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CENTRO DE INVESTIGACION Y DE ESTUDIOS AVANZADOS DEL I.P.N.
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21212

Irapuato, Gto., September 8, 1995
UNIDO Contract No 92/0555
(CRP/MEX 91-02)

Dr. V. Koloskov
Contracts Officer, Contracts Unit
Purchase and Contract Branch
Operational Support Division

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

ALEJANDRO BLANCO-LABRA

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

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

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Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

Protein Related to Plant Resistance to Insects Throug 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 mail address :
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Abstract:

ABSTRACT

Plant proteinaceus 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 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 origen. 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.

OBJECTIVES/METHODOLOGY

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 objetive. We thing 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 technics, 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, gödöllö 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, withe originaly proposed objetives, we think they are withing the reasonable pourpouses of the proponsal. .

Part 3

Work plan and time schedule
(originally envisaged)

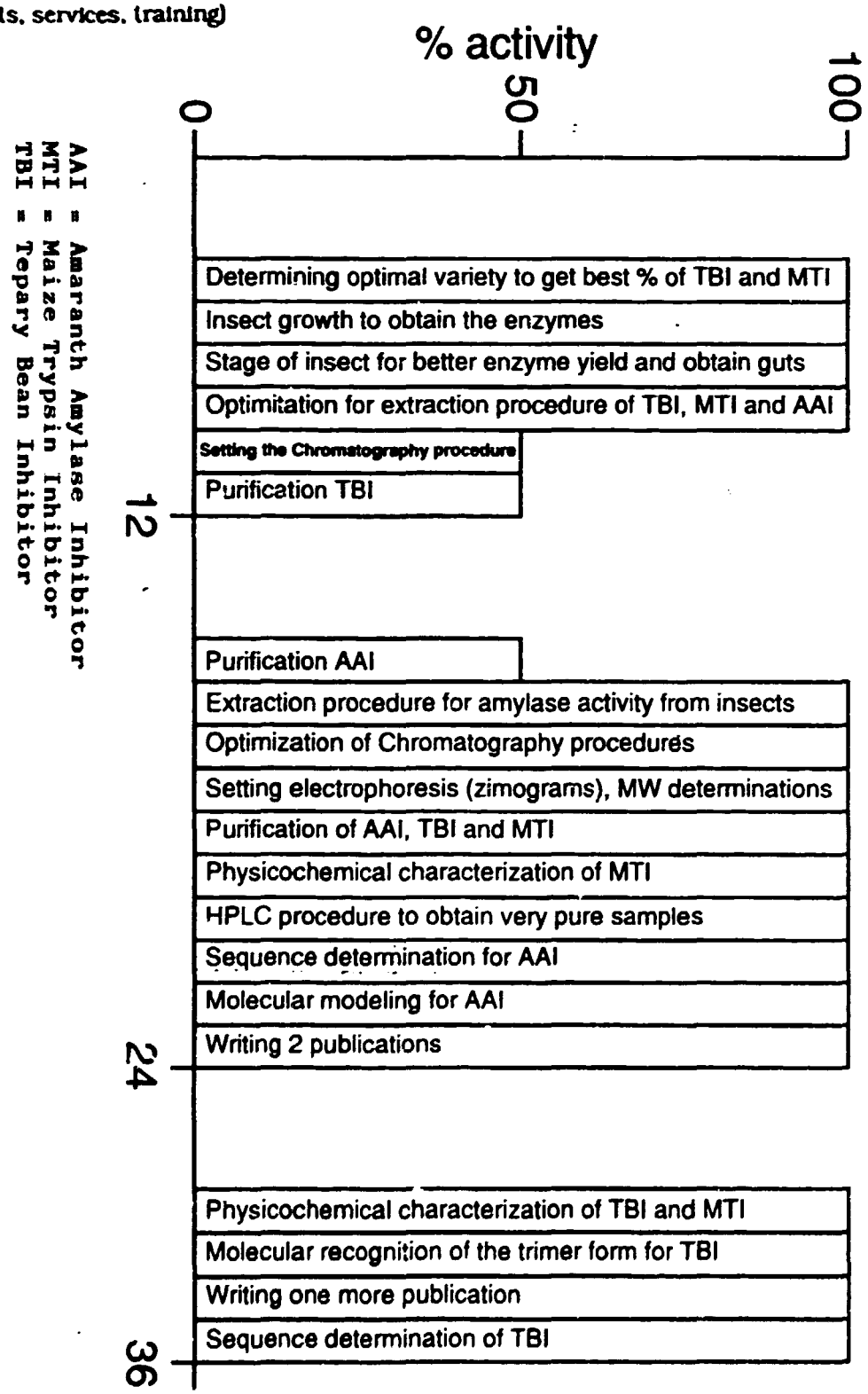
Work plan and time schudele

The original plan did not covered as may aspect as we worked. However, since we could made participate to several studentes, this facilitated our more comprehensive work. We then covered some work withy amaranth and maize, which were not originaly included.

The participation of good graduate students, plus the continuos support from this grant, allowed us to purify and study no only the two inhibitor 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.

Work plan and time schedule (actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)



Networking

Very good research relationships were stablished with different groups.

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

2. A very good collaboration has been stablished with a Brazilian cientist 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.
  Journal of Food Biochemistry 19 (1995), pp 2

- A Novel α-Amylase Inhibitor from Amaranth (*Amaranthus hypochondriacus*) Seeds.
  The Journal of Biological Chemistry. Vol. 269 No. 38 pp23675-23680, (1994).

- 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

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 3,188.20
2) consumables	US$	2) consumables	US$ 8,917.04
3) training	US$	3) training	US$ - - -
4) literature	US$	4) literature	US$ 622.56
5) miscellaneous	US$	5) miscellaneous	US$ 27,272.20
TOTAL GRANT	**US$.....**	**TOTAL**	US$ 40,000.00

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 objetives for the whole country. It has been pointed than one of the most necessary components for development, is to have well trained personel. Therefore, we emphasized most on this point.

Student name	**Level**	**Finishing date**
Jorge Campos	MSc	Sept. 1994
Lorena Sandoval	Ph.D.	Expected for Dic. 1995
Isabel Mosqueda	BSc	Expected for Dic. 1995
Juan F. Gómez	MSc	Expected for Sept. 1996
Jorge Campos	Ph.D.	Expected for Nov. 1996
Manuel Vázquez	Ph.D.	Expected for Nov. 1996
Silvia Valdés	Ph.D.	Expected for Nov. 1997

Short training

Arnubio Valencia (from Universidad, Manizalez, Colombia)	- 5 month training (from 2 Enero to 2 Junio 1995) (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).

XX National Congres Mexican Biochemical Society Oct. 1994.

- 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

CONCEPT	APORTATION USD	TRANSFER	PAID 1ST REPORT	PAID 2ND REPORT	CREDIT USD
SMALL EQUPMENT	3,500.00	(311.80)	2,203.76	984.44	00.00
TRAINING	8,200.00	(8,200.00)	00.00	00.00	00.00
LITERATURE	900	(277.44)	226.74	395.82	00.00
OTHER COSTS	23,400.00	3.872.20	16,815.34	10,456.86	00.00
SUPPLIES	4,000.00	4,917.04	4,226.52	4,690.52	00.00
T O T A L	40,000.00	00.00	23,472.36	16,527.64	00.00

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
UNIDAD IRAPUATO

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CONTRACT: 91/264

FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995

SUPPLIES

	SUPPLIER	ORDER	INVOICE	AMOUNT MEXICAN PESOS
1.-	THE TRUSTEES OF. 750 DLLS.	1752		N$ 2,582.70
2.-	ROA FORWARDING FLETE 218 DLLS.	1311	1311	751.58
3.-	PAPEL. CORNEJO		13295	32.98
4.-	ASTROFOTO		5307	58.21
5.-	FOTO IMAGEN		4942	26.00
6.-	REPRES. R.C.M.	2404	6733	665.81
7.-	SEMILLAS Y CEREALES	87	130	265.00
8.-	PRICE CLUB	SP-130	1440	20.20
9.-	ANGEL DELGADO LOPEZ	87	16	18.00
10.-	DAM-ESPLA	193	1146	110.00
11.-	PAPEL. CORNEJO	87	14983	26.52
12.-	GTO. DE IMPORTACION	918-93	2321	63.60
13.-	QUIMICA VALANER	190	2015	211.20
14.-	REPRES. R.C.M.	191	7483	1,076.94
15.-	PROESBA	614	5331	61.05
16.-	SIGMA CH. 35.20 DLLS.	192	950555832	220.00
17.-	SERV. PROF. JONHSON	2434	11710	82.69
18.-	RENATO ORTIZ	SP-3024	14046	1,579.24
19.-	RYSE DE IRAPUATO	1038	21451	259.51
20.-	IND. KEM DE LEON	1088	10630	657.72
21.-	DAM-ESPLA	1085	2168	230.00
22.-	DAM-ESPLA	1086	2168	149.50
23.-	IND. KEM DE LEON	1116	10686	786.36
24.-	PAPEL. CORNEJO	1089	18123	32.19
25.-	ACCESOLAB	1119	9445	537.56
26.-	ENC. FELIPE QUIROZ	1220	1345	207.00
27.-	ENC. FELIPE QUIROZ	1159		331.20
28.-	REPRES. R.C.M.	1113	9124	1,252.93
29.-	CONSORCIO CIENTIFICO	1114	12550	1,907.85
30.-	PAPEL. CORNEJO	1233	18779	367.56

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

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CONTRACT: 91/264

FINANCIAL STATEMENT FROM NOVEMBER 1994 TO AUGUST 1995

SUPPLIES

	SUPPLIER	ORDER	INVOICE		AMOUNT MEXICAN PESOS
31	ARTES GRAFICAS CASTORES	1239	582		138.00
32	ROPA LA CENTRAL	SP-2525	42545		18.60
33	FOTO IMAGEN		6649		50.00
34	FOTO IMAGEN		6611		50.00
35	CONSORCIO CIENTIFICO	1115	13101		76.48
	TOTAL			N$	14,904.08
				USD	4,690.52

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

	SUPPLIER	ORDER	INVOICE		AMOUNT MEXICAN PESOS
1.-	BICOR DISENO (PARTIAL)	1427	772	N$	3,128.44
	TOTAL			N$	3,128.44
				USD	984.44

LITERATURE

	SUPPLIER	ORDER	INVOICE		AMOUNT MEXICAN PESOS
1.-	FOOD NUTRITION PRESS	SP-2530	12315	N$	1,257.31
	TOTAL			N$	1,257.31
				USD	395.82

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

OTHER COST

	SUPPLIER	ORDER		AMOUNT MEXICAN PESOS
1	DR. ALEJANDRO BLANCO L.	SEP-94 TO MAY-95	N$	9,567.60
2	SILVIA VALDES R.	SEP-94 TO MAY-95		5,760.00
3	ALICIA CHAGOLLA	SEP-94 TO MAY-95		4,550.39
4	NORMA MARTINEZ	SP-2688		1,250.00
5	DR. ALEJANDRO BLANCO			108.50
6	DR. ALEJANDRO BLANCO	SP-3174		1,641.85
7	ELIZABETH MENDIOLA	SP-3433		493.67
8	DR. ALEJANDRO BLANCO	SP-3417		170.00
9	DR. ALEJANDRO BLANCO	SP-3598		562.50
10	ELIZABETH MENDIOLA	SP-3668		201.00
11	DR. ALEJANDRO BLANCO	SP-18		112.00
12	DR. ALEJANDRO BLANCO	SP-130		87.40
13	DR. ALEJANDRO BLANCO	SP-1526		95.00
14	DR. ALEJANDRO BLANCO	SP-3801		399.00
15	ELIZABETH MENDIOLA	SP-3802		238.00
16	ENLACES TERRESTRES	SP-2230		95.00
17	JORGE CAMPOS	SP-2163		100.00
18	DRA. ALEJANDRA COVARRUBIAS	SP-2220		705.46
19	DRA. ALEJANDRA COVARRUBIAS	SP-2541		2,491.32
20	DR. ALEJANDRO BLANCO	SP-2018		1,652.30
21	DR. ALEJANDRO BLANCO	SP-2526		2,945.58
	TOTAL		N$	33,226.57
			USD	10,456.86

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:

Monosaccharide	Moles/mole inhibitor
Xilose	1
Acetyl Xilose	2
Mannose	12
Glucose	16
Glucose acetamide	4

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 Ki= 2.2 x 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 trough 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 where 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.

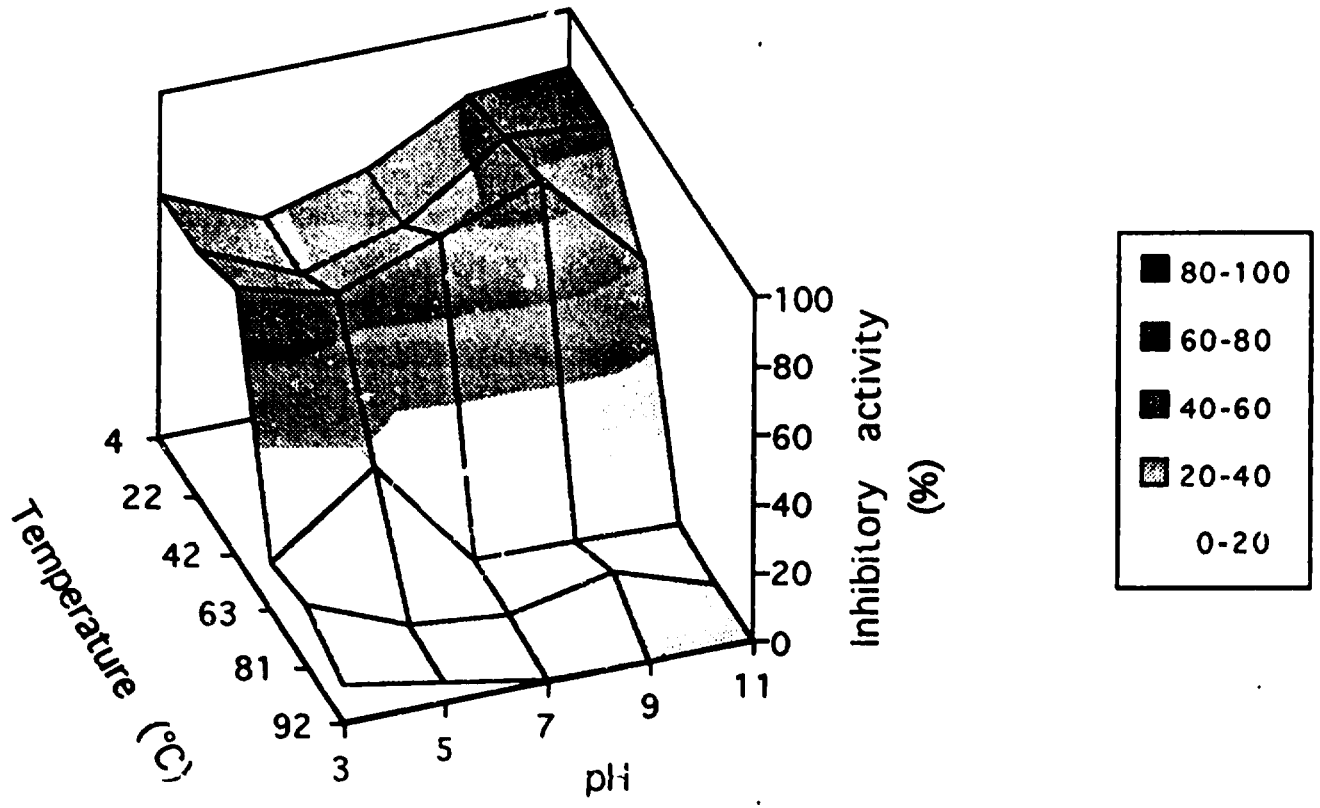

Fig. 1: Temperature and pH effect on TAI stability. Samples of inhibitor (1 μg/ 40 μL) were treated for 60 min at different pH and temperature conditions. After the treatments samples were assayed for inhibitory activity.

Table 1. Effect of TAI on α-amylases from different sources at 30 and 60 min incubation time (E+i).

Source	Inhibition (%)*	
	30 min	60 min
Porcin Pancreatic	1.70	0.00
Human Salivary	7.05	9.15
Bacillus subtilis	10.13	8.77
Aspergillus oryzae	8.13	8.00
Barley´s malt	0.00	0.00
Tribollium castaneum	100.00	100.00

* Based on 100% inhibition of *T. castaneum* α-amylase activity.

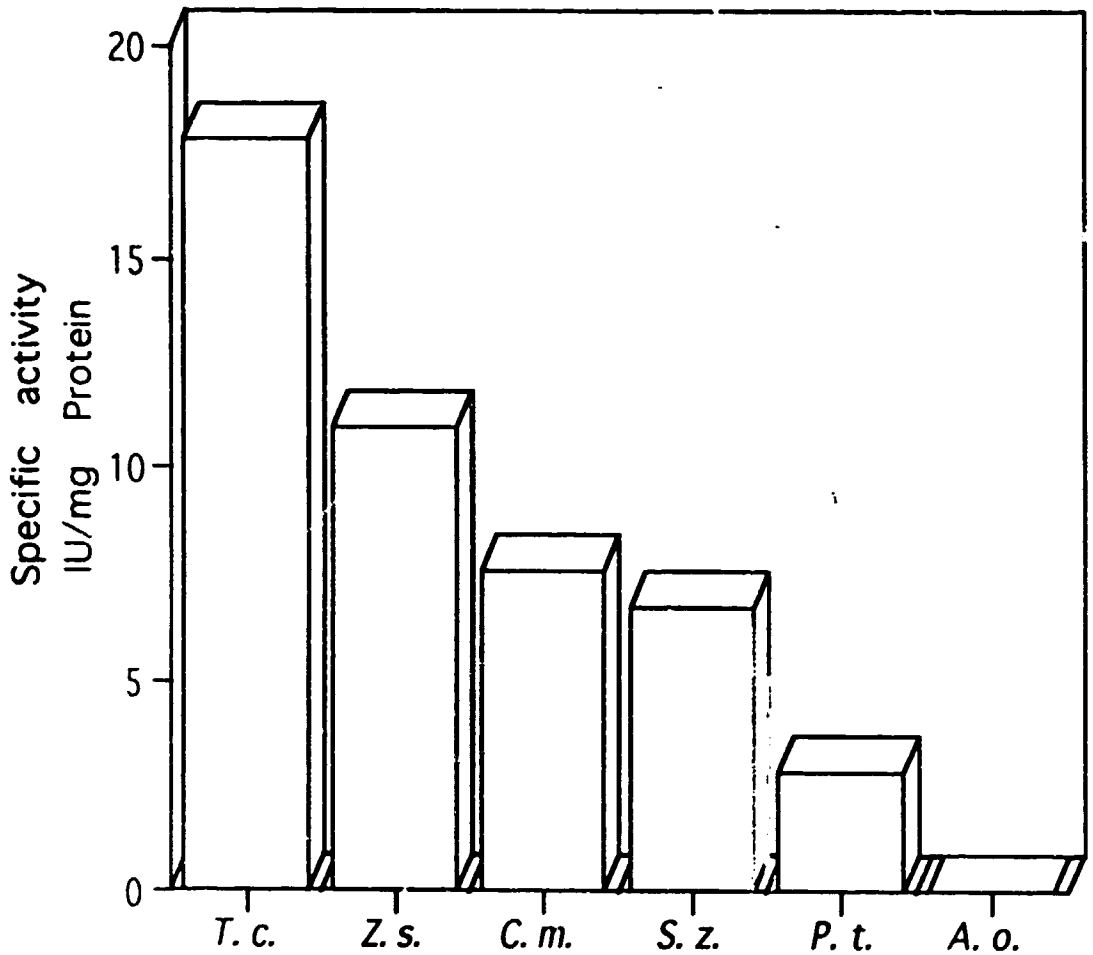

Fig. 2: Inhibitory activity of TAI against enzymes extracted from larvae of different insects. *T. c. Tribolium castaneum; Z. s. Zabrotes subfasciatus; C. m. Callosobruchus maculatus; S. z. Sitophilus zeamais; P.t. Prostephanus truncatus,* and *A. o. Acanthoscelides obtectus.*

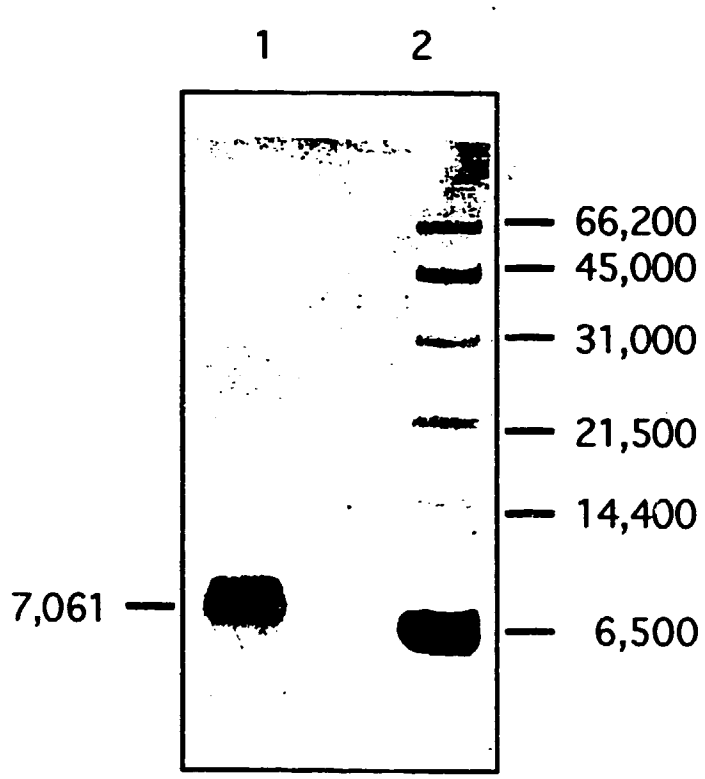

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the inhibitor purified by RP-HPLC. Lane 1, Inhibitor purified by reverse phase HPLC. Lane 2, Molecular weight (Mr) markers; bovine albumin (66,200), egg albumin (45,000), carbonic anhydrase. (31,000), soybean trypsin inhibitor (21,500) and aprotinin (6,500).

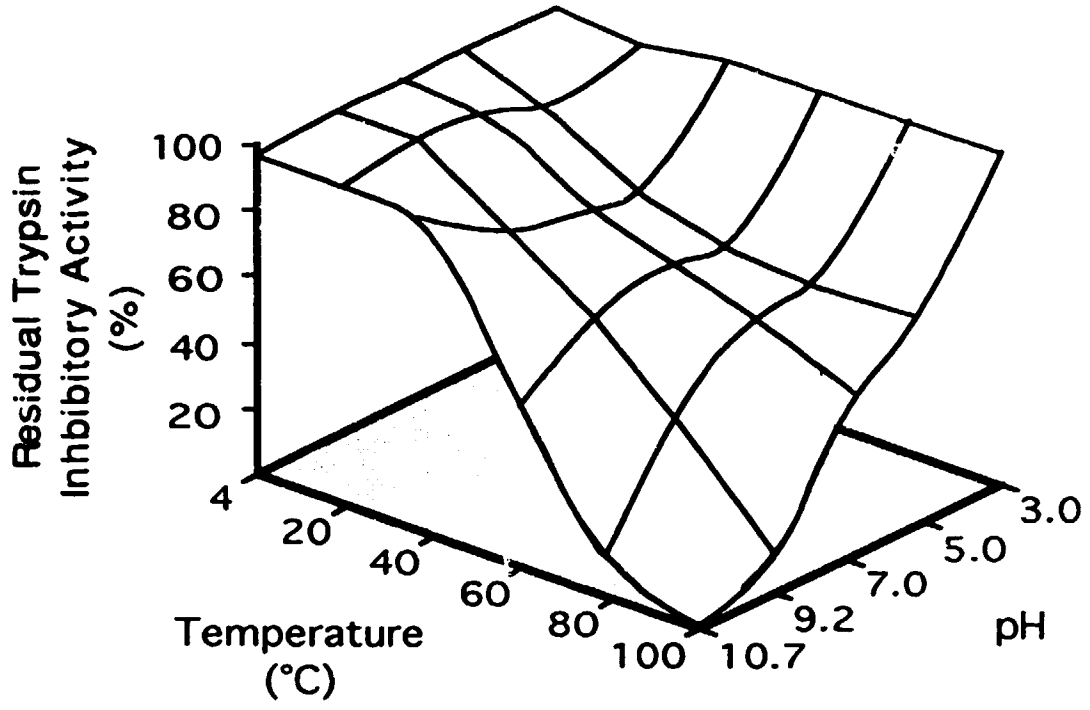

Figure 4. Trypsin inhibitory activity stability response surface for tepary bean inhibitor with variations in pH and temperature. Samples of inhibitor (0.1 mg mL-1) were treated for 60 min at different pH and temperature conditions. After treatment, samples were assayed for trypsin inhibitory activity.

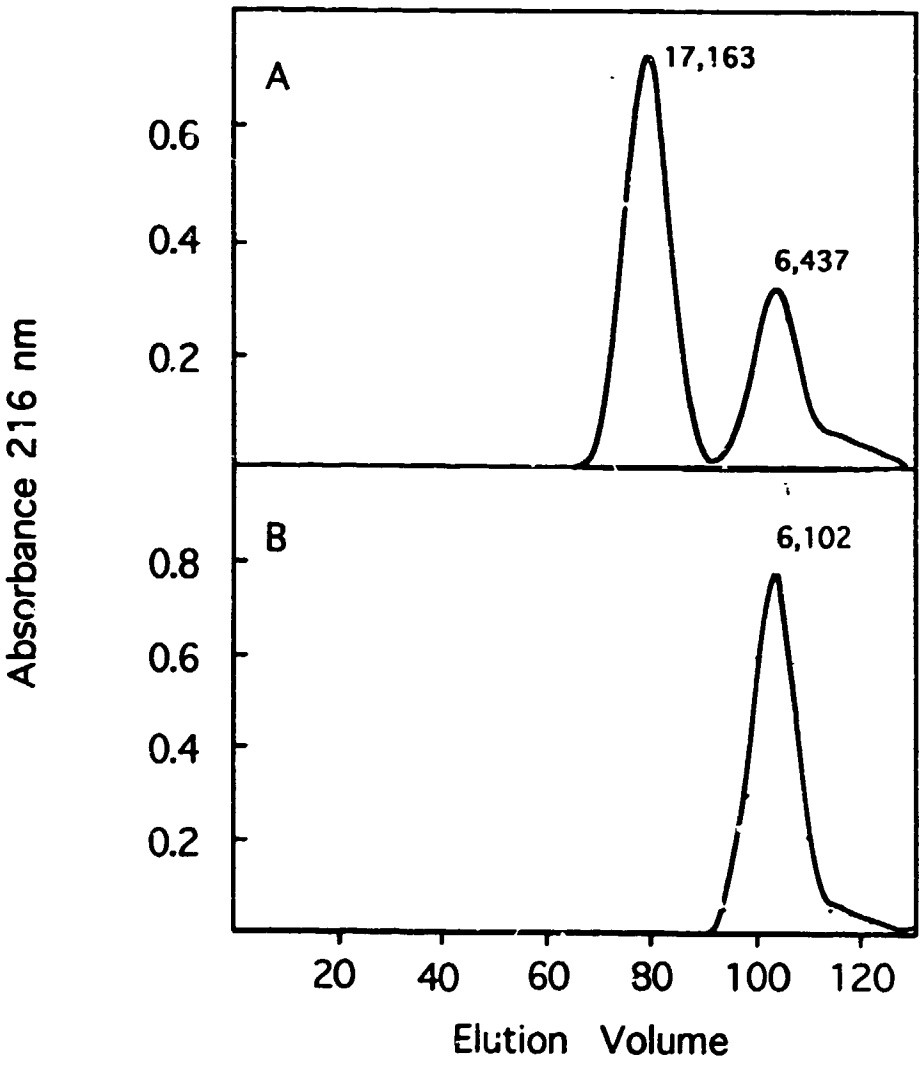

Figure 5. Molecular weight estimation by gel filtration chromatography (G-75). A Sephadex column (1.6 X 57.7 cm) was equilibrated with 0.05 M sodium phosphate and 0.01 M NaCl buffer. A) Elution profile of inhibitor under native conditions. B) Elution profile in the presence of 3 M guanidine, acting as a denaturing agent.

DOCUMENTS

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

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

AGRADECIMIENTOS

Deseo expresar mi agradecimiento al financiamiento recibido por parte de las siguientes instituciones:

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August 2, 1995

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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,

Lawrence I. Gilbert
Executive Editor

LIG:pc

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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 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 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. HPLC[1]-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 × 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 × 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 × 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: Against insect amylases	Against mammalian or other amylases	Against proteases	Members of the group with other activities[a]
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 phenylisothiocyantate, 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. Afterwards, 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 cyles 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, Cβ-Cβ 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 Cβ-Cβ 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 NH_4HCO_3 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.

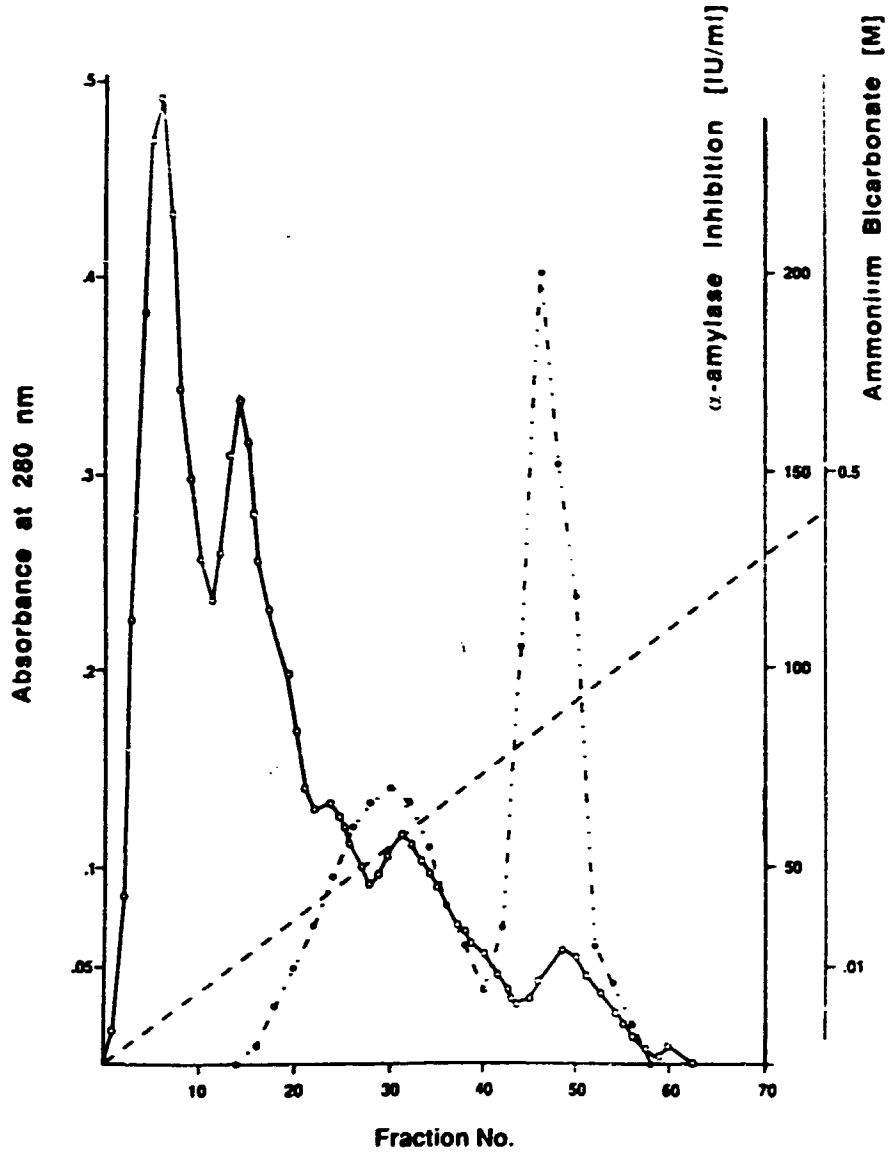

FIG. 1. **Purification of the amaranth α-amylase inhibitor AAI with ion exchange chromatography on DEAE-Sepharose CL-6B column.** *Continuous line*, absorbance at 280 nm; *dashed line*, inhibitory activity against *T. castaneum* α-amylase expressed as inhibitory units/ml (22).

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. 3*A*. The

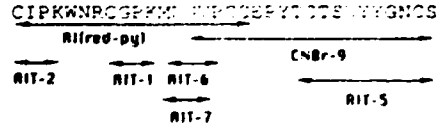

FIG. 2. **The sequence of AAI as determined by automated Edman sequencing.** The *arrows* denote the peptides obtained by enzymatic and chemical cleavage and isolated by reverse phase HPLC before sequencing.

results indicate that Cys^3 and Cys^{31} are connected by a disulfide bond. Furthermore, both Cys^1 and Cys^{32} form a disulfide bond either with Cys^{17} or with Cys^{18}, respectively. The exact placement of these disulfide bonds could not be determined from these experiments since the enzymatic cleavage of the Cys^{17}–Cys^{18} peptide bond cannot be accomplished. The two theoretically possible disulfide bonding patterns are shown in Fig. 3*B*.

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

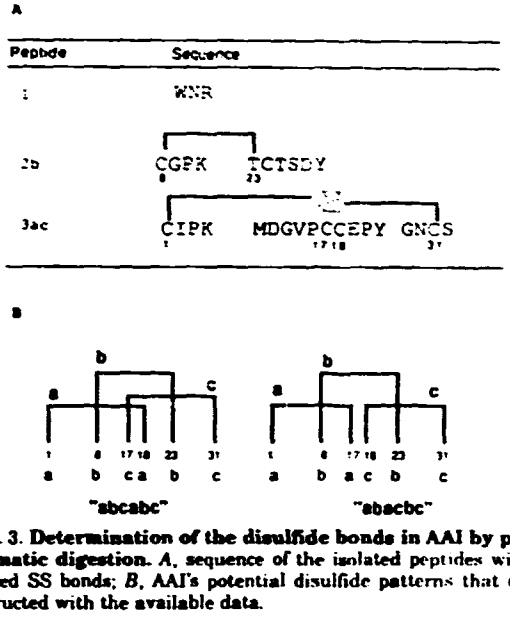

FIG. 3. Determination of the disulfide bonds in AAI by partial enzymatic digestion. *A*, sequence of the isolated peptides with the deduced SS bonds; *B*, AAI's potential disulfide patterns that can be constructed with the available data.

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

Modeling—The superimposition of the structures and th alignment of the structurally conserved regions was used t design a structural template for AAI. Assignment of two disu fide bridges in AAI was, in principle, an open question, sinc the connectivity of adjacent cysteines 17 and 18 could not b directly determined by chemical means. Theoretically there ar two possibilities to form SS bridges using our connectivity dat (Fig. 3*B*). (i) The "abcabc" topology is characteristic of group structures, and was also found by chemical means in ω-conc toxin (48); (ii) The abacbc topology, on the other hand, has no yet been found experimentally in short proteins (49). On th basis of the chemical evidence (48) and of the convincing sim ilarity of AAI to group I proteins (Fig. 4), we chose the abcab topology for our modeling studies.

We made a structural template in which the conformation o the first amino acids of (CPRILMR) followed that of the ET structure, while the rest of the molecule was modeled on th cellulose binding domain of *T. reesei* cellobiohydrolase Sinc the three-dimensional coordinates of the cellobiohydrolase I cellulose binding domain are not published, we used the struc ture of the cellobiohydrolase I cellulose binding domain (1cbl (40)), which is reported to be identical with the former. To build this structure we had to introduce deletions in the (Thr^{17}-Val^{18}) and (Val^{27}-Leu^{28}) positions, respectively (numbering of the 1cbl structure). The sequence alignment between the AAI and th template (Fig. 6) resulted in sequence identity for 11 out of 3 residues (34%). The model was finally constructed through residue by residue replacement. The disulfide bridge originall absent in the *1cbh* framework was built and the model wa refined by energy minimization and molecular dynamics to giv 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 th ProsaII (36, 51) program. The program calculates the Cβ-C pair interaction energy for each residue in the sequence, an correctly folded proteins produce smooth energy plots with neg ative values (36). The AAI model gave an energy profile wit values corresponding to those of native structures (Fig. 8). Th energy profile had no positive regions that would indicate mis folded parts in the model. Also, the so-called *Z*-score or normal ized energy value (36) was −3.9, which is within the range o the values expected for native proteins of this length (36 When tested with the AAI sequence, all other known structure gave higher *Z* scores indicating that the model presented her fits the sequence better than any of the other structures (no shown).

DISCUSSION

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

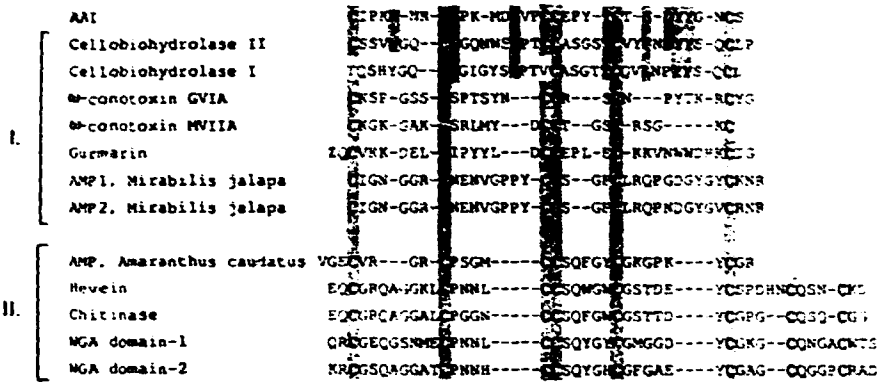

Fig. 4. Sequence alignment of AAI with protein sequences of similar cysteine patterns. The sequences are taken from the Swiss-Prot data bank (44) as follows: *Cellobiohydrolase II*, (exoglucanase II, EC 3.2.1.91) from *T. reesei*, GUX2_TRIRE, 27–63; *Cellobiohydrolase I*, exoglucanase I (EC 3.2.1.91) from *T. reesei* (40), GUX1_TRIRE, 479–533); *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*).

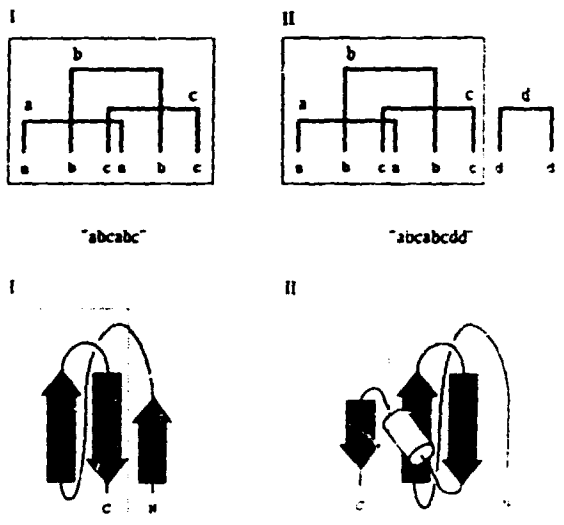

Fig. 5. Disulfide patterns (*top*) and folding pattern (*bottom*) of proteins distantly related to AAI. *I* and *II* correspond to the groups shown in Fig. 4. The disulfide and folding patterns were deduced from known structural data in each group.

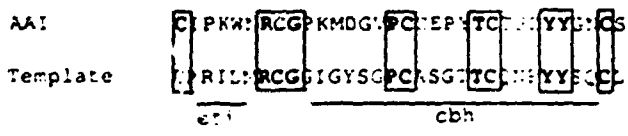

Fig. 6. The structural template used for the modeling AAI. *eti* is the *Ecballium elaterium* trypsin inhibitor structure (50), *cbh* is the cellulose binding domain of cellobiohydrolase I (40).

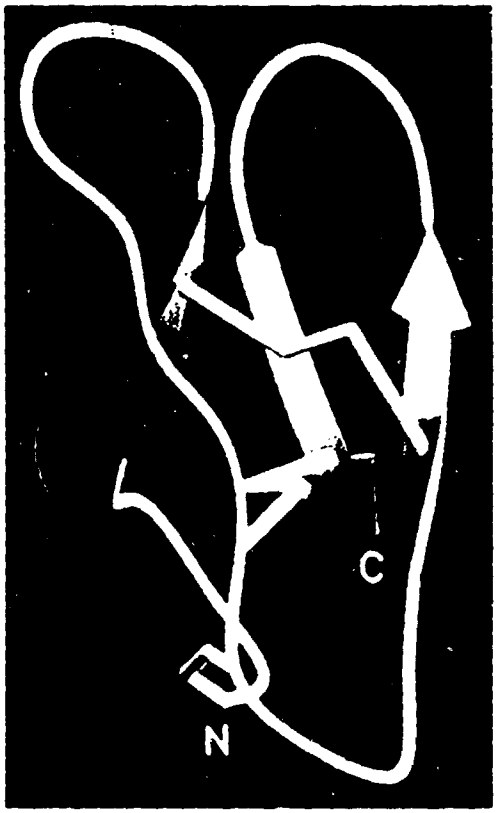

Fig. 7. The schematic model of the AAI structure. Disulfide bridges are shown in *yellow*, β-sheets are *green*, irregular conformations are shown in *white*. The illustration was generated with the program SETOR (52) using the AAI coordinates obtained by homology modeling and molecular dynamics refinement.

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), venoms (ω-conotoxin), and antimicrobial peptides. Using th known three-dimensional structures we built a model based o the similarity of AAI to the squash family of trypsin inhibitor ω-conotoxin, and to the cellulose binding domain of cellobioh drolase. Nguyen and associates introduced the term "knottii for this group of structures, based on a "knotlike" feature in tl three-dimensional fold (47). This feature is retained in tl model of AAI. AAI is the first α-amylase inhibitor described this group.

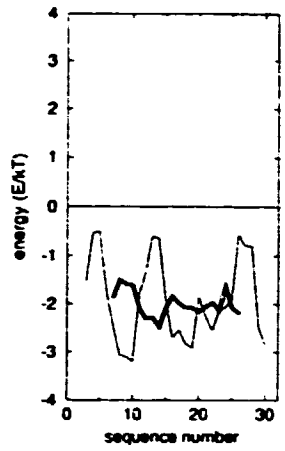

FIG. 8. **Energy plot for the refined AAI model as generated with the ProsaII (36) program.** The curves are smoothed (*thin line*, window size of 5; *thick line*, window size of 13). Energies are represented in units of E/kT.

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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FURTHER CHARACTERIZATION OF THE 12 kDa PROTEASE/ ALPHA AMYLASE INHIBITOR PRESENT IN MAIZE SEEDS

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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;

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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 Halim *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 seeds 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 insectary at CINVESTAV-Irapuato Unit.

Insect cultures of *Prostephanus truncatus* and *Sitophilus zeamais* were maintained on whole maize seeds, while *Tribolium castaneum* was grown on maize flour. *Callosobruchus maculatus* cultures were maintained on whole chick pea seeds; *Zabrotes subfasciatus* and *Acanthoscelides obtectus* cultures were grown on whole navy beans. All cultures were maintained in a growth chamber at 28C with a relative humidity of 65–75%.

The fungi *Aspergillus niger* and *Aspergillus fumigatus* were grown on potato-dextrose agar at 28C for 4–7 days, in order to obtain enough sporulation. The cultures were then maintained at 4C, and transferred to a new medium every two months.

Bovine trypsin (Type I), N-α-benzoyl-L-arginine ethyl ester (BAEE) and the amylases from *Bacillus subtilis* and *Aspergillus oryzae* were from Sigma Chemical Co.; Sephadex G-75 was from Pharmacia Fine Chemicals. Acrylamide and bisacrylamide were from Bio-Rad. 4,N-N-Dimethyl-aminoazobenzene-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 m*M* acetate buffer, pH 5.5, containing 1 m*M* calcium chloride (1:5, w/v), and continuously stirred for 12 h at 4C. The supernatant was separated by centrifugation (10,000 × g, 1 h, at 4C).

Ammonium Sulfate Precipitation

Proteins of the supernatant above mentioned were precipitated with 60% $(NH_4)_2SO_4$ for 12 h at 4C, and then centrifuged at 10,000 × g for 1 h. The precipitate was collected, redissolved 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 m*M* 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 30C. Separation was performed at a flow rate of 5 ml/min, using a linear gradient of acetonitrile and 0.1% TFA (0–60% 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 SDS 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 carboxymethylated inhibitor was subjected to micro sequence analysis using the 4-N,N-dimethylaminoazo-benzene-4'-isothiocyanate (DABITC)/phenylisothiocyanate (PITC), double coupling method of Chang *et al.* (1978).

Larval Crude Enzymes

The acetone-defatted larvae of the insects *P. truncatus*, *S. zeamais*, *T. castaneum*, *C. maculatus*, *Z. subfasciatus* and *A. obtectus* 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 m*M* succinic acid, pH 6.5, solution. The enzymes of *S. zeamais*, *T. castaneum*, *Z. subfasciatus*, *C. maculatus* and *A. obtectus* were extracted with 0.2 *M* succinic acid buffer, pH 4.5. The suspensions were centrifugedd at 10,000 × g for 10 min at 4C. The supernatants served as the soruce 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 Curl (1972), were inoculated with the spore suspension. 1,000 spores per ml of medium were added and incubated 48 h at 28C with continuous stirring (100 rpm).

The mycelia obtained were separated by centrifugation at 10,000 × g for 15 min, and the enzymes were extracted by homogenization with glass beads in 50 m*M* Tris-HCl, pH 8.6, buffer solution, in a 1:4 (v/v) ratio. The suspensions were centrifuged at 10,000 × 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 30C 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 BAEE as substrate. The reaction rate in the absence of inhibitor was determined at 30C according to the method described by Schwertz and Takenaka (1955). The preincubation of 25 µl of bovine trypsin (200 µg/ml) and crude protease extract from *P. truncatus* with the inhibitor was done in 2.8 ml of 0.15 *M* Tris (hydroxymethyl aminomethane) buffer solution, pH 8.1, containing 50 m*M* $CaCl_2$. 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 *Sitophilus zeamais* was evaluated by incubating it (up to 100 µg of protein) with the crude protease extract (0.2 ml). The residual proteolytic activity was measured at pH 2.5, using hemoglobin (0.5%) 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 absorpuon units under the previously described assay conditions.

The effect of the inhibitor against the proteases extracted from larvae of *C. maculatus*, *Z. subfasciatus* and *A. obtectus* was determined by the modified method of Kakade *et al.* (1969) and Lenney (1975). The crude extracts of protease (100 µl) were preincubated with the inhibitors (100–200 µg) 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 µg 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 µg of protein) with the protease extracts (200 µl) at pH 8.6. 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 fo 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 30C, in a total volume of 0.7 ml. Following the preincubation step, a 0.125% (w/v) starch solution (0.5 ml) was added, the reactions proceeded for 3 min, after which the reaction was stopped by the addition of 5 ml of an acidic iodine (5%)-iodate (0.5%) solution diluted 100 fold with water. The absorbance of the solutions was measured at 580 nm against a blank containing only the buffer (1.2 ml) and the iodine-iodate solution. The amylolytic activity was calculated on the basis on the absorbance difference between the solution containing the enzyme (or the enzyme plus inhibitor), and the control solution containing undigested starch. A unit of enzymatic activity was defined as the decrease in one unit of absorbancy under the described conditions. A unit of enzyme inhibitory 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 μl) 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 centrifugating 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 inhibitor activity against amylases from *T. castaneum* and *C. maculatus* larvae 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 6.0; 0.04 *M* succinic acid, pH 4.5 and 6.5; 0.1 *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 30C, 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.

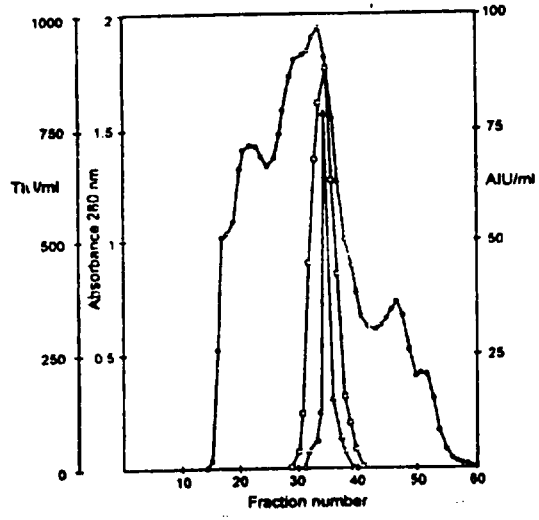

FIG. 1. GEL FILTRATION OF SEPHADEX G-75 COLUMN OF THE 60% $(NH_4)_2SO_4$ FRACTION OF THE MAIZE EXTRACT

The gel filtration column (1.6 × 160 cm) eluted with 10 mM ammonium bicarbonate, at a flow rate of 15 ml/h. -o-, absorbance at 280 nm; -□-, trypsin inhibitor units/ml (TIU/ml); -△-, amylase inhibitor units/ml (AIU/ml).

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 inhibitor 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).

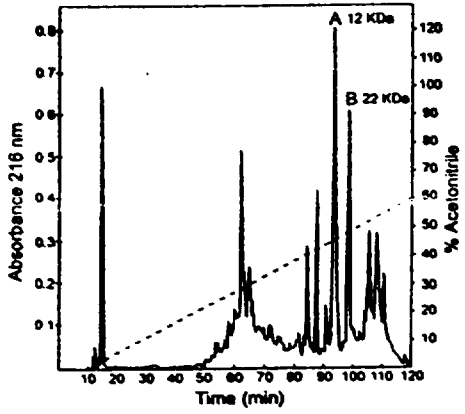

FIG. 2. REVERSE PHASE HPLC CHROMATOGRAM OF THE ACTIVE FRACTION OBTAINED FROM THE SEPHADEX G-75 CHROMATOGRAPHY

A 10 mg sample was dissolved in 100 μl guanidine-HCl and injected into a Vydac C-18 (22 × 250 mm) column. Elution was performed by using a linear gradient of acetonitrile and 0.1% aqueous TFA (0–60%) in 110 min, at a flow rate of 5 ml/min.

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 (Houseman and Thie 1993), was weakly inhibited. The crude fungal proteases extracted from the mycelia of *A. niger* and *A. fumigatus* 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 proteses such as BAEE, TAME and BAPNA.

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

HPLC purified inhibitor was assayed against each enzyme at least in duplicate. Each value represents the mean of three replicates.

SOURCE	PROTEASE		
	E UA ml^{-1}	E+I UA ml^{-1}	INHIBITION* UI mg^{-1}
Bovine trypsin*,†	228.8 ± 3.5	101.7 ± 3.8	2,104.4 ± 9.8
INSECTS			
Prostephanus truncatus†,‡	100.9 ± 0.8	53.5 ± 1.4	116.2 ± 3.5
Sitophilus zeamais	1.5	1.5	0
Tribolium castaneum	5.3 ± 0.5	5.3 ± 0.5	0
Callosobruchus maculatus	0.7 ± 0.1	0.7 ± 0.1	0
Acanthoscelides obtectus	0.9	0.9	0
Zabrotes subfasciatus	5.5	5.5	0
FUNGI			
Aspergillus niger‡,§	0.13	0.06	3.5 ± 0.1
Aspergillus fumigatus‡,§	0.20	0.11	2.4 ± 0.2

*Units of inhibition per mg of maize inhibitor.
†Method of Schwertz and Takenaka, 1955 (using BAEE 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 proteinase activity present in the *T. castaneum* extract is due to an aspartic acid proteinase. In the other cases, aminopeptidases 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. obtectus* (Weiman and Nielsen 1987) and *Z. subfasciatus* (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 elevent 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 proteases from *P. truncatus* were preincubated and assayed against the 12 kDa inhibitor (1.5 μg and 20 μg of protein, respectively) at different pHs, they showed a maximum inhibition at pH 8.0 and 7.5, respectively (Fig. 3A and 3B).

The pH effect of the reaction medium on the inhibition of amylases of *T. castaneum* and *C. maculatus* is shown in Fig. 4A and 4B. In both cases the maximum inhibition was obtained at pH 5.0.

TABLE 2.
EFFECT OF 12 kDa MAIZE INHIBITOR AGAINST AMYLASES FROM DIFFERENT SOURCES
HPLC purified inhibitor was assayed against each enzyme at least in duplicate. Each value represents the mean of three replicates

SOURCE	AMYLASE		
	E UA ml^{-1}	E+I UA ml^{-1}	INHIBITION* UI mg^{-1}
Human saliva	67.6 ± 0.3	67.6 ± 0.3	0
INSECTS			
Prostephanus truncatus	3.8	3.8	0
Sitophilus zeamais	24.0 ± 0.2	24.0 ± 0.2	0
Tribolium castaneum [*1]	97.6 ± 4.5	56.8 ± 3.9	101.7 ± 9.7
Callosobruchus maculatus [*1]	4.4 ± 0.1	1.9 ± 0.1	49.1 ± 2.8
Acanthoscelides obtectus	57.7 ± 1.1	57.7 ± 1.1	0
Zabrotes subfasciatus	87.1 ± 0.3	87.1 ± 0.3	0
FUNGI AND BACTERIA			
Aspergillus niger	0.2	0.2	0
Aspergillus fumigatus	0.2	0.2	0
Aspergillus oryzae	68.6 ± 0.5	68.6 ± 0.5	0
Bacillus subtilis	166.6 ± 1.5	166.6 ± 1.5	0

* Units of inhibition per mg of maize inhibitor.
[*1] Method of Bird and Hopkins 1954 (using starch as substrate)
E=Enzyme E+I=Enzyme plus inhibitor

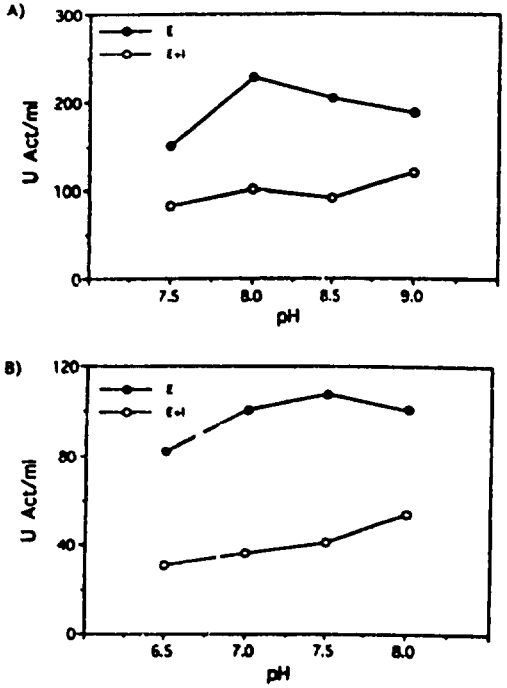

FIG. 3. pH EFFECT ON THE PROTEOLYTIC ACTIVITY AND INHIBITION BY THE 12 kDa MAIZE α-AMYLASE/TRYPSIN INHIBITOR (A) Bovine trypsin and (B) crude larval extract from *P. truncatus*. ●, units activity/ml of enzyme and ○, units activity/ml of enzyme plus inhibitor.

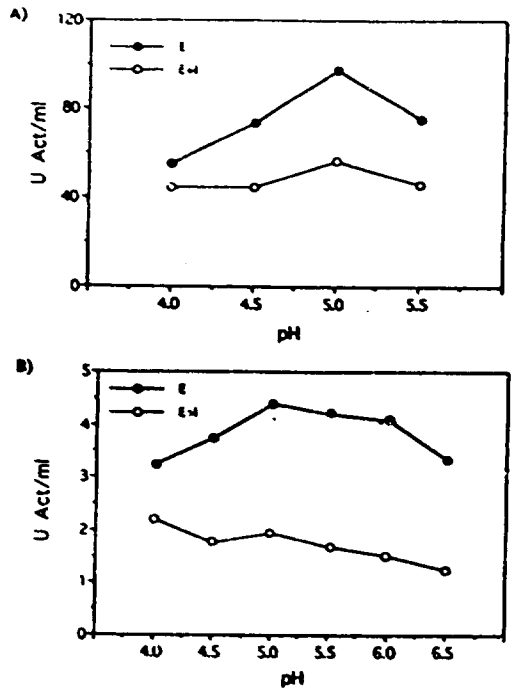

FIG. 4. pH EFFECT ON THE AMYLOLYTIC ACTIVITY AND INHIBITION BY THE 12 kDa MAIZE α-AMYLASE/TRYPSIN INHIBITOR (A) Crude larval extracts from *T. castaneum* and (B) crude larval extracts from *C. maculatus*. ●, units activity/ml of enzyme and ○, units activity/ml of enzyme plus inhibitor.

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 (Cristeller *et al.* 1992).

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 zeamatin, 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 zeamatin and the 22 kDa inhibitor still remains to be established. However, a purified protein, with 99% homology wiht 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, particulary *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 deleterious effect on the fungi or on the insects whose enzymes are sensitive to it.

ACKNOWLEDGMENTS

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Alejandro Blanco

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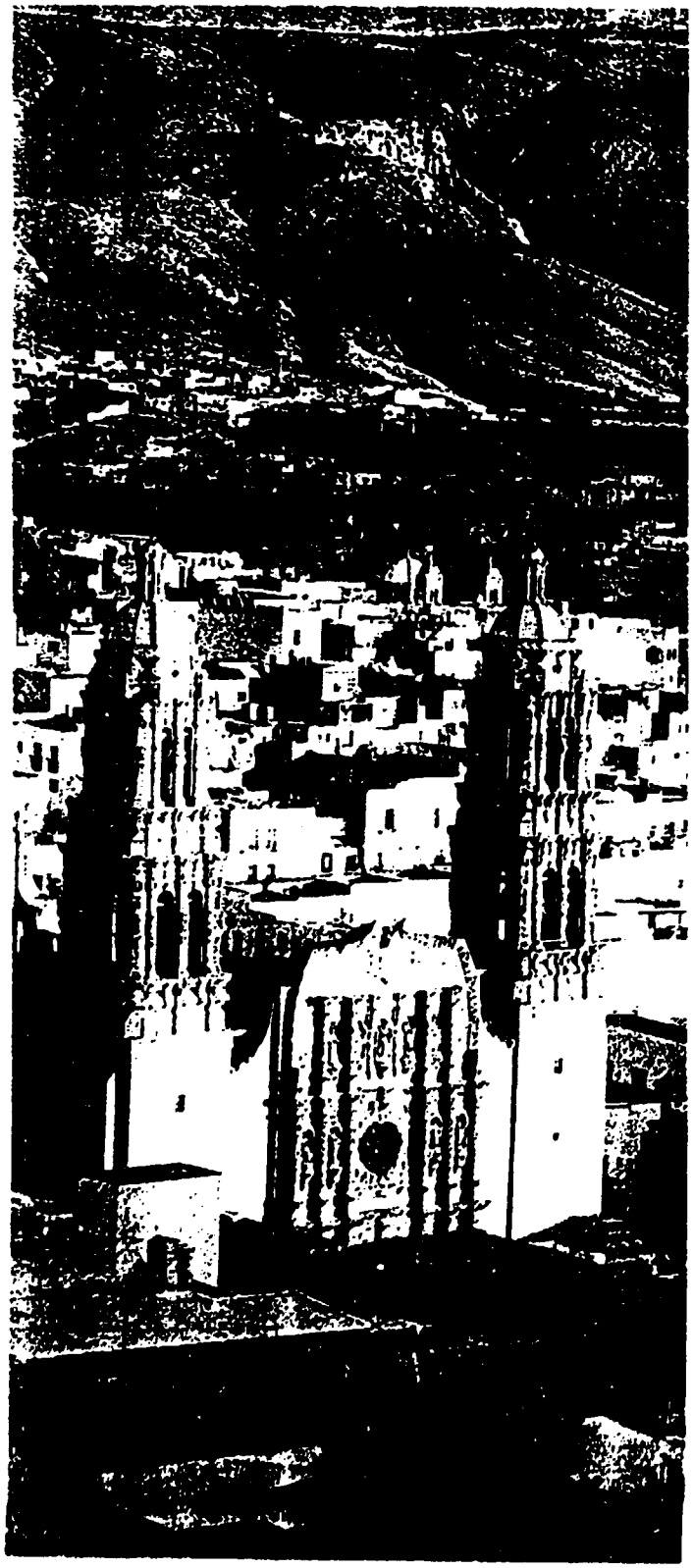

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CONGRESO NACIONAL

ZACATECAS, ZAC.
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OCTUBRE 30 - NOVIEMBRE 4
1994

PURIFICACION DE LECTINAS DE COLEOPTILO DE MAIZ *Zea maye* L. POR CROMATOGRAFIA DE INTERCAMBIO IONICO.

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Las lectinas de maíz han sido reportadas como inestables, es decir, pierden rapidamente su actividad y por esta razón su purificación es dificil (1).En este reporte se presenta una metodología para purificar lectinas de coleoptilo de maíz activas y estables, por cromatografía de intercambio iónico. 80 gr de coleoptilo se homogenizaron con un buffer de boratos (w/v). se precipitó dos veces con acetona fría al 66% y se disolvió en 15 ml de un buffer de tricina 30 mM pH 8.5. Se aplicarón 14 mg de proteína de la preparación a una columna de intercambio iónico (Econo-pac Q Bio-Rad).

Se obtuvieron 2 fracciones, una eluída con 40% de actividad con 5.6 mg de proteína, y otra con un gradiente de NaCl de 0-0.3 M con 42 % 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 sacarosa. El grado de purificación de la fracción eluída con el gradiente es del 70 % y su rendimiento fué del 66 %. El análisis electroforético muestra dos bandas de alto peso molecular y al adsorber la lectina con eritrocitos humanos "O" una de las bandas desaparese. Esto sugiere que la preparación contiene una contaminación con otra proteína.

1.- Jankovic,M., Cuperlovic,M., and Majdukovic,L. (1990) Plant Physiology 93: 1659-1662.

EFECTO DE FENILALANINA, ACIDO CINAMICO Y ACIDO P-CUMARICO SOBRE LA ACUMULACION DE CAPSAICINOIDES Y LIGNINAS EN SUSPENSIONES CELULARES DE CHILE *Capsicum annuum* L.

Muñoz-Palomino,Hector Enrique
Ochoa Alejo Neftali

CENTRO DE INVESTIGACION Y DE ESTUDIOS AVANZADOS DEL I P N UNIDAD IRAPUATO Km 9.6 del Libramiento Norte Carretera Irapuato-Leon. Apdo. Postal 629. Irapuato Gto. Mexico

En los frutos de chile se sintetizan y acumulan los capsaicinoides, metabolitos secundarios que le confieren la pungencia característica. Estos compuestos son el producto de 2 rutas biosintéticas: La de los fenilpropanoides iniciando con la fenilalanina como precursor, y la de los ácidos grasos ramificados partiendo de valina o leucina. Los intermediarios de la ruta de los fenilpropanoides son los ácidos cinámico, p-cumárico, cafeico y ferúlico que pueden generar la vainillilamina que se condensa con diferentes ácidos grasos ramificados para dar los distintos capsaicinoides. Células de chile cultivadas in vitro producen bajos niveles de capsaicinoides. Una estrategia para aumentar la producción de ellos en los cultivos celulares es la adición de precursores. En este trabajo se investigó el efecto de la fenilalanina, ac. cinámico y p-cumárico sobre la producción de capsaicinoides y de ligninas, compuestos que también se derivan de la ruta de los fenilpropanoides.
Se utilizaron suspensiones celulares de chile (*Capsicum annuum* L. var. tampiqueño 74, tipo serrano) cultivadas en medio MS líquido con 6.25 µM de ácido 2,4-diclorofenoxiacético y 2.66 µM de benziladenina. La fenilalanina se incorporó rápidamente en compuestos insolubles, pues no se detectó en forma libre en las células y en el medio de cultivo después de 3 días de incubación. No se observó incremento en los niveles de fenilpropanoides ni de capsaicinoides al adicionar este aminoácido.
Al adicionar ác. cinámico a las suspensiones solo se incrementó el nivel de ác. cafeico y no de capsaicinoides. Tampoco el ác. p-cumárico estimuló la acumulación de los capsaicinoides. La acumulación de lignina se incrementó notablemente en presencia de ac. cumárico y en menor proporción en fenilalanina y ac. cinámico. Estos resultados sugieren que los precursores adicionados al medio de cultivo se utilizan principalmente para la biosíntesis de ligninas y no de capsaicinoides.
Hall,R.D. *et al.* (1987). The accumulation of phenylpropanoid and capsaicinoid compounds in cell cultures and whole fruits of the chilli pepper, *Capsicum annuum*. Plant Cell & Organ Culture. 8 (161-176).

RELACION ENTRE PROPIEDADES FUNCIONALES Y ESTRUCTURA SECUNDARIA DE GLOBULINAS DE AMARANTO Y DE OTRAS PLANTAS.

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INTRODUCCION: Existen algunos estudios sobre caracterización bioquímica y fisicoquímica de globulinas de amarante basados en solubilidad, patrones electroforéticos, coeficientes de sedimentación y composición de aminoácidos. A la fecha no existen datos acerca de la estructura secundaria de estas proteínas. En este trabajo el principal objetivo fue investigar la estabilidad estructural de las globulinas de amarante en comparación con las de soya y arroz.
MATERIAL Y METODOS: Se efectuaron determinaciones de intensidad de fluorescencia, estudios cinéticos, calorimetría diferencial de barrido (DSC) y dicroismo circular (CD). Estas mediciones se emplearon para seguir el desenrollamiento de las globulinas de una estructura nativa a una al azar.
RESULTADOS Y DISCUSION: El desenrollamiento progresivo se midió como una función de la intensidad de la fluorescencia. Los espectros de las proteínas nativas fueron típicos del contenido de triptofano (picos cerca de 335 nm), variando según la fuente de globulina. A la longitud de onda de 360 nm aparece un hombro a 309 nm. Las mediciones cinéticas mostraron que la desnaturalización de globulina en presencia de cloruro de guanidina fue similar a aquella con urea pero con cambios más dramáticos. Se necesitaron concentraciones más bajas de GdnCl para iniciar el decremento en intensidad fluorescente. Los estudios de CD de la estructura secundaria exhibieron decrementos en el contenido de alfa-hélice de la globulina desnaturalizada en comparación con la nativa. La menor estabilidad estructural de la proteína desnaturalizada se mostró también por una temperatura de desnaturalización más baja. Se observaron diferencias menores en el contenido de alfa-hélice y estabilidad térmica de las globulinas de soya y arroz comparativamente con las de amarante. La estructura secundaria fue computarizada por matriz de punto y análisis de hidropatía. El perfil de desenrollamiento de las globulinas se demostró por las propiedades fluorescentes intrínsecas de los residuos de triptofano, por los cambios en el contenido de alfa-hélice y por la estabilidad térmica, todo lo cual puede ser usado para predecir los cambios funcionales de proteínas en un sistema alimentario específico.

AISLAMIENTO Y ESTRUCTURA DE UN NUEVO INHIBIDOR DE α-AMILASAS DE *Amaranthus hypochondriacus*.

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Un inhibidor de α-amilasas (AAI), presente en semillas de *Amaranthus hypochondriacus*, una variedad mexicana de Amaranto, fue aislado purificado a homogeneidad y secuenciado en un secuenciador automático por el método de Edman. La localización de sus puentes disulfuro fue determinada usando fragmentación enzimática y química combinada con secuenciación N-Terminal.

AAI es un polipéptido de 32 residuos cuya secuencia de aminoácidos no presenta homologías cuando se comparó con secuencias de los bancos de datos tanto de proteínas (Swiss-Prot, PIR como de DNA (Genbank).
Sin embargo, cuando se analizó la conectividad de sus puentes disulfuro, se encontró que ésta tiene relación con un grupo de inhibidores de proteasas y con un grupo de neurotoxinas con propiedades neurotóxicas.

En base a ésta característica estructural fue posible construir un modelo de la estructura tridimensional de AAI, usando la técnica de reemplazamiento de cadenas laterales y finalmente, refinando el modelo utilizando técnicas de Dinámica Molecular.

Este trabajo fue financiado por la C.E.E., proyecto Multinacional de Biotecnología-Alimentos y por el I.C.G.E.B.

001

PURIFICACION Y CARACTERIZACION PARCIAL DE UN INHIBIDOR DE PROTEASAS PRESENTE EN FRIJOL TEPARI (*Phaseolus acutifolius*)

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Irapuato. Gto. México

El frijol tépari, es una leguminosa considerada que presenta una gran tolerancia a condiciones ambientales adversas, así como una elevada resistencia al ataque de insectos y microorganismos. Se ha reportado que las causas de esa resistencia, se deben a la presencia de fitatos, lectinas e inhibidores de proteasas.

A partir de harina de semilla de frijol tépari, se llevó a cabo la purificación de una proteína con actividad de inhibidor de proteasas. El aislamiento y la purificación se logró por medio de precipitación fraccional con (NH4)2S4, cromatografía de filtración en gel, cromatografía de intercambio iónico y cromatografía de líquidos de alta resolución fase reversa (RPLC). La pureza del inhibidor fue evaluada por medio de cromatografía en RPLC y por electroforesis en gel de poliacrilamida en condiciones desnaturalizantes. El peso molecular, determinado por cromatografía de filtración en gel en condiciones nativas y desnaturalizantes y por electroforesis en condiciones desnaturalizantes, demostró que el inhibidor tiene un peso molecular aparente de 7.1 KDa. y existe en equilibrio con su forma trimérica de 21 KDa. La actividad inhibitoria se presenta únicamente en la forma trimérica. Este equilibrio monómero-trímero podría representar un mecanismo novedoso de regulación de la actividad de este inhibidor.

La proteína aislada, fue caracterizada como un inhibidor de amplio espectro, que reconoce principalmente serín proteasas como quimotripsina, tripsina y proteasas tipo tripsina, así como tambien reconoce aspártico y cisteín proteasas de larvas de diferentes insectos, que son plagas de cultivos importantes, principalmente leguminosas y cereales. Esta proteína no contiene residuos de carbohidratos y presenta una alta estabilidad, ya que fue capaz de soportar un tratamiento de 1 hr a 100°C a pH 3 sin pérdida considerable de actividad.

La cinética de este inhibidor muestra una estequiometría contra tripsina pancreática de 1:1, y su constante de disociación es $K_i = 10^{-9}$. Esto indica como una interacción muy fuerte (tight binding) en la formación del complejo Enzima-Inhibidor.

Este trabajo fue financiado por la O.E.A., proyecto Multinacional de Biotecnología-Alimentos y por el I.C.G.E.B. proyecto UNIDO GE/GLO/89/003.

002

ESTUDIOS SOBRE LA RELACION POLINIZACION DEL [illegible]
[illegible] A TRAVES DE EXTRACTOS DE POLINIA
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Centro de Investigación [illegible]
Cerdenas 97310 [illegible]

El [illegible]
Actualmente en [illegible]
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003

PURIFICACION Y CARACTERIZACION PARCIAL DE UNA ASPARTICO PROTEASA DIGESTIVA DE *Tribolium castaneum* (Herbst).(Coleoptera:Tenebrionidae).

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Tribolium castaneum (Herbst) conocido comúnmente como gorgojo rojo de la harina, es un insecto de distribución mundial, que generalmente ataca a los granos almacenados de insectos, por lo que se le considera una plaga importante.

Es de importancia caracterizar las enzimas digestivas de los insectos que atacan a los granos, ya que esto abre la posibilidad para la utilización de inhibidores de proteasas como un mecanismo natural de defensa de las plantas.

Se encontró una proteína ácida de tipo aspártico proteasa en los intestinos de la larva del insecto, la cual es activa a un pH de 6.0 mostrando una actividad proteolítica máxima a pH 3.0. La enzima resultó ser muy lábil ya que pierde actividad aún a 4°C, pero es estable a -80°C.

La purificación se llevó a cabo, en extractos de intestinos y posteriormente en extractos de larva completa utilizando cromatografía de filtración en gel (columna de Sephadex G-75), de la cual se obtuvieron dos picos colectándose el pico de mayor actividad proteolítica, posteriormente se pasó por una columna de Q-Sepharose FF a pH 6.0, se obtuvo un pico activo, con un peso molecular aproximado de 30 KDa. La purificación final fue de 34 veces a partir del extracto crudo y debido a su inestabilidad, solo se recupera un 5% de la actividad total. Al incubar la proteasa 15 min a diferentes temperaturas se observa que a 62°C pierde 25% de la actividad, mientras que a 50°C pierde 80% de la actividad original.

Con objeto de caracterizar el tipo de proteasa, se probaron diferentes inhibidores: Cisteína (E-64, IAA), Serina (PMSF), Metalo (EDTA), Aspártico (Pepstatina A), Inhibidor de proteasa de Amaranto (IPA) e Inhibidor de proteasa de frijol Tepari (IPT). Resulto positiva la inhibición sólo frente a Pepstatina A, lo que sugiere que la enzima aislada es una aspártica proteasa (Catepsina D ó Pepsina).

En base a su habilidad para hidrolizar hemoglobina en mayor proporción que albumina, además de su pH óptimo de actividad y su inhibición por Pepstatina A, la enzima se caracterizó como aspártico proteasa de tipo Catepsina D.

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PROPAGACION DE *Epidendrum ibaguense* POR TECNICAS DE CULTIVO DE TEJIDOS VEGETALES

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La familia de las orquídeas incluye 500 géneros y más de 15 mil especies [illegible] E. ibaguense es una de las más [illegible] de Colombia [illegible] en el estado de San Luis Potosí [illegible] especies de orquídeas [illegible]

Las principales causas que han provocado el número de estas especies amenazadas o en peligro de extinción son: la [illegible] y el comercio de especies que son recolectadas y en el peor de los casos [illegible] de un ejemplar [illegible] destrucción de hábitat natural [illegible]

En Japón, Estados Unidos, Europa y Canadá [illegible] un enorme mercado [illegible] de orquídeas originarias de México. Según los datos de "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES), en 1985 [illegible] 2,777,774 [illegible] de orquídeas. La propagación [illegible] de estas especies que presentan [illegible] que requieren muchos años para [illegible]

El Cultivo de Tejidos Vegetales constituye un [illegible] que permite conservar y propagar el material vegetal ya que en [illegible] diferentes asociados con los métodos de propagación [illegible] Se estudiaron por lo menos 35 especies de orquídeas que incluyen 21 géneros se han propagado mediante Cultivo de Tejidos. Esta propagación se ha logrado tanto [illegible] híbridos comerciales como de híbridos silvestres.

Con el fin de incrementar [illegible] existente de las orquídeas del estado de San Luis Potosí, se [illegible] de interés comercial [illegible] desarrollaron con una solución de [illegible] Murashige Skoog para la germinación. Después de 3 meses [illegible] aproximadamente 1 cm de diámetro [illegible] medios con diferentes combinaciones de auxinas y citocininas. Se obtuvo una elevada proliferación de brotes con combinaciones de [illegible] de benciladenina y 1 mg/l de ácido naftalenacético (ANA). Los brotes se transfirieron posteriormente a medios con diferentes concentraciones de auxinas para favorecer el enraizamiento. Utilizando una concentración de ANA [illegible] mg/l se logró un [illegible] y poca formación de callo. Posteriormente los brotes se transfirieron a medio de [illegible] para promover su crecimiento.

ESTRUCTURAS Y FUNCION DE INHIBIDORES DE ENZIMAS DE PLANTAS.

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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 cisteínas con el grupo de las curtatoxinas.

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EFECTO 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.