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Interaction of proteases with legume seed inhibitors. Molecular features [1]

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SUMMARY. After having found that raw black beans (*Phaseolus vulgaris*) were toxic, while the cooked ones constitute the basic diet of the underdeveloped peoples of the world, in the sixties, our research directed by Dr. Jaffé, concentrated mainly around the detection and identification of the heat labile toxic factors in legume seeds. A micromethod for the detection of protease inhibitors (PI) in individual seeds was developed, for the purpose of establishing that the multiple trypsin inhibitors (TI) found in the Cubagua variety were expressions of single seeds and not a mixture of a non homogenous bean lot. Six isoinhibitors were isolated and purified, all of which were «double-headed» and interacted with trypsin (T) and chymotrypsin (CHT) independently and simultaneously, as shown by electrophoresis of their binary and ternary complexes with each and both enzymes. However, their affinity for the enzymes, including elastases, was rather variable, as well as their amino acid composition which consisted of 51 units for inhibitor V, the smallest, and 83 amino acids for inhibitor I, the largest.

A low molecular weight protein fraction that inhibited subtilisin (S), but recognized neither T, CHT nor pancreatic elastase was detected in 63 varieties of *Phaseolus vulgaris* as well as in broad beans (*Vicia faba*), chick peas (*Cicer arietinum*), jack beans (*Canavalia ensiformis*), kidney beans (*Vigna aureus*),etc., It was absent though, in soybeans (*Glycine max*), lentils (*Lens culinaris*), green peas (*Pisum sativum*), cowpea (*Vigna sinensis*) and lupine seeds (*Lupinus sp*).

Subtilisin inhibitors (SI) were isolated from black beans, broad beans, chick peas and jack beans. Their Mr is between 8-9KD and they show a rather high stability in the presence of denaturing agents. They are specific toward microbial proteases, in addition to subtilisins, Carlsberg and BPN', they inhibit the alkaline protease from *Tritirachium album* (Protease K), from *Aspergillus oryzae* and one isolated from *Streptomyces griseus*, but do not interact with either animal digestive or plant thiol enzymes.

RESUMEN. Interacción de proteasas con inhibidores de semillas de leguminosas. Caracterización molecular del proceso. Luego de encontrar que las semillas crudas de *Phaseolus vulgaris* eran tóxicas, mientras que las cocidas eran la base de la dieta en muchos países en vías de desarrollo, en los años sesenta nuestras actividades de investigación bajo la supervisión del Dr. Jaffé se centraron en la detección e identificación de factores de naturaleza termolábil en las semillas de leguminosas. Se desarrolló un micrométodo para la detección de inhibidores de proteasas en semillas individuales. Se encontró que las múltiples formas de los inhibidores de tripsina (TI)encontradas en la variedad Cubagua constituían en realidad isoformas y no eran el producto de un lote de semillas no homogéneo. Se aislaron y purificaron seis isoinhibidores de «doble cabeza» capaces de interactuar con la tripsina (T) y la quimotripsina (CHT)de manera simultánea e independiente, lo cual se evidenció aislando los complejos binarios con cada enzima y los ternarios con ambas proteasas. Sin embargo, su afinidad por las enzimas, incluyendo las elastasas fue muy variable, así como también su composición de aminoácidos, desde 51 en el isoinhibidor más pequeño (V) hasta 83 en el más grande (I).

Una fracción proteica de bajo peso molecular, capaz de inhibir a la subtilisina pero que no reconocía a T ni a CHT ni a la elastasa pancreática, fue detectada en 63 variedades de *P. vulgaris*, así como en habas (*Vicia faba*), garbanzos (*Cicer arietinum*), haba de burro (*Canavalia ensiformis*), frijoles (*Vigna aureus*), etc. No se encontró el inhibidor en otras especies como la soya (*Glicine max*), lentejas (*Lens culinaris*), frijoles (*Vigna sinensis*) y lupino (*Lupinus sp*).

El inhibidor de subtilisina (SI) fue aislado de caraotas, habas, garbanzos y habas de burro. Sus Mr se encuentran entre 8-9 KD y tienen una gran estabilidad frente a agentes denaturalizantes. Los SI mostraron afinidad por proteasas microbianas, además de las subtilisinas Carlsberg y BPN, ellos inhibieron a la proteasa alcalina de *Tritiraquium album* (Proteasa K), de *Aspergillus oryzae* y una aislada de *Streptomyces griseus*, pero no interactuaron ni con las enzimas digestivas de animales ni con las tiólicas de plantas. Los SI reaccionaron con S a través del «mecanismo convencional» de interacción de T con TI. Este enfoque fue usado para identificar el sitio reactivo del SI de caraotas negras como un enlace ALA-LEU(ILE); se encontró,

[1] This paper is dedicated to Dr. Werner G. Jaffé and was presented at a symposium organized to celebrate his 80th birthday. It covers part of the work I had the privilege and the pleasure to share with the Maestro in the last 30 years.

SI react with S by the «standard mechanism» proposed for the interaction of T with TI, and their reactive sites are split and resynthesized as in TI. This latter method was used to identify the reactive site of black bean SI against S as an ALA-LEU(ILE) bond and a LYS-VAL bond split by TPCK-trypsin, the target enzyme of which is still unknown. Structural differences among the SI isolated from different legume species were suggested by variations in specific activities, in stability under denaturing conditions and mainly in immuno-chemical assays.
The nutritional significance of bean TI was focused on by developing alternative methods to detect their toxicity.

además, un enlace LYS-VAL roto por la TPCK-tripsina, cuya enzima blanco se desconoce. La variación en la actividad específica, su estabilidad frente a agentes desnaturalizantes y los resultados de pruebas inmunoquímicas sugieren diferencias estructurales entre los SI aislados de varias leguminosas.
La significación nutricional de los TI de caraotas fue ensayada usando métodos alternativos a fin de determinar su toxicidad.

For enzymes to interact with substrates structural compatibility is a necessary requirement. Proteases degrade proteins into peptides according to the specificity of the enzyme. Natural protease inhibitors are proteins whose structure is recognized but not degraded by the enzyme. They form complexes with the latter, blocking it from interacting with substrate molecules.

This paper is limited to the discussion of the relations of two animal proteolytic enzymes: trypsin (T) and chymotrypsin (CHT) and a bacterial one: subtilisin (S), with their natural inhibitors isolated in our laboratory from legume seeds.

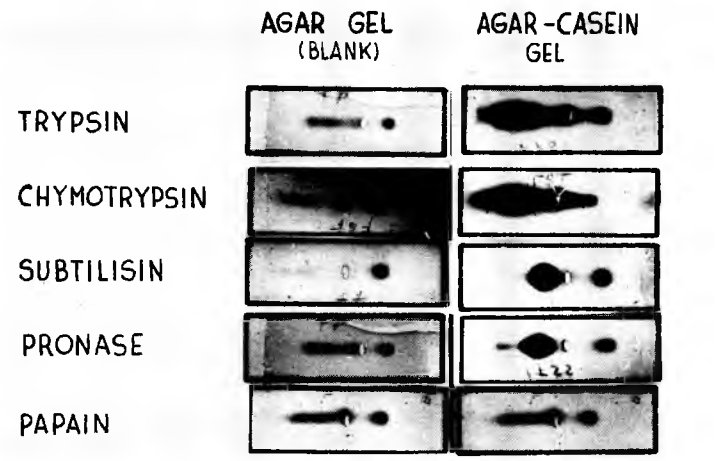
In the sixties, Osawa and Laskowski demonstrated that the interaction of T with the Kunitz soybean inhibitor and with chicken ovomucoide involves the cleavage of a single peptide bond of the arg-x or lys-x type, which affects their activity. Based on these initial observations the authors proposed that one of these bonds on the molecular surface of the natural inhibitors constitutes the site of interaction with the enzyme [1].

Both method and the «reaction mechanism» hypothesis started many of the researchers assisting at this meeting, to look into the molecular interaction of proteases with their inhibitors, and into the study of the reactive sites of those isolated from soybeans (*Glycine max*), black and garden beans (*Phaseolus vulgaris*), broad beans (*Vicia faba*), jack beans (*Canavalia ensiformis*), etc.

In our laboratory, at the Universidad Central de Venezuela, the research efforts concentrated around the study of those heat-labile toxic factors from black beans (*Phaseolus vulgaris*), that inhibit proteolytic enzymes. Seven T inhibitory fractions were detected in aqueous extracts of the cv Cubagua on disc gel electrophoresis. It was necessary to elucidate whether these activities were due to a mixture of inhibitors originating from different seeds or to isoinhibitors, the genetic expression of each single seed. In order, to find the correct answer the development of a micro-method capable of detecting inhibitors in single seeds was required.

At that time, Eglis González, Dr. Jaffé's student, and Antonio Callejas, the best technician we ever had, started to screen 12 legume species for proteolytic enzyme inhibitors [2]. The method used consisted of electrophoretic separation of seed protein extracts on agar-casein gel plates. The gels were then covered with filter paper slips soaked in a protease solution, which during incubation digested casein, except in the areas where inhibitor(s) blocked degradation. These spots became visible after coloring with amido black (figure 1).

FIGURE 1
Electrophoretic and inhibition patterns of protease inhibitors on agar gel plates



The method was then adjusted to the microgram scale and the bean lots were analyzed seed by seed. The results inferred that the lots were homogeneous and the T inhibitors (TI) in multiple forms were present in each and every seed [3].

The screening of 63 varieties of Phaseolus vulgaris showed the omnipresence of TI. However, their migration and inhibition patterns varied greatly within the species (figure 2), (2).

Six isoinhibitors were purified to electrophoretic homogeneity from cv cubagua their specificity spectra (table I), amino acid composition (table II) and active sites against T and CHT were determined. All inhibitors showed to be double headed, having independent reactive sites. Their binary complexes with either T or CHT and a ternary complex with both enzymes were identified on acetate cellulose membrane after electrophoresis (figure 3).

FIGURE 2
Variability of protease inhibitors in single *Phaseolus vulgaris* seeds

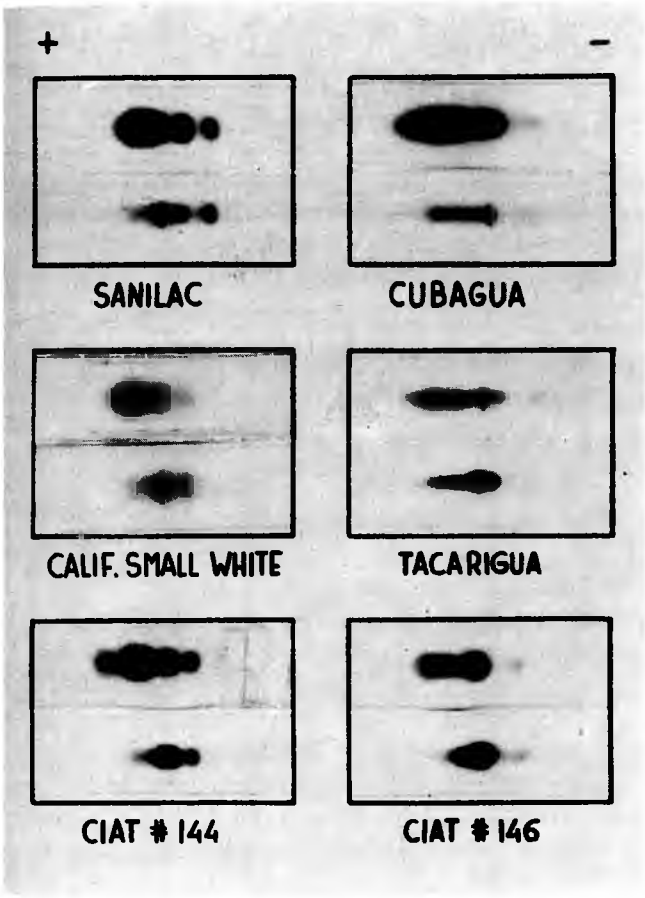


FIGURE 3
Acetate cellulose electrophoresis of black bean trypsin inhibitor (ITC_1) complexes with trypsin (lanes 3 and 4), chymotrypsin (lane 7) and both enzymes (lanes 5 and 6)

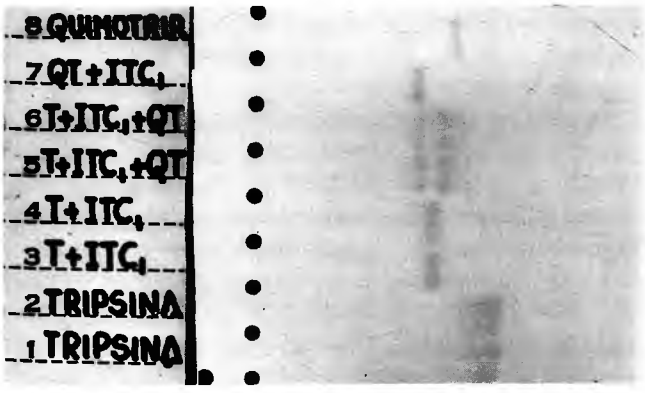


TABLE I
SPECIFICITY SPECTRUM OF BLACK BEAN ISOINHIBITORS

INHIBITOR #	ug I*/nmol T	ug I*/nmol CHT	ug I*/nmol HLE	ug I*/nmol BPE
I	14.5	64.0	14.1	16.8
II	12.0	11.0	23.3	85.0
III	7.1	7.8	12.5	47.5
IV	3.0	9.2	9.2	11.0
V	4.5	8.3	14.6	26.0
VI	4.2	8.9	14.6	26.0

* Micrograms of inhibitor required to inhibit 1 ug of the indicated enzyme. BAPNA was used as substrate for trypsin (T), GPNA for chymotrypsin (CHT) and Succ-(ala)3-p-NA for human leucocyte elastase (HLE) and bovine pancreatic elastase (BPE).

TABLE II
AMINO ACID COMPOSITION OF BLACK BEAN ISOINHIBITORS

AMINO ACID	INHIBITOR					
	I	II	III	IV	V	VI
LYS	5	5	3	3	3	3
HIS	2	3	2	2	2	2
ARG	3	2	2	2	2	2
ASP	7	8	6	7	7	7
THR	7	5	3	4	3	4
SER	13	12	8	8	7	8
GLU	8	8	5	5	5	5
PRO	6	6	5	4	4	4
GLY	10	5	1	3	1	1
ALA	5	5	3	3	3	2
CYS	4	8	8	8	8	10
VAL	2	1	-	1	-	-
MET	1	-	-	-	-	-
ILE	3	3	3	2	2	3
LEU	4	3	2	2	2	2
TYR	2	2	1	2	1	2
PHE						
TOTAL	83	78	54	57	51	55

During the isolation of TI from black beans, Selma Olivares and later Fanny Locker, two of my thesis students, found in the eluate of the bean extract, on an ion exchange column a low

molecular weight protein fraction that inhibited subtilisin, but recognized neither T, CHT nor pancreatic elastase [4]. The subtilisin inhibitor (SI) activity was also detected on agar-casein gel electrophoresis in the 63 varieties of phaseolus vulgaris mentioned earlier, as well as in broad beans (*Vicia faba*), chick peas (*Cicer arietinum*), jack beans (*Canavalia ensiformis*), kidney beans (*Vigna aureus*),etc., It was absent though, in soybeans (*Glycine max*), lentils (*Lens culinaris*), green peas (*Pisum sativum*), cowpea (*Vigna sinensis*) and lupine seeds (*Lupinus sp*)[2].

The first SI was isolated from black beans (cv. Cubagua) in collaboration with Hugo Abreu. It contains 82 amino acids, has two disulfide bridges and a Mr of 9KD. Its stability in the presence of denaturing agents is similar to that of TI from legumes [4].

The inhibitors purified later on, with Juscelino Tovar, Pilar Lorenzo and Elena Pinelli, from chick peas, broad beans and jack beans have similar molecular weights and show the same characteristic stability with only slight variations. For example, the chick pea inhibitor is the only one denatured by 5% TCA at room temperature, while all others are resistant [5,6].

We named the above inhibitors SI because commercially available subtilisin Carlsberg was used to detect and estimate them, just as bovine T is used in the TI assays. It is clear though, that in neither case the enzyme is necessarily the principal target of the inhibitor.

SI studied in our laboratory are rather specific toward bacterial serine proteases. In addition to subtilisins, Carlsberg and BPN', they inhibit the alkaline protease from *Tritirachium album* (Protease K), that of *Aspergillus oryzae* and one isolated from *Streptomyces griseus*. Inhibition level varies, however, from one seed species to the other indicating structural differences between the inhibitors. These apparently do not interfere with enzyme recognition, but can affect negatively the stability of the enzyme-inhibitor complex. It could cause an increase in the dissociation constant of the complex and in the amount of inhibitor required for complete inhibition [7].

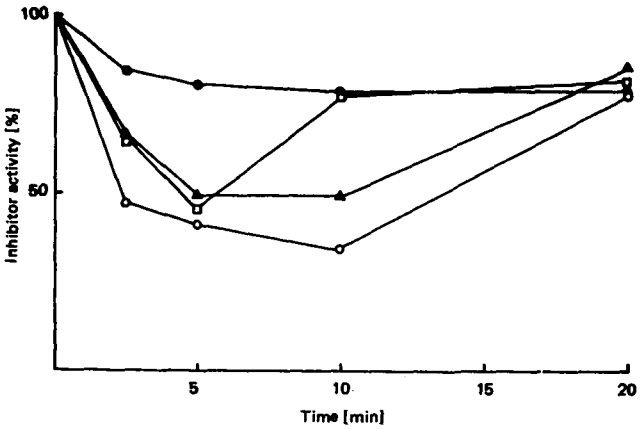
An unusual feature was detected in the chick pea SI, namely that it recognizes the structural differences existing between subtilisin Carlsberg and BPN'. It inhibits the former six times stronger, probably due to the higher dissociation constant of the BPN'-SI complex [5].

Specificity studies of the inhibitors indicate that no interactions occur with either CHT, or plant thiol enzymes, or microbial acid proteases. Their capacity to inhibit T was slight or not detectable [6]. However, a «silent» reactive site against T was found to be present in the black bean SI [8].

Using the Laskowski method for the identification of active sites on inhibitor surfaces, it was shown that the «anti-subtilisin» site of the black bean inhibitor is ala-leu(ile) bond. When SI is modified (SIs) by selective hydrolysis of this bond it loses activity, which is restored upon resynthesis [8]. Figure 4 depicts the modification of four inhibitors by S as a function of incubation time. Activity decreases at the begining of the period and tends to increase, reaching an equilibrium at about 80% of the original value after 20 min. This pattern suggests that SI react with S according to the «standard reaction mechanism» proposed by Osawa and Laskowski [1] for the interaction of T with its natural inhibitors.

FIGURE 4

Reactive site modification of subtilisin inhibitors. *Phaseolus vulgaris* (●), *Cicer arietinum* (Δ), *Canavalia ensiformis* (○) and *Vicia faba* (□). Cleavage of the active site was performed under the following conditions: Subtilisin: inhibitor molar ratio 1:1, pH 8 and dissociation of the complex in 15% acetic acid.



In order to be able to compare the splitting of the active site bond the original method had to be modified. Instead of catalytic amounts of S, equimolar enzyme:inhibitor ratio was applied, and the acid incubation medium was changed to pH 8. Under these conditions the bond on all four inhibitors was split and resynthesized. Due probably to structural differences, modification with S of the chick pea SI occurs only at neutral or alkaline pH, while that of black bean preferably in acid solution; those from broad beans and jack beans react at both pH values.

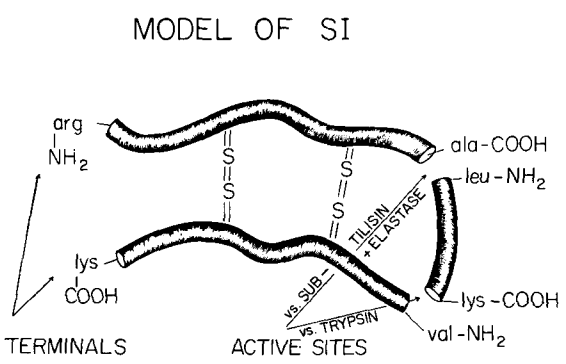
Two interesting molecular features of the black bean SI should be recalled. Although it does not inhibit pancreatic elastase, it interacts with human leucocyte elastase (HLE) in a 1:1 molar ratio. This inhibition is reverted by adding increasing amounts of S to the incubation mixture, suggesting that both enzymes compete for the same reactive site of SI [8].

The second unusual property was found when the black bean SI was incubated with catalytic amounts of TPCK-trypsin and a lys-val bond was split. The T modified SI (SIt) loses some S inhibitory activity. However, when the new lys terminal is cleaved by carboxypeptidase B, activity is abolished. Considering the fact that black bean SI shows only a very slight activity against T, it was concluded that the molecule possesses a «silent» reactive site recognized by T [8].

It is impossible to conclude whether the two reactive sites of the black bean SI overlap or are independent, for the

«target» enzyme for the «trypsin-site» has not yet been found. They might be close to one another, because cleavage of lys from the «trypsin-site» inactivates interaction with S. Alternately, lys may be important for the formation of the S-SI complex, or else due to the presence of only 2 disulfide bonds per molecule, as compared to 7 in the Bowman-Birk inhibitor, conformational changes occur upon lys cleavage causing the observed inactivation. Based on these data a structural model for the black bean SI has been proposed (figure 5).

FIGURE 5
Hypothetical model of black bean subtilisin inhibitor, indicating active sites, terminals and disulfide bonds



When the four SI inhibitors isolated from different legume species were further compared, immunochemical differences were detected. Antibodies raised against jack bean SI, which recognized SI from other species and varieties of the Canavalia genus, did not interact with the inhibitors purified from either black beans, broad beans or chick peas.

In spite of their structural differences and based on similarities in specificity, «reaction mechanism», heat stability and molecular weight, we suggested the possibility of classifying the SI from legume seeds into a separate «family» of protease inhibitors [6].

In order to determine the effect of an antinutritional factor in a certain animal, quantities in the order of grams are required. As the amounts of inhibitor present per 100 g seed are on the mg scale, we looked at alternative methods able to predict possible toxic effects on an organism. Based on the hypothesis that the relative sensitivity of a pancreatic protease of an animal to an inhibitor can affect the entire digestive process, we compared the activity of 7 inhibitor preparations on T and CHT from 12 animal species, including bovine and human [9].

Interactions varied widely from one species of animal to another, as well as from one seed species to another. From our in vitro results we could not predict the in vivo nutritional effect of the TI tested. However, it was clearly shown that assessing TI content of a foodstuff with bovine T is an unwise practice, for what can look like low activity with this enzyme may affect seriously the digestive process of an animal from another species fed the TI containing diet [9].

The other alternative for the study of antinutritional effects is the use of small model organisms requiring minute amounts of a purified inhibitor. Several years ago the thesis of J. Szwarcbort, established a correlation between the toxicity of black bean proteins in mice and in rice weevils (*Sitophylus oryzae*). Recently we used this system to determine the toxicity of different antinutritional factors in jack bean flour [10].

The half-life of young adult weevils, on artificial seeds made from peeled dry pea flour, is 183 days, as compared to 5.3 days on jack bean flour seeds and about 10 days without food. Surprisingly, the cooking of the jack bean did not improve survival, suggesting that a synergistic effect of the different toxic factors causes the premature death of the insects.

The addition of TI to the pea meal, in a concentration equivalent to the one in the jack bean seed, caused only a very slight decrease in survival, probably due to an excess of nutrients in the basic pea diet [10].

This hypothesis is supported by the fact that adults have a low amino acid requirement, allowing survival in defatted, deproteinated corn starch. Addition of casein to this basal diet, however, produces an increase in the insect mean body weight. This might be hindered by TI. At the present, the characteristics of the minimal basic diet are being determined, in order to start a systematic study of the effect of purified antinutritional factors even when available only in small amounts.

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