*M* IPTG (Fig. 3A).

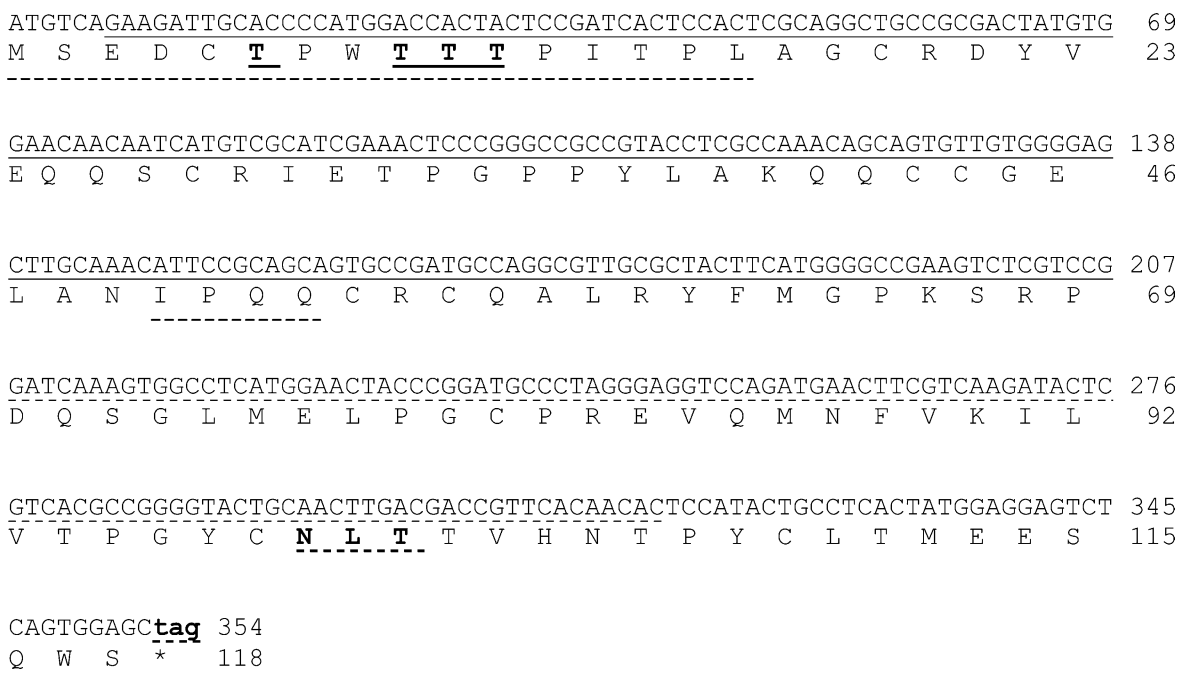

Fig. 1. Nucleotide sequence of *BIII* gene and deduced amino acid sequence. The underline nucleotide sequence indicates fragment of the *BIII* gene utilized for cloning in *E. Coli*. O-β-GlcNAc attachment sites in α-amylase sequences (bold and underlined) were predicted by YinOYang 1.2 Prediction Server (http://www.cbs.dtu.dk/services/YinOYang/). The dotted line indicates the α-amylase inhibitory motif that interacts with enzyme.

SDS-PAGE analysis of total protein extracts from induced bacterial clones showed a protein with an apparent molecular mass of 27.5 kDa, in agreement with the expected size of the recombinant protein when fused with thioredoxin. This band did not exist in extracts from non-induced cells (Figure 3A, lane 2). Treatment with 0.2 m*M* IPTG, 3.5 m*M* betain and 1 *M* sorbitol for 1 hr at 26°C reduced the number of inclusion bodies and increased the amount of recombinant protein in soluble extracts. Digestion with enterokinase was used to eliminate any influence of thioredoxin on the activity of recombinant fusion protein. The recombinant protein was purified (Fig. 3B), refolded, cleaved and used in enzyme inhibition assays.

3.2. Inhibitory Activities In Vitro and In Vivo

The activity of pBIII towards porcine pancreatic amylase and insect α-amylase (AoA, ZSA and AgA) was determined *in vitro*. Inhibition was observed against insect α-amylases, but no inhibition effect was observed against the porcine enzyme at the same concentration. The inhibition of insect α-amylases was ~40%, which was similar to that described for this protein purified from rye seeds (Iulek *et al*., 2000; Oliveira-Neto *et al*., 2003). pBIII had no inhibitory activity against any of the serine proteinases tested (*S. frugiperda* and *A. grandis*) trypsin-like proteinases, bovine chymotrypsin and trypsin, and *Streptomyces* proteinases.

To confirm the insecticidal activity of the inhibitor in insect pests, the recombinant protein was tested against *A. grandis* by incorporating different amounts of the protein (0.05%, 0.2%, 0.45% and 0.89%, w/v) into an artificial diet. The recombinant protein caused mortality of all concentrations tested. Significant mortality (83%) was observed at the highest concentration of recombinant protein tested (ANOVA $F = 6.4$; gl = 6.3; $p = 0.04$; Student–Newman–Keuls test $p < 0.05$). The same concentration of BIII reduced the body weight of the insects by 13.3–38.6% (Figure 4), but no effect in the body size, longevity and fertility of adult were found (data not shown). The high mortality found with the highest concentration of *BIII* tested hindered the formation of viable mating couples and made it difficult to evaluate fertility and longevity. The inhibitor did not cause any deformities in the larvae, pupae and adult insects.

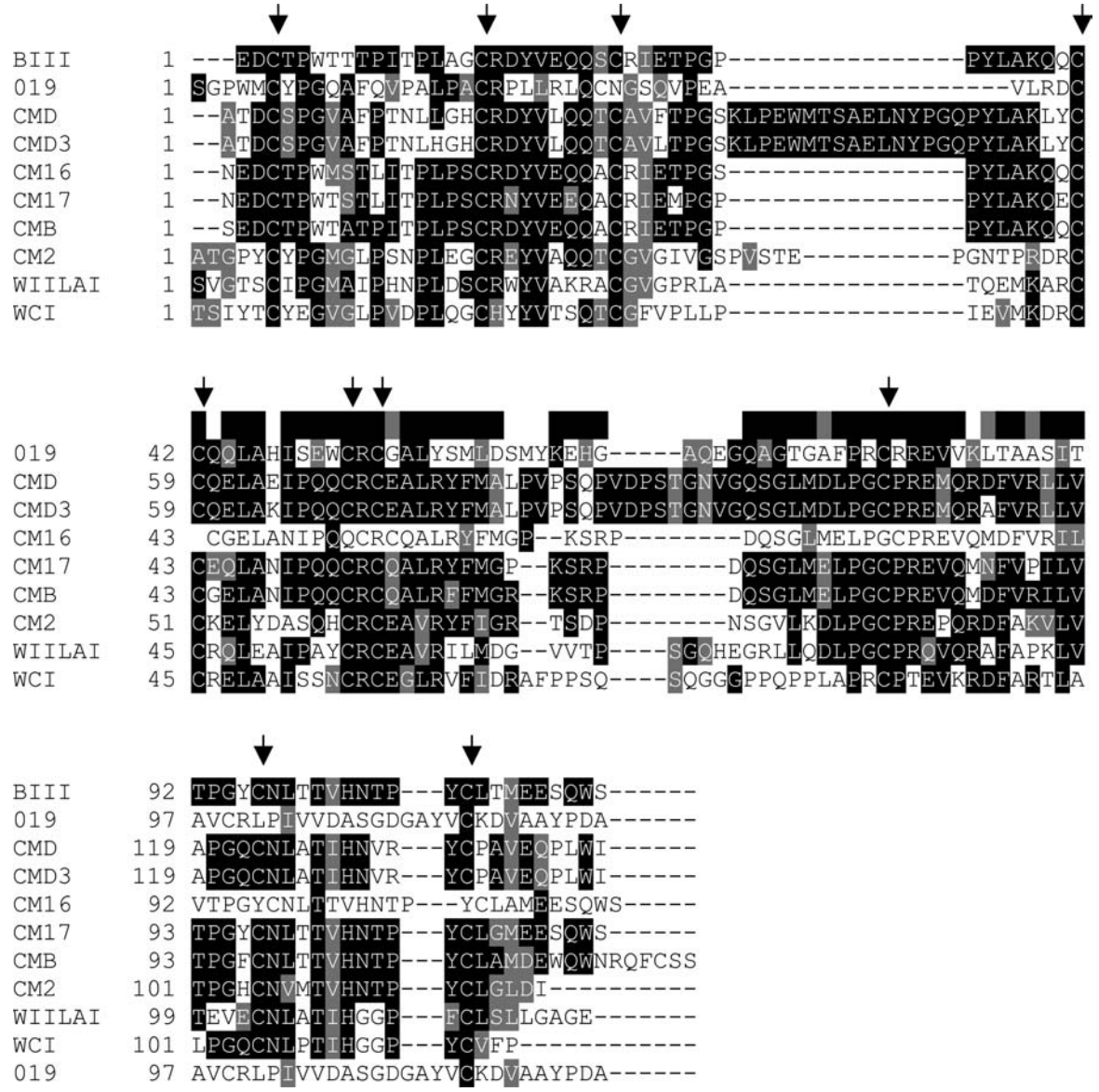

Fig. 2. Multiple sequence alignment of deduced amino acids sequences of *BIII* with several α-amylases/trypsin inhibitor and trypsin inhibitor from cereal kernels. Sequences used are:CMd, CMD3 and CMb:α-amylase/trypsin inhibitor from barley; CM16, CM2 and CM 17:α-amylase/trypsin inhibitor from wheat. 0.19:α-amylase inhibitor from wheat. WIILAI:α-amylase/trypsin inhibitor from finger millet; WCI:Trypsin inhibitor from wheat. Black arrows show the conserved cysteine residues. Black shading indicates residues that are identical in BIII, while similar residues are gray. The alignment was generated using ClustalW 1.6, together with the Boxshade server (http://ulrec3.unil.ch/software/BOX_html).

4. DISCUSSION

Alpha-amylases are ubiquitous proteins that play a key role in carbohydrate metabolism in various insects, especially those, such as seed weevils, that feed on starchy seeds during the larval and/or adult stages and depend on their α-amylases for survival (Franco *et al*., 2000; MacGregor *et al*., 2000). Because of their important biochemical roles in insect growth and development, there is an interest in isolating and identifying proteinaceous inhibitors that act against these α-amylases (Svensson *et al*., 2004).

In this report, we decribe the molecular cloning of *BIII*, an α-amylase inhibitor that has been purified and partially characterized from rye seeds (Iulek *et al*., 2000). DNA sequencing showed that *BIII* gene exactly matched the previously reported

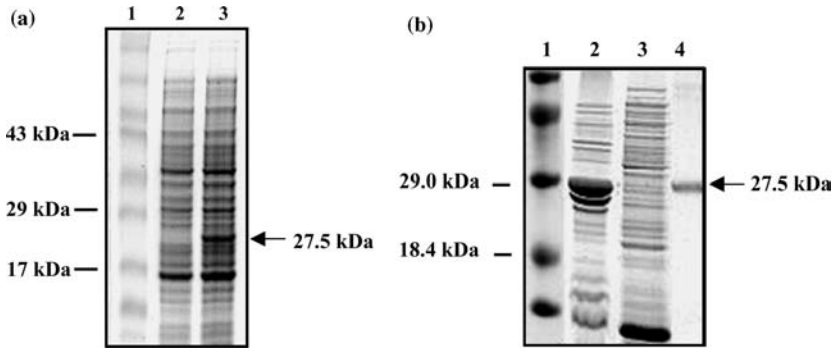

Fig. 3. (a) SDS-PAGE analysis of induced *E.coli* cells crude extract grown for 3 hr at 37°C. Lane 1:Molecular mass marker. Lanes 2:Non-induced extracts after 3 hr. Lane 3:Induced extracted after the same time. (b) Expression and purification of recombinant domain in *E.coli* system. Lane 1:molecular mass marker. Lane 2:Soluble fraction extract. Lane 3:Wash with 250 m*M* imidazole Lane 4-purified recombinant protein. Expression was induced with 0.5 m*M* IPTG after 3 hr at 28°C and purified in affinity chromatography using Ni-column. The total protein extracts were analyzed by 12% SDS-PAGE and stained with Coomassie blue. Arrow indicates recombinant protein with 27.5 kDa.

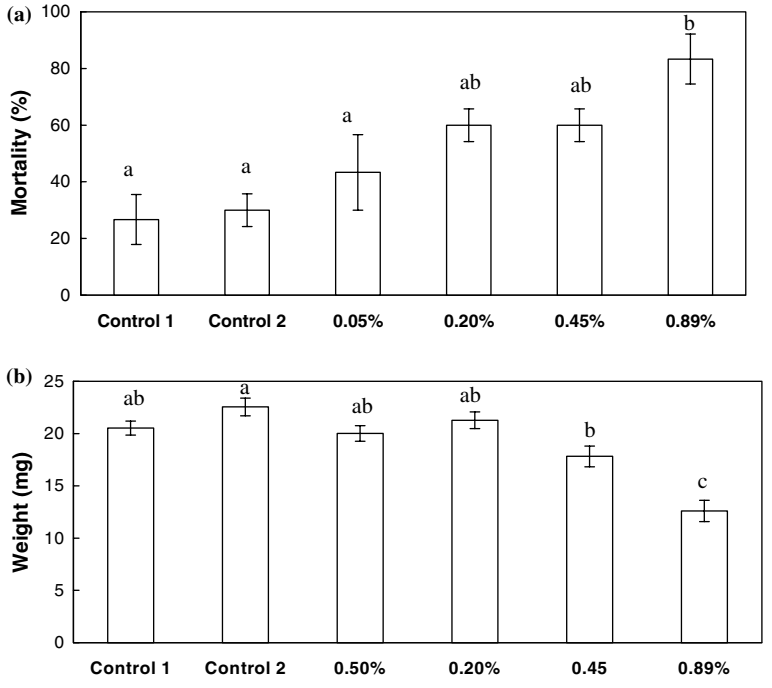

Fig. 4. Mortality (a) and adult weight (b) of the boll weevil developed in artificial diet in the presence of different concentrations of recombinant protein pBIII. Control 1:artificial diet containing water, Control 2:artificial diet added with *E. coli* BL21 (DE3) pRYL no IPTG induced. Columns in each graphic followed by same letters ar not significantly different (Student–Newman–Keuls tests, p).

partial N-terminal peptide sequence (Iulek *et al.*, 2000). This was confirmed when the molecular mass (~13 kDa) obtained by SDS-PAGE (Iulek et al., 2000) was compared to the theoretical molecular mass predicted from the translated *BIII* DNA. Sequence analyses of BIII showed that this α-amylase inhibitor is the first member of the CM protein group cloned from rye seeds (Figure 2). CM proteins have been purified from wheat (CM1, CM2, CM3, CM16 and CM17) and barley seeds (CMa to CMe) (Barber *et al.*, 1986a; García-Maroto *et al.*, 1991; Halford *et al.* 1988). The BIII sequence showed 10 half-cysteines capable of forming a disulfide bond. The cysteines at positions 4 and 18 are absolutely conserved in the cereal bifunctional-inhibitor (Strobl *et al.*, 1998), and in other families of CM protein inhibitors (Lyons *et al.*, 1987; Medina *et al.*, 1993). This finding suggests that disulfide

bonds are important in establishing the active conformation of these inhibitors (Laskowski and Kato, 1980).

A truncated version of BIII, pBIII, containing several structural regions responsible for inhibitory activity after heterologous expression, was used in our tests. This is not the first time that a CM protein has been expressed in a heterologous system. A cDNA of CM16 was cloned in *E. coli* and, after purification; the recombinant protein was characterized biochemically (Lullien *et al.*, 1994). Another CM protein, CMe, one of the best characterized members of this family, was also expressed in tobacco (Lara *et al.*, 2000) and rice (Alfonso-Rubí *et al.*, 2003). The pBIII recombinant protein accumulated in inclusion bodies, which are less susceptible to proteolytic attack. The presence of these structures drastically reduced the solubility of the recombinant protein, making it necessary refold the protein before determining the inhibitory activity of pBIII.

Enzyme assays confirmed the specificity of pBIII in inhibiting insect α-amylases. Some α-amylase inhibitors show strict target enzyme specificity and recognize only one out of several closely related isozymes (Franco *et al.*, 2000; Weselake *et al.*, 1983). Other inhibitors have high affinity for both mammalian and insect α-amylases. For example, 0.53 and 0.28 from wheat kernels, which belong to the CM protein family (Franco *et al.*, 2000), as well a knotting-like inhibitor α-AAI from *Amaranthus* seeds (Pereira *et al.*, 1999) and a lectin-like inhibitor, α-AI2, from *P. vulgaris* seeds (Grossi-de-Sá *et al.*, 1997) show specificity for insect α-amylases. This desirable characteristic of BIII was also observed by Iulek *et al.* (2000), who reported the ability of this protein to strongly inhibit ZSA, AoA and AgA, with only low activity against mammalian α-amylases.

Threading analyses of BIII, using the Bio-Meta server, showed that this protein had striking structural homology to the RBI inhibitor from *E. coracana* seeds. A molecular model of BIII was obtained by molecular replacement in the complex formed by this inhibitor and α-amylase from *T. molitor* guts (TMA). In this BIII–TMA complex, the inhibitor binds to the active site of the enzyme (Fig. 5, green residues). In particular, three functional segments can be identified in BIII that interact with the α-amylase in a very specific manner. Segment 1, comprising the N-terminal residues Ser1-Thr8, protrudes like an arrowhead into the TMA substrate-binding groove and directly targets the active site of the enzyme (blue region in Fig. 5), the collar region of BIII (blue region in Fig. 5), which stabilizes the complex around the arrowhead (Fig. 5), and the C-terminal residues, which may also prevent the access of substrate by steric hindrance, as seen in the RBI–TMA complex (Strobl *et al.*, 1998).

An important consideration was to compare the inhibitory efficiency of natural and recombinant pBIII. As previously observed (Iulek *et al.*, 2000), 25 μg of protein was sufficient to inhibit ZSA by 85%. In contrast, 50 μg of recombinant pBIII caused only 21% inhibition. This lower potency could be explained by the absence of the N-terminal serine. When the structure of the RBI–TMA complex was analyzed (Strobl *et al.*, 1998), this serine was found to be involved in numerous hydrogen bonds with residues in the active site of the cognate enzyme. Garcia-Maroto *et al.* (1991) observed similar results, with a decrease in inhibitor activity and in the formation of enzyme-inhibitor complex when site-directed mutagenesis altered the N-terminal sequence anterior to the first cysteine.

Another important question addressed by molecular modeling was the specificity for insect amylases. A comparison of the modeled interfaces of the BIII–TMA and 0.19-TMA/HSA complexes showed a single difference in the sequence of protein 0.19, which inhibits porcine pancreatic α-amylase efficiently (Franco *et al.*, 2000), compared to that of BIII, which inhibits the same enzyme with a lower affinity (Iulek *et al.*, 2000). At position 47, protein 0.19 has a His residue whereas BIII has an Asn (Fig. 2). An Asn at this position would hinder binding to mammalian α-amylase because of its proximity to Glu349 of the porcine enzyme, thereby significantly reducing the specificity of pBIII for mammalian α-amylases.

The modeling of complexes between wheat inhibitors and insect amylases supports the finding that inhibitors with even 98% sequence identity can have different specificities and biochemical properties (Franco *et al.*, 2000). The previous finding that BIII does not inhibit proteinases (Iulek *et al.*, 2000) was confirmed here, at least for serine proteinases. This lack of effect could be explained by the absence of a loop constructed by residues Arg34-Leu35. This loop occurs in RAGI inhibitors and has been identified as the reactive site of bifunctional inhibitors (Campos and Richardson, 1983; Lyons *et al.*, 1987). The *BIII* sequence was submitted to GeneBank as a member

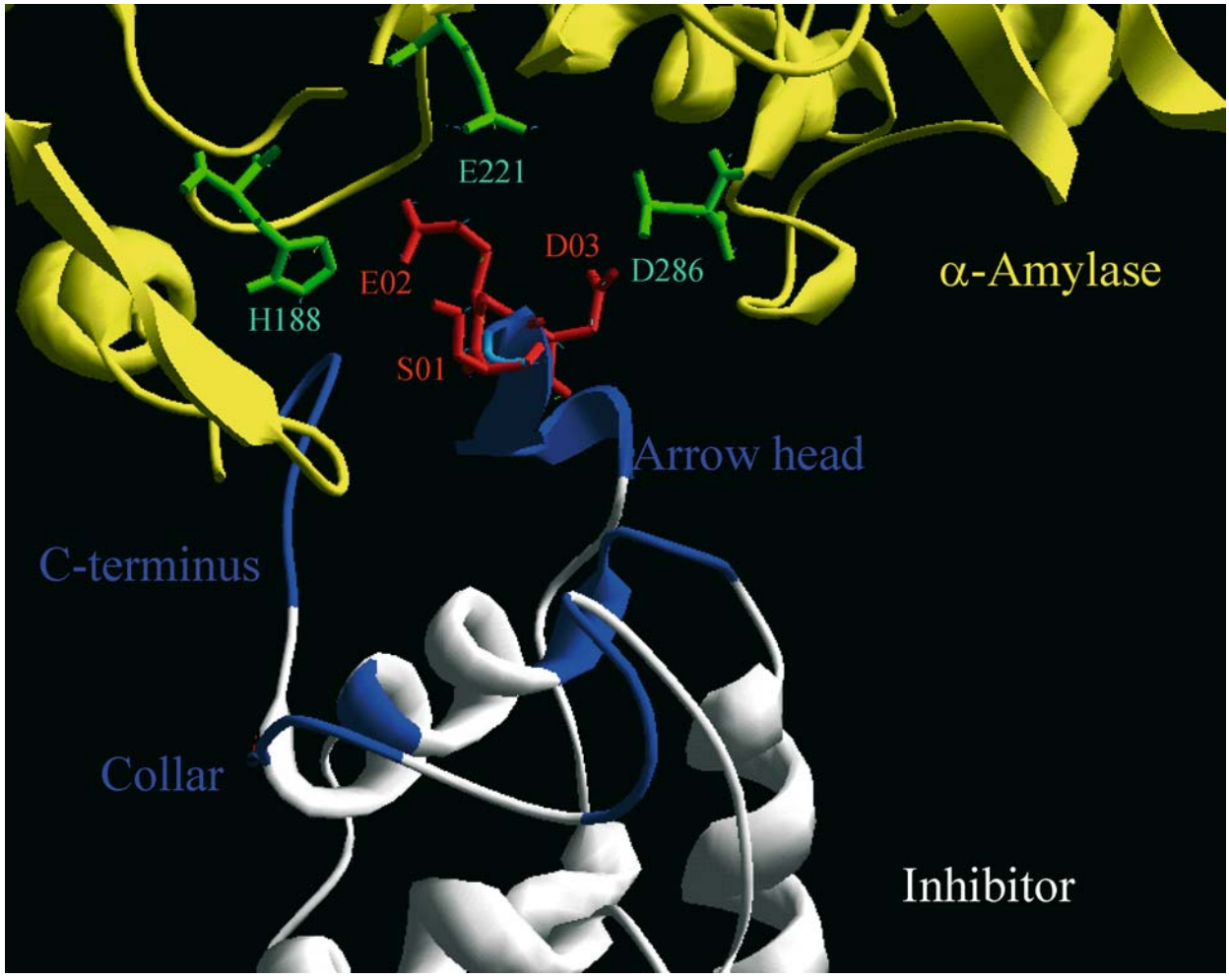

Fig. 5. Structural model of BIII (white) complexed to TMA (yellow). Red (inhibitor) and green (enzyme) side chains are involved in inhibition process as well the blue regions. Model was drawn using SPDViewer 3.7.

of the bifunctional α-amylase/trypsin inhibitor family. However, pBIII was unable to inhibit certain enzymes in these two widely divergent groups. This finding suggests that most of the GeneBank assignments are based solely on homology to the only member of the family for which both activities have been demonstrated. The results presented here suggest that caution should be exercised in drawing functional conclusions solely from sequence information, particularly for this family.

The presence of α-amylases in the alimentary tract of *A. grandis* (Oliveira-Neto *et al.*, 2003), *A. obtectus* and *Z. subfasciatus* (Grossi-de-Sá *et al.*, 1997; Silva *et al.*, 2000), and the reliance upon these enzymes for feeding, may provide an efficient strategy for controlling these pests with an amylase inhibitor. The bioassay showed that pBIII adversely affected the boll weevil mortality and adult weight at all concentrations tested (Fig. 4). Similar results were observed in bioassays using α-AI1 against *Callosobruchus maculatus* and *Callosobruchus chinensis*, in which artificial seeds containing inhibitor at a concentration of 0.2% adversely influenced the larval development and an inhibitor concentration of 1.0% resulted in 100% mortality (Ishimoto and Kitamura, 1989).

The cotton boll weevil larvae that survived the treatment with pBIII developed into adults with a reduced weight. Insects with a reduced size or weight generally have lower than normal longevity and fertility. However, we were unable to confirm this hypothesis because it was impossible to successfully pair adults obtained at higher concentrations of pBIII. α-amylase inhibitors are generally assumed

to be effective inhibitors of larval development because they inhibit larval digestive amylases. Larvae that were fed a diet containing recombinant protein at a very early stage suffered strong adverse effects, probably because they were unable to hydrolyze the starch in the artificial diet. Furthermore, several reports have demonstrated a decrease in fertility and an increase in deformities in insects fed a diet containing solely proteinase inhibitors (De Leo and Gallerani, 2002; Franco *et al*., 2004; Rahbe *et al*., 2002). However, our results showed no deleterious effects of pBIII on fertility and/or metamorphosis.

Different strategies have been used to screen for α-amylase inhibitors in plants. Artificial entomopathogenic proteins were recently obtained by applying an *in vitro* evolutionary strategy using phage display and DNA shuffling technologies (Campos *et al*., 2004; Melo *et al*., 2003). Additionally, the use of a scaffold of the cellulose binding domain from *Trichoderma reesei* cellobiohydrolase Cel7A (Lehtiö *et al*., 2000) or of dromedary VHH antibodies raised against PPA have provided novel possibilities for discovering weapons against pests and pathogens (Desmyter *et al*., 2002). Numerous genes conferring resistance to pests have been incorporated into crop plants (Schuler *et al*., 1998; Carlini and Grossi-de-Sá, 2000), including α-amylase inhibitors. Transgenic pea plants transformed with two α-amylase inhibitors, α-AI1 and α-AI2, from *P. vulgaris* showed expression levels of 0.8–1.0% of the total protein in seeds, and showed complete protection against the pea weevil *Bruchus pisorum*, the cowpea weevil *C. maculatus* and the azuki bean weevil *C. chinensis* (Ishimoto *et al*., 1996; Morton *et al*., 2000). Furthermore, when a CM protein (CMe), which showed trypsin activity inhibitory, was expressed in tobacco and rice, a significant reduction in the survival of the rice weevil *Sitophilus oryzae* was observed, indicating the ability of CM inhibitors to control a coleopteran pest (Alfonso-Rubí *et al*., 2003; Lara *et al*., 2000). Several studies have demonstrated the potential of proteinase inhibitors against the boll weevil (Franco *et al*., 2003, 2004). The α-amylase inhibitor described here could be potentially useful in producing transgenic plants resistant to insect pests. This inhibitor could be used in association with other insecticidal proteins such as proteinase inhibitors or Bt toxin. Such an approach could improve the control of insect pests and decrease the development of resistance.

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