

A Novel α -Amylase Inhibitor from Amaranth (*Amaranthus hypocondriacus*) Seeds*

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The major α -amylase inhibitor (AAI) present in the seeds of *Amaranthus hypocondriacus*, a variety of the Mexican crop plant amaranth, is a 32-residue-long polypeptide with three disulfide bridges. Purified AAI strongly inhibits the α -amylase activity of insect larvae (*Tribolium castaneum* and *Prostephanus truncatus*) and does not inhibit proteases and mammalian α -amylases. AAI was sequenced with the automated Edman method, and the disulfide bridges were localized using enzymatic and chemical fragmentation methods combined with N-terminal sequencing. AAI is the shortest α -amylase inhibitor described so far which has no known close homologs in the sequence data bases. Its residue conservation patterns and disulfide connectivity are related to the squash family of proteinase inhibitors, to the cellulose binding domain of cellobiohydrolase, and to ω -conotoxin, i.e. a group of small proteins termed "knottins" by Nguyen, D. L., Heitz, A., Chiche, L., Castro, B., Boigegrain, R., Favel, A., and Coletti-Previero, M. ((1990) *Biochimie* 72, 431–435) The three-dimensional model of AAI was built according to the common structural features of this group of proteins using side-chain replacement and molecular dynamics refinement techniques.

Enzyme inhibitors are important tools of nature for regulating the activity of enzymes in cases of emergency. Plant seeds are known to produce a variety of enzyme inhibitors that are thought to protect the seed against insects and microbial pathogens. Proteinase inhibitors are the best studied of this group (1); expression of proteinase inhibitor genes in transgenic plants provides protection against pathogens (for a review, see Ryan (2)). Comparatively less is known about the inhibitors of α -amylase which might, on the other hand, be equally attractive candidates for conferring pest resistance to transgenic plants since many of them inhibit both proteinases and α -amylase.

The structure of α -amylase inhibitors is quite variable (Table I) (see also Richardson (3) for a review); they belong to families that contain proteins of seemingly quite unrelated activity, among which are many proteinase inhibitors. Several of the structurally related proteins play a role in the stress response of plants (proteinase inhibitors, osmotin, salt-induced pro-

teins). It is an important feature of the plant α -amylase inhibitors that their inhibitory activity can be species-specific. For example, members of the cereal family of amylase/protease inhibitors are active against insect α -amylases but do not seem to inhibit the α -amylases present in the digestive system of mammals.

Here we report on the purification of a new type of α -amylase inhibitor isolated from the seeds of *Amaranthus hypocondriacus* which strongly inhibits the α -amylase of the larvae of the red flour beetle (*Tribolium castaneum*) and of the grain borer (*Prostephanus truncatus*). The primary structure of this small protein is not closely related to any other known protein. Its disulfide topology and residue conservation patterns, however, are similar to those of a group of proteins that include members of the squash family of proteinase inhibitors (1, 18, 19), as well as ω -conotoxins (20, 21), which contain three conserved disulfide bridges and an array of three β sheets.

EXPERIMENTAL PROCEDURES

Materials—Seeds of *A. hypocondriacus* line 53 were kindly provided by the Mexican National Institute for Research in Forestry and Agriculture in Celaya Gto., Mexico. Sephadex G-75 and DEAE-Sepharose CL-6B were obtained from Pharmacia Biotech Inc. α -Chymotrypsin and trypsin were from Serva; cyanogen bromide and vinyl pyridine were from Aldrich. All chemicals used were of analytical or sequencing grade. HPLC¹-grade acetonitrile and trifluoroacetic acid were obtained from Aldrich.

α -Amylase Assay—Crude extracts of larval α -amylases of *T. castaneum* and *P. truncatus* were extracted as described previously (22). The activity of α -amylase was determined using nitrosalicylic acid according to the Bernfeld method (8).

Purification of the α -Amylase Inhibitor—A crude extract of α -amylase inhibitor was obtained from 100 g of ground defatted amaranth seeds as described previously (22). The supernatant was precipitated by the addition of ammonium sulfate. The precipitate in the range of 35–65% saturation was collected and redissolved in 0.01 M ammonium bicarbonate. This material was fractionated on a Sephadex G-75 column (1.6 \times 160 cm) preequilibrated with the same solution. Fractions inhibiting the α -amylase activity of *T. castaneum* larvae were pooled and lyophilized. This material was redissolved in 5 ml of 0.02 M ammonium bicarbonate buffer, pH 8.3, and chromatographed on a (20 \times 2.6 cm) DEAE-Sepharose CL-6B column preequilibrated with the same buffer using a linear gradient of ammonium bicarbonate (0.02–0.5 M). The active fractions eluted were lyophilized and subjected to reverse phase HPLC on a preparative Vidac C18 column (22.5 \times 250 mm, 10 mm particle size) using a model 1050 Hewlett Packard HPLC system. The two solvents used were 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). A linear gradient was used for elution (flow rate, 5 ml/min) in which the solvent composition changed from 0 to 80% B in 120 min.

Amino Acid Analysis—Amino acid composition of the peptides was determined using a Waters workstation and PicoTag HPLC system

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¹ The abbreviations used are: HPLC, high performance liquid chromatography; SCR, structurally conserved region; SD, steepest descent; CG conjugate gradient; AAI, α -amylase inhibitor.

TABLE I
A classification of α -amylase inhibitors

Based on a classification by Richardson (3), completed with recent data.

Class	Source ^a	Size [aa]	1/2-Cys	Inhibitory activity			Members of the group with other activities ^a
				Against insect amylases	Against mammalian or other amylases	Against proteases	
Kunitz type	Barley (4), wheat (5), rice (6)	176–180	2–4	+	+	+	Miraculin (7)
Cereal type	Wheat (3), barley (3), indian finger millet (8)	124–160	10	+	–	+	ND ^b
γ -Purothionin type	Sorghum (9)	47–48	8	+	–	–	γ -Purothionins (10)
Ragi I-2 type	Indian finger millet (11)	95	7	+	–	–	Phospholipid transfer proteins
Legume lectin type	Common beans (12)	246		+	+	–	Legume lectins (13)
Thaumatin type	Maize (14)	173–235	10–16	+	+	+	Pathogenesis related protein (15) osmotin (16) thaumatin (17)
Prokaryotic	Actinomycetes	75–120	4	+	+	–	ND

^a Numbers in parentheses are references.

^b ND, no data.

(Millipore Waters Chromatography Corp). The peptides were hydrolyzed by 6 N HCl in the presence of crystalline phenol at 110 °C for 18 h, and the amino acids were analyzed after derivatization with phenylisothiocyanate, as described elsewhere (23). Free sulfhydryl groups were determined as described by Hampton *et al.* (24).

Sequence Determination—Samples reduced and pyridylethylated (24) were digested separately with trypsin and CNBr, as previously described (25, 26). The resulting peptides were purified by narrow bore reverse phase HPLC on an analytical Aquapore OD300 column (220 × 2.1 mm, 7-mm particle size, Applied Biosystems) using gradients of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. The pyridylethylated protein and the peptides were sequenced using an Applied Biosystems protein sequencer (model 471A) employing a Edman degradation sequenator program (27).

Determination of the Disulfide Bridges—To determine the connectivity of the disulfide bridges, the native protein dissolved in 0.1 M ammonium acetate, pH 5.2, was digested by the simultaneous addition of trypsin and chymotrypsin, respectively, using an E/S ratio of 1:1:20 (w/w) at 37 °C for 44 h. Peptides from the digest were purified by narrow bore reverse phase HPLC and were analyzed for amino acid composition and sequence.

Sequence Similarity Searches—Sequence similarities between the amaranth α -amylase inhibitor and other known proteins were searched (March, 1994) using the NCBI BLAST (28) and the EMBL BLITZ (29, 30) electronic mail servers. All other computer analyses of the sequence were carried out using the GCG package of sequence analysis (31).

Modeling—All modeling procedures, including energy minimization and molecular dynamics, were performed using the INSIGHT II/DISCOVER software (Biosym Technologies Inc., San Diego, CA) implemented on a Silicon Graphics Indigo workstation. Energy calculations were carried out using the CVFF force field (32).

The structure of the amaranth α -amylase inhibitor was modeled according to similarity with known structures. The atomic coordinates for all proteins were taken from the Brookhaven Protein Data bank. The structures were superimposed manually on the graphics display in order to determine the structurally conserved regions (SCRs) (33). The sequences were also aligned manually, minimizing the number of gaps and avoiding gaps within SCRs (see Fig. 6). The template structure used for model building was derived from this alignment. The model was then built manually by side chain replacement, as follows. Identical residue pairs were assumed to have the same conformations as in the template. Side chains of differing residues were replaced, overlapping the common heavy atoms, and were visually inspected in order to ascertain that no serious steric clashes occurred. Replacement of non-proline by proline residues and vice versa was followed by a local energy minimization of 100 steepest descent cycles in order to obtain a correct geometry. Deletions were introduced using INSIGHT's biopolymer module. Each residue was treated separately. As the first step, the residue was deleted and connectivity was reestablished, in a first step, with 100 cycles of localized steepest descents (SD) energy minimization and 200 cycles of conjugate gradient (CG) minimization constrained to the 2 flanking residues on each side of the deletions. And in a second step the procedure was repeated including 4 flanking residues. Finally, the connectivity of the disulfide bonds was reestablished, and the structure was refined by energy minimization and molecular dynamics. No cross terms were used, and a harmonic bond stretching potential was applied.

A nonbonded cutoff value of 10 Å was used together with a distance-dependent dielectric constant. Dynamics was performed using a time step of 1 fs, and the temperature was kept constant by coupling to a thermal bath with a constant of 0.1 ps (34). The refinement was a modification of the procedure described by Du and co-workers (35). An initial minimization was performed with 200 cycles of SD minimization followed by 200 cycles of CG minimization. Afterward, the model was put into a 50-Å diameter sphere of preequilibrated water and the system was subjected to 5 ps dynamics at 200 K and 200 cycles of SD minimization keeping the protein atoms fixed, in order to allow badly placed water molecules to move. This was followed by a 5 ps dynamics at 200 K keeping only the SCRs fixed. The refinement proceeded with a low temperature simulated annealing starting at 340 K and lowering the temperature to 260 K using 20-K steps. The system was kept for 5 ps at each temperature. The system was minimized using 200 cycles of SD minimization and 200 cycles of CG minimization. Finally, the protein-solvent system was subjected to a 100-ps molecular dynamics simulation at 300 K, allowing all atoms to move. The resulting structure was subjected to 200 steps of SD energy minimization and to a CG minimization until the maximum energy derivative was lower than 0.1 kcal/Å.

The model was evaluated using the ProsaII program (36). The program uses mean force potentials derived from known protein structures (36) in order to calculate the energy of a new structure. Two kinds of potentials, C β -C β pair interaction potentials and surface potentials, are used for this purpose. As the surface potentials are not recommended for small proteins (37) (in fact the surface potential calculations failed to recognize the native structure in 8 out of 10 small disulfide-rich proteins tested by us), we based the analysis of the AAI structure solely on the C β -C β interaction potentials. The program was used with standard parameters as provided by the author.

RESULTS

Purification of *A. hypocondriacus* α -Amylase Inhibitor—The crude extract prepared by succinic acid extraction contained inhibitors of both α -amylase and trypsin (data not shown). When subjected to gel filtration on a Sephadex G-75 column, the fractions inhibiting α -amylase of the larval enzyme of *T. castaneum* eluted essentially as a single peak. These fractions were lyophilized and subjected to anion exchange chromatography on a DEAE-Sepharose CL-6B column. No inhibitory activity was found in the unbound fraction and a linear NH₄HCO₃ gradient allowed for the separation of two peaks showing inhibitory activity (IEX-1 (*left*) and IEX-2 (*right*), in Fig. 1). The respective fractions were pooled and subsequently subjected to reverse phase HPLC. IEX-1 yielded several peaks with α -amylase inhibitory activity that were not analyzed further in this study. Reverse phase HPLC of the IEX-2, on the other hand, gave one major peak eluting at 25% acetonitrile, which we called amaranth α -amylase inhibitor (AAI), and subjected to sequencing.

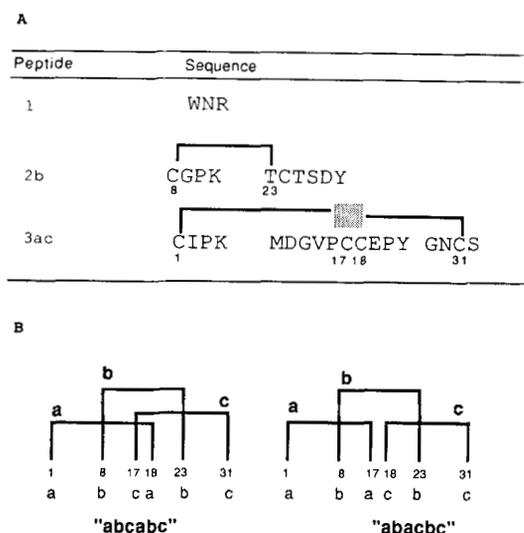


FIG. 3. Determination of the disulfide bonds in AAI by partial enzymatic digestion. A, sequence of the isolated peptides with the deduced SS bonds; B, AAI's potential disulfide patterns that can be constructed with the available data.

that this behavior is characteristic of short and compositionally biased query sequences such as AAI (39). In order to increase the sensitivity of the search, we selected a subset of the data base in which cysteine residues were distributed in a way similar to AAI. This search allowed us to tentatively identify a group of short cysteine-rich proteins and protein domains of different organisms, including various carbohydrate-binding proteins (cellobiohydrolase, wheat germ agglutinin, hevein, chitinase), toxins (conotoxins), antimicrobial peptides from *Amaranthus* (AMP), and the sweet taste-suppressing protein, gurmarin (Fig. 4). The best similarity (40% residue identity) was found in the case of the cellulose binding domain of cellobiohydrolase II from *Trichoderma reesei*. The homologous cellulose binding domain of cellobiohydrolase I is also included in the alignment. In this structure one disulfide bridge (denoted *a* in Fig. 3) is missing.

Given that the three-dimensional structure of several of these proteins is already known from x-ray and NMR studies, we could classify the structures in two groups based on disulfide topologies (Fig. 5, top) and folding patterns (Fig. 5, bottom). Group I contains the squash family of trypsin inhibitors, the cellulose binding domain of cellobiohydrolase and ω -conotoxin (1cco, 1cbh, 1cti, 2eti). As the structures in this family contain a characteristic knotlike arrangement, Nguyen *et al.* (47) suggested the "knottin" name for this family of proteins. Group II contains chitin-binding domains of wheat germ agglutinin and hevein (1hev and 9wga). In group I (topology *abcabc* in Fig. 5, top) there are three disulfide bridges, whereas in group II (topology *abcabcd* in Fig. 5, top), there are four. When viewed in the sequence context, three of these are in a topological arrangement seen in group I (*i.e.* *abcabc*), and one (bridge *d*) is outside. A comparison of the available three-dimensional structures revealed that a sheet composed of three short β -strands is present in both structural groups (Fig. 5, bottom). The disulfide bridges are, however, arranged in a different manner within the two groups. In group I all three disulfide bridges take part in the "reinforcement" of the sheet structure. In group II the three short β -strands are present but bridge *c* is connected to a short helical segment (absent in group I) connecting two strands of the sheet, while the fourth bridge *d* connects two ends of the C-terminal strand (symbols shown in Fig. 5, top). The common element of the two folding patterns is a short β -hairpin-like structure with an irregular N-terminal exten-

sion (boxed in Fig. 5, bottom). The three-strand arrangement is seemingly common to both folding patterns, however, while the third strand is located at the N terminus of the common pattern in group I, in the group II structures it is at the C terminus. Though the strands of the sheet are short, the three-strand arrangement can be seen on all but one of the structures related to AAI. The only exception is the carboxypeptidase A inhibitor (4cpa), in which no regular secondary structures can be detected even though its overall folding pattern is clearly related to the other structures in group I.

Modeling—The superimposition of the structures and the alignment of the structurally conserved regions was used to design a structural template for AAI. Assignment of two disulfide bridges in AAI was, in principle, an open question, since the connectivity of adjacent cysteines 17 and 18 could not be directly determined by chemical means. Theoretically there are two possibilities to form SS bridges using our connectivity data (Fig. 3B). (i) The "abcabc" topology is characteristic of group I structures, and was also found by chemical means in ω -conotoxin (48); (ii) The abacbc topology, on the other hand, has not yet been found experimentally in short proteins (49). On the basis of the chemical evidence (48) and of the convincing similarity of AAI to group I proteins (Fig. 4), we chose the abcabc topology for our modeling studies.

We made a structural template in which the conformation of the first amino acids of (CPRILMR) followed that of the ETI structure, while the rest of the molecule was modeled on the cellulose binding domain of *T. reesei* cellobiohydrolase. Since the three-dimensional coordinates of the cellobiohydrolase II cellulose binding domain are not published, we used the structure of the cellobiohydrolase I cellulose binding domain (1cbh (40)), which is reported to be identical with the former. To build this structure we had to introduce deletions in the (Thr¹⁷-Val¹⁸) and (Val²⁷-Leu²⁸) positions, respectively (numbering of the 1cbh structure). The sequence alignment between the AAI and the template (Fig. 6) resulted in sequence identity for 11 out of 32 residues (34%). The model was finally constructed through a residue by residue replacement. The disulfide bridge originally absent in the 1cbh framework was built and the model was refined by energy minimization and molecular dynamics to give the structure shown in Fig. 7.

The reliability of the model was tested by the knowledge-based mean field approach of Sippl, as implemented in the ProsaII (36, 51) program. The program calculates the C β -C β pair interaction energy for each residue in the sequence, and correctly folded proteins produce smooth energy plots with negative values (36). The AAI model gave an energy profile with values corresponding to those of native structures (Fig. 8). The energy profile had no positive regions that would indicate misfolded parts in the model. Also, the so-called Z-score or normalized energy value (36) was -3.9, which is within the range of the values expected for native proteins of this length (36). When tested with the AAI sequence, all other known structures gave higher Z scores indicating that the model presented here fits the sequence better than any of the other structures (not shown).

DISCUSSION

Amaranthus seeds seem to contain a number of α -amylase inhibitors that can be separated by ion-exchange chromatography and reverse phase HPLC. Here we report on the purification and the structure of AAI, the most abundant α -amylase inhibitor of amaranth seeds that accounts for more than half of inhibitory activity measurable in crude extracts. This protein shows strong α -amylase inhibitory activity against one of the most important pests of maize, *P. truncatus* (larger grain borer), and a pest of wheat flour, *T. castaneum* (rust-red beetle).

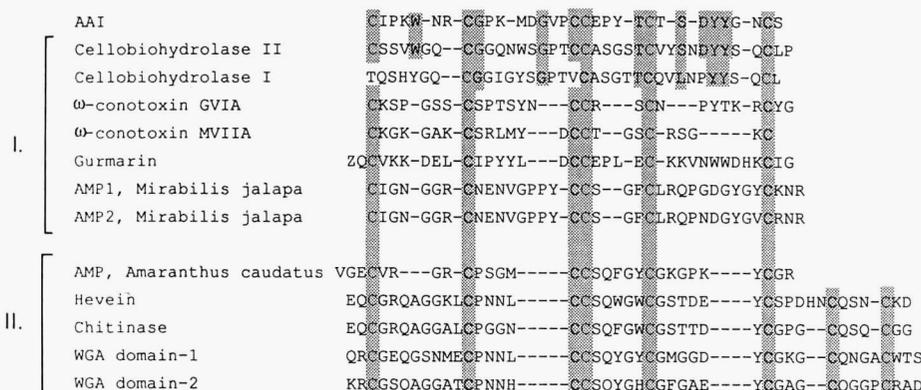


FIG. 4. Sequence alignment of AAI with protein sequences of similar cysteine patterns. The sequences are taken from the Swiss-Prot data bank (44) as follows: *Cellobiohydrolase II*, (exoglucanase II, EC 3.2.1.91) from *T. reesei*, GUX2_TRIRE, 27–63; *Cellobiohydrolase I*, exoglucanase I (EC 3.2.1.91) from *T. reesei* (40), GUX1_TRIRE, 479–593; ω -conotoxin GVIA, snake venom of *Conus geographus*, CXO6_CONGE, 46–73; ω -conotoxin MVIIA, snake venom of *Conus magus* (41), CXOA_CONMA, 1–25; Gurmarin, sweet taste-modifying protein from *Gymnema sylvestre* (42), GUR_GYMSY, 1–35; AMP, antimicrobial peptide from *Amaranthus caudatus*, AMP_AMACA, 1–30; AMP1, antimicrobial peptide from *Mirabilis jalapa* (43), AMP1_MIRJA, 1–37; AMP2, antimicrobial peptide from *A. caudatus* (44), AM2_MIRJA, 1–36; Hevein, chitin-binding lectin from *Hevea brasiliensis*, HEVE_HEVBR, 18–60; Chitinase, basic endochitinase I from rice (45), CHI1_ORYS, 19–61; WGA, wheat germ agglutinin (46), AGI1_WHEAT, 27–69 (domain 1), 70–112 (domain 2).

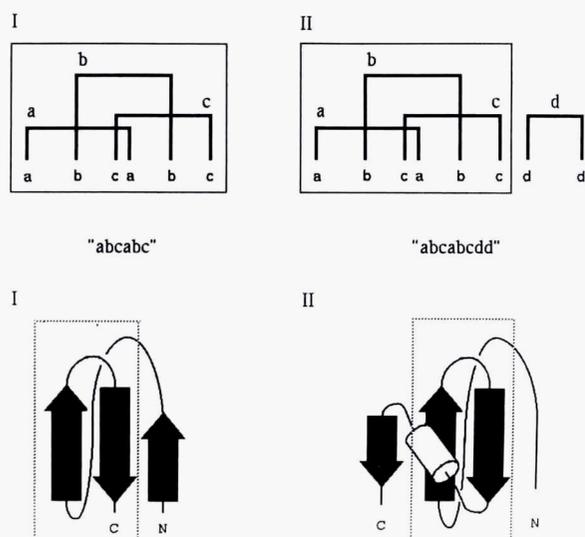


FIG. 5. Disulfide patterns (top) and folding pattern (bottom) of proteins distantly related to AAI. I and II correspond to the groups shown in Fig. 4. The disulfide and folding patterns were deduced from known structural data in each group.

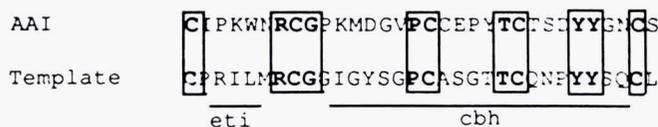


FIG. 6. The structural template used for the modeling AAI. eti is the *Ecballium elaterium* trypsin inhibitor structure (50), cbh is the cellulose binding domain of cellobiohydrolase I (40).

The inhibitory activity, according to our preliminary results, is absent or minimal against human or bovine α -amylases, thus this protein appears to be an ideal candidate for conferring insect resistance upon transgenic plants.

AAI is a 32-residue peptide containing 6 cysteines. The first residue of the sequence is not methionine, therefore AAI is probably synthesized as part of a larger precursor. The sequence of AAI shows no obvious similarities with any of the known proteins. Spurious similarities and an examination of the residue conservation pattern allowed us to identify a group of structurally related proteins which contains sugar binding proteins (wheat germ agglutinin and cellobiohydrolase), ven-

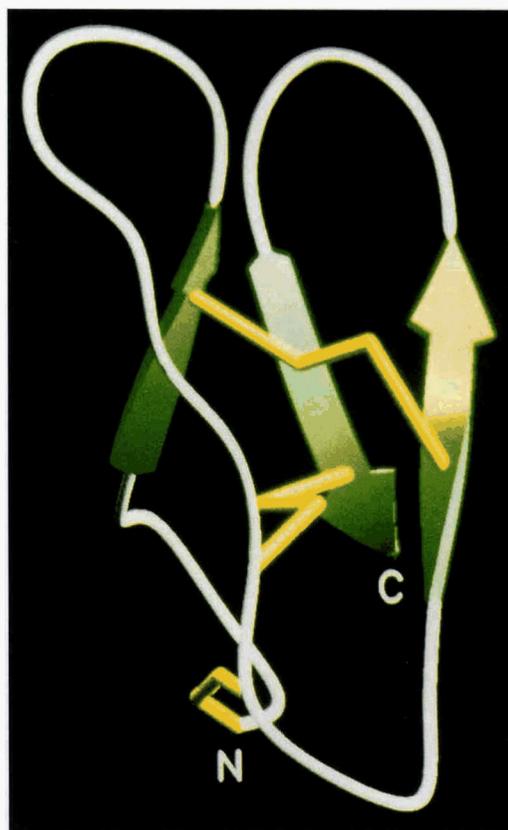


FIG. 7. The schematic model of the AAI structure. Disulfide bridges are shown in yellow, β -sheets are green, irregular conformations are shown in white. The illustration was generated with the program SETOR (52) using the AAI coordinates obtained by homology modeling and molecular dynamics refinement.

oms (ω -conotoxin), and antimicrobial peptides. Using the known three-dimensional structures we built a model based on the similarity of AAI to the squash family of trypsin inhibitors, ω -conotoxin, and to the cellulose binding domain of cellobiohydrolase. Nguyen and associates introduced the term “knottin” for this group of structures, based on a “knotlike” feature in the three-dimensional fold (47). This feature is retained in the model of AAI. AAI is the first α -amylase inhibitor described in this group.

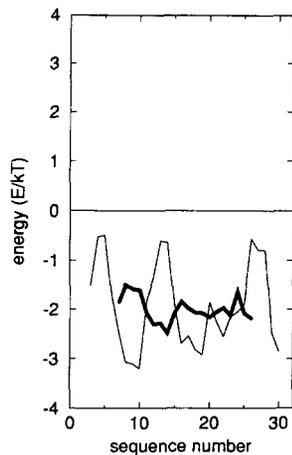


FIG. 8. Energy plot for the refined AAI model as generated with the ProsaII (36) program. The curves are smoothed (thin line, window size of 5; thick line, window size of 13). Energies are represented in units of E/kT.

AAI seems to be the shortest of the peptide α -amylase inhibitors described so far, and, in spite of its overall similarity to the squash family of proteinase inhibitors, AAI does not seem to inhibit proteases. Even though AAI has some potential similarity to other small proteins, which allowed us to build a three-dimensional model of this inhibitor, we tend to believe that the similarities are structural rather than evolutionary. In other words, short peptides may not have too many stable conformations for accommodating three disulfide bridges, therefore a similar fold may arise as a result of convergent evolution. Finally we mention that AAI seems to be a good core structure for protein engineering studies since several of the related proteins are known to be stable and to refold correctly from the reduced state *in vitro* (47).

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