THE MECHANISM OF THE PROTEOLYTIC INHIBITORS PRESENT IN SOYBEANS

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SUMMARY

1. A mechanism for the inhibitor action has been advanced. The inhibitor has been shown to act as a promoter to the enzyme trypsin in bringing about a reversal of proteolysis, from the intermediate products of protein break down.

2. The rate of release of methionine, lysine and phenylalanine has been studied. These results indicate that the inhibitor affects the availability of all the aminoacids in general, but the rate of release of methionine is affected to a greater extent than others. The implication of this observation has been fully discussed.

The presence of substances exerting inhibitory influence on the utilisation of protein has attracted the attention of several nutritionists in recent years. The isolation of the inhibitor in soybeans was first reported by Ham, *et al.* (1944) and subsequent researches were directed mainly in counteracting the adverse influence of the inhibitor. Thus it was shown by Evans, *et al.* (1946) that autoclaving the raw beans for 30 minutes at 15 lb. pressure resulted in an improvement in its nutritive value. The close association of the availability of sulphur amino acid with the nutritive value of soyprotein was very early recognised. The poor nutritive value of raw bean was shown by Melnick, *et al.* (1948) to be due to the slow rate of liberation of methionine with consequent lack of supplementation of the same with the other amino acids. Desikachar, *et al.* (1950) reported that germination of raw bean was followed by improved methionine availability in spite of the fact that there was no change in the inhibitor concentration during the process of germination.

Though the problem has been studied extensively with regard to its several aspects, very little work has been done on the mode of action of the inhibitor. So far no suitable mechanism arising out of experimental observation has been put forth which could explain all the scientific data accumulated in the field in a satisfactory manner. This investigation was therefore undertaken to throw some light on this aspect of soy inhibitors.

EXPERIMENTAL

Pretryptic Digestion and Influence of Inhibitor.—It was reported by Leiner, *et al.* (1949) and subsequently by Viswanatha and De (1951) that the prepeptic digestion of raw soy protein would result in methionine availability and digestibility equal to that of autoclaved protein. These results were interpreted to mean that proteolytic inhibitors in soybean are only *antiproteinase of trypsin* in nature having no influence on the peptidase activity of the enzyme. This view was later substantiated by further experimental findings (Results under publication).

Similar studies were extended to predigestions with trypsin. Casein was selected as the substrate for this purpose. 1 gm. portions of casein (B.D.H.) was transferred to 100 c.c. conical flask and dissolved in sodium hydroxide-phosphate buffer (pH 8·0). 5 ml. of trypsin solution (containing 100 mg. of enzyme) in the same buffer was added to each flask. Toluene was added to each flask, well corked and placed in an incubator at 37° C. At the end of definite intervals of digestion periods, inhibitor extract at the same pH was added and its influence studied. Suitable controls, one without any inhibitor and one with the inhibitor present from the commencement of proteolysis were run. Blank for inhibitor sample-enzyme reaction was also carried out.

The influence of the inhibitor on the proteolysis was studied by determining the degree of digestion one hour after the addition of the inhibitor. The degree of digestion was measured by formol titration method. The percentage inhibition was calculated by comparing the degree of digestion of the experimental with the control digestion, *i.e.*, without the inhibitor. The results are tabulated in Table I.

TABLE I. *Table showing the Influence of Pretryptic Digestion on the Inhibitor Action*

Period of digestion Hrs.	Inhibition per cent.					
	1 hr.	2 hrs.	3 hrs.	5 hrs.	7 hrs.	24 hrs.
0 hr control inhibition	47·0	47·2	40·8	44·0	41·3	22·9
1 hr. predigestion ..	..	23·6	21·2	22·8	22·0	19·7
2 hrs. ,, ..	..	..	34·4	30·8	24·3	23·1
4 hrs. ,, ..	..	..	..	27·6	23·8	23·0
6 hrs. ,, ..	..	..	..	..	21·5	21·5

Effect of Inhibitor and Enzyme on the Rate of Release of Amino Acids.— Experiments were next planned to determine whether the sulphur amino acids were the only ones to be affected by the inhibitor or whether the other amino acids are also affected as well. For this purpose, the rate of liberation of some essential amino acids, such as Lysine, Methionine and Phenylalanine were studied under varying concentrations of enzyme and the inhibitor.

One gm. portions of casein was weighed into five 100 c.c. conical flasks. To the first three flasks inhibitor sample (at pH 8·0) was added at levels of 5 ml., 10 ml. and 15 ml. respectively. To each of them 10 c.c. of trypsin solution (corresponding to 100 mgm. trypsin) was added. The final volume was made upto 60 c.c. by the addition of buffer (pH 8·0). The other two flasks, had the inhibitor concentration at the same level, 5 ml., but the enzyme concentration was varied (5 ml. and 15 ml.). The final volume was again brought up to 60 c.c. by the addition of buffer.

Suitable controls with and without the inhibitor and blanks for the enzyme-inhibitor reaction were run along with the experiment. The reaction was allowed to proceed at 37° C. after the addition of toluene to each flask and corking them. The experiment was done in duplicates.

The degree of digestion and the rate of release of amino acids were studied at two intervals, namely 2 and 4 hours after digestion. The degree of digestion was recorded by Srensen's Formol method. The amino acids were assayed microbiologically by the method of Bartonwright (1946). The results of the experiment are presented in tables.

Results

The results of pretryptic digestion experiments show that predigestion given to protein has brought down the inhibitory influence. It is interesting to note that one hour pretryptic digestion has resulted in a fall of inhibition as measured one hour after the addition of inhibitor while two hours predigestion records a slight rise in inhibition.

The observations relating to the rate of release of amino acids show that the liberation of all the amino acids is affected by the inhibitor. But a study of the ratios of the rate of release of amino acids indicates that methionine is affected much more than the other two amino acids.

Discussion

Results with pretryptic digestion confirm our previous finding, namely,

TABLE II. *Table showing the Nitrogen, Phenylalanine, Lysine and Methionine Content of Casein*

Protein	Nitrogen %	Lysine %	Methionine %	Phenylalanine
Casein ..	14·6	7·0	2·48	5·4

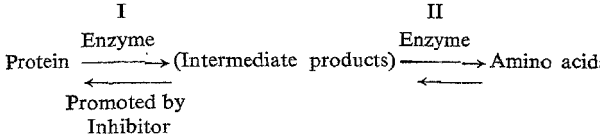
TABLE III. *Table showing the Rate of Reslease of Lysine, Methionine and Phenylalanine with Varying Amounts of Inhibitor and Enzyme*

Sl. No.	Inhibitor concn. c.c.	Enzyme concn. c.c.	Period of Digestion hrs.	% digestion	Lysine Released		Methionine Released		Phenylalanine Released	
					mg.	%	mg.	%	mg.	%
1	5	10	2	11·6	1·5	2·14	1·0	6·4	6·2	11·5
2	10	10	do	10·6	1·2	1·71	1·2	4·8	6·0	11·1
3	15	10	do	9·8	1·0	1·50	0·80	3·0	5·7	10·6
4	5	5	do	8·9	0·9	1·1	1·1	4·4	2·8	5·2
5	5	15	do	17·0	2·5	3·6	3·1	12·4	8·2	15·2
6	0	10	do	20·5	3·1	4·4	6·2	24·8	12·2	22·2
1	5	10	4	17·8	2·2	3·14	3·4	13·6	8·4	15·5
2	10	10	do	16·9	2·0	2·9	2·7	10·8	7·6	14·1
3	15	10	do	15·8	1·8	2·6	1·8	7·2	6·9	13·0
4	5	5	do	11·6	1·6	2·3	1·5	6·0	6·0	10·7
5	5	15	do	24·0	4·0	5·7	5·2	20·8	9·6	17·7
6	0	10	do	30·5	5·0	7·0	10·0	40·0	20·0	37·3

TABLE IV. *Table showing the Ratics of Rate of Release of Phenylalanine Methioniueand Lysine : Methionine Expressed in Terms of Grom Mols.*

Sl. No.	Period of digestion hrs.	Lysine liberated g. mol. × 10^{-4}	Methionine liberated g. mol. × 10^{-4}	Phenylalanine liberated g. mol. × 10^{-4}	Lysine : Methionine	Phenylalanine : Methionine
1	2	0·103	0·117	0·346	8 · 9	3 : 1
2	2	0·082	0·087	0·335	1 : 1	4 : 1
3	2	0·073	0·055	0·210	4 : 3	6 : 1
4	2	0·061	0·08	0·156	3 : 4	2 : 1
5	2	0·171	0·226	0·458	3 : 4	2 : 1
6	2	0·212	0·462	0·610	2 : 1	4 : 1
1	4	0·15	0·250	0·47	3 : 5	9 : 5
2	4	0·137	0·197	0·423	7 : 10	21 : 10
3	4	0·128	0·131	0·391	1 : 1	9 : 3
4	4	0·11	0·109	0·335	1 : 1	3 : 1
5	4	0·274	0·38	0·536	14 : 19	27 : 19
6	4	0·343	0·73	1·120	2 : 1	4 : 3

that the soy inhibitor is only an antiproteinase of trypsin having no effect on the peptidase activity of the enzyme. The slight rise in the inhibition at the end of 2 hours predigestion could be accounted for on the basis that the reverse reaction occurs at a considerably fast rate. The mechanism of the inhibitor could be schematically represented as follows:



The inhibitor being antiproteinase in character inhibits the 1st stage of protein breakdown. This might be brought about by the reversal of forward reaction, *i.e.*, synthesis of protein from the intermediate products by the enzyme under the influence of inhibitor. The inhibitor acts as a promoter to the catalyst, enzyme (trypsin), in bringing about a synthesis of protein from the intermediate products.

This mechanism of inhibitor action accounts for the observed rise in inhibition at the end of 2-hour pretryptic digestion followed by the addition of inhibitor, inhibition being measured one hour after the addition of the inhibitor. It may be stated that one hour pretryptic digestion results in the breakdown to a certain extent of the protein into intermediate products. So the inhibition is reduced considerably since inhibitor being an antiprotenase in nature does not inhibit further digestion of these products. The reverse reaction will also be taking place but not to an appreciable degree since the concentration of intermediate products will not be much at the end of 1-hour digestion. At the end of 2-hour digestion, the concentration of intermediate products would have considerably increased and hence the backward reaction proceeds faster than in the previous case and subsequent increase in inhibition is noticed. In the case of longer predigestion periods, the concentration of intermediate products of protein digestion is very large, the backward reaction, *i.e.*, synthesis of protein and forward reaction, namely, breakdown of these intermediate products into amino acids would take place together, the equilibrium gradually shifting towards the forward direction. Hence there will be a gradual decline in inhibition with increasing periods of predigestion.

This mechanism for inhibitor action is substantiated by the fact that inhibitor-enzyme reaction is not one of competitive nature but an equilibrium reaction, an observation made by Bcrchers, *et al.* (1947).

The above mechanism for inhibitor action would account for all the data collected so far in the field, like the favourable effect of prepeptic digestion, germination, etc. Leiner, *et al.* (1949) observed during their *in vitro* studies that methionine availability and digestibility of raw soybeans were improved by prepeptic digestions. But the *in vivo* experiments were not in agreement with the above results. In the case of animals fed raw soyprotein, though there is prepeptic digestion in the stomach before being acted upon by trypsin in the intestines, methionine is not available to the body. To account for this anomaly Leiner, *et al.* (1949) assumed the presence of a new factor similar to the inhibitor but not responding to prepeptic digestion, and having adverse effect on the utilisation of soyprotein. So far the isolation of such a factor has not been reported. These differences between *in vivo* and *in vitro* findings could be easily explained on the basis of the above mechanism in the following manner. The intermediate products of protein digestion, *e.g.*, peptides, polypeptides, etc., are converted back into protein stage by the enzyme under the influence of the inhibitor, in the body. This "make and break" of proteins continues with the gradual shifting of the equilibrium towards the forward reaction with the result there would be a delayed release of amino acids. This delayed release and consequent inadequate supplementation of amino acids would render the whole protein unavailable for tissue protein synthesis.

The rate of release of amino acids studied in the present experiment indicates that the liberation of all the amino acids is adversely affected by the inhibitor, *i.e.*, the inhibition is of general nature. But the ratios of rate of release of phenylalanine : methionine, and lysine : methionine indicate that methionine release is affected to a somewhat greater extent than the other amino acids. It is quite probable that methionine peptides find a preference over the other peptides in the above inhibitor mechanism. In this connection mention can be made of the observation made by Almquist, *et al.* (1951) that the inhibitor mechanism is of general nature. The amino acids present in marginal level are affected further by the inhibitor, with the result an amino acid deficiency is created with respect to such amino acids. So the inhibitor may be said to affect the availability of all the amino acids, the effect being more acute in the case of amino acids present in marginal and suboptimal amounts. It is quite likely that the peptides of these marginally present amino acids are mostly converted back into protein stage. So the close association of sulphur amino acids with the inhibitor function is only a chance finding since methionine is the limiting amino acid in most of the leguminous proteins and also in the sample of casein used in this experiment. Further work on these lines is under progress.

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