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RAPID COMMUNICATION

Cloning and Sequence Analysis of the Amylase Gene from the Rice Pest *Scirpophaga incertulas* Walker and its Inhibitor from Wheat (Variety MP Sehore)

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Abstract: *Scirpophaga incertulas* Walker (Lepidoptera: Pyralideae), commonly known as yellow stem borer, is a predominant monophagous pest of rice, which causes 5% to 30% loss of the rice crop. We report for the first time, the cloning and sequence analysis of the amylase gene of this pest. The cloned gene translates into a protein of 487 amino acids having a predicted molecular weight of 54,955 daltons and a theoretical pI of 5.9. The 3D structure of the amylase is predicted from its amino acid sequence by homology modeling using the structure of the amylase from *Tenebrio molitor* L (Coleoptera: Tenebrionidae). We also report the purification of a dimeric α-amylase inhibitor from a local variety of wheat MP Sehore that is specific for the amylase of this pest and does not inhibit human salivary amylase or porcine pancreatic amylase. The gene encoding this inhibitor has been cloned and its sequence has been analysed to find a possible explanation for this specificity.

Keywords: *S. incertulas* walker, α-amylase, cDNA cloning, homology modeling, wheat dimeric α-amylase inhibitor, sequence analysis

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Introduction

Scirpophaga incertulas Walker (Lepidoptera: Pyralidae), commonly known as yellow stem borer, is a predominant monophagous pest of rice, which causes 5% to 30% loss of the rice crop.[1] The larvae feed voraciously on stem parts and complete their entire growth and development within the stem and hence are not easily accessible to sprayed pesticides. Controlling yellow stem borer by insecticides is uneconomical and ecologically unsafe. Therefore, the development and use of rice varieties tolerant to this pest has been receiving increasing attention in recent years. No completely known source of resistance to this insect pest has been identified, in spite of screening all the germplasm at the International Rice Research Institute (IRRI) in the Philippines.[2,3] Moreover, tolerance to yellow stem borer is polygenic in nature[2–4] due to which, during breeding, the tolerance trait gets diluted on repeated selection. Hence, in such cases, where a source of total resistance is unavailable, the strategy to increase the host plant resistance/tolerance has been through genetic engineering, by developing rice varieties expressing genes encoding insecticidal toxins.

In case of *S. incertulas* Walker (Lepidoptera: Pyralidae), considerable success has been achieved using *Bt* genes.[5–27] Though presently larvae of this rice pest grown on rice varieties transformed with *Bt* genes have shown 100% mortality, there is a need to explore alternate strategies to attain durable resistance, because a major problem that could result from the extensive use of insect-resistant transgenic plants is the evolution of resistance to Bt toxin as has been observed in different pest populations.[28–37] It is quite possible that *S. incertulas* also may develop mechanisms to overcome the toxic effects of Bt toxins. Hence, it is necessary to develop such alternate strategies wherein the probability of resistance evolving could be substantially reduced and resistance would be durable.

α-amylases play a central role in the carbohydrate metabolism in those insects that live on seeds and plant parts. The larvae which feed voraciously on the stem parts and complete their development within the stem depend to a large extent on their α-amylases for their survival.[1,8] Hence, proteinaceous inhibitors to the α-amylases would be attractive candidates for the control of yellow stem borer larvae. Purified monomeric and dimeric α-amylase inhibitor fractions from a local variety of wheat seeds (variety MP Sehore) specifically inhibit the α-amylase activity from this pest. These characteristics would make these inhibitors attractive candidates for genetic engineering to develop rice transgenics with improved tolerance to *S. incertulas*. The reported monomeric class of α-amylase inhibitors from wheat has been shown to specifically inhibit the amylases from insects,[38] whereas the reported dimeric α-amylase inhibitors from wheat inhibit insect amylases and also human salivary amylase and porcine pancreatic amylase.[38–41] However, the dimeric inhibitor fraction isolated from MP Sehore is specific for the *S. incertulas* amylase and does not inhibit porcine pancreatic amylase and human salivary amylase.

An understanding of the molecular basis of the specificity of inhibition between the α-amylase of this rice pest and these wheat inhibitors is an important step before initiating work for developing transgenic rice expressing such an inhibitor. As a first step towards this goal, we describe the cloning and sequence analysis of the cDNA of the α-amylase from this rice pest. The three-dimensional structure of the amylase has been predicted from its translated amino acid sequence, by homology modeling, using the structure of the amylase from *Tenebrio molitor* (Coleoptera: Tenebrionidae). The gene encoding the dimeric α-amylase inhibitor from wheat variety MP Sehore has been cloned and its sequence has been compared with the sequence of the reported dimeric inhibitor, which has been modeled with the amylase from human saliva and yellow mealworm, to find a possible explanation for this specificity.

Materials and Methods

Collection of larvae of *S. incertulas* Walker (Lepidoptera: Pyralidae)

Live larvae in the third and fourth instar stage were collected from the rice fields at Roha and Alibag (Raigad district) in Maharashtra State, India. Live larvae were dissected from infested rice stems and immediately immersed in RNAlater reagent (Qiagen) and stored at –70°C, in aliquots of 10 larvae (~100 mg)/vial.

Preparation of crude α-amylase from *S. incertulas* Walker

One gram of insect larvae was crushed in liquid nitrogen in a mortar and pestle. The powdered material

was suspended in five volumes of 150 mM NaCl saline or 150 mM Tris-HCl pH 7.5 and centrifuged at 10,000 rpm for 10 min at 4°C in a RC5-Sorvall centrifuge. The pellets were discarded and the supernatant was assayed for enzyme activity. It was observed that extraction with 150 mM NaCl yielded 37% more enzyme activity as compared with 150 mM Tris-HCl pH 7.5. So, all further extractions were carried out in 150 mM NaCl. The NaCl extract containing the crude amylase enzyme was dialyzed against 10 mM sodium-phosphate buffer (pH 7.0) and then concentrated by lyophilization to one fourth its volume and dissolved in 10 mM sodium-phosphate buffer pH 7.0 and then re-dialyzed against the same buffer. The recovery on lyophilization was 88%. As the extract was highly pigmented, the pigment was removed using ethyl alcohol as follows: 10 ml of the concentrated extract was precipitated by addition of three volumes of chilled ethanol. The precipitate was centrifuged at 10,000 rpm in a RC5 Sorvall centrifuge at 4°C and the resultant precipitate was suspended in a total volume of approximately 16 ml in four consecutive steps of 4 ml each. This solution was lyophilized, dialyzed against 10 mM sodium-phosphate buffer pH 7.0 and once again subjected to ethyl alcohol precipitation to remove the residual pigment. The removal of pigment was monitored spectrophotometrically at 410 nm. The recovery of the enzyme was 78%. This partially purified enzyme was used for preliminary characterization of its properties.

Assay for amylase activity

The amylase activity was determined by the dinitrosalicylic acid (DNS) method.[42] The reaction mixture contained 0.25 ml of suitably diluted enzyme in sodium-phosphate buffer (50 mM, pH 8.0) and 0.25 ml of (1% w/v) starch solution for amylase activity. The reaction was terminated by the addition of 0.5 ml DNSA. The tubes were heated in a boiling water bath for 5 min and the color intensity was read at 540 nm after dilution with 5 ml of distilled water. A standard glucose curve was used for calculating enzyme activities. The linearity curve of the enzyme was determined for calculation of unit activity. One IU of amylase activity is defined as the amount of enzyme required to produce 1 µmole of glucose/min.

Isolation and purification of the monomeric and dimeric α-amylase inhibitors from wheat (Variety: MP Sehore)

Extraction of α-amylase inhibitors from wheat flour

Extraction of wheat α-amylase inhibitors was carried out as described.[43] In a preliminary experiment it was observed that a 150 mM NaCl solution was more effective in extraction of the α-amylase inhibitors from wheat flour than 100 mM Tris/HCl buffer pH 7.6. Hence, 150 mM NaCl was used for extraction of the α-amylase inhibitors. Hundred grams of finely ground whole wheat flour (Variety MP Sehore) was stirred in 300 ml of 150 mM NaCl solutions at room temperature for 3 h. The suspension was then centrifuged for 10,000 rpm for 20 min in a RC-Sorvall centrifuge at 4°C. The supernatant liquid was subjected to ammonium sulphate fractionation to isolate the α-amylase inhibitors.

Ammonium sulphate precipitation of α-amylase inhibitors

The crude α-amylase inhibitor solution was first precipitated with 0–0.4 M $(NH_4)_2SO_4$ to remove non-proteinaceous components. The resultant supernatant liquid after centrifugation was salted out with 0.4–2.0 M $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10,000 rpm for 10 min and dissolved in one tenth the volume of double distilled water and dialyzed against water for 24 h with four changes. The inhibitor fraction was concentrated by lyophilization. Since the crude preparation had endogenous α-amylase activity, the inhibitor solution was boiled for 1 min at 100°C to inactivate the endogenous α-amylase activity.

Purification of α-amylase inhibitors by FPLC Superose-12 Gel permeation column chromatography

The crude ammonium sulphate fraction of the wheat α-amylase inhibitor containing 6 mg protein and 7000 units of inhibitor activity in 300 µl was loaded on a FPLC-Superose 12 gel permeation column (10 × 300 mM). Phosphate buffer pH 7.4 of composition 137 mM-NaCl, 3 mM-KCl, 1 mM-KH_2PO_4, 8 mM-Na_2HPO_4 was used. The flow rate of the column was maintained at 18.5 ml/h. A total of twenty-five fractions of one ml each were collected and the individual fractions were assayed for inhibitor activity.

The FPLC profile revealed the presence of five distinct peaks (Fig. 1a). Inhibitor activity was detected only in peak three and no activity was detected in any of the other peaks. Native PAGE of the third peak showed the presence of two bands corresponding to the monomer and dimer. In order to separate the dimer and monomer, the individual FPLC fractions of peak three were collected from several runs, dialyzed, lyophilized and loaded on Sephadex G-50 gel permeation column chromatography. The buffer system used was the same as described for FPLC. The flow rate of the column was maintained at 15 ml/h. Two peaks were obtained as shown in Figure 1b. The two peak fractions were analyzed by native PAGE. The two samples were found to be homogenous and corresponded to the dimer and monomer. (Fig. 1c). Other FPLC fractions were processed similarly.

Assay for inhibitor activity

The assay for determining inhibitor activity was carried out essentially as described for amylase activity excepting that the reaction mixture contained suitable aliquots of seed extract containing inhibitor. One unit of inhibitor activity is defined as the amount of inhibitor required to inhibit 50% of enzyme activity. Commercial dimeric α-amylase inhibitor from wheat (Sigma Catalogue No. A1520) was used as a control as it inhibits human salivary α-amylase (Sigma Catalogue No. A0521) and porcine pancreatic α-amylase (Sigma Catalogue No. A3176).

PAGE electrophoresis

Non-denaturing gel electrophoresis was performed according to the procedure described by Davis[44] using a 10% separating gel at pH 8.9. Bromophenol blue was used as the tracking dye.

Molecular weight determination of the inhibitors by MALDI-TOF

Gel electrophoresis was performed.[44] After electrophoresis the protein bands were excised from the gel and transferred into two separate vials. Sufficient extraction solution (100 µl) consisting of a mixture of formic acid/glass distilled water/2-propanol (1:3:2) was added to the crushed gel to completely cover the gel pieces. The tubes were closed and vigorously shaken at room temperature for 4 to 8 h. After vortexing,

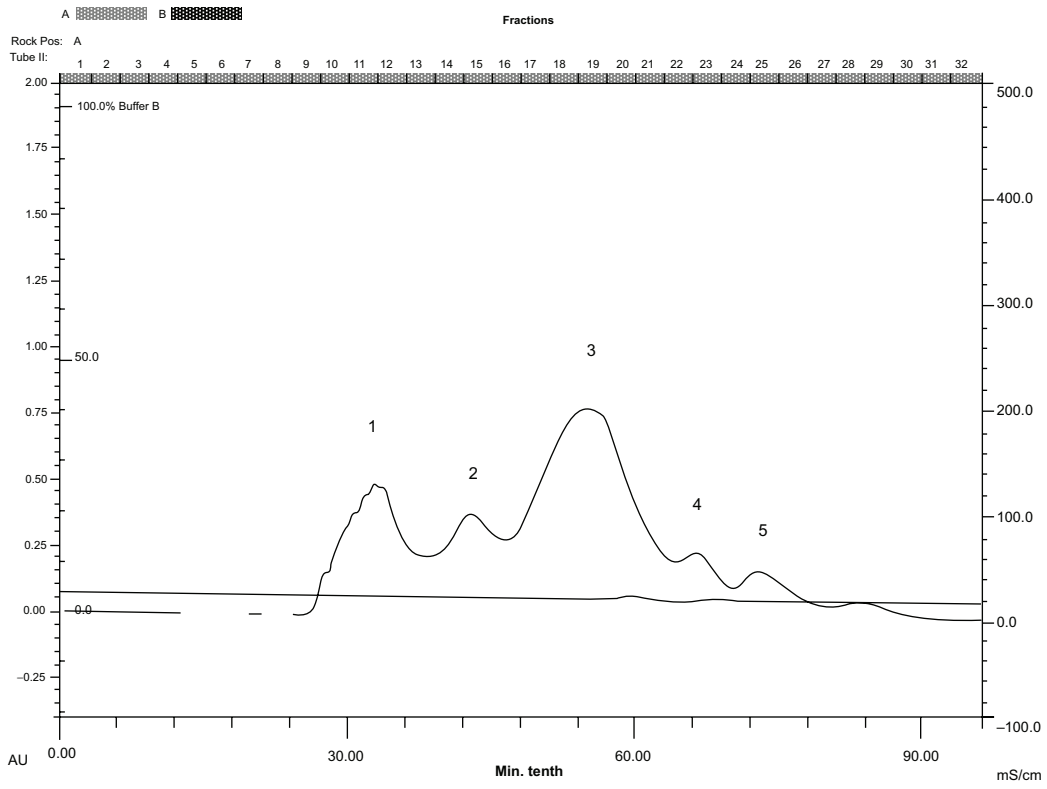

Figure 1a. Profile of FPLC- gel permeation column chromatography (Superose 12 prep. Grade).

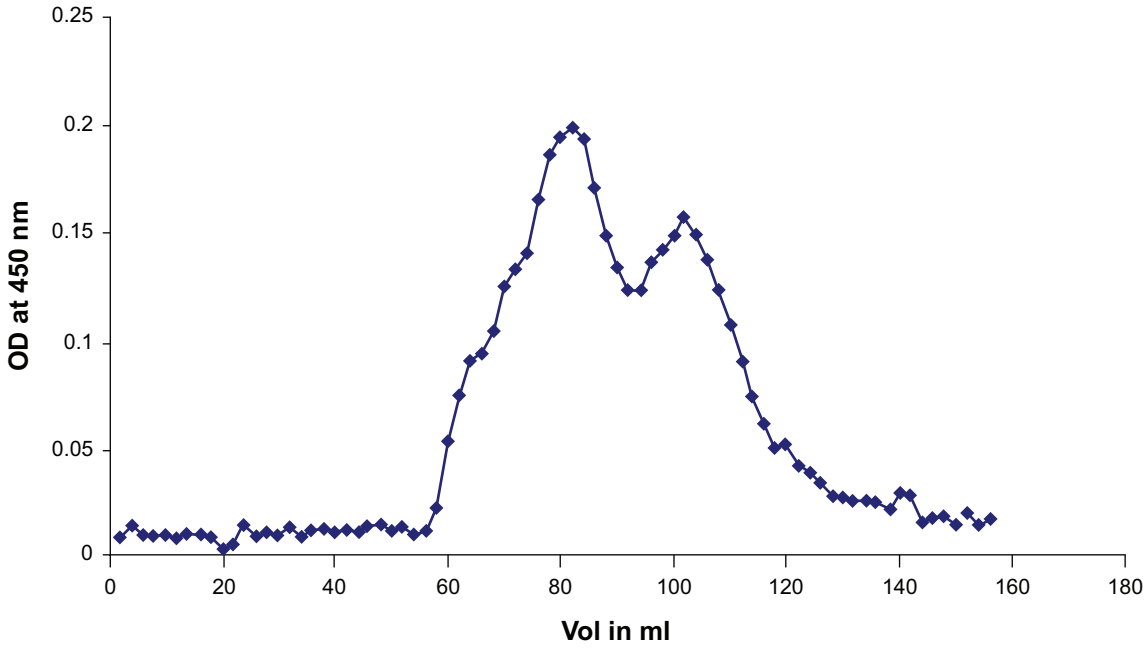

Figure 1b. Profile of G 50 gel permeation column chromatography.

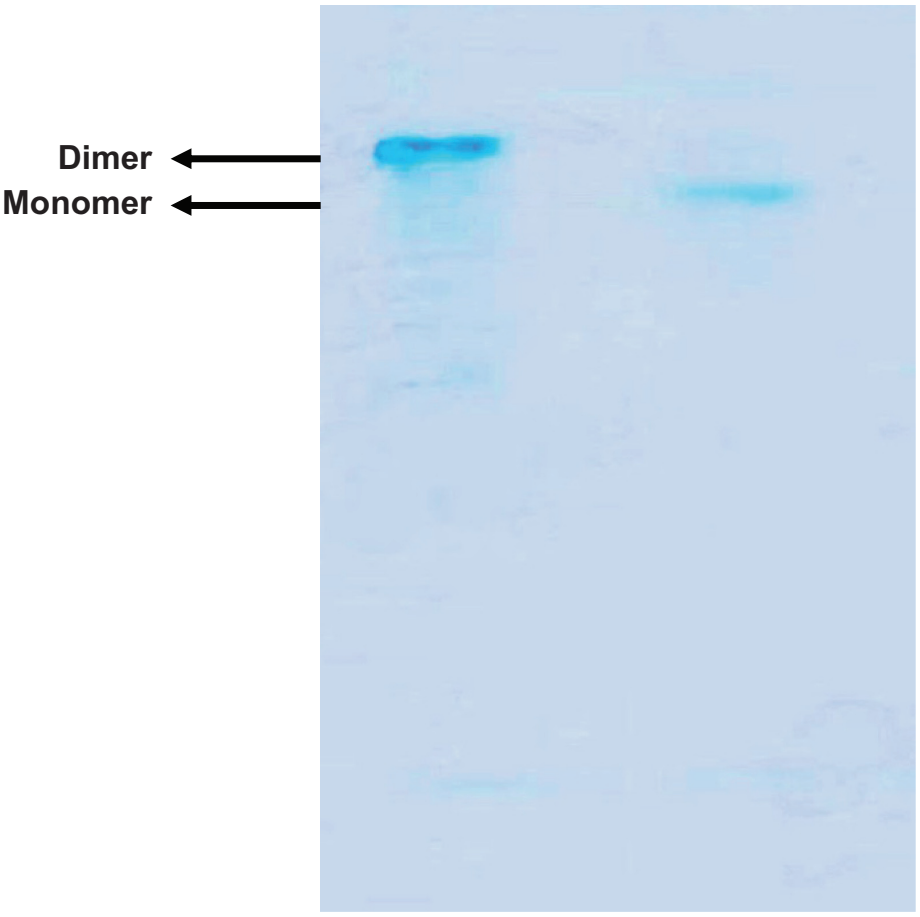

Figure 1c. Native PAGE of purified monomeric and dimeric α-amylase inhibitors.

the sample was microfuged. The supernatant was retrieved with a micropipette. The crushed gel was washed once with an equal volume of fresh extraction solution and the washes were combined with the supernatant. The two supernatants were concentrated and subjected to MALDI-TOF.[45] MALDI-TOF analysis showed that the molecular weights of the dimer and monomer were 13,330 and 13,159 Daltons respectively, suggesting that band 1 was a dimer and band 2 was a monomer.

Cloning of the gene encoding the dimeric inhibitor from wheat (Variety: MP Sehore)

For amplifying the dimeric α-amylase inhibitor from wheat (variety MP Sehore), forward and reverse primers were designed according to Wang et al.[46] Total DNA to be used as a template in the PCR reaction was isolated according to Dellaporta et al.[47] The conditions for amplification were the same as described by Wang et al.[46] The amplification product was cloned into pCR4 TOPO vector (Invitrogen) and was transformed into *E. coli* Top10 cells (Invitrogen) as per the manufacturer's instructions. The positive clones were sequenced and the amino acid sequence of the dimeric α-amylase inhibitor was deduced from the nucleotide sequence using the 'ORF Finder' program at the NCBI site (http://www.ncbi.nlm.nih.gov/projects/gorf). The Gene Bank accession number of the dimeric α-amylase inhibitor from wheat variety MP Sehore is GQ374443 (Fig. 2). The first thirty amino acids represent the signal sequence as determined by SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

RNA extraction

100 mg of larvae preserved in RNAlater (Qiagen) were used each time for extraction of RNA. Total RNA was extracted from the larvae using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The quality of the RNA preparation was judged by formaldehyde agarose gel electrophoresis.[48]

Reverse transcription—polymerase chain reaction and cDNA cloning

The full length cDNA of the α-amylase gene, henceforth designated as SiAmy, was cloned as follows:

The following four pairs of forward (F1–F4) and reverse primers (R1–R4) were designed, based on the conserved regions of insect alpha-amylases:

SiAmy F1–5′GTGCACCTCTTCGAATGG 3′
SiAmy R1–5′CTCAAATCGGCTGGCCAC 3′
SiAmy F2–5′GGGTGTCGTGGTTGTCGACGAA 3′

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  1 atgctcgtggcgacacccatagcggccgagtacgacgcatggagc
    M  L  V  A  T  P  I  A  A  E  Y  D  A  W  S
 46 gttaacagtggtccctggatgtgctatccagggtatgcctttaag
    V  N  S  G  P  W  M  C  Y  P  G  Y  A  F  K
 91 gtgccagcgctccctggctgtcgtccagtgctgaagctccagtgc
    V  P  A  L  P  G  C  R  P  V  L  K  L  Q  C
136 aatggcagccaggtgcccgaggctgtcctaagggactgctgccag
    N  G  S  Q  V  P  E  A  V  L  R  D  C  C  Q
181 cagctcgcccacatcagcgagtggtgcaggtgcggggccctctac
    Q  L  A  H  I  S  E  W  C  R  C  G  A  L  Y
226 agcatgttggacagcatgtataaggagcatggcgcgcaggaggga
    S  M  L  D  S  M  Y  K  E  H  G  A  Q  E  G
271 caggcagggacaggagcgttcccacgctgccggagggaggtggtg
    Q  A  G  T  G  A  F  P  R  C  R  R  E  V  V
316 aagctgacggcggcgagcatcacggcggtctgcaagctacccatc
    K  L  T  A  A  S  I  T  A  V  C  K  L  P  I
361 gtcattgatgcgtctggagatggagcgtatgtctgcaagggtgtg
    V  I  D  A  S  G  D  G  A  Y  V  C  K  G  V
406 gccgcatacccggacgtctag 426
    A  A  Y  P  D  V  *
```

Figure 2. DNA sequence and the deduced protein sequence of the dimeric α-amylase inhibitor from wheat variety MP Sehore (Gene Bank Accession Number GQ374443). The first thirty amino acids (bold and highlighted in red) represent the signal peptide.

SiAmy R2–5′GACGTTGTGTTCAACCATATGGCTG 3′
SiAmy F3–5′GTCCATCTATTCGAATGG 3′
SiAmy R3–5′TCCAAATCTGCAGGCCAC 3′
SiAmy F4–5′GTTCATCTTTTTGAGTGG 3′
SiAmy R4–5′TGCAGATCTGCCGGCCAC 3′

Total RNA (~1 µg) was reverse transcribed at 50°C for 30 min, using Superscript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) as per the manufacturer's instructions. The RT reaction was followed by one cycle of denaturation at 94°C for 2 min. The RT template was then subjected to 30 cycles of polymerase chain reaction. Each cycle comprised of 94°C for 30s, 50°C for 1 min and 68°C for 1 min. Final extension was done at 68°C for 5 min. The PCR was carried out in a Techne (Progene) thermal cycler.

Of the four pairs of primers, only SiAmy F1–SiAmy R1 gave a 545 bp amplification product. The 545 bp PCR products was cloned into pGEM-T vector (Promega) and transformed into NovaBlue competent cells (Novagen) according to the Manufacturer's instructions. Positive clones were sequenced in an automated DNA Sequencer. The BLAST search of the DNA sequences and translated amino acid sequence revealed that the inserts of the clones showed significant sequence homology to other insect α-amylases. Based on the sequence of the 545 bp insert, the following three pairs of forward and reverse primers were designed, to be used for rapid amplification of cDNA ends (RACE-PCR).

Race F1–5′AGCTAACCGGCCGTGGTGGGAGC GGTACCAA 3′
Race F2–5′GCGGTACCAACCCATTTCGTACAAA CTCA 3′
Race F3–5′ACTCACCACACGATCAGGTGACAA CA 3′
Race R1–5′CTGCGTCTATCCTGAAACCAGCAA CTCCTA 3′
Race R2–5′ACAAGCTCGCAATTTCGAACCCTC 3′
Race R3–5′CGCAATTTCGAACCCTCCAAGCAT 3′

The 5′ and 3′ ends of SiAmy cDNA were amplified using the 5′/3′ RACE kit from Invitrogen, according to the manufacturer's instructions. The PCR products were cloned into pCR4-TOPO vector and transformed into TOP 10 chemically competent cells (Invitrogen) according to the manufacturer's instructions. Positive clones from the 5′ and 3′ RACE were sequenced to obtain the sequence of the full length alpha-amylase gene. The forward (SiAmy CF1) and reverse (SiAmy CR1) primers were designed based on the complete gene sequence derived from the 5′ and 3′ RACE PCR and the complete gene of 1506 bp was amplified using these primers.

SiAmyCF1: 5′ATGATTCCTCTAATATTACTAGCC GTG3′
SiAmyCR1: 5′TAGCCGGCTCTGAGGACCAAT3′

Sequence analysis and homology modeling

The cloned gene sequence was translated into the protein sequence using the 'ORF Finder' program at the NCBI site (http://www.ncbi.nlm.nih.gov/projects/gorf). The signal peptide cleavage site was determined using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The theoretical pI and Mw (molecular weight) were computed using the 'Compute pI/Mw tool' option on the ExPasy server (http://www.expasy.ch/tools/).

Sequence analyses were performed with the BLAST program of the National Centre for Biotechnology Information (NCBI), NIH, Bethesda, MD, USA. Sequence alignments were obtained using CLUSTALW.[49] The numbering of SiAmy is based on the mature protein (excludes the 15 residue signal peptide). The phylogenetic analysis was performed using the TreeTop Phylogenetic Tree Prediction Program (GeneBee—Molecular Biology Server).[50,51] The phylogenetic tree was built using Phylip program option in the TreeTop Phylogenetic Tree Prediction Program and a bootstrap value of 100 was used.

The three-dimensional structure of the amylase of *S. incertulas* Walker (Lepidoptera: Pyralidae) was predicted from its amino acid sequence by homology modeling (http://www.predictprotein.org/) Structural model used, closest to this amylase, was that of the amylase from *T. molitor* L. (pdb:1jae). Display of the structures and superposition were carried out using QUANTA (Accelrys, USA) on a silicon graphics workstation running operating system iris 6.3. The geometry of the predicted model was checked using the program PROCHECK.[52] When the model was checked against ideal geometry

parameters more than 99% of the residues were falling in the allowed regions of the Ramachandran plot. More than 99% of bond lengths and more than 91% of bond angles were within limits. The overall G factor –0.13 (dihedrals: –0.07, covalent: –0.21) output by the program shows that the model has acceptable geometry.

Results and Discussion

Specificity of the dimeric α-amylase inhibitor from wheat variety MP Sehore

The activity of the purified dimeric α-amylase inhibitor from the above variety was evaluated against human salivary α-amylase and porcine pancreatic α-amylase (Table 1). The results presented in Table 1 clearly demonstrate that the dimeric α-amylase inhibitor from wheat variety MP Sehore is specific for the *S.incertulas* amylase.

Cloning and sequence analysis of SiAmy cDNA

Forward primers SiAmyF1-4 and reverse primers SiAmyR1-4 for RT-PCR were designed based on the short conserved amino acid sequences VHLFEWR/K and KHMWPA/G which are found in insect alpha-amylases.[53–55] Of the four pairs of primers used for the RT-PCR reaction, only the primer pair SiAmyF1-SiAmyR1 amplified a 545 bp fragment. The derived amino acid sequence of this fragment showed significant homology to other insect alpha-amylases. Primers for 5′ and 3′ RACE were designed based on this primary fragment and the fragment was extended in both directions by RACE-PCR. SiAmyCF1 and SiAmyCR1 were designed based on the 5′ and 3′ RACE derived sequences and the complete gene of 1506 bp was amplified. Translation of this sequence gave a protein with an open reading frame of 502 amino acids, which showed significant homology with α-amylases from other insects. This clone was designated as SiAmy (Gene Bank accession number EF672102). (Fig. 3). The signal peptide cleavage site was found using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Cleavage of the 15 amino acid long signal peptide would leave a mature protein of length 487 amino acids with a predicted molecular weight of 54,955 Daltons and theoretical pI of 5.39.

A phylogenetic tree was generated using Phylip in the TreeTop Phylogeny prediction program (Fig. 4). SiAmy shows 69%–73% identity with the α-amylases from other lepidopterans and 49%–59% identity with flies and beetles. (Fig. 4).

Figure 5a shows the clustal alignment of SiAmy with the α-amylase from *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) (TMA) and Figure 5b shows the clustal alignment of SiAmy with the α-amylases from a few lepidopterans as well as with TMA and porcine pancreatic amylase (PPA). In the sequence alignment of SiAmy and TMA (Fig. 5a) as well as in the superposition of structures (Fig. 6), the three active site residues (Asp 185, Glu 222 and Asp 287) of *T. molitor* amylase perfectly match with the corresponding residues (Asp 196, Glu 233 and Asp 298) of *S. incertulas* amylase (The numbering in SiAmy excludes the signal peptide). This modeling and comparison has helped us to identify the putative active site residues of *S. incertulas* amylase enzyme. A comparison with the α-amylases of PPA, TMA and other insect α-amylases shows that His 104, His 200 and His 297 involved in substrate binding, Asn 103, Arg 157, Asp 166 and His 200 involved in Ca^{2+} binding and Asn 296 and Arg 194 involved in Cl^- binding are conserved in SiAmy. His 319 (in PPA) which is conserved in all mammalian amylases is present as His 303 in SiAmy.

Table 1. Specificity of the wheat dimeric alpha-amylase inhibitor from wheat variety MP Sehore.

Source	*S. incertulas* α-amylase (units/mg)	Porcine α-amylase (units/mg) (Sigma catalogue No. A3176)	Human α-amylase (units/mg) (Sigma catalogue No. A0521)
Commercial wheat inhibitor (dimer) (Sigma Catalogue No. A1520)	81.08	83.32	72.18
G-50 Purified monomer	1589.42	ND	ND

Abbreviation: ND, No Inhibition detected.

```
   1 atgattcctctaatattactagccgtggccgtatttgctcgtgcg
     M  I  P  L  I  L  L  A  V  A  V  F  A  R  A
  46 gctaacgattacaagaatccttactatgtgccaggcaggtcagtg
     A  N  D  Y  K  N  P  Y  Y  V  P  G  R  S  V
  91 aatgtccacctcttcgagtggaagtgggaggatatagctgaagag
     N  V  H  L  F  E  W  K  W  E  D  I  A  E  E
 136 tgtgagcgctttttaggacccaatggttttggtggggtacagatt
     C  E  R  F  L  G  P  N  G  F  G  G  V  Q  I
 181 tcaccaccaaacgaaaacgtgataatatggtcagctaaccggccg
     S  P  P  N  E  N  V  I  I  W  S  A  N  R  P
 226 tggtgggagcggtaccaacccatttcgtacaaactcaccacacga
     W  W  E  R  Y  Q  P  I  S  Y  K  L  T  T  R
 271 tcaggtgacaacacgcagctggccaacatgttgaggcgatgtaac
     S  G  D  N  T  Q  L  A  N  M  L  R  R  C  N
 316 aacgttggagtcagaatatatgtcgatgctgtcatcaatcatatg
     N  V  G  V  R  I  Y  V  D  A  V  I  N  H  M
 361 actggggatcctccagagaatgtggggactgccggcagtactgct
     T  G  D  P  P  E  N  V  G  T  A  G  S  T  A
 406 accttcaatgaatggcactaccctactgtaccgtacagaagagaa
     T  F  N  E  W  H  Y  P  T  V  P  Y  R  R  E
 451 cattttaactggccctcttgtggcatcgatggtaccgactaccaa
     H  F  N  W  P  S  C  G  I  D  G  T  D  Y  Q
 496 accaatgcttggagggttcgaaattgcgagcttgttggtttaaaa
     T  N  A  W  R  V  R  N  C  E  L  V  G  L  K
 541 gatttagatcagtctattgatcatgtgcgcaatatgattgtggat
     D  L  D  Q  S  I  D  H  V  R  N  M  I  V  D
 586 ttcatgaacactctgattgatttaggagttgctggtttcaggata
     F  M  N  T  L  I  D  L  G  V  A  G  F  R  I
 631 gacgcagcaaaacacatgtggccagaagatctccgaataatctac
     D  A  A  K  H  M  W  P  E  D  L  R  I  I  Y
 676 gatcgactacataatctgagcacagaccatggcttcccattgaat
     D  R  L  H  N  L  S  T  D  H  G  F  P  L  N
 721 gcccgcccatacatttaccaagaagtcattgattatggtggcgaa
     A  R  P  Y  I  Y  Q  E  V  I  D  Y  G  G  E
 766 gctgtcagtagagaagagtatactccaattggagctgtgactgag
     A  V  S  R  E  E  Y  T  P  I  G  A  V  T  E
 811 ttcaaggctggcatggagttgtctaactgcttcagaggacataat
     F  K  A  G  M  E  L  S  N  C  F  R  G  H  N
 856 cagctccgatggctcgtcagctggggagcgccgttcggattgctg
     Q  L  R  W  L  V  S  W  G  A  P  F  G  L  L
 901 gagagcagagacgctttgacgtttattgataatcacgacaatgaa
     E  S  R  D  A  L  T  F  I  D  N  H  D  N  E
 946 agaggtcacggaggtggtggcggtgtgcttacttacaagcaacct
     R  G  H  G  G  G  G  G  V  L  T  Y  K  Q  P
 991 agaccatataaagcagctatagccttcctgttagcacatccttat
     R  P  Y  K  A  A  I  A  F  L  L  A  H  P  Y
1036 ggagaaccacaaatcatgagtagtttcgagttttgggacccagaa
     G  E  P  Q  I  M  S  S  F  E  F  W  D  P  E
1081 ataggtccgccaatgaatagtcagggtaacataatatccccttct
     I  G  P  P  M  N  S  Q  G  N  I  I  S  P  S
1126 atcaactcagatggcacttgtggtaacgggtggatatgccagcac
     I  N  S  D  G  T  C  G  N  G  W  I  C  Q  H
1171 aggtggcgacagatctactccatggtgtcgttcaggaatgccgct
     R  W  R  Q  I  Y  S  M  V  S  F  R  N  A  A
1216 ggcagcgggggccttaacaactggtgggacaatggttccaaccaa
     G  S  G  G  L  N  N  W  W  D  N  G  S  N  Q
1261 atagcattttgccgcggagaaagcgcttttatagcatttaacaat
     I  A  F  C  R  G  E  S  A  F  I  A  F  N  N
1306 gacagccgggatttaaacgaaaatatgcagacatgtctcccagct
     D  S  R  D  L  N  E  N  M  Q  T  C  L  P  A
1351 gggcagtattgcgacgtgatcacaggcgagagaaacggaagttcc
     G  Q  Y  C  D  V  I  T  G  E  R  N  G  S  S
1396 tgctccggtaaggtggtgacggtgggagagaacgggtgggctcat
     C  S  G  K  V  V  T  V  G  E  N  G  W  A  H
1441 gtctacgtaggagcgcaagactacgatatgatgttagcaatacat
     V  Y  V  G  A  Q  D  Y  D  M  M  L  A  I  H
1486 attggtcctcagagccggctatga 1509
     I  G  P  Q  S  R  L  *
```

Figure 3. cDNA sequence and the deduced protein sequence of the *S. incertulas* α-amylase SiAmy (Gene Bank Accession Number EF672102). The first fifteen amino acids (bold and highlighted in red) represent the signal peptide. The three amino acids (bold and highlighted in yellow) are conserved in α-amylases and are part of the active site.

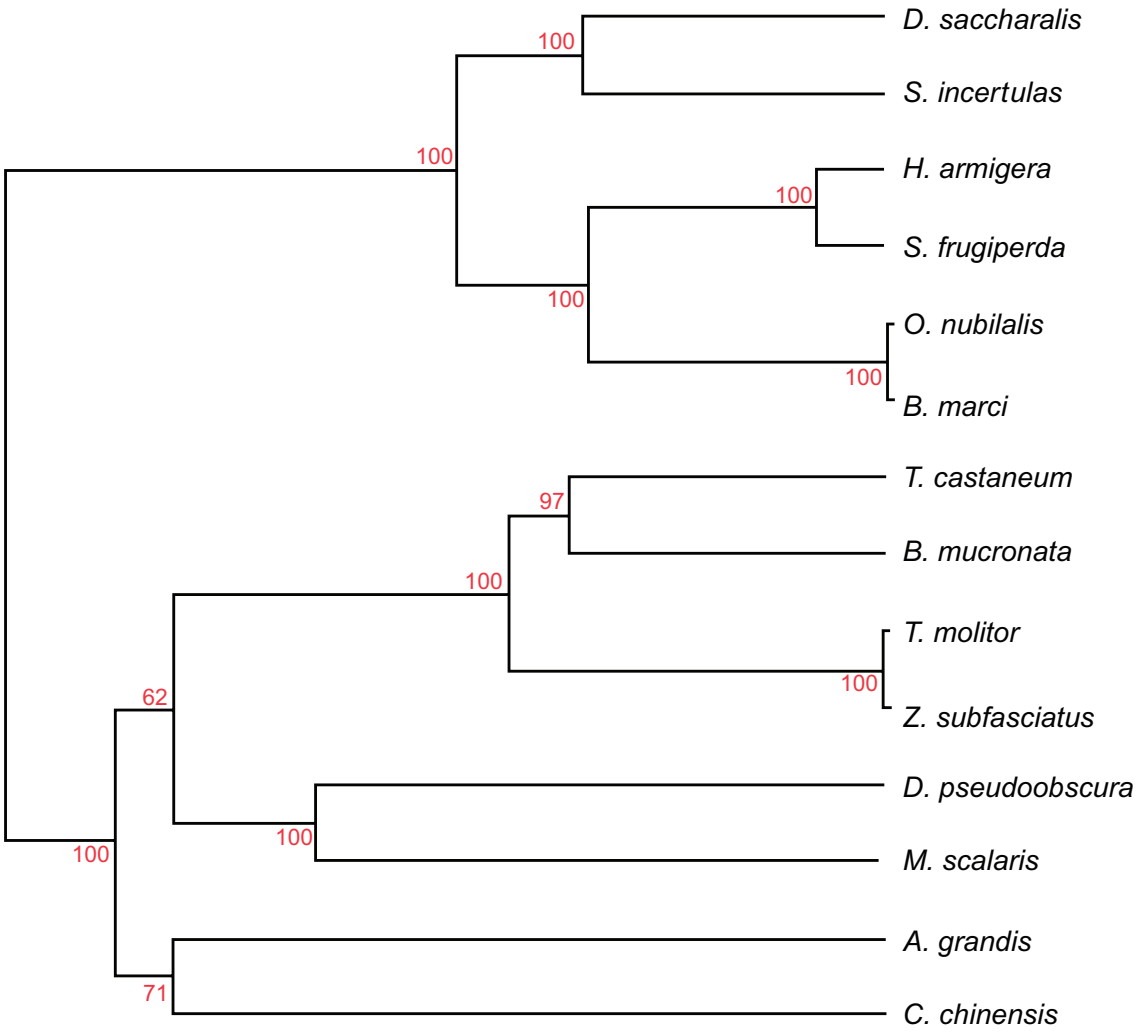

Figure 4. Phylogenetic tree of α-amylases of the following insects. *Diatraea saccharalis* (AY333761), *S. incertulas* Walker (EF672102), *Helicoverpa armigera* (EF600049), *Spodoptera frugiperda* (AA013754), *Ostrinia nubilalis* (AAA03715), *D. pseudoobscura* (X76240), *Megaselia scalaris* (AA017923), *Bibio marci* (AAL92553), *Tribolium castaneum* (U04271), *Blaps mucronata (*AF462603), *Tenebrio molitor* (P56634), *Zabrotes subfasciatus* (AAF73435), *Callosobruchus chinensis* (AB11048), *Anthonomus grandis (*AF527876*)*. A bootstrap value of 100 was used and the bootstrap values are expressed in percentages at the nodes.

This histidine residue is conserved in lepidopterans and flies but not in beetles. In vertebrates, beetles and flies, the Cl^- ion is coordinated by six ligands i.e. Arg 210(PPA) and Arg 351(PPA) in a bidentate mode and Asn 312(PPA) in a unidentate mode.[56] In bacterial α-amylases, Arg 351(PPA) is replaced by a lysine residue.[57] In the α-amylases of lepidopterans including SiAmy, Arg 351/Lys 351 is replaced by a glutamine residue (Q 334 in SiAmy). It has been suggested that such a replacement by a glutamine residue makes the α-amylase independent of chloride ions for activity and also shifts the optimum pH towards alkaline values[58,62] which is an advantage for lepidopterans whose gut environment is alkaline. The α-amylases of beetles and flies have either Arg or Lys in this position, which is suitable for activity in an acidic gut environment. Also, the binding of chloride ion to α-amylases has been suggested to broaden the pH activity profile such that the pH optimum shifts from acidic values to slight alkaline values.[58.62]

A glycine rich 'flexible loop' (residues 318–323 in PPA) which forms the surface edge of the substrate binding cleft in PPA,[59] is also present in SiAmy (residues 302–308) and other lepidopterans (Fig. 5b), but is absent in TMA. This loop has been suggested to hold residues assisting catalysis in the appropriate orientation and to induce a trap-release mechanism of substrate and products.[59–61] Though the amylases of *S.incertulas* Walker (Lepidoptera: Pyralidae) appear to be chloride independent (unpublished

```
S.INCERTULAS       1 ANDYKNPYYVPGRSVNVHLFEWKWEDIAEECERFLGPNGFGGVQISPPNE     50
                     .|:..:..||:..||||||||.|||:|||||||.|.|||||||||||||
T.MOLITOR          1    EKDANFASGRNSIVHLFEWKWNDIADECERFLQPQGFGGVQISPPNE     47

S.INCERTULAS      51 NVIIWSANRPWWERYQPISYKLTTRSGDNTQLANMLRRCNNVGVRIYVDA    100
                     .::  :..|||||||||:||.:.|||||.:...:|.||||:.||||||||
T.MOLITOR         48 YLV--ADGRPWWERYQPVSYIINTRSGDESAFTDMTRRCNDAGVRIYVDA     95

S.INCERTULAS     101 VINHMTGDPPENVGTAGSTATFNEWHYPTVPYRREHFNWPSCGIDGTDYQ    150
                     |||||||  ...|||:||:|..:..:||.|||....|:.| |.::  :||
T.MOLITOR         96 VINHMTG--MNGVGTSGSSADHDGMNYPAVPYGSGDFHSP-CEVN--NYQ    140

S.INCERTULAS     151 TNAWRVRNCELVGLKDLDQSIDHVRNMIVDFMNTLIDLGVAGFRIDAAKH    200
                      :|..||||||||||:||:|..|:||.:::|:||.:||||||||:|||||
T.MOLITOR        141 -DADNVRNCELVGLRDLNQGSDYVRGVLIDYMNHMIDLGVAGFRVDAAKH    189

S.INCERTULAS     201 MWPEDLRIIYDRLHNLSTDHGFPLNARPYIYQEVIDYGGEAVSREEYTPI    250
                     |.|.||.:|:..|.||:||:||...|||:|||||||.||||:|:.|||..
T.MOLITOR        190 MSPGDLSVIFSGLKNLNTDYGFADGARPFIYQEVIDLGGEAISKNEYTGF    239

S.INCERTULAS     251 GAVTEFKAGMELSNCFRGHNQLRWLVSWGAPFGLLESRDALTFIDNHDNE    300
                     |.|.||:.|:.|.|.|:|.|||:.|.:||..:||||..||:.|:|||||:
T.MOLITOR        240 GCVLEFQFGVSLGNAFQGGNQLKNLANWGPEWGLLEGLDAVVFVDNHDNQ    289

S.INCERTULAS     301 RGHGGGGGVLTYKQPRPYKAAIAFLLAHPYGEPQIMSSFEFWDPEIGPPM    350
                     |  .||..:||||.|:|||.||||:||||||..:|||||:|.|.:.|||.
T.MOLITOR        290 R--TGGSQILTYKNPKPYKMAIAFMLAHPYGTTRIMSSFDFTDNDQGPPQ    337

S.INCERTULAS     351 NSQGNIISPSINSDGTCGNGWICQHRWRQIYSMVSFRNAAGSGGLNNWWD    400
                     :..||:|||.||.|.||.||::|:|||||:|.||.||||.....:.|||.
T.MOLITOR        338 DGSGNLISPGINDDNTCSNGYVCEHRWRQVYGMVGFRNAVEGTQVENWWS    387

S.INCERTULAS     401 NGSNQIAFCRGESAFIAFNNDSRDLNENMQTCLPAGQYCDVITGERNGSS    450
                     |..||||||.||...|:||.|.. |||:|:.|.||||.|||||:||.:|.|
T.MOLITOR        388 NDDNQIAFSRGSQGFVAFTNGG-DLNQNLNTGLPAGTYCDVISGELSGGS    436

S.INCERTULAS     451 CSGKVVTVGENGWAHVYVGAQDYDMMLAIHIGPQSRL    487
                     |:||.||||:||.|.:.:|:.:.|.:||||:..:.
T.MOLITOR        437 CTGKSVTVGDNGSADISLGSAEDDGVLAIHVNAKL      471
```

Figure 5a. ClustalW alignment of the *S. incertulas* amylase SiAmy (EF672102) with the amylase of *Tenebrio molitor* (TMA) (P56634). The three catalytic residues D196, E233 and D298 of SiAmy and the corresponding ones in *T. molitor* amylase (TMA) are highlighted in yellow. The numbering of SiAmy excludes the signal peptide.

results), residues Ser 354, His 400 and Glu 42 in PPA which are strictly conserved in chloride-dependent α-amylases, are present as Ser 337, His 375 and Glu 30 in SiAmy. This triad has been shown to mimick the active site of proteases and lipases and was first described in the α-amylase from *Alteromonas haloplanctis*.[57] Though there is no evidence for a catalytic role, this triad has been suggested to play a pivotal role in stabilizing the structure of chloride-dependent α-amylases.[62]

Four disulfide bonds required for proper folding of the SiAmy structure are formed by the conserved cysteines ie. C31–C89, C142–C159, C367–C373 and C439–C451. These bonds are found in all chloride-dependent α-amylases.[62] The C409–C432 disulfide bond in SiAmy (absent in TMA) bridges the β-strands in domain C and has been implicated in improving the rigidity of the globular C domain.[62] As seen in Figure 5b, the sequence DGX_1X_2AIH in the C-terminal tail does not completely follow the general consensus.[63] In SiAmy and lepidopterans, the glycine is replaced with methionine which is also a non-polar amino acid. The two amino acids preceeding AIH are hydrophobic i.e. Valine, Leucine or Methionine. In SiAmy and the α-amylases of *Diatraea saccharalis* and *Ostrinia nubilalis*, AIH is followed by hydrophobic amino acids isoleucine or valine. In case of *Helicoverpa armigera* and *Spodoptera frugiperda*, AIH is followed by threonine, a hydrophilic amino acid. The next three amino acids are not necessarily hydrophilic.

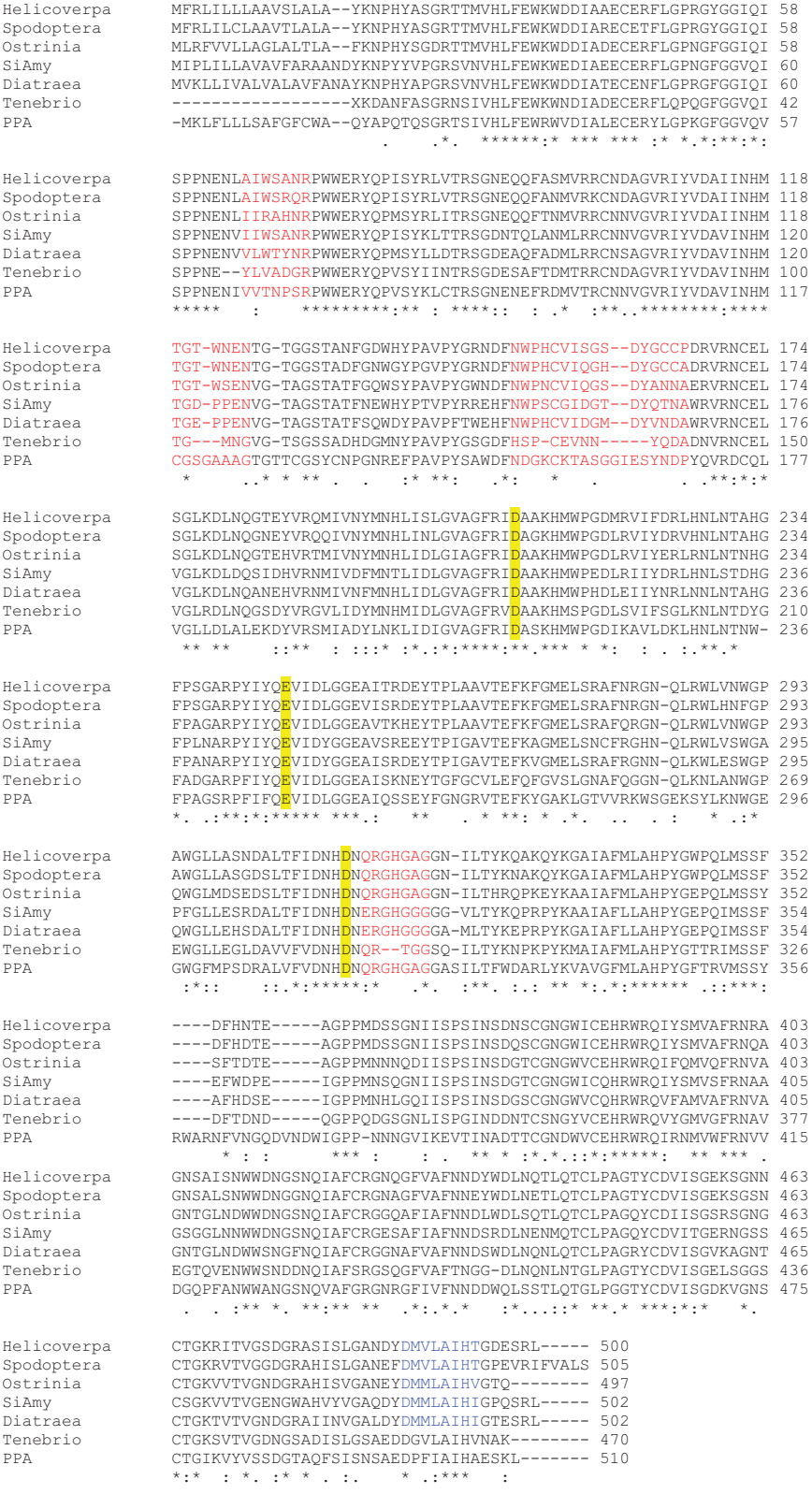

Figure 5b. ClustalW alignment of the α-amylases of *Diatraea saccharalis* (AY333761). *S. incertulas* Walker (EF672102), *Helicoverpa armigera* (EF600049), *Spodoptera frugiperda* (AA013754), *Ostrinia nubilalis* (AAA03715), *Tenebrio molitor* (P56634) and Porcine Pancreatic Amylase (AFO64742). The four regions (numbering based on *S. incertulas* amylase, excluding the signal peptide) shown in red (1) 53–59, (2) 106–112, (3) 138–152 and (4) 300–306 correspond to insertions in *S. incertulas* amylase and form part of loops surrounding the catalytic site. The three catalytic residues D 196, E 233 and D 298 of *S.incertulas* amylase (SiAmy) and the corresponding ones in *T. molitor* amylase (TMA) are highlighted in yellow. The amino acid residues in the C-terminal tail of the lepidopteran amylases are highlighted in blue.
Symbols *conserved residue; substitution by similar amino acid; substitution by non-similar amino acid.

Homology modeling

The sequence alignment of *S. incertulas* Walker (Lepidoptera: Pyralidae) amylase (SiAmy) with the sequence of the amylase from *Tenebrio molitor* L. (TMA) used for homology modeling is shown in Figure 5a. The chain length (487) of SiAmy is longer than that of TMA (471). Consequently, in addition to N- and C-terminals there are four major regions in the sequence wherein SiAmy has insertions of amino acids compared to TMA. Manual superposition using QUANTA (Accelrys) of the modeled structure of SiAmy on TMA shows that the sizes of four loops in *S. incertulas* Walker (Lepidoptera: Pyralidae) have increased due to insertion of additional residues (Fig. 4).

Based on Blastp, SiAmy shows only 56% homology to the amylase of *T. molitor*. The only insect amylase whose 3D structure is well studied is that of *T. molitor*. The 3D structure of *T. molitor* amylase complexed with various inhibitors is also well studied. The maximum homology between SiAmy and the amylases from other lepidopterans is 76%. Several of the pests, which infest several crops, are lepidopterans. Hence, a 3D

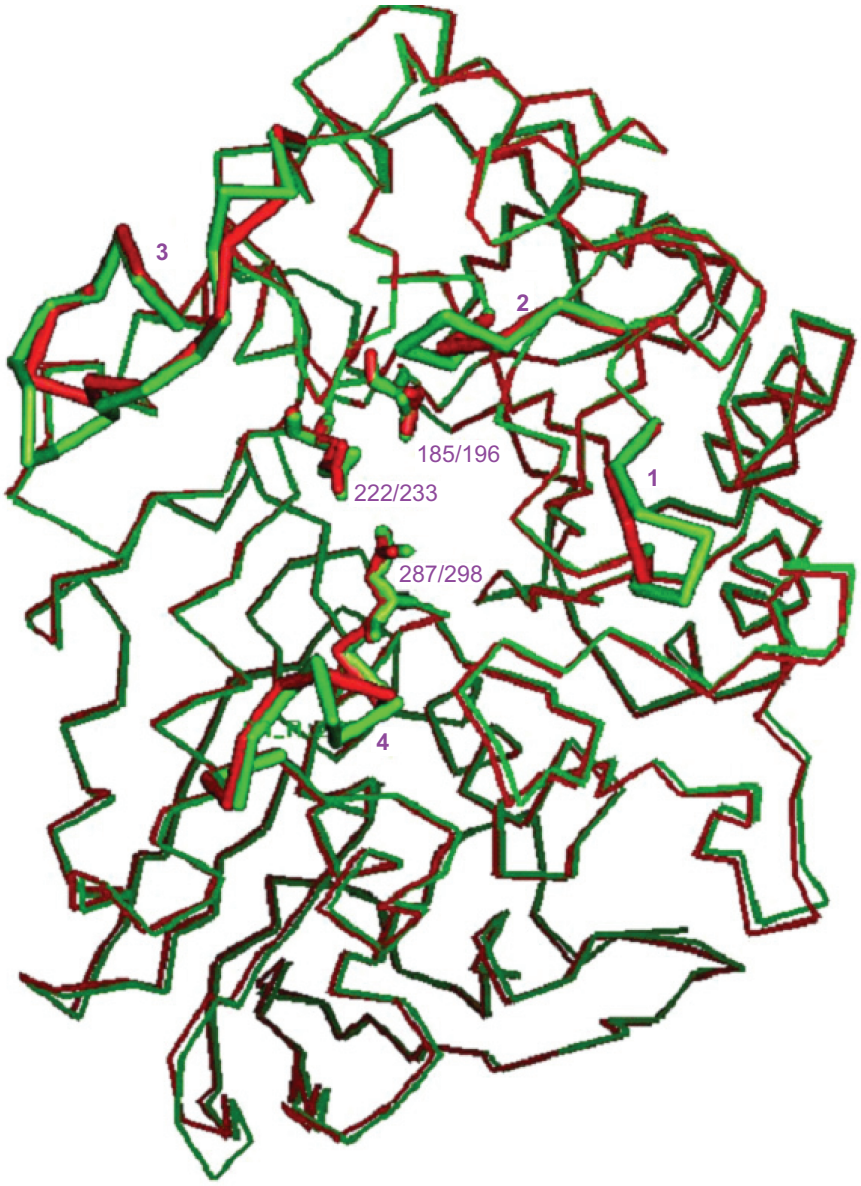

Figure 6. Superposition of the modeled structure of *Scirpophaga incertulas* Walker amylase (green) on the reported structure of *Tenebrio molitor* L. α-amylase (red, pdb: 1jae). The four loop regions (1) 53–59 (2) 106–112 (3) 138–152 (4) 300–306 where loops deviate due to insertion of residues are shown liquorized (numbering based on SiAmy excluding the 15 residue signal peptide). Superposition of three active site residues are also shown (residue numbers correspond to TMA/SiAmy) (Refer Fig. 5a also).

model developed for SiAmy will serve to compare lepidopteran amylases more accurately.

The wheat inhibitor reported here is sequentially placed in the class 0.19 dimer type inhibitors of wheat. Sequence comparison showed that it differs from the dimeric α-amylase inhibitor from *Triticum turgidum* subsp. *dicoccoides* (GeneBank ACP40697.1) by just three amino acids. Similarly it differs by 8 residues with respect to sequence of 0.19 inhibitor structure reported in protein data bank (1HSS) and also by the presence of 17 extra residues at the N-terminus. The wheat inhibitor characterized here has an extra C-terminal valine residue compared to others of this type. We have experimental evidence to show that the inhibitor protein isolated by us is specific for insect amylases and does not inhibit human salivary amylase (HAS) (Table 1). It is reported that among the wheat amylase inhibitors 0.19 type class shows wider specificity for both insect and mammalian α-amylases whereas 0.28 class is more specific for insect amylases.[38] Comparative modeling studies between 0.19 and 0.28 types inhibitors for insect and mammalian amylases[64] have shown that the residues 104–107, VVDA of 0.19 mutated to a shorter PNP in 0.28 in an insertion loop which essentially may be determining the exclusive specificity of latter type for insect amylase. Similar conclusion was drawn in a different modeling study using four different inhibitors from wheat.[39] We have found out that the residues of loop region 104–107 in our inhibitor are VIDA. However, it will be hard to believe that this single change of valine to isoleucine alone can change it to become non-inhibitory for human salivary amylase. Although both N- and C-terminal residues are reported to be important,[64] in a proposed global mechanism of inhibition by this class of inhibitors, whether the extra valine at C-terminus in our inhibitor decides its non-specificity for HAS is not clear from the present information. Further structural studies only can provide an exact description of the inhibitor-amylase interactions and specificity.

An ideal inhibitor should be able to specifically inhibit the insect amylases. We have identified and purified a dimeric inhibitor from a local variety of wheat which unlike other dimeric inhibitors reported to date, does not inhibit porcine α-amylase or human salivary α-amylase but specifically inhibits the *S. incertulas* amylase. Elucidation of the molecular basis of specificity of interaction between SiAmy and the wheat inhibitor will not only help understand the specificity of the wheat inhibitor but can help to design inhibitors specific for other lepidopteran amylases.

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Disclosures

The authors report no conflicts of interest.

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