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Dear Dr. Koloskov,

I am herewith sending you the FINAL REPORT AND THE FINANCIAL STATEMENT.
I hope you will find the progress made, sufficient to fulfill the expectations.
I would like to express my gratitude to the UNIDO program and the ICGEB that made possible to work in this area, and to establish close collaboration with other groups.
In case any further clarification had to be done, please contact me, and I will be happy to do it.
Sincerely yours

[Signature]

ALEJANDRO BLANCO-LABRA
Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 92/0555
ICGEB ref. #: CRP/MEX 91-02

Project initiation: 27 October 1994
Project termination: 31 August 199
Title of Project

Protein Related to Plant Resistance to Insects Through Enzyme Inhibitors

Keywords:

Plant Resistance, Plant Defense Mechanisms, Enzyme Inhibitors

UNIDO contract #: 92/0555

ICGEB ref.: CRP/MEX 91-02

Project initiation: 27 October 1994

Project termination: 31 August 1995

Principal Investigator's name: Alejandro Blanco Labra

Affiliate Centre:

Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato

Km. 9.6 Libramiento Norte, Carr. Irapuato-León

Apdo. Postal 629, 36500 Irapuato, Gto. MEXICO

Telephone no. (462) 516-00

Fax no. (462) 459-96 and 458 46

Telex no.

Email address

Abstract:

Plant proteinaceous enzyme inhibitors are considered to be part of the constitutive and inducible array of defense mechanisms of plants against the attack by insects and microbial pests, mainly fungi. The work involved in the present project has been concerned with the study of three different inhibitors, isolated from seeds of maize, tepary beans and amaranth. The three of them were purified and studied. The amylase inhibitor presented two different inhibitory activities, against amylases and against proteinases. The tepary beans inhibitor showed a new type of regulatory activity though the formation of a trimer form (active), versus a monomer form (inactive). This inhibitor also presents a wide type of activity, since it recognized proteinases from different origin. Its completed amino acid sequence was determined. The amylase inhibitor from amaranth, is a small peptide (32 amino acid residues), whose whole sequence was determined, showing a high cystein content. Based on similarities to its disulfide bridge pattern, a molecular model was built, being this the first molecular model reported for an amylase inhibitor. Finally the amylase from the guts of the insect P. truncatus, was isolated and purified. It was characterized as an acidic D-cathepsin type proteinase, with a maximal activity at pH 3 and with an apparent molecular weight of 22 kDa.
The objectives proposed at the beginning of the project were met. During the progress of the present project, some new aspects appeared which were considered important, but they were all within the context of the main objective. We think that the results were extended in the light of new data obtain during the development of this project.

New materials from amaranth and maize were included, they were purified and tested also as possible factors of the plant defense mechanisms. The purification and characterization of the proteinase from *Tribolium castaneum* was also included, since it was considered important to have at least one purified enzyme for the characterization of the different inhibitors.

Methodology.

As for the methodology used, this was very much the same as originally described, mostly protein purification techniques, together with enzymes characterization, and we could finally include the sequencing of two different enzyme inhibitors, one of them with the help of Dr. Pongor from the ICGEB, and the affiliated center Institute of Biochemistry and Protein Research, Gödöllő at Hungary, and the other at the University of California Davis.
RESULTS

Most insect enzymes are not very well known, the majority have not been purified and characterized. It was then thought to be important, to extract purify and characterize a digestive enzyme from the gut of T.C.

The study of this enzyme will allow us to design a better strategy for the use of enzyme inhibitors as part of the defense mechanisms of plants.

It also necessary if we are to characterize their sinetic constants.

As planned, we also isolated enzyme inhibitors from plants which are known to be resistant to certain insects attack. Three different inhibitors were studied.

Comparing the results we obtained, with the originally proposed objectives, we think they are within the reasonable purposes of the proposal.
Work plan and time schedule

The original plan did not cover as many aspects as we worked. However, since we could make participation to several students, this facilitated our more comprehensive work. We then covered some work with amaranth and maize, which were not originally included.

The participation of good graduate students, plus the continuous support from this grant, allowed us to purify and study not only the two inhibitors from tepary bean seeds and one insect enzyme, but two other inhibitors from maize and from amaranth seeds. It also provided us the opportunity to begin some molecular modeling and we are now in the process to isolate the gene from amaranth seed inhibitor. Beside that, we are studying in amaranth the protease inhibitor location within the plant tissue.
Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

% activity

Determining optimal variety to get best % of TBI and MTI
Insect growth to obtain the enzymes
Stage of insect for better enzyme yield and obtain guts
Optimization for extraction procedure of TBI, MTI and AAI

Setting the Chromatography procedure
Purification TBI

Purification AAI
Extraction procedure for amylase activity from insects
Optimization of Chromatography procedures
Setting electrophoresis (zymograms), MW determinations
Purification of AAI, TBI and MTI
Physicochemical characterization of MTI
HPLC procedure to obtain very pure samples
Sequence determination for AAI
Molecular modeling for AAI
Writing 2 publications

Physicochemical characterization of TBI and MTI
Molecular recognition of the trimer form for TBI
Writing one more publication
Sequence determination of TBI
Networking

Very good research relationships were established with different groups.

1. With Dr. Pongor at ICGEB and with Alejandro Blanco. A good collaboration was done, which allowed us to publish the results in a paper in J. Biol. Chem.

2. A very good collaboration has been established with a Brazilian scientist Dr. José Xavier Filho from the Universidade Estadual do Norte Fluminense (UENF), with whom we continue to work in collaboration.

3. Very helpful was the collaboration with Dr. John R. Whitaker from the University of California, Davis.

PUBLICATIONS.

- Further characterization of the 12 kDa Protease/alpha amylase inhibitor present in maize seeds.

- A Novel α-Amylase Inhibitor from Amaranth (Amaranthus hypochondriacus) Seeds.

- Purification and characterization of a digestive cathepsin D proteinase isolated from Tribolium castaneum larvae (Herbst).
  Insect Biochemistry and Molecular Biology

- This work has already been accepted for publication (acceptation letter is enclosed) on August 2, 1995, in the Journal: (acceptation letter is enclosed).
### Part 4

**STATEMENT OF EXPENDITURES**

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Please itemize the following budget categories (if applicable)

**Capital equipment**

1. Vacuum pump Trademark Kidney Mod. KUC-5
2. Phast System Cat. 18-1018-23

**Training** (provide names, duration of training, host laboratory)

(Seed attached sheet)

**Literature**

* Please do not send invoices/receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.

** except for invoices that are required in connexion with paragraph 5. of the Contract.
Students trained under this program.

It is important to emphasize that student training is one of our main objectives for the whole country. It has been pointed than one of the most necessary components for development, is to have well trained personnel. Therefore, we emphasized most on this point.

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<td>Lorena Sandoval</td>
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<td>Isabel Mosqueda</td>
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<td>Manuel Vázquez</td>
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<tr>
<td>Silvia Valdés</td>
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Short training

Arnubio Valencia - 5 month training (from 2 Enero to 2 Junio 1995)
(from Universidad, Manizalez, Colombia)
(under a fellowship from International Net of Biology)
Work presented in Congresses.

VI Plant Biochemistry Meeting:

1. Effect of temperature on the stability of the protease inhibitor (7 kDa) in amaranth seeds.

2. Structure and function of plant enzyme inhibitors. (Simposium).


- Isolation and structure of a new amylase inhibitor from Amaranthus.

- Purification and partial characterization of a protease inhibitor from tepary bean.
CENTRO DE INVESTIGACION Y ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL UNIDAD IRAPUATO

PROJECT: PROTEIN RELATED TO PLANT RESISTANCE TO INSECTS THROUGH ENZYME INHIBITORS

CONTRACT: 91/264

FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995

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DR. ALEJANDRO BLANCO LABRA

M. EN C. RAMON GARCIA FERRER ADMINISTRATOR

FIRST APORTATION 13,333 USD, 3.092 Mx PESOS FOR DOLLAR
SECOND APORTATION 13,333 USD, 3.075 Mx PESOS FOR DOLLAR
THIRD APORTATION 13,334 USD, 3.200 Mx PESOS FOR DOLLAR

AVERAGE FIRST REPORT 3.0835
AVERAGE SECOND REPORT 3.1775
**CENTRO DE INVESTIGACION Y ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL**
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**FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995**

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CENTRO DE INVESTIGACION Y ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL
UNIDAD IRAPUATO

PROJECT: PROTEIN RELATED TO PLANT RESISTANCE TO INSECTS THROUGH ENZYME INHIBITORS

CONTRACT: 91/264

FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995

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**CENTRO DE INVESTIGACION Y ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL UNIDAD IRAPUATO**

**PROJECT**: PROTEIN RELATED TO PLANT RESISTANCE TO INSECTS THROUGH ENZYME INHIBITORS

**CONTRACT**: 91/264

**FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995**

**EQUIPMENT**

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**PROJECT:** PROTEIN RELATED TO PLANT RESISTANCE TO INSECTS THROUGH ENZYME INHIBITORS

**CONTRACT:** 91/264

**FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995**

**OTHER COST**

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<tr>
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<tr>
<td>DR. ALEJANDRO BLANCO</td>
<td>SP-2526</td>
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</table>

**TOTAL**

<table>
<thead>
<tr>
<th>AMOUNT</th>
<th>N$</th>
<th>USD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33,226.57</td>
<td>10,456.86</td>
</tr>
</tbody>
</table>
RESEARCH PROGRESS

In this three years project, considerable advances have been obtained in our group in the understanding of insect enzymes, and their possible control by different proteinaceous inhibitors extracted from different sources. The study has covered also the knowledge on structural insight, trying to correlate the function of a particular inhibitor, with the molecule structure.

INHIBITORS PRESENT IN TEPARY BEAN SEEDS

We have been involved in the study of two different inhibitors, one which inhibits alpha-amylases, and another one specific for serin proteases of the type of trypsin.

**ALPHA AMYLASE INHIBITOR:**

We isolated and purified to homogeneity, by HPLC and PAGE, an alpha amylase inhibitor. The purification of this protein was specially difficult, considering that another very closely related protein is present in the seed ( De Mejia, E. G., C. N. Hankins., O. Paredes-Lopez, and L. M. Shannon. 1990. The lectins and lectin-like proteins of tepary beans (*Phaseolus acutifolius*) and tepary-common bean (*Phaseolus vulgaris*) hybrids. J. Food Biochem., 14, 117. ). The other protein is a lectin which agglutinates red cells, however our pure protein was proof to be free of any agglutinating activity.

The inhibitor is a glycoprotein with an apparent molecular weight of 37.7 kDa, as determined by SDS-PAGE. The glucoprotein consist of 17% carbohydrate content. Gas Chromatography-Mass spectrometry analysis, showed that the carbohydrate moiety consist of the following monosaccharides:

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Moles/mole inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xilose</td>
<td>1</td>
</tr>
<tr>
<td>Acetyl Xilose</td>
<td>2</td>
</tr>
<tr>
<td>Mannose</td>
<td>12</td>
</tr>
<tr>
<td>Glucose</td>
<td>16</td>
</tr>
<tr>
<td>Glucose acetamide</td>
<td>4</td>
</tr>
</tbody>
</table>

The inhibitor showed high sensibility to thermal treatment, loosing a high percent of its activity even at low temperature (fig 1).

The characterization towards amylases from different origin was measured. It showed to be active mainly against amylases extracted from different insects (table 1 and fig. 2 ). Two of those insects are important
plagues to the common bean (*P. vulgaris*), which made this results interesting, considering that tepary bean can be cross with common bean.

**PROTEINASE INHIBITOR**

A proteinase inhibitor was extracted through selective precipitation, and purified to homogeneity as shown by SDS-PAGE and HPLC. The kinetic characterization showed it to be a strong trypsin type inhibitor, with a tight binding constant of $K_i = 2.2 \times 10^{-9}$ according to the method of Bieth (Bieth, J. 1974 In Proteinases Inhibitors, Bayer Symposium V, Springer-Verlag: New York. pp 463-469), with a non-competitive type of inhibition. This protein is free of carbohydrates, and its relative MW was 7 kDa (fig. 3). It presented very high thermostability, being able to stand high temperatures, in the order of 95°C for one hour, without a considerable loss of activity (fig. 4).

When the stoichiometry was measured, it was observed that only the trimer form was active. This was demonstrated after a Sephadex chromatography in not denaturant conditions, were it was possible to isolate two molecular forms, one corresponding to the monomer and one corresponding to the trimer. In this case only the trimer form was active (fig. 5). To our knowledge, although there are several examples of different types of oligomeric forms for some inhibitors, all the oligomers are always active, and this is the first time in which one of this type of inhibitors can regulate its activity through oligomerization, indicating that this could be a new type of control mechanism.

Characterization of the type of enzyme inhibited:

The activity of the inhibitor was tested against enzymes from different source, including animal trypsin and chymotrypsin, and different type of proteases and amylases, these last ones because there have been reports about double activity in some inhibitors. The results obtained were quite unusual, since it was able to inhibit enzymes from different types.

This research is continuing now and we think in very short time we will be able to write the first publication.

**12 kDa AMYLASE- PROTEASE INHIBITOR FROM MAIZE SEEDS.**

Accepted for publication in the Journal of Food Biochemistry.

A 12 kDa protease/amylase inhibitor was purified from maize seeds. It was characterized as a bifunctional inhibitor, since it arrested the activity of both amylases and proteinases. Eight different proteinases extracted from insects and fungi which attack grains during storage, were
tested with this inhibitor. Bovine trypsin and trypsin like proteases from
the insect *P. truncatus*, and from the fungi *A. niger* and *A. fumigatus*,
were recognized by this inhibitor as for the amylases, out of eleven
enzymes tested, only the one from *T. castaneum* and *C. maculatus*, were
recognized.

**MOLECULAR MODELING OF THE AMARANTH AMYLASE INHIBITOR.**

This was published in the Journal of Biological Chemistry. A family of amylase inhibitors from amaranth, have been isolated and are
now under investigation. The major peptide, is a 32 residue long peptide,
with 3 disulfide bridges and a very strong activity against insect*”
amylases from *P. truncatus*, *T. castaneum, and C. maculatus*. Computer
analysis of 3-D related structures, showed that this inhibitor belongs to a
group of small proteins named "knottins". Using the common structural
features of this group we built a 3-D model structure of the inhibitor.

**PURIFICATION AND CHARACTERIZATION OF a DIGESTIVE AMYLASE
EXTRACTED FROM THE INSECT Tribolium castaneum**

Accepted for publication in the journal: Insect Biochemistry and Molecular
Biology.

This insect is an important secondary pest, which attack several
economically important grains. Its amylase has shown to be sensible to
different amylases inhibitors, therefore, it is important to learn more
from the interaction of this enzyme with the inhibitors, in order to learn
more about the mechanisms of reaction of both the enzyme, and the
inhibitor. We isolated and purified the enzyme, which was characterized
as a D-cathepsin type of proteinase.
Fig. 1: Temperature and pH effect on TAI stability. Samples of inhibitor (1 µg/40 µL) were treated for 60 min at different pH and temperature conditions. After the treatments samples were assayed for inhibitory activity.
Table 1. Effect of TAI on \( \alpha \)-amylases from different sources at 30 and 60 min incubation time (E+i).

<table>
<thead>
<tr>
<th>Source</th>
<th>Inhibition (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Porcin Pancreatic</td>
<td>1.70</td>
</tr>
<tr>
<td>Human Salivary</td>
<td>7.05</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>10.13</td>
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<tr>
<td>Aspergillus oryzae</td>
<td>8.13</td>
</tr>
<tr>
<td>Barley’s malt</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Tribolium castaneum</em></td>
<td>100.00</td>
</tr>
</tbody>
</table>

* Based on 100% inhibition of *T. castaneum* \( \alpha \)-amylase activity.
Fig. 2: Inhibitory activity of TAI against enzymes extracted from larvae of different insects. T. c. Tribolium castaneum; Z. s. Zabrotes subfasciatus; C. m. Callosobruchus maculatus; S. z. Sitophilus zeamais; P.t. Prostephanus truncatus, and A. o. Acanthoscelides obtectus.
Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the inhibitor purified by RP-HPLC. Lane 1, Inhibitor purified by reverse phase HPLC. Lane 2, Molecular weight (Mr) markers; bovine albumin (66,200), egg albumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and aprotinin (6,500).
Figure 4. Trypsin inhibitory activity stability response surface for tepary bean inhibitor with variations in pH and temperature. Samples of inhibitor (0.1 mg mL\(^{-1}\)) were treated for 60 min at different pH and temperature conditions. After treatment, samples were assayed for trypsin inhibitory activity.
Figure 5. Molecular weight estimation by gel filtration chromatography (G-75). A Sephadex column (1.6 X 57.7 cm) was equilibrated with 0.05 M sodium phosphate and 0.01 M NaCl buffer. A) Elution profile of inhibitor under native conditions. B) Elution profile in the presence of 3 M guanidine, acting as a denaturing agent.
DOCUMENTS
PURIFICACION Y CARACTERIZACION
DE UN INHIBIDOR DE PROTEASAS PRESENTE EN
FRIJOL TEPARI (Phaseolus acutifolius)

TESIS QUE PRESENTA

JORGE EDUARDO CAMPOS CONTRERAS

PARA OBTENER EL GRADO DE
MAESTRO EN CIENCIAS
CON ESPECIALIDAD EN

BIOTECNOLOGIA DE PLANTAS

IRAPUATO, GUANAJUATO, MEXICO
1994
Deseo expresar mi agradecimiento al financiamiento recibido por parte de las siguientes instituciones:

Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca de estudiante de maestría.

Organización de Estados Americanos (OEA) a través del proyecto Multinacional de Biotecnología-Alimentos.

International Centre for Genetic Engineering and Biotechnology (ICGEB) a través del proyecto UNIDO GE/GLO/89/001.
ESTE TRABAJO FUE REALIZADO EN EL CENTRO DE INVESTIGACION Y ESTUDIOS AVANZADOS DEL I.P.N., UNIDAD IRAPUATO, EN EL DEPARTAMENTO DE BIOQUIMICA Y BIOTECNOLOGIA, BAJO LA DIRECCION DEL DR. ALEJANDRO BLANCO LABRA.
August 2, 1995

Dr. Alejandro Blanco-Labra
Apartado Postal 629
C. P. 36500 Irapuato, Gto.
México

Dear Dr. Blanco-Labra:

I am most pleased to inform you that your revised manuscript “Purification and characterization of a digestive ...” (6017) has now been accepted for publication in our journal. We have chosen the manuscript with one table. It will be some months before galley proofs arrive.

Sincerely yours,

[Signature]

Lawrence I. Gilbert
Executive Editor

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

(Received for publication, May 4, 1994, and in revised form, June 14, 1994)

Alicia Chagolla-Lopez††, Alejandro Blanco-Labra‡, András Patthy, Roberto Sánchez∗∗,**, and Sándor Pongor††

From the International Centre for Genetic Engineering and Biotechnology, 34012 Trieste, Italy. †Centro IN PNP ap 629 Irraputo Gto. Mexico, and Institute of Biochemistry and Protein Research, Agricultural Biotechnology Center, 2101 Gdodlo, Hungary

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 celllobiohydrolase, and to α-conotoxin, i.e. a group of small proteins termed "knottins" by Nguyen, D. L., Heitz, A., Chiche, L., Castro, B., Boige-grain, 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; 11 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 proteinase and α-amylase.

The structure of α-amylase inhibitors is quite variable. Table 1 (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 proteins). It is an important feature of the plant α-amylase inhibitors that their inhibitory activity can be species-specific. For example members of the central 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 and N-terminal conotoxins (14, 15, 19); as well as α-conotoxins (26, 21), which contain three conserved disulfide bridges and an array of three β sheets.

EXPERIMENTAL PROCEDURES

Materials—Seeds of A. hypochondriacus 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. "Hymotrypsin and trypsin were from Serva, cyanogen bromide and vinyl pyridine were from Aldrich. All chemicals used were of analytical or sequencing grade. HPLC-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 nitroprussic acid as a substrate at the Berndfeld method B.

Purification of the α-Amylase Inhibitor—A crude extract of an 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 v ammonium bicarbonate. This material was fractionated on a Sephadex G-75 column (1.6 x 160 cm) in 0.02 v ammonium bicarbonate buffer, pH 8.3, and chromatographed on a 20 x 2.6 cm DEAE-Sephrose CL-6B column preequilibrated with the same buffer using a linear gradient of ammonium bicarbonate 0-2.0 v. The active fractions eluted were lyophilized and subjected to reverse phase HPLC on a preparative Vydac C18 column (22.5 x 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 Protag HPLC system.

The abbreviations used are: HPLC, high performance liquid chromatography; NMR, structurally conserved region; SD, steepest descent; C-terminal, C-terminal; CL-68, C-terminal.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Recipient of an International Centre for Genetic Engineering and Biotechnology fellowship.

‡‡ Recipient of an International Centre for Genetic Engineering and Biotechnology fellowship.

** The correspondence should be addressed to Dr. Sándor Pongor, Tel: 39-18-3757390; Fax: 39-49-226-555; E-mail: pongor@fjg.tnado.it.
A Novel Amaranth Seed α-Amylase Inhibitor

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

### Table 1

<table>
<thead>
<tr>
<th>Class</th>
<th>Source*</th>
<th>Size (Da)</th>
<th>1-2-Cys</th>
<th>Against α-amylases</th>
<th>Against maltase or other α-amylases</th>
<th>Against protease</th>
<th>Members of the group with other activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kunze type</td>
<td>Barley</td>
<td>176-190</td>
<td>2-4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Miraculin</td>
</tr>
<tr>
<td>Cereal type</td>
<td>Wheat</td>
<td>124-190</td>
<td>10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>ND</td>
</tr>
<tr>
<td>Finger millet (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>ND</td>
</tr>
<tr>
<td>γ-Purothionin type</td>
<td>Sorghum</td>
<td>47-48</td>
<td>8</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>ND</td>
</tr>
<tr>
<td>Rag 1-2 type</td>
<td>Indian</td>
<td>95</td>
<td>7</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>ND</td>
</tr>
<tr>
<td>Legume lectin type</td>
<td>Common</td>
<td>246</td>
<td>10-16</td>
<td>*</td>
<td></td>
<td>*</td>
<td>ND</td>
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<tr>
<td>Thaumatin type</td>
<td>Maize</td>
<td>173-235</td>
<td>10-16</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>ND</td>
</tr>
<tr>
<td>Prokaryotic</td>
<td>Actinomyces</td>
<td>75-120</td>
<td>4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are references.
* 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 (22). 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 ODS2 column (25) or 7-µm particle size, Applied Biosystems: 50-µmol 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 an Edman degradation sequencing protocol (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 1:20 (w/w) at 37 °C for 4 h.** Peptides from the digestion 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 BLASTZ (29, 30) electronic mail servers.** All other computer analyses of the sequences were carried out using the UCSC 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 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 II's biopolymer module. Each residue was treated separately. As the first step, the residue was deleted and connectivity was restored, in a first step, with 100 cycles of localized steepest descent (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 restored, 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 coworkers (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 prequenched 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 slowly placed water molecules to move. This was followed by 3 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/A.

The model was evaluated using the Prowall program (36). The program uses mean force potentials derived from known protein structures (36) in order to calculate the energy of a new structure. To avoid potentials, CB-CB 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 AAL structure solely on the CB-CB interaction potentials. The program was used with standard parameters as provided by the author.

**RESULTS**

**Purification of A. hypochondriacus 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-Sephadex A-1.5-B column. No inhibitory activity was found in the unbound fraction and a linear NH₂HCO₃ 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.
Specificity of AAI—Crude extracts of amaranth seeds inhibit both trypsin (22) and insect amylases. Purified AAI showed inhibitory activity against the larval α-amylase of *T. castaneum* and that of *P. truncatus*. On the other hand, AAI does not inhibit human or bovine saliva α-amylases in an appreciable manner (the species-specificity studies will be published elsewhere).

**Amino Acid Sequence and Disulfide Topology of AAI**—As the protein showed a high percentage of cysteine with no free sulfhydryl groups, the samples were subjected to reduction and pyridylethylation. Digestion of the reduced and pyridylethylated protein with trypsin and cyanogen bromide resulted in seven overlapping peptides that were separated by reverse phase HPLC and sequenced with automatic Edman degradation. Notable features of the AAI sequence (Fig. 2) are the high content of cysteine (six) and proline (four) within 32 residues.

Disulfide bridges were determined through partial double digestion with trypsin/chymotrypsin at low pH for 44 h. On reverse phase HPLC, the digestion mixture yielded six main peaks (TCA1–TCA6), one of them (TCA5) being identical to the native protein according to amino acid composition. The other peaks were analyzed through sequencing as well as by amino acid analysis, the deduced sequences are shown in Fig. 3A. The results indicate that Cys1 and Cys11 are connected by a disulfide bond. Furthermore, both Cys1 and Cys11 form a disulfide bond either with Cys37 or with Cys39, respectively. The exact placement of these disulfide bonds could not be determined from these experiments since the enzymatic cleavage of the Cys1–Cys11 peptide bond cannot be accomplished. The two theoretically possible disulfide bonding patterns are shown in Fig. 3B.

Sequence and Structural Similarities—When the sequence was compared with all known proteins using the FASTA (38), the BLAST (28) and the BLITZ (29, 30) programs, no convincingly significant homologies could be detected and the top list of homologies substantially varied depending on the choice of search parameters (data not shown). It has to be mentioned...
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, gymarin (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 \( \mu \)-conotoxin (1occ, 1eb1, 1cti, 2eti). As the structures in this family contain a characteristic knotlike arrangement, Nguyen et al. (47) suggested the "knotting" name for this family of proteins. Group II contains chinin-binding domains of wheat germ agglutinin and hevein (1hev and 1wga). In group I (topology abca\( c \) in Fig. 5, top) there are three disulfide bridges, whereas in group II (topology abcabc\( d \) 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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-hairpin-like structure with an irregular N-terminal extension (boxed in Fig. 5, bottom). The three-strand arrangement 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 inhibitor (4pep), in which no regular secondary structure 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 structures, and was also found by chemical means in \( \mu \)-conotoxin (48); (ii) The absbc topology, on the other hand, has not 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 CPRLMIR followed that of the ET 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 I cellulose binding domain are not published, we used the structure of the cellobiohydrolase I cellulose binding domain (1cb1 (40)), which is reported to be identical with the former. To build this structure we had to introduce deletions in the Thr57-Val93 and (Val127-Leu171) positions, respectively (numbering of the 1cb1 structure). The sequence alignment between the AAI and the template (Fig. 6) resulted in sequence identity for 11 out of 31 residues (34%). The model was finally constructed through residue replacement. The disulfide bridge originally absent in the 1cb1 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 database mean field approach of Sippl, as implemented in the Prosali (36, 51) program. The program calculates the \( \bar{C}B \)-Ca 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. 6). 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 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 \( \alpha \)-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 \( \alpha \)-amylase inhibitor of amaranth seeds that accounts for more than half of inhibitory activity measurable in crude extracts. This protein shows strong \( \alpha \)-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).
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 celllobiohydrolase), venoms (α-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 inhibitor α-conotoxin, and to the cellulose binding domain of celllobiohydrolase. Nguyen and associates introduced the term "knottin" for this group of structures, based on "knoodle" feature in the three-dimensional fold (47). This feature is retained in the model of AAI. AAI is the first α-amylase inhibitor described this group.
A Novel Amaranth Seed α-Amylase Inhibitor

Fig. 8. Energy plot for the refined AAI model as generated with the PROSII (36) program. The curves are smooth (thin line, window size of 5; thick line, window size of 13). Energies are represented in units of kcal/mol.

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).

Acknowledgments—We thank Dr. Manfred Sippl for providing us with the PROSAII program. The protein sequencing laboratory is supported by the endowment grant of the Agricultural Biotechnology Center, Gázdó, Hungary.

REFERENCES
ABSTRACT

A 12 kDa protease/α-amylase inhibitor was purified from maize seeds and studied. Its trypsin- and amylase-inhibitor activities against enzymes from different origins were determined, as well as its optimal pH for inhibition. Eight different proteases, extracted from insects and fungi which attack grains during storage, were tested with the inhibitor. Bovine trypsin and trypsin-like proteases from the insect P. truncatus, and the fungi A. niger and A. fumigatus, were recognized by this inhibitor. Out of 11 α-amylases tested, only those from T. castaneum and C. maculatus were recognized by this inhibitor. The optimal pH's for the inhibition of trypsin and the trypsin-like protease from P. truncatus were 8.0 and 7.5, respectively. The optimal pH activity was 5.0 for the inhibition of amylases from T. castaneum and C. maculatus.

INTRODUCTION

Plant proteinaceous enzyme inhibitors of enzymes have been extensively investigated. They are part of the storage (reserve) proteins of the seeds, and they are also considered to be part of the constitutive and inducible array of defense mechanisms of plants against attack by insects and microbial pests (Ryan 1973). Both the proteins and their genes are being actively studied (Hilder et al. 1987;
Ryan 1978. Wen et al. (1992) reported the isolation of the gene encoding the 12 kDa protease inhibitor from maize seeds. This inhibitor was previously investigated in opaque-2 maize seeds by Halm et al. (1973a). The data indicate that the maize inhibitors are slowly inactivated by heat and that they consist of multiple components. Mahoney et al. (1983) reported the complete amino acid sequence, and some of the properties of the inhibitor. Its double specificity to inhibit the activity of proteases and amylases has been studied (Chen et al. 1992).

We previously reported the presence of another bifunctional inhibitor in maize seeds (Richardson et al. 1987), a 22 kDa protein, which also inhibits proteases and amylases. In this paper, we report on the characterization and inhibitory specificity of the 12 kDa maize seed inhibitor.

MATERIALS AND METHODS

Maize seeds (Zea mays; line B8) were provided by the National Institute for Research in Forestry and Agriculture, Celaya Unit (Mexico). Insects were provided by the institute at CINVESTAV-Irapuano Unit.

Insect cultures of Prostephanus truncatus and Sitophilus zeamais were maintained on whole maize seeds, while Tribolium castaneum was grown on maize flour. Collosochrobus maculatus cultures were maintained on whole chick pea seeds. Zabrotes subsectanus and Acanthoscelides obtectus cultures were grown on whole navy beans. All cultures were maintained in a growth chamber at 28°C with a relative humidity of 75.75.

The fungi Aspergillus niger and Aspergillus japonicus were grown on potato-

28°C - dehydrate agar at 28°C for 4-7 days, in order to obtain enough sporulation. The cultures were then maintained at 4°C, and transferred to a new medium every two months.

Bovine trypsin (Type I), Nα-benzoyl-L-arginine ethyl ester (BAEE) and the amylase from Bacillus subtilis and Aspergillus oryzae were from Sigma Chemical Co.; Sephadex G-75 was from Pharmacia Fine Chemicals. Acrylamide and bis-acrylamide were from Bio-Rad. 4, N,N'-Dimethyl-aminooacetobenzen-4'-isothiocyanate, phenyl isothiocyanate, and trifluoroacetic acid were sequence grade from Pierce. All other chemicals were reagent grade from J.T. Baker.

Extraction of Inhibitors

Maize seeds were ground to pass a 1 mm mesh screen. The flour was defatted by continuously stirring in acetone for 15 min at room temperature. The acetone was decanted and the flour again washed for three more times.

Defatted maize flour was suspended in 20 mM acetate buffer, pH 5.5, containing 1 mM calcium chloride (1.5 μl), and continuously stirred for 12 h at 4°C.

The supernatant was separated by centrifugation (10,000 × g, 1 h, at 4°C).

Ammonium Sulfate Precipitation

Our procedure was performed with 60% (NH₄)₂SO₄ of 12 h at 4°C, and then centrifuged at 10,000 × g for 1 h. The precipitate was collected, redisolved in water, dialyzed extensively against water and lyophilized.

Gel Filtration

Two hundred mg of the lyophilized protein were dissolved in 8 ml of water and centrifuged to remove a small amount of insoluble particles. This solution was applied to a Sephadex G-75 column (1.6 × 166 cm) previously equilibrated with 10 mM ammonium bicarbonate. The collected fractions (7 ml) were assayed for protein content and for inhibitory activity against amylase from T. castaneum and against bovine trypsin. The active fractions were pooled and lyophilized.

Reverse Phase HPLC

The lyophilized powder was dissolved in 6 M guanidine HCl solution (100 mg/ml) and 100 μl were injected into a high pressure liquid chromatography instrument, fitted with a preparative μ-Bondapak C-18 column (22 × 250 mm), maintained at 30°C. Separation was performed at a flow rate of 5 ml/min. using a linear gradient of acetonitrile and 0.1% TFA (from 60% in 110 min). The fractions corresponding to the eluted peaks were collected and freeze dried. The powders were then dissolved in a minimum volume of water and their inhibitory activity was measured.

Electrophoresis

Purity of the inhibitor was evaluated by 5% polyacrylamide gel electrophoresis as described by Laemmli (1970), using 15% polyacrylamide gels.

N-Terminal Amino Acid Sequence

Disulfide bonds of the inhibitor were reduced and S-carboxymethylated in 6 M guanidine HCl in 0.1 M Tris, pH 8.6, as described by Crestfield et al. (1963).
The S. castanea methyleated inhibitor was subjected to microsequence analysis using the 3-8-N dimethylalanine-benzene-4-nitroaniline (DABITC)/phenylisothiocyanate (PITC), double coupling method of Chang et al. (1978).

Larval Crude Enzymes

The active digestive larvae of the insects P. truncatus, S. zeamais, T. castaneum, C. maculatus, Z. subfuscans, and A. obliqua were homogenized with different buffer solutions in a 1.5 (w/v) ratio, to extract the amylases and the proteases. In the case of P. truncatus enzymes, they were extracted with a 0.1 M NaCl in 40 mM succinic acid, pH 6.5, solution. The enzymes of S. zeamais, T. castaneum, Z. subfuscans, C. maculatus and A. obliqua were extracted with 0.2 M succinic acid buffer, pH 4.5. The suspensions were centrifuged at 10,000 \times g for 10 min at 4°C. The supernatants served as the source of proteases and amylases.

Fungal Crude Enzymes

The spores obtained from PDA cultures of A. niger and A. fumigatus were suspended in 10 ml of sterile water. Erlenmeyer flasks with corn meal medium, prepared as in Johnson and Cull (1972), were inoculated with the spore suspension. 1,000 spores per ml of medium were added and incubated 48 h at 28°C with continuous stirring (100 rpm).

The mycelia obtained were separated by centrifugation at 10,000 \times g for 15 min, and the enzymes were extracted by homogenization with glass beads in 50 mM Tris-HCl, pH 8.0. buffer solution, in a 1:4 (w/v) ratio. The suspensions were centrifuged at 10,000 \times g for 20 min and the enzyme activities were measured in the supernatant. Controls were done in the same manner, without adding the inhibitor.

Protease Inhibitor Assay

The inhibitory activities against bovine trypsin and proteases extracted from the different insects were determined by preincubating the 12 kDa inhibitor at 30°C for 3 min with the different protease extracts. Preincubation was followed by determination of the residual proteolytic activity.

Bovine trypsin and trypsin-like proteases from P. truncatus larvae were assayed using BAEF as substrate. The reaction rate in the absence of inhibitor was determined at 30°C according to the method described by Schwert and Takanaka (1955). The preincubation of 25 \mu l of bovine trypsin (200 \mu g/ml) and crude protease extract from P. truncatus with the inhibitor was done in 2.0 ml of 0.15 M Tris (hydroxymethyl ammonium) buffer solution, pH 8.4, containing 50 mM CaCl₂. The amount of inhibitor added was adjusted so as to yield 50% residual proteolytic activity. One unit of enzymatic activity was defined as the amount of enzyme that catalyzed an increase of 0.01 absorption units per min at 253 nm under the assay conditions. Similarly, one unit of inhibitor activity was defined as the amount of inhibitor that inhibited one unit of enzyme activity.

The activity of the inhibitor against proteases extracted from S. zeamais was evaluated by incubating it (up to 100 \mu g of protein) with the crude protease extract (0.2 ml). The residual proteolytic activity was measured at pH 2.5, using hemoglobin (0.3%) as substrate, according to the method described by Lenney (1975). One unit of enzymatic activity was defined as the amount of enzyme that catalyzed an increase of 0.01 absorption units under the previously described assay conditions.

The effect of the inhibitor against the proteases extracted from larvae of C. maculatus, Z. subfuscans and A. obliqua was determined by the method of Kakade et al. (1969) and Lenney (1975). The crude extracts of protease (100 \mu l) were preincubated with the inhibitors (100–200 \mu g) and the residual activities were measured with casein at pH 6.5, and with hemoglobin at pH 2.5.

The residual activities of T. castaneum proteases after preincubation with the inhibitor (up to 200 \mu g of protein) were assayed with casein at pH 6.0 by the method of Kakade et al. (1969).

The inhibitory activity against proteases extracted from A. niger and A. fumigatus was evaluated by incubating the 12 kDa inhibitor (up to 20 \mu g of protein) with the protease extracts (200 \mu l) at pH 8.0. The residual activity was measured according to the method of Kakade et al. (1969).

Effect of pH on Inhibition of Proteases

The effect of pH on the inhibitory activity was assayed by preincubating and measuring the activity of each of the proteases with different pH buffer solutions (0.2 M citric acid, pH 2.5, 0.2 M and 0.03 M succinic acid, pH 6.0 and 6.5, respectively; 0.1 M Tris, pH 7.0, 7.5 and 8.6; 0.15, 0.2 and 0.5 M Tris, pH 8.0, 8.5 and 9.0, respectively). In all the buffer solutions used, the ionic strength was adjusted to 0.15 with NaCl. After 3 min preincubation time, the residual proteolytic activity was measured at each of the indicated pH's.

Amylase Inhibitor Assay

The inhibition by the amylases was assayed according to the method of Bird and Hopkins (1954). The inhibitory activities against larval amylases were measured using buffers of different pH values. Crude larval enzymatic extracts
(0.002 to 0.05 ml) were preincubated for 3 min at each pH, with different amounts of purified 12 kDa inhibitor at 30°C, in a total volume of 0.7 ml. Following the preincubation step, a 0.125 M (w/v) starch solution (0.3 ml) was added, the reactions proceeded for 3 min, after which the reaction was stopped by the addition of 5 ml of 3 M acetic acid, saline (5%) isoelectroelectric solution, and the absorbance was measured at 540 nm by the spectrophotometer. The absorbance of the solutions was measured at 500 nm against a blank containing only the buffer (1.2 ml) and the saline solution. The amylolytic activity was calculated on the basis of the absorbance difference between the solution containing the enzyme (or the enzyme plus inhibitor), and the control solution containing undigested starch. A unit of amylolytic activity was defined as the decrease in one unit of absorbance under the described conditions. A unit of amylolytic activity was considered as the amount of inhibitor which inactivated one unit of enzymatic activity.

The mycelia extracts prepared as described before were concentrated to 50% volume by ultrafiltration. The concentrated amylase solutions (500 ml) were incubated with different amounts of 12 kDa inhibitor (10–50 µg of protein). The residual activity was measured as described before.

Human saliva amylase was prepared by centrifuging a saliva sample at 10,000 × g for 10 min. The inhibitory activity was determined by incubating a 10 µl aliquot (diluted 1:5) with the different inhibitor solutions. A. oryzae and B. subtilis amylases were dissolved in de-ionized water to give concentrations of 0.61 and 0.07 mg/ml, respectively. Ten µl of these solutions were assayed against different amounts (1–10 µg) of the 12 kDa inhibitor.

Effect of pH on Inhibition of α-Amylase by Amylase Inhibitor

The effect of pH on the inhibition activity against amylases from T. castaneum and C. maculatus larva was evaluated as follows: a sample of 12 kDa inhibitor (0.8 or 2 µg) was preincubated with 2 and 40 µl of amylase of the crude extracts from T. castaneum and C. maculatus, respectively. The preincubation medium was made up to 700 µl with buffer solutions of different pH values (0.2 M succinic acid, pH 4.0 and 4.5; 0.04 M succinic acid, pH 5.0 and 5.5). In all the buffer solutions used, the ionic strength was adjusted to 0.15 with NaCl. After a preincubation time of 3 min at 30°C, the residual amylolytic activity was measured as described before.

Protein

In most cases, protein concentration was determined by the method of Lowry et al. (1951). Protein concentrations in fractions from gel filtration columns and RP-HPLC columns were estimated from the absorbance at 280 and 235 nm, respectively.

RESULTS AND DISCUSSION

Purification

Ammonium sulfate at 60% saturation precipitated most of the inhibitor activity from maize extracts. In a typical experiment, 500 g of maize flour, after precipitation, dialysis and lyophilization of the supernatant yielded approximately 0.625 g of the crude precipitate containing the inhibitor. The precipitate was subjected to gel chromatography on Sephadex G-75. Figure 1 shows the elution profile of a typical separation. The inhibitory activity was eluted as a single peak. Determinations of the inhibitory activity against bovine trypsin and Tribolium castaneum amylase confirmed that the inhibitory activities against both enzymes were present in the same fractions. These fractions (indicated by the bar in Fig. 1) were pooled and lyophilized.
Further purification of the inhibitory activity was performed by RP-HPLC using an increasing acetonitrile gradient (Fig. 2). Protein absorbance and inhibitory activity against bovine trypsin and T. castaneum amylase showed that proteins lacking inhibitory activity were eluted first, followed by the protein inhibitor. The major inhibitory activity was located in peak A, which eluted at 46% acetonitrile, and in peak B, which eluted at 49% acetonitrile. Both peaks showed inhibitory activity against bovine trypsin and T. castaneum amylases. Some other small peaks eluting at 41–46% acetonitrile also had the same inhibitory activities (data not shown); however, due to their low recoveries only the two larger peaks were further studied.

The active fractions obtained after RP-HPLC were pooled, lyophilized and analyzed by SDS-PAGE; they were homogeneous with a single protein band after they were run in gels with different polyacrylamide concentrations. The molecular weight of the protein eluted at 46% acetonitrile was estimated to be 12,000 and 22,000 for the protein eluted at 49% acetonitrile as determined by molecular weight markers (data not shown).

The homogeneity of the 12 kDa inhibitor was confirmed further by analyzing the N-terminal amino acid sequence, which showed the single N-terminal amino acid sequence S-A-G-T-S-C-V. These results are in agreement with the sequence reported for a trypsin Hageman factor inhibitor isolated from opaque-2 maize seeds by Mahoney et al. (1983).

Specificity
The 12 kDa inhibitor purified by HPLC was assayed against proteases and amylases from different sources. It was shown (Table 1) that the inhibitor strongly inhibited bovine trypsin. Also, the protease activity from the insect P. truncatus, which has been shown to be a trypsin-like enzyme (Housman and Thie 1993), was weakly inhibited. The crude fungal proteases extracted from the mycelia of A. niger and A. flavus were slightly inhibited. These fungal proteases, which are not typical trypsin-like enzymes, have an optimal activity at pH 8.6 and are unable to hydrolyze specific substrates for trypsin-like proteases such as BTEE, TAME and BAPNA.

**TABLE 1.**
**EFFECT OF 12 kDa MAIZE INHIBITORS AGAINST PROTEASES FROM DIFFERENT SOURCES**

<table>
<thead>
<tr>
<th>PROTEASE</th>
<th>SOURCE</th>
<th>INHIBITION (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>E+I</td>
<td></td>
</tr>
<tr>
<td>Bovine trypsin</td>
<td>228.8 ± 3.3</td>
<td>101.7 ± 3.8</td>
</tr>
<tr>
<td>INSECTS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. truncatus</td>
<td>100.9 ± 0.8</td>
<td>59.5 ± 1.4</td>
</tr>
<tr>
<td>S. zeamaisa</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>T. castaneum</td>
<td>5.3 ± 0.5</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>C. maculatus</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Z. subscutata</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>FUNGI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>A. flavus</td>
<td>0.20</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Units of inhibition per mg of maize inhibitor.
**Method of Schwartz and Tabakova, 1955 (using BTEE as substrate).
***Method of Kakade et al. 1969 (using casein as substrate).
E = Enzyme; E + I = Enzyme plus inhibitor.*
Several other proteases were not inhibited by the 12 kDa maize inhibitor, such as the enzymes extracted from the larvae of the other five insects tested. It has been shown in our laboratory that the main protease activity present in the T. castaneum extract is due to an aspartic acid proteinase. In the other cases, ammopeptidases have been reported from S. zeamais by Baker (1982), and aspartic and cysteine proteinases from C. maculatus (Gatehouse et al. 1985; Silva and Xavier-Filho 1991), A. obesus (Weiman and Nielsen 1987) and Z. subfuscans (Lemos et al. 1990, Silva and Xavier-Filho 1991). These data confirm that the 12 kDa maize inhibitor is a specific inhibitor directed mainly against trypsin-like proteinases, since none of the insects whose enzymes are known to be different from trypsin were inhibited.

In the case of the amylases, only two out of the eleven enzyme tested were inhibited by this inhibitor; these were the ones extracted from T. castaneum and C. maculatus (Table 2). These data confirm the bifunctionality of the 12 kDa maize inhibitor previously reported by Chen et al. (1992). When bovine trypsin and trypsin-like proteinases from P. truncatus were preincubated and assayed against

![Graph](image)

**FIG. 3. pH EFFECT ON THE PROTEOLYTIC ACTIVITY AND INHIBITION BY THE 12 kDa MAIZE α-AMYLASE/TRYPSIN INHIBITOR**

(A) Bovine trypsin and (B) maize larval extract from P. truncatus. 0, units activity of enzyme; ε, units activity of enzyme plus inhibitor.

**TABLE 2.**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>E +</th>
<th>E -</th>
<th>INHIBITION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UAc ml⁻¹</td>
<td>UAc ml⁻¹</td>
<td>UAc ml⁻¹</td>
</tr>
<tr>
<td>Human saliva</td>
<td>67.6 ± 0.3</td>
<td>67.6 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td>INSECTS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procepha tus truncatus</td>
<td>3.8</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>Steptopus zeamais</td>
<td>24.0 ± 0.2</td>
<td>24.0 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>Tribolium castaneum ‡</td>
<td>97.6 ± 4.5</td>
<td>56.8 ± 3.9</td>
<td>101 ± 2.9</td>
</tr>
<tr>
<td>Callosobruchus maculatus ‡</td>
<td>4.4 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>49 ± 2.8</td>
</tr>
<tr>
<td>Arrenescutes obscurus</td>
<td>57.7 ± 1.1</td>
<td>57.7 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td>Leucotes subsitatus</td>
<td>87.1 ± 0.3</td>
<td>87.1 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td>FUNGUS AND BACTERIA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>68.5 ± 0.5</td>
<td>68.5 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>166.6 ± 1.5</td>
<td>166.6 ± 1.5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Units of inhibition per mg of maize inhibitor.
† Method of Bird and Hummela 1954 (using starch as substrate).
‡-enzyme; ε-enzyme plus inhibitor.
It becomes evident that, in order to think of using this type of inhibitor as a possible tool to improve plant resistance to insect or microbial attack, it is necessary to consider in advance the specific type of enzymes of the particular target organism (Ristlaller et al., 1993).

Halim et al. (1973b) reported the suppression of fungal growth by relatively small samples of maize trypsin inhibitor. They also proposed that the resistance to ear rot in maize could be associated with the trypsin inhibitor, since they had previously detected trypsin inhibitory activity in maize silks. However, due to the impurity of the sample they used, it is impossible to conclude which inhibitor might be responsible for the detected effect, or whether it could be due to a different compound.

More recently Vigers et al. (1991) showed that a protein, named zeamin, with a similar N-terminal amino acid sequence to the 22 kDa maize inhibitor, but lacking inhibitory activity, was also active against some fungi by altering their membrane permeability. The difference between zeamin and the 22 kDa inhibitor still remains to be established. However, a purified protein, with 99% homology with the 22 kDa maize inhibitor, inhibited the growth of the agronomically important pathogens of potato wilt (Fusarium oxysporum) and tomato early blight (Alternaria solani) (Huynh et al., 1992). Here we show that the 12 kDa maize inhibitor is also active, although with lower activity than with the other recognized enzymes, against the fungal enzymes extracted from A. niger and A. fumigatus. These two fungi are known to be a serious problem for different grains during storage, particularly A. fumigatus. This is one of the fungi responsible for the production of aflatoxins, which represents one of the major problems for human and animal health when infested grain is consumed.

The 12 kDa maize trypsin inhibitor seems to be an attractive target directed toward increasing the defensive system of some plants against the attack of a wider group of pests, since it is relatively selective and environmentally safe. It is also a useful biochemical marker for plant breeding programs, as well as a potential tool for genetic engineering. It can either be expressed in plants that lack it, or else it can be expressed in larger quantities in grains such as the maize studied in the present work, in order to increase its detrimental effect on the fungi or on the insects whose enzymes are sensitive to it.

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SOCIEDAD MEXICANA DE BIOQUIMICA

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Las lectinas de peúca han sido reportadas como inestables, es decir, pierden rápidamente su actividad y por esta razón su purificación se dificulta. Libera una metodología para purificar lectinas de coleoptilo de maíz inactivas y estables, por cromatografía de intercambio iónico. 80 gr de coleoptilo se homogéneizaron con un buffer de boratos (v/w), se precipitaron dos veces con acetonía fría al 66° y se disolvieron en 1:1 ml de buffer de tricina 30 mm pH 8.5. Se aplicaron 1 mg de proteína de la preparación a una columna de intercambio iónico (Ionomac 0 Bio-Rad).

Se obtuvieron 2 fracciones, una eluida con 40% de actividad con 5.6 mg de proteína, y otra con un gradient de HCl de 0-0.3 M con 47% de actividad con 5.5 mg de proteína.

Las dos fracciones mantuvieron su actividad por más de dos semanas cuando se les agregó 0.5 M de escarcha. El grado de purificación de la fracción eluida con el gradient es del 70% y su rendimiento fue del 66%. El análisis electroforético mostró dos bandas de proteínas de peso molecular similar al adosar la lectina con eritrocitos humanos 0" una de las fracciones. Estimamos que la preparación contiene una competencia con otra proteína.


RELACIÓN ENTRE PROPIEDADES FUNCIONALES Y ESTRUCTURA SECUNDARIA DE GLOBULINAS DE AMORTEY DE OTRAS PLANTAS.

Isolación y caracterización de las globulinas.

Elaboración y Estructura de un Nueva Inhibidor de n-Amilas de Amaranthus hypochondriacus.

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Un inhibidor de n-amilas (AAT), presente en semillas de Amaranthus hypochondriacus, una variedad mexicana de Amaranth, fue analizado detalladamente para determinar su secuencia automático por el método de Edman. La localización de las puentes disulfuro fue determinada usando fragmentación enzimática, y química combinada con secuencia N-Terminal.

AAT es un polipeptido de 32 residuos cuya secuencia de aminoácidos no presenta similitudes cuando se compara con secuencias de los bancos de datos de proteínas (SwissProt, PDB) como de DNA (GenBank).

En base a esta característica molecular, fue posible construir un modelo de la estructura tridimensional de AAT, basado en técnicas de reemplazamiento de cadenas laterales. Finalmente, refino el modelo utilizando técnicas de Dinámica Molecular.

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El frigón tèplic es una lechuga comun que presenta una útil resistencia a la salinidad. Esta lechuga es de la familia de las Lactucae. A partir de hierba de esta lechuga se prepara un polvo de semillas que se utiliza en la preparación de la sal de la lechuga. El frigón tèplic es capaz de producir una sal de alta calidad que se utiliza en la industria alimentaria. El polvo de semillas de frigón tèplic se prepara mediante procesos enzimáticos que permiten su extracción. Luego, el polvo se seca y se vende en las tiendas de alimentos. La lechuga frigón tèplic es una planta de rápido crecimiento que puede ser cultivada en diferentes condiciones climáticas. Es una planta resistente a la salinidad y a las sequías, lo que la hace ideal para cultivos en áreas con recursos hídricos escasos. El frigón tèplic se consume crudo o cocido y es utilizado en ensaladas, salsas y como condimento. Su alto contenido en fibra y vitamina C, lo convierte en un alimento saludable y nutricionalmente valioso.
Amaranto es uno de los cereales más antiguos en el continente americano. Su presencia en México se remonta a más de 4,000 años A.C., en la región de Tehuacán Puebla. La semilla del amaranto, contiene inhibidores proteicos de enzimas, que han sido identificados como parte de los mecanismos de defensa de las plantas, contra el ataque de insectos y hongos. Dos inhibidores de enzimas, uno de proteasas tipo tripsina y uno de amilasa, han sido estudiados en nuestro laboratorio. Su caracterización y la determinación de su secuencia, indican que se trata de una proteína de 7,000 KD, la cual pertenece a la familia II de inhibidores de la papa. La estructura molecular de su sitio activo es prácticamente idéntica en una región de 9 aminoácidos, a la del inhibidor de semillas de calabaza. En el caso del inhibidor de amilasa, éste es menor que el de proteasas con sólo 32 aminoácidos. Su secuencia no se parece a ninguna previamente reportada, y sólo buscando por homología lejana, se encontró similitud por patrón de cisteínes con el grupo de las curtatoxinas.
EFFECTO DE LA TEMPERATURA SOBRE LA ESTABILIDAD DEL INHIBIDOR DE PROTEASAS (7 KDa) DE SEMILLAS DE AMARANTO.

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La estabilidad del inhibidor proteico de proteasas (7 KDa) extraído de semillas de amaranto (Amaranthus hypochondriacus) y purificado por cromatografía de líquidos de alta resolución se probó a diferentes tratamientos térmicos, sometiendo el inhibidor puro y después el inhibidor en presencia de la enzima (tripsina bovina).

Se pudo comprobar que el inhibidor presenta una elevada estabilidad en un rango de temperaturas, que van de 4°C a 90°C por períodos de hasta 5 minutos. Después de ese tiempo la estabilidad disminuye principalmente a elevadas temperaturas (90°C).

Se pudo observar que tanto para la enzima pura, como para el complejo Enzima-Inhibidor la estabilidad a la temperatura presenta una tendencia similar, ya que en ambos casos, a una temperatura de 70°C, se observa un marcado descenso en la actividad.

Sin embargo, cuando el inhibidor se encuentra presente, este le confiere una estabilidad a la enzima, ya que el porcentaje de pérdida de actividad enzimática, es menor para el complejo Enzima-Inhibidor.