cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the α-amylases of the Western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract

We report the characterization and cDNA cloning of two α-amylase isozymes from larvae of the Western corn rootworm (*Diabrotica virgifera virgifera* LeConte). Larvae raised on artificial media have very low levels of amylase activity, and much higher levels are found in larvae raised on maize seedlings. At pH 5.7, the optimum pH for enzyme activity, the α-amylases are substantially but not completely inhibited by amylase inhibitors from the common bean (*Phaseolus vulgaris*) and from wheat (*Triticum aestivum*). Using the reverse transcriptase polymerase chain reaction (RT-PCR), we cloned two cDNAs with 83% amino acid identity that encode α-amylase-like polypeptides. Expression of one of the two cDNAs in insect cells with a baculovirus vector shows that this cDNA encodes an active amylase with a mobility that corresponds to that of one of the two isozymes present in larval extracts. The expressed enzyme is substantially inhibited by the same two inhibitors. We also show that expression in *Arabidopsis* of the cDNA that encodes the amylase inhibitor AI-1 of the common bean results in the accumulation of active inhibitor in the roots, and the results are discussed with reference to the possibility of using amylase inhibitors as a strategy to genetically engineer maize plants that are resistant to Western corn rootworm larvae. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: α-amylase; Rootworm; cDNA cloning

1. Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are a family of enzymes that catalyze the hydrolysis of the α-(1,4) glycosidic linkages in starch and related compounds. α-Amylases from insects and mammals have been characterized from a biochemical, molecular and structural point of view in considerable detail (see, for example, Qian et al., 1993; Grossi de Sa and Chrispeels, 1997; Strobl et al., 1998). Because α-amylases play a central role in carbohydrate metabolism, organisms with a starch-rich diet depend on the effectiveness of their amylases for survival. This is certainly the case for insects that are serious agricultural pests because they consume starch-rich plant organs such as seeds and roots. The Western corn rootworm (WCRW), *Diabrotica virgifera virgifera* LeConte (Coleoptera:Chrysomelidae), is a pest of maize in the United States and Europe in fields where crop rotation is not practiced. The greatest damage results from the underground feeding of the larvae, with only slight damage caused by the adults above ground. Larval root feeding decreases plant vigor by reducing the amount of water and nutrients supplied by the root system to the developing maize shoot. Extensive root damage weakens the root system and makes the plants more susceptible to lodging.

All plants possess a certain degree of resistance to insects, thus only a limited range of herbivores are able to feed on each individual species. At the same time, plants within one species or genus vary in their level of insect resistance, a fact used by plant breeders to increase the insect resistance of crop cultivars. Resistance to insects can also be introduced with transgenes, and recent commercial releases of genetically engineered crops have included transgenic maize, cotton and potato that express *Bacillus thuringiensis* (Bt) toxins (reviewed...
2.1. Insects

WCRW-resistant maize plants. To the prospect of using genetic engineering to create gene encoding AI-1 and discuss our results in relation to preparation of maize-reared larvae, incubated eggs were germ/casein-based diet (Marrone et al., 1985). For the hatch in plastic containers and larvae transferred with preparation of diet-reared larvae, eggs were allowed to hatch in soil at 25°C, 50% RH for 14–16 days post-oviposition. For the preparation of diet-reared larvae, eggs were transferred to two other legumes, Pisum sativum and Vigna angularis, and seeds of the transgenic plants showed complete resistance against azuki bean weevils, pea weevils and cowpea weevils (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996). The purpose of the present work was to characterize α-amylase(s) of WCRW with the ultimate goal of finding inhibitors that might be used to genetically engineer maize to be less susceptible to this pest. Since plant roots are starch-storing organs we assume that insects that eat roots need α-amylases to obtain nutrition from the roots.

This study shows that extracts of WCRW larvae contain two major α-amylase isozymes, and by reverse transcriptase polymerase chain reaction (RT-PCR) we were able to obtain two different full-length cDNA-derived amino acid sequences with extensive homology to other insect α-amylases. Two well-characterized inhibitors, AI-1 of P. vulgaris (Moreno and Chrispeels, 1989; Le Berre-Anton et al., 1997) and dimeric α-amylase inhibitor of Triticum aestivum (WI) (Maeda et al., 1985), were found to inhibit the activity of the two α-amylases of total larval extracts in vitro (60 to 75% inhibition). We demonstrate that active bean AI-1 accumulates in roots of transgenic Arabidopsis thaliana transformed with the gene encoding AI-1 and discuss our results in relation to the prospect of using genetic engineering to create WCRW-resistant maize plants.

2.2. α-Amylase inhibitors

Dimeric α-amylase inhibitor from wheat (Triticum aestivum) was purchased from Sigma Chemicals, St Louis, MO. α-Amylase inhibitor from P. vulgaris cv Greensleeves was purified from total bean extracts as described (Powers and Whitaker, 1977), with minor modifications.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from second and third instar larvae using the RNAeasy Total RNA kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer’s instructions. Poly(A)+RNA was purified from total RNA using Dynabeads Oligo(dT)25 (Dynal A.S., Oslo, Norway) according to the manufacturer’s instructions. mRNA (1 μg) was used to synthesize cDNA with Moloney murine leukemia virus reverse transcriptase (MoMLV-RT) (Gibco BRL) according to the manufacturer’s protocol and oligo(dT)25 primer.

2.4. 5’/3’ RACE PCR and cDNA cloning

To clone the full-length cDNA coding for putative α-amylase of WCRW (Dvα1) we used the same strategy that was employed to clone α-amylase from Zabrotes subfasciatus and has been described in detail (Grossi de Sa et al., 1997). To clone the 546 bp central fragment, two primers amy1 (5′ATCGTCAGCTGTCGATGG3′) and AS1 (5′GTGGCCGGCCACATGTG3′) were designed, which corresponded to short sequences conserved in a number of insect α-amylases. Amplification by the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable thermal controller (MJ Research, Inc., Watertown, MA, USA) using Taq polymerase (Perkin Elmer, Norwalk, CT, USA) under the following conditions: 2 min at 94°C then 30 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C plus an extension step for 5 min at 72°C. The 5′ and 3′ ends were amplified using a 5′/3′ RACE kit (Boehringer Mannheim) according to the manufacturer’s instructions. To amplify the 5′ end of Dvα1, cDNA specific nested primers AS2 (5′TCGGTTAGATATCTTCCCAC3′), AS3 (5′CTGGTTGATATCTTCCCAC3′) and
AS4 (5’CATTAATCCCCAGCATGTG3’) and oligo dT-Anchor primer provided by the kit were used. For 3’ end amplification the specific S3 (5’TAGAAACTGTGGCATTCCG3’) and oligo dT-Anchor primer provided by the manufacturer were used. Two specific primers, Sense1 (5’CAGGAATAGTCGTAAACACGATAACG3’) and Antisense1 (5’CGGATCCTTTTACAATTTGGGCATGTAATAGC3’), were used to amplify a full-length cDNA coding for Dva1 with Pfu I polymerase (Stratagene, San Diego, CA, USA) under the following conditions: 2 min at 94°C then 45 s at 94°C, 1 min at 50°C, 2 min 30 s at 72°C plus an extension for 5 min at 72°C. A set of six degenerate primers was designed to clone the central fragment of the cDNA coding for Dva2: three primers in the sense orientation, DEGS1 (5’GTCCATCTATTTGCGTTGACATGAATAGC3’), DEGS2 (5’GTGCACCTCTTCGAATGG3’) and DEGS3 (5’GTTCATCTTTTGTAGTG3’); and three in the antisense orientation, DEGAS1 (5’TCCAAATCGTGGCCGCCAC3’), DEGAS2 (5’CTCAAATCGGTCTCAATGG3’) and DEGAS3 (5’TGAGATCTCCCGGCCAC3’). The 548 bp product of PCR amplification was cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and inserts of 42 recombinant plasmids were sequenced to pick up a clone divergent from Shuster and Gifford (1962) with some modifications as described by Chrispeels and Varner (1967). We defined 1 unit of α-amylase as that amount of enzyme in total larval extract that decreases the A₂₅₀ of 400 μl of a 0.125% substrate solution of soluble starch by 75%. Up to this point the reaction rate is linear with time. One unit of α-amylase activity was incubated with substrate in a total volume of 1.2 ml of phosphate buffer pH 5.8 at 30°C for 25 min following by the addition of 5 ml of iodine reagent (Chrispeels and Varner, 1967).

To measure inhibitor activity, 1 unit of α-amylase activity was preincubated with different amounts of purified inhibitor at room temperature for 30 min prior to addition of substrate solution. To study the possible additive inhibitory effect, 1 unit of enzyme was incubated either with a given amount of each inhibitor for 30 min at room temperature or just with one inhibitor for 15 min followed by the addition of the same amount of the second inhibitor with subsequent incubation for another 15 min prior to the addition of the substrate solution. The α-amylase assay according to Bernfeld (1955) was also used and 1 unit of α-amylase activity was defined as the amount of enzyme that produced 1 μg of reducing sugar per minute at 30°C.

2.7. Detecting α-amylase activity and its inhibition in gel

To detect the α-amylase activity in a gel, two different electrophoresis systems were used. The first was a native 8% PAGE gel containing 0.5% soluble starch. After running, the gel was incubated for 30 min in 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.5 containing 20 mM NaCl and 0.1 mM CaCl₂. After quick rinsing with the same buffer without starch, the gel was stained with 3% KI/I₂ solution. Another system used for detection of α-amylase activity was isoelectric focusing in the pH range 3–9 (Pharmacia Biotech). Protein samples were run on pre-made minigels for the GelPhast system according to the manufacturer’s instructions for IEF 3–9 gels and incubated afterwards in 2% of substrate solution at room temperature for 30 min. After extensive washes in 100 mM phosphate buffer pH 5.8 containing 20 mM NaCl and 0.1 mM CaCl₂ the minigel was stained in the iodine solution.

2.8. Expression of Dva1 cDNA in insect cells Sf9

cDNA coding for putative α-amylase of WCRW Dva1 was expressed in a baculovirus expression system (Invitrogen, Carlsbad, CA, USA). The clone was inserted in pBlueBac 4.5 expression vector in NheI-HindIII sites and was cotransfected with viral linear Bac-N-Blue DNA using Sf9 insect cells culture in a log phase according to the manufacturer’s protocol. Six blue recombinant viral plaques were selected after plaque assay, and after viral amplification they were checked for α-amylase expression. One recombinant virus was chosen for α-amylase expression.
2.9. Expression of αAI-1 inhibitor from P. vulgaris in Arabidopsis thaliana

cDNA coding for αAI-1 inhibitor of α-amylase from P. vulgaris was expressed in A. thaliana under the CaMV35S constitutive promoter inserted in a pBII121 binary vector (Clontech, CA, USA). The construct was mobilized from E. coli strain DH5α to Agrobacterium tumefaciens C50 strain by direct transformation (An et al., 1988) and used to transform A. thaliana (var. Columbia) (Horsh et al., 1985).

2.10. Total protein extracts from leaves and roots of transgenic A. thaliana

Leaves and roots of transgenic A. thaliana plants were homogenized with an extraction buffer containing 50 mM TRIS–HCl, pH 7.4, 1% β-mercaptoethanol, 10% glycerol, 0.1% Triton X-100 and proteinase inhibitors cocktail (0.1 mg/ml pepstatin A, 0.03 mM leupeptin, 0.37 mg/ml aprotinin, 0.18 mg/ml phenylmethyl-sulfonyl fluoride) using a 1:4 (plant wt-buffer vol) ratio. After centrifugation at 14 000 rpm in an Eppendorf bench centrifuge at 4°C for 30 min, the supernatant was collected. Extracts were analyzed by 15% SDS–PAGE according to Laemmli (1970) and by immunoblotting using a polyclonal rabbit serum raised against deglycosylated red kidney bean αAI-1. Assays of the expressed inhibitor combined with total larval extracts were carried out as described above.

2.11. Binding experiments with Sepharose 4B beads

Pancreatic porcine α-amylase (PPA) was coupled to Sepharose 4B beads (Pharmacia-Biotech) according to the manufacturer’s instructions. Binding of 20 µl of total root extracts of transgenic A. thaliana to 0.1 ml of beads slurry, elution of bound polypeptides and analysis by the immunoblot technique using antibody raised against bean AI-1 were carried out as described (Grossi de Sa et al., 1997).

3. Results

3.1. Pattern of α-amylase activity of total WCRW larval extracts

Two different electrophoretic systems were used to visualize larval α-amylases: native polyacrylamide gels and isoelectrofocusing gels with a pH gradient from 3 to 9 (Fig. 1). In both systems, two major α-amylase activity bands with quite different electrophoretic mobilities were detected in extracts from larvae fed on maize sprouts and maize roots. We designated these two activities as DvAmy1 and DvAmy2. In a gel with a pH gradient, staining for amylase activity shows two bands, one that runs as an acidic form (DvAmy2) and one as a basic form (DvAmy1). In total extracts of larvae fed on an artificial diet, the α-amylase activity was extremely low and was represented by one activity band with a similar electrophoretic mobility as the DvAmy2 band (Fig. 1). In a native PAGE system, two to three minor α-amylase isoforms could be detected with mobilities between DvAmy1 and DvAmy2 (Fig. 1A). These results show that WCRW larvae have two major and possibly some minor α-amylases isozymes.

3.2. Cloning of two cDNAs encoding putative α-amylases from D. v. virgifera

Conserved regions in nine α-amylases were used to design oligonucleotide primers (amy1 and As1) that were used to amplify a DNA fragment of 546 bp using a cDNA library synthesized from poly(A)+RNA of WCRW. The fragment showed high sequence similarity with α-amylase sequences and was extended in both directions. A DNA fragment, designated Dva1 and containing an open reading frame encoding a predicted protein of 482 amino acids with a calculated pf of 6.98, was obtained. To clone other α-amylase cDNAs, the same strategy was applied with three other sense and three antisense primers. The 548 bp product of PCR amplification was cloned and inserts of 42 recombinant plasmids were sequenced. Two kinds of fragments were identified: the first class had fragments identical to Dva1 and the second class had a sequence that was 85% identical to Dva1. This dissimilar clone was used for 5’/3’ RACE PCR and another full-size cDNA (designated Dva2) was obtained with an open reading frame of 481 amino acids and a calculated pf 5.38. Dva1 and Dva2 share 83% identity at the amino acid level. Their sequence comparison is shown in Fig. 2. The two proteins have putative signal peptide cleavage sites 20 and 19 amino acid residues in length, respectively. The GenBank accession numbers are AF208002 and AF208003. The deduced amino acid sequences of Dva1 and Dva2 are homologous to other known α-amylase sequences from insects and mammals (Fig. 3). They share 51% sequence identity with PPA; DVA1 and DVA2 have 62% and 63% sequence identity with Z. subfasciatus amylase, and 66% and 70% with the T. castaneum enzyme, respectively. An aspartic acid residue and two glutamic acid residues (numbered Asp185, Glu219 and Glu284 in PPA) that are known to form the active center of PPA (Qian et al., 1994) are conserved in both WCRW sequences. Moreover, three conserved histidine residues (His99, 189 and 283) that are believed to be involved in substrate binding are also conserved.
Fig. 1. Zymograms to visualize α-amylase isozymes in larval extracts of WCRW. (A) 8% native polyacrylamide gel, containing 0.5% soluble starch; (B) Isoelectrofocusing gel pH 3–9. Two major activity bands are designated as DvAmy1 and DvAmy2. 1, Purified PPA; 2, Extract prepared from larvae fed on maize sprouts; 3, Extract prepared from larvae fed on maize roots in soil; 4, Extract prepared from larvae fed on an artificial diet.

Fig. 2. Comparison of deduced amino acid sequences of two cDNAs (Dva1 and Dva2) cloned from WCRW D. v. virgifera and encoding two putative α-amylases. Sequences have 83% identity.
Fig. 3. Amino acid sequence comparison of seven $\alpha$-amylases from different sources. Dva1 and Dva2, D. v. virgifera; Tca, Tribolium castaneum (U04271); Zsa, Zabrotes subfasciatus; Dma, Drosophila melanogaster (L22730); Aga, Anopheles gambiae (L04753); PPA, porcine pancreatic (P00690). (*) Conserved histidine residues necessary for substrate binding; (–) the active site residues of PPA (Asp, Glu and Asp).

### 3.3. Inhibition of $\alpha$-amylase activity of larval extracts of WCRW by two plant inhibitors

One of the goals of the present work was a search for potential inhibitors of the WCRW $\alpha$-amylases. Total seed extracts of different dicot and monocot plants were tested for the presence of inhibitors (data not shown). In addition, the effect of two well-characterized inhibitors, the wheat amylase inhibitor (WI) and AI-1 from the common bean, were tested. Effects of these two inhibitors on total WCRW amylase activity are shown in Fig. 4. WI was more active against total $\alpha$-amylase compared to AI-1. WI was twice as active as AI-1 at low levels (2.5 μg); at higher concentrations (15 μg), inhibition of $\alpha$-amylase activity by WI was 76%, whereas inhibition caused by AI-1 was 43%. Higher levels of inhibitors did not increase the extent of inhibition.

A possible additive effect of WI and AI-1 was examined...
by adding the inhibitors sequentially at 15 min intervals. Two amounts of each inhibitor (0.5 and 1 μg) were tested and an additive effect was obtained (Fig. 5). Additive effects were only observed if the level of inhibition was low (10 to 30%) for the first inhibitor to be added. When more of the first inhibitor was added initially so that the percent inhibition was large, there was no additive effect.

3.4. Heterologous expression of Dva1 cDNA in Sf9 insect cells

Dva1 was expressed in Sf9 insect cells using a baculovirus expression vector. Six recombinant viruses were amplified. Infected cells and supernatants were checked for the presence of α-amylase activity by using the Bernfeld assay, and by immunoblotting using an antiserum against α-amylase of Z. subfasciatus. A recombinant virus (N5) with the highest expression level was chosen to produce the recombinant enzyme for further analysis. Dva1 α-amylase in an IEF gel (pH 3–9) was indistinguishable from DvAmy1 present in WCRW larval extracts (Fig. 6). Dva1 produced in insect cells migrated as a doublet, possibly caused by an early translational termination product or the result of a posttranslational modification. The Sf9 cells, either by themselves or infected with control baculovirus, did not produce amylase sufficient for detection in these gels. The effect of WI and AI-1 on this Dva1 enzyme expressed in the baculovirus system was examined (Fig. 7). Inhibition by AI-1 plateaued out at 60% and by WI at 75%.

3.5. Inhibition of α-amylase activity of total larval extracts by AI-1 and WI in an in-gel assay

Results of an in-gel assay of the inhibitory effect of AI-1 and WI on α-amylase activity in extracts containing different isozymes are shown in Fig. 8. Extracts of larvae reared on maize sprouts and maize roots were run side by side with purified pancreatic porcine amylase (PPA) as a control on IEF gels (Fig. 8A). To assess the effect of inhibitors, the gels were incubated in a buffer (pH 5.8) containing 5 μg/ml of purified AI-1 or WI, prior to incubation with 2% soluble starch (Fig. 8B and C respectively). After staining the gel with iodine reagent, the extent of the inhibition was estimated as a decrease in the size of the white activity bands in comparison with a control gel that was not treated with inhibitor. Both inhibitors appear to inhibit the two amylases of the WCRW similarly, but the qualitative nature of this assay does not allow us to determine if one isozyme is
inhibited more than the other. The partial inhibition noted before (Fig. 4) is apparently not caused by the complete inhibition of one of the isozymes and lack of inhibition of the other one.

3.6. Expression of bean inhibitor αAI-1 in A. thaliana plants results in accumulation of active protein in roots

AI-1 is a seed protein that accumulates in protein storage vacuoles where it is proteolytically processed by a specific protease. Such processing is required for activity (Pueyo et al., 1993). To investigate whether active AI-1 can accumulate in roots, which are the major targets of the WCRW, Arabidopsis plants were transformed with a chimeric construct of AI-1 under the CaMV35S constitutive promoter. Ten independent transgenic lines were obtained and three of them were chosen for further analysis. An immunoblot of extracts containing soluble root proteins of four independent transgenic lines is shown in Fig. 9. The pattern of polypeptides that crossreacted with the antiserum against bean AI-1 is identical to that of AI-1 purified from bean seeds (lane 1). The same result was obtained when soluble proteins from Arabidopsis leaves were tested with antiserum (data not shown).

To determine if this AI-1 expressed in Arabidopsis roots is active we performed two tests: binding to a PPA affinity column, and inhibition of amylase activity in extracts of WCRW larvae. The binding assays showed that the expressed inhibitor bound to and could be eluted from a PPA affinity column made by linking PPA to Sepharose 4B (data not shown). In an in vitro inhibition assay, the recombinant AI-1 from the Arabidopsis roots was shown to inhibit the α-amylase activity of total larval extracts of WCRW. The inhibition was comparable to that obtained with AI-1 purified from seeds of the common bean. The results presented in Fig. 10 show that amounts of root extract that contained about 2 μg of expressed inhibitor gave an inhibition of approximately 20%, similar to that obtained for 2 μg of pure inhibitor. Because we used total root extracts, the assay conditions did not permit to test larger amounts (necessitating larger volumes) to achieve a higher level of inhibition.
Fig. 9. Immunoblot of total root extracts of transgenic *A. thaliana* lines expressing AI-1 under the 35S constitutive promoter. Antibody raised against AI-1 from *Phaseolus vulgaris* was used. M, molecular weight markers (kD); 1, Purified AI-1 (1 µg); 2, Extract from transgenic line transformed with the empty vector pBI121; 3–5, Extracts from three different transgenic lines expressing αAI-1. 20 µg of total protein has been loaded on lanes 2–5.

Fig. 10. Inhibition of α-amylase activity of total larval extracts of WCRW by αAI-1 either purified from *P. vulgaris* (■) or present in root extracts of *A. thaliana* expressing AI-1 under the constitutive CaMV 35S promoter (△). Each point represents an average of three independent experiments.

4. Discussion

4.1. WCRW larvae contain multiple α-amylases

We present the derived amino acid sequences of two α-amylase cDNAs of WCRW larvae and document the presence of two different isoforms in larval extracts. The amylases appear to have an acidic pH optimum, similar to the proteases found in this insect (Gillikin et al., 1992). Visualization of the amylase isoforms by two different methods indicates that the WCRW larvae contain two major isozymes, similar to findings with *D. melanogaster* (Doane, 1969), *Sitophilus zeamais* (Baker, 1983; Cheng et al., 1992) and *Prostephanus truncatus* (Vázquez-Arista et al., 1999). However, the isoforms of these other insects do not differ so widely in their isoelectric points. Some other insects have been reported to contain a single isozyme, including *C. chinensis* (Podoler and Applebaum, 1971, *Tenebrio molitor* (Buonocore et al., 1976) and *Z. subfasciatus* (Campos et al., 1989). The number of isozymes may depend on the sensitivity of the methods used. For example, Grossi de Sa and Chrispeels (1997) found only a single α-amylase isozyme in gut extracts of *Z. subfasciatus*, but Silva et al. (1999) found three isozymes by using a less-denaturing gel electrophoresis system. In addition, the number of isozymes may depend on the particular insect strain (Baker, 1987).

It is particularly interesting that WCRW larvae fed on an artificial diet contain much reduced levels of amylase, and that the acidic isoform is completely absent from larvae fed on an artificial diet. Since the artificial medium does not contain any starch, we propose that starch in the maize tissues induces the presence of α-amylase in the larvae. This finding indicates that it may be best to study digestive enzymes in larvae that have been reared on their normal food sources rather than on an artificial medium. A similar conclusion was reached by Valaitis et al. (1999) who observed that Western spruce budworm larvae collected in the field had much higher levels of midgut carboxypeptidase than laboratory-reared larvae.

Two cDNAs (Dva1 and Dva2) that share 83% amino acid identity and encode putative α-amylase isoforms were isolated. Most strains of *Drosophila* have two closely linked genes encoding two α-amylase isozymes (Boer and Hickey, 1986; Gemmill et al., 1986). The rice weevil *Sitophilus oryzae* also has two amylase isozymes (Baker et al., 1990), and the multiple isozymes found in *S. zeamays* are encoded by at least two genes (Baker and Halliday, 1989). In contrast, only one gene is present in *T. molitor* (Strobl et al., 1998). Expression of Dva1 in insect cells with a baculovirus vector allowed us to characterize the gene product. Dva1 encodes an active α-amylase that appears to correspond to DvAmy1, the basic isoform, as judged by its co-migration with DvAmy1. The calculated pI (6.98) is not as basic as its mobility in the IEF gel would suggest. We did not succeed in expressing Dva2 in the same manner and do not know if it corresponds to DvAmy2. Dva2 was the only other amylase-like sequence detected by PCR screening of the cDNA products made with larval mRNA. We therefore suspect that it probably encodes DvAmy2. The calculated isoelectric point (5.38) is not as low as the isoelectrofocusing indicates for DvAmy2. To confirm that the two cDNAs encode the two identified isoforms
it will be necessary to purify the isoforms and determine a partial amino acid sequence. Additionally, further work with WCRW may reveal that the minor isozymes are encoded from other \(\alpha\)-amylase genes, or represent post-translational modification of Dva1 and Dva2 encoded polypeptides.

The derived amino acid sequences for a number of \(\alpha\)-amylases of different origin have been determined and they share considerable identity. Dva1 and Dva2 share the highest identity with other insect amylases. Sequence comparisons also show that all regular secondary structure elements defining the general fold of mammalian \(\alpha\)-amylases are most likely conserved in the insect enzymes. Three residues (Asp197, Glu233 and Asp300) define the catalytic site of the PPA enzyme (Qian et al., 1994). Analysis of the 3D structure of the \(\alpha\)-amylase of the yellow mealworm (\textit{Tenebrio molitor}) (Strobl et al., 1998) indicates that similar residues (Asp185, Glu222 and Asp287) are conserved and take part in the catalysis. The same amino acids are also conserved in Dva1 and Dva2. Four His residues (101, 201, 299 and 305) of PPA have been shown to form hydrogen bonds with the carbohydrate inhibitor acarbose (Qian et al., 1994; Gilles et al., 1996) and in the insect enzymes three of the four His residues are conserved (Fig. 3).

4.2. Proteinaceous amylase inhibitors inhibit the \(\varepsilon\)-amylases

Proteinaceous \(\alpha\)-amylase inhibitors are widespread in plants and are found primarily in starch-storing seeds and roots, consistent with the idea that they evolved as a plant-defense strategy. They exert their action by slowing down the digestion of the plant material ingested by the plant pest. Insects usually evolve together with their food sources, so inhibitors of a particular plant species are often not effective against the insects that attack that plant species. Well-characterized \(\alpha\)-amylase inhibitors are found in plants such as the common bean (Powers and Whitaker, 1977), wheat (Garcia-Olmedo et al., 1986) and amaranth (Chagolla-Lopéz et al., 1994), and these have been shown to be active against some insect and mammalian \(\alpha\)-amylases. Total larval extracts of WCRW were tested in two different ways: in liquid assays and by incubating gels with an inhibitor solution prior to development of the amylase activity. The latter method allowed us to see if only one or both major amylases were inhibited by the inhibitors. We found that both inhibitors inhibit the enzyme activity at pH 5.6 by about 75% and that the effects of the inhibitors are additive when they are present at suboptimal concentrations. However, when the inhibitors are present at higher concentrations the two inhibitors together do not raise the total inhibitory level above 75–80%. At lower concentrations, WI was found to be more active against total WCRW \(\alpha\)-amylase activity than bean AI-1. When present in artificial seeds, inhibitors of digestive enzymes cause a slowdown in the rate of growth of bruchid larvae, which take more time to progress from one instar to the next (Huesing et al., 1991; Shade et al., 1994; Pueyo et al., 1995). Moreover, at lower concentrations inhibitors can have additive effects (Pueyo et al., 1995). Unfortunately, there is no simple way to test these two amylase inhibitors on the development of WCRW larvae because the larvae need to develop normally on maize sprouts or roots; rearing them on artificial media without starch as the sole energy source is not a valid way to test the inhibitory activity of amylase inhibitors. As there is no starch-based artificial diet for WCRW larvae, there seems to be no alternative to testing the effect of these inhibitors on larval development other than expressing the inhibitor genes in transgenic plants.

4.3. Transgenic plants for insect control

There have been rapid developments in the technology to transfer insect resistance genes of different origin (bacterial, plant or animal) into plants in order to increase the level of insect resistance. Approximately 40 different genes conferring insect resistance have been incorporated into crops, and insect-resistant crops have been commercialized in several countries (Schuler et al., 1998). Expression in pea and azuki bean of the cDNA coding for bean AI-1 inhibitor under a seed specific promoter made the seeds of these legumes resistant to certain species of Bruchidae whose digestive amylases are inhibited by this inhibitor (reviewed in Chrispeels et al., 1998). One of the unique features of AI-1 is that it needs to be proteolytically processed to be active (Pueyo et al., 1993). This proteolytic processing step is catalyzed by a specific protease that cleaves on the carboxyl side of a specific Asn residue. This enzyme is present in the protein storage vacuoles of seeds which are devoid of the general proteases found in leaves and roots. When the gene encoding a vacuolar protein is expressed in a heterologous plant, the protein will be transported to the vacuoles of the cells in the various organs. Since vacuoles of cells in leaves and roots contain abundant general endopeptidases, unlike the protein storage vacuoles of seeds, we wanted to determine if active AI-1 would accumulate in leaves and roots, or if the inhibitor would be degraded by the vacuolar proteases. The inhibitor detected in leaf and root tissues of transgenic plants was correctly processed but not degraded by the general proteases (Fig. 9). The inhibitor bound to an \(\alpha\)-amylase affinity resin and inhibited \(\alpha\)-amylase activity present in WCRW larval extracts \textit{in vitro} (Fig. 10). The same strategy needs to be applied to the gene coding for the WI to show if active inhibitor could accumulate in roots. Previously it has been demonstrated that expression of the cDNA of WI in \textit{E. coli} gave rise to an active protein (Garcia-Maroto et al., 1991). The final goal will be to
construct transgenic maize plants expressing both inhibitors in roots and using them in a bioassay with WCRW larvae to confirm their possible role as plant defense proteins. Successful results have in the past been obtained with inhibitors that completely inhibited their target enzymes (Ishimoto and Kitamura, 1989) but recent results show that even partial inhibition can give substantial control of insect pests (Morton et al., 2000).

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