Digestibility and Proteinase Inhibitory Action of a Kidney Bean Globulin

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A globulin fraction, isolated from black beans (*Phaseolus vulgaris*), was resistant to hydrolysis by pepsin, trypsin, chymotrypsin, papain, ficin, hurain, and subtilisin. After denaturation of the globulin by heat or urea, only slight hydrolysis by enzymes could be detected. The activity of all seven proteinases on their respective substrates was inhibited by the bean globulin. Preincubation of enzyme and globulin enhanced the inhibitory effect. When heat denatured, insoluble globulin was stirred with papain, proteolytic activity was diminished. The name globulin proteinase inhibitor is proposed for this factor. Adsorption with bentonite and celite of a crude bean extract eliminated the specific trypsin inhibitor activity but not that of the unspecific proteinase inhibitor. Trypsin inhibitor and proteinase inhibitor activities could also be separated by dialysis against distilled water, the former remaining in solution while the latter precipitated. The possible nutritional significances of these observations are discussed.

The low digestibility of raw beans (*Phaseolus vulgaris*) was observed in animal tests many years ago (Johns and Finks, 1920). The detection of specific trypsin inhibitors in beans (Bowman, 1944) seemed to offer a plausible explanation, but later investigations shed doubt on the role of this factor (Pusztai, 1967). When Jaffé and Hannig (1965) studied some purified bean protein fractions, they found several to be very resistant toward the hydrolytic activity of pepsin and papain, an observation that could not be explained by the existence of the trypsin inhibitor.

In the present investigation, the authors tried to gain more detailed insight into the relation between digestibility and enzyme inhibition of bean proteins.

EXPERIMENTAL

Separation from Trypsin Inhibitor. Black kidney beans (*Phaseolus vulgaris*) of the variety Cubagua were used. Extracts were prepared by suspending 10 grams of finely ground seeds in 100 ml. of 1% NaCl solution and stirring at 4° C. for 24 hours. After centrifugation, protein concentration was determined in the supernatant by the method of Lowry (1951) and was adjusted to 1.5% for the experiments on removal of the trypsin inhibitor by the method of Kunitz (1946). Two milligrams of a 1 to 1 mixture of bentonite (U.S.P.) and celite (Johns-Manville Corp.) were suspended in each milliliter of the extract previously adjusted to pH 4 with hydrochloric acid. After being mechanically stirred for 30 minutes, the extract was filtered and the treatment repeated after the protein concentration and the trypsin inhibitor activity had been determined.

Preparation of Bean Globulin. A bean globulin fraction was prepared according to Goa and Strid (1959) by dissolving the water-insoluble proteins of the original salt extract of bean meal in 0.1*M* phosphate buffer at pH 7.0 with 0.5*M* NaCl, and dialyzing against 0.1*M* acetate buffer, pH 4.1, with 0.2*M* NaCl. The precipitate was discarded and the solution dialyzed against distilled water. The precipitated globulins were treated once again in the same way and finally dryed by lyophylization. This fraction, called *E*, amounted to about 30% of the extractable proteins. It is soluble in 0.5*M* NaCl. It is not completely homogeneous, but can be purified further by free flow electrophoresis (Jaffé and Hannig, 1965). The preparation thus obtained shows a single band in paper and immunoelectrophoresis and was used for verification of results previously obtained with the less pure fraction.

Enzyme Activity Determination. The following enzymes were used: trypsin, 3800 N.F. unit per mg.; pepsin crystallized × 3; papain crude; bromelin, 1200 gelatin digesting units; ficin crude; subtilisin; hurain (Seidl and Gaede, 1961). The substrates are indicated in Tables I–IV.

For measuring the activity of trypsin, papain, ficin, bromelin, subtilisin, and hurain on fraction *E* (Table V), a 1% solution of the substrate in phosphate buffer pH 7, containing 0.5*M* NaCl, was used. Pepsin activity was measured on a 1% hemoglobin solution in 0.06*N* HCl and 0.5*M* NaCl. Digestion time was 18 hours at 25° C. Optical density of the trichloroacetic acid (TCA) soluble products was read at 280 mμ in a Zeiss PMQ II spectrophotometer. Enzyme concentration was adjusted in each case so that with 1% casein as substrate the optical density of the TCA filtrate would be 0.500 ± 0.020 after 30 minutes of incubation. Incubation time with papain was 10 minutes.

For protein denaturation, the method of Anson (1938) was applied. Final urea concentration was 6.6*M* and protein concentration 1%. Heat denaturation was performed by boiling a 1% solution in 0.1*M* phosphate buffer pH 7, containing 0.5*M* NaCl for 60 minutes in a water bath. All digestion experiments of heat denatured protein were performed with magnetic stirring.

Enzyme Inhibition. Determination of enzyme inhibitor activity was performed with a modification of Kunitz's method (1947), using 0.1 ml. of enzyme solution, 0.9 of a suitable bean extract or solution of fraction *E*, and 3 ml. of 1%

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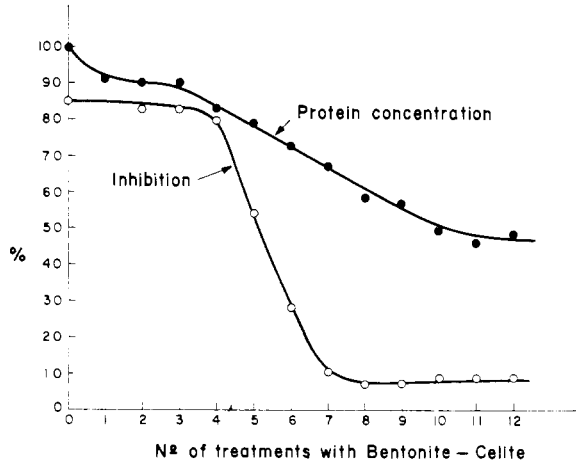

Figure 1. Effect of repeated treatments with bentonite and celite mixture on protein concentration and trypsin inhibitor activity of a bean extract

Original protein content of bean extract was 1.5%. After each treatment, protein and trypsin inhibitor were determined in aliquots by methods described in the text. Inhibition is expressed as per cent of the activity reduction of 75 μg. of trypsin on casein by 0.6 mg. of soluble bean proteins

casein solution at pH 7.0. If not otherwise stated, enzyme and inhibitor were incubated for 10 minutes before the addition of the substrate. After incubation for 30 minutes at 25° C. (10 minutes for papain), 2 ml. of 20% TCA solution were added and the UV absorption of the filtrates read at 280 mμ. To check the inhibition results, the following alternative methods were used with trypsin and papain: formol titration with casein digestion according to Soerensen (1907) and hydrolysis of *N*-α-tosyl-L-arginine amide (TSAA) (Schwert *et al.* 1948). For quantitative comparison of the inhibition of the different enzymes 6 mg. of fraction *E* in 4 ml. of enzyme substrate solution were used.

The degree of inhibition was defined by comparison with the enzyme activity in absence of the inhibitor; this was considered to be 100%, and hydrolysis obtained in the presence of the inhibitor was calculated as a fraction of this value.

For the experiment of Table IV, a solution of 6.5 mg. per ml. of fraction *E* was heat treated as described above. Part of the solution was used without further treatment (*A*) and part was centrifuged. The clear supernatant was called *B*-1 and the precipitate was resuspended in the original volume of buffer and called *B*-2. Ten milligrams of crude papain in 0.1 ml. of $5 \times 10^{-3} M$ KCN solution were incubated with 0.9 ml. of solutions *A*, *B*-1, *B*-2, or buffer, respectively. After 15 minutes they were centrifuged, and the clear supernatants were added to casein substrate and incubated for 10 minutes for the determination of the remaining papain activity.

RESULTS

Separation from Trypsin Inhibitor. The first three treatments of kidney bean extracts with bentonite-celite resulted in a slight reduction of the protein content without decrease in trypsin inhibitor activity. Between the fourth and seventh treatments, this activity was reduced to a residual value which remained constant after five more adsorptions, although the protein content was further diminished (Figure 1). This residual activity was called proteinase inhibitor because its action could be demonstrated on all enzymes studied. Trypsin inhibitor and proteinase inhibitor activities could also be separated by dialysis against distilled water, the former remaining in solution while the latter was detected in the globulin fraction *E* (Table I).

Table I. Inhibitory Effect of Crude Extracts, Water Soluble Proteins, and Purified Globulin Fraction *E* from Kidney Beans on Casein Digestion by Papain and Trypsin

Enzyme	Bean Fraction	Protein, mg.	Inhibition, %
Papain	Crude extract in 1% NaCl	6.40	21
	Water sol. proteins	10.00	0
	Fraction *E*	4.00	30
Trypsin	Crude extract in 1% NaCl	0.30	30
	Water sol. proteins	0.14	30
	Fraction *E*	4.00	36

Table II. Enzyme Inhibition by Bean Globulin *E* Measured with Different Methods

Substrate	Enzyme	Method	Globulin *E* Added, Mg.	Activity, %
Casein	Trypsin	Absorption at 280 mμ	4	65
Casein	Trypsin	Formol titration	4	57
TSAA	Trypsin	NH_3 microdiffusion	4	65
Egg albumin	Papain	Absorption at 280 mμ	4	68
Casein	Papain	Absorption at 280 mμ	4	63

Table III. Effect of Preincubation of Proteolytic Enzymes and Bean Globulin Fraction *E* on Inhibition[a]

Enzyme	Additions	Activity, %
Papain	Casein	100
Papain	Casein and fraction *E* simultaneously	88
Papain	Fraction *E* and 10 min. later casein	61
Pepsin	Hemoglobin	100
Pepsin	Hemoglobin and fraction *E* simultaneously	92
Pepsin	Fraction *E* and 10 min. later hemoglobin	72

[a] Concentration of inhibitor was 6 mg. in final volume.

Proteinase Inhibition. The globulin fraction *E* inhibited the hydrolytic activity to a similar degree when measured with different substrates. Casein digestions determined by UV absorption of the TCA soluble portion, by formol titration, or by TSAA hydrolysis were all reduced to a similar extent. The same was true for the cleavage of casein or urea denatured egg albumin (Table II).

All seven proteinases studied were inhibited by the bean globulin. Ficin and bromelin were most susceptible to inhibition. Their activity was reduced to 40% of their respective original values. The activities of trypsin, papain, and hurain were reduced to 60% of the hydrolytic action in absence of the inhibitor. Subtilisin, with a mere 15% and pepsin with 24% inhibition were the least affected by fraction *E*.

The degree of inhibition was markedly greater when the respective enzyme was preincubated with the inhibitor prior to the addition of the substrate, than when inhibitor and substrate were added simultaneously to the enzyme solution, as shown by the results presented in Table III.

Table IV. Action of Boiled Bean Globulin *E* on Papain Activity[a]

15 Min. Preincubation with	Activity, %
Buffer	100
Boiled fraction *E* (*A*)	68
Precipitate from boiled fraction *E* (*B*-1)	63
Supernatant (*B*-2)	96

[a] Ten mg. of crude papain in 0.1 ml. of KCN solution were incubated with 0.9 ml. of the respective solutions or suspensions prepared as explained in the text, centrifuged, and the proteolytic activity in the supernatants was tested with casein as substrate.

The high protein concentration of the inhibition experiments resulted in a somewhat higher viscosity of the solution. In a separate assay, it was shown that this did not affect the results, as the proteolysis of casein with papain was not reduced in the presence of polyvinylpyrrolidon in a highly viscose solution.

Heat denatured fraction *E* retained the inhibitory activity. This was demonstrated by incubating a papain solution with a suspension of boiled globulin. After centrifugation, the enzyme activity in the supernatant had diminished considerably when tested against casein as substrate (Table IV).

Digestibility of Bean Globulin. Native fraction *E* was very resistant to the digestion by all the enzymes studied. Heat denaturation caused the susceptibility to increase slightly, but only in the case of urea denatured fraction *E* digested by pepsin or hurain, a significant hydrolytic action was evident which was still small compared to casein digestion (Table V).

DISCUSSION

Resistance to Proteolysis. The present results show that the bean globulin is resistant to the action of all of the proteases studied and moreover that it inhibits the activity of all these enzymes toward their substrates. The resistance of the globulin toward the hydrolytic action of the enzymes was remarkable and most pronounced toward tryptic digestion, which was not enhanced at all after denaturation (Table V). In this context it may be recalled that the arginine and the lysine contents of this protein is normal (Jaffé and Hannig, 1965).

Comparison of Fraction E and Trypsin Inhibitor. The unspecific inhibitory activity of fraction *E* in contrast to that of the specific trypsin inhibitor, the fact that it was not eliminated from a bean extract by repeated treatment with bentonite-celite which abolished the specific trypsin inhibitor effect, and that the former precipitated from a salt-free solution together with the globulins, while the latter remained in solution, can all be taken as evidence for the existence of two different inhibitory factors. The term globulin proteinase inhibitor is proposed to distinguish it from the classical trypsin inhibitor and from the inhibitor of Pusztai (1968).

The proteinase inhibitor is much less active on a weight basis than the trypsin inhibitor. In the experiments, the results of which are presented in Table I, 0.3 mg. of the protein in the crude bean extract containing both the trypsin and the proteinase inhibitors, reduced the proteolytic activity of trypsin nearly as much as 4 mg. of globulin fraction *E*. Papain, however, was not significantly inhibited by the former, indicating that it did not contain a more active proteinase inhibitor. The low activity of the proteinase inhibitor compared to that of the trypsin inhibitor is probably the reason that it escaped attention until now.

The insoluble heat denatured globulin inhibits papain probably by adsorption (Table IV). This may indicate that the inhibitory effect is due to the formation of a rather stable complex between the enzyme and the globulin.

Other Bean Proteinase Inhibitors. A few published reports are related to the present observations. Abramova and Chernikow (1964) mention an inhibitory activity of kidney bean globulins on trypsin and chymotrypsin. A protease inhibitor active on trypsin, chymotrypsin, elastin, and plasmin but inactive on pepsin, papain, and subtilisin has been isolated from kidney beans by Pusztai (1968). It was a small protein with a molecular weight of about 10,000 and is therefore quite different from our inhibitor. High resistance of the proteins from field beans (*Dolichos lablab*) (Phadke and Sohonie, 1961) and of double beans (*Phaseolus lunatus*) to digestion by pepsin and trypsin were reported by Inamdar and Sohonie (1961). Heat denaturation enhanced the attack of trypsin very little but made the bean proteins more susceptible to the combined action of pepsin and trypsin. Matsubara *et al.* observed that lima bean proteins inhibit the proteolytic action of subtilisin (1958).

Nutritional Significance. Nitrogen retention in rats fed raw kidney beans is very low (Kakade and Evans, 1966). The explanation of this fact is difficult, because of the simultaneous presence in the beans of trypsin inhibitors, hemagglutinins, and possibly of other, unrecognized factors which probably interfere with amino acid absorption (Liener, 1962). Even after proper heating the apparent protein digestibility of black beans is rather low, although considerably improved over the uncooked beans (Jaffé, 1950). This may be related to the low digestibility of the bean globulin described in this paper.

From the data published by Wagner and Riehm (1967) on the trypsin inhibitor from navy beans, it would appear that its concentration in the crude bean extract is in the order of 2%, while that of globulin fraction *E* is about 30%. This relative abundance and its resistance to the attack of digestive enzymes indicate that the inhibitory activity of this compound on protein digestion may be of a certain importance, notwithstanding its low activity, as compared on a weight basis with trypsin inhibitor.

Recent observations of Jaffé and Vega (1968) point to the existence of an inhibitory effect of raw beans on intestinal proteolysis not caused by trypsin inhibitor. In assays with young rats kept on a diet containing 40% of raw bean meal

Table V. Hydrolysis of Native and Denatured Globulin Fraction *E* by Proteinases[a]

Bean Globulin	Trypsin	Hurain	Pepsin	Papain	Ficin	Bromelin	Subtilisin
Native	40	60	350	100	130	20	90
Urea denatured	50	≫2000	≫2000	1345	1200	1250	1700
Heat denatured	60	560	850	730	1125	900	1400

[a] Values are expressed in optical density × 10^3 at 280 mμ of TCA filtrates after 18-hr. incubation at 25° C. at the proper pH. Corresponding values for casein digestion would be about 18,000. Quantities of each enzyme used were chosen to produce similar action on casein as described in the text.

of a cultivar with very low specific trypsin inhibitor and hemagglutinin activity, growth and nitrogen retention were low as compared to the control animals fed the corresponding ration prepared with heated beans. A casein supplement did not improve weight gain, but supplementation with digested casein did. The low trypsin inhibitor contents of the beans used and the fact that trypsin inhibitor does not interfere significantly with *in vivo* digestion in rats (Pusztai, 1967) ruled it out as a possible cause. The globulin proteinase inhibitor described in the present paper may offer an explanation.

The most abundant protein in kidney beans is a globulin soluble only in NaCl solutions of high concentration (Danielsson, 1950). It would be interesting to know whether it has inhibitory characteristics similar to those studied in the present paper. Both globulins account for about 80% of bean proteins and therefore could well exert a significant action on intestinal digestion.

Characterization of Globulin Fraction *E*. Fraction *E* is one of the legume globulins studied by Danielsson (1950), Goa and Strid (1959), and others. Its amino acid composition and the sugars it contains have been described by Jaffé and Hannig (1965). Its molecular weight is about 200.000 (Camejo, 1961) and varies with the pH and the concentration of the solution. After 12 to 20 washings with distilled water, it goes into solution and may be precipitated by bi- or trivalent metal ions (Jaffé and Hannig 1965). Fraction *E* and the crystalline bean protein of Bourdillon (1949) cannot be distinguished by immunological methods (Palozzo and Jaffé, 1969). However, three of the nineteen peptides, detected by paper chromatography and electrophoresis of the pepsin digests of both proteins, are different (Valbuena, 1967). The globulins from different bean varieties or cultivars may differ from each other in physicochemical characteristics (Jourbet, 1957) and in immunochemical properties (Palozzo and Jaffé, 1969).

It will be interesting to explore to what structural features of the bean globulin the resistance to proteolysis may be related. It can not be due to S-S bridges as no cystine has been found in this protein (Jaffé and Hannig, 1965).

We have not yet studied in detail the behavior of other bean proteins when exposed to proteinases. Preliminary observations indicate that most of them seem to be resistant to proteolysis.

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