THE COMPARATIVE RESISTANCE TO PEPSIN OF SIX NATURALLY OCCURRING TRYP SIN INHIBITORS*

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(Received for publication, June 28, 1955)

The wide-spread distribution of trypsin inhibitors throughout the vegetable and animal kingdoms (1) makes elucidation of their physiological function of great interest. From this point of view, resistance to peptic digestion becomes an important property, since only an inhibitor resistant to pepsin is likely to produce any physiological effect when ingested by a normal animal.

The soy bean inhibitor is the only inhibitor which has been studied in detail. Kunitz (2, 3) investigated the digestion of native and heat-denatured soy bean trypsin inhibitor by 0.01 per cent pepsin at pH 3 and pH 2 at 30°. The denatured inhibitor was rapidly digested at both pH values. The native inhibitor remained totally active after 7 hours exposure to pepsin at pH 3, whereas, at pH 2, it was digested at an almost constant initial rate, about 40 per cent of the inhibiting activity being lost after 150 minutes incubation. While rate measurements have not been made with other trypsin inhibitors, Fraenkel-Conrat et al. (4) incubated solutions of inhibitor preparations with 0.0025 per cent pepsin in 0.01 N HCl at 40° for 24 hours, and found that the amorphous Lima bean inhibitor retained 93 per cent of the original activity, soy bean inhibitor 1 per cent, and ovo-mucoid 2 per cent. Incubation with acid alone gave values of 100, 55, and 100 per cent activity retained, respectively.

The present paper describes a study of the resistance to peptic digestion of six inhibitors, four of which have not been investigated before.

EXPERIMENTAL

Materials—Three samples of crystalline trypsin were used, two prepared by the method of Kunitz and Northrop (5) as modified by McDonald and Kunitz (6), and one prepared from trypsin-inhibitor complex after dissociation of the complex with trichloroacetic acid (5). The pepsin was a commercial preparation (Armour), which was stated to contain 13.5 per cent nitrogen and 0.22 proteolytic unit per mg. of nitrogen. A check of

* Aided by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.
the activity by the hemoglobin digestion method (7) indicated 0.17 unit per mg. of nitrogen.

The six inhibitors studied have been described previously: partially purified bovine blood plasma inhibitor (8), crystalline Kazal's inhibitor from pancreas (9), ovomucoid (10), crystalline soy bean inhibitor (11), crystalline bovine colostrum inhibitor (12, 13), and the crystalline pancreatic inhibitor of Kunitz and Northrop (5). The italicized names will be used subsequently.

Casein (14), human hemoglobin (15), and α- p-toluenesulfonyl L-arginine methyl ester hydrochloride (TAME) (16) were used as substrates.

Solutions of pepsin, blood plasma inhibitor, and ovomucoid were made on the basis of weight. Solutions of trypsin and the remaining inhibitors were prepared on the basis of optical density at 280 μ, the optical factors previously described (1) being used.

Digestion with Pepsin—Suitable concentrations of inhibitor (equivalent to about 50 γ of trypsin per ml.), previously warmed to 37°, were added to pepsin solutions similarly equilibrated, so that the final pepsin concentration was 0.01 per cent and the final HCl concentration was 0.032 N (pH 1.5). To stop the digestion at desired intervals, 2 ml. aliquots were removed into 10 ml. volumetric flasks containing 1 ml. of 0.2 M borate buffer, pH 7.6, and NaOH equivalent to the HCl. At least two, and usually three or four, digestions were performed for each inhibitor, and the trypsin-inhibiting activity remaining in the digests was determined with aliquots of each digest at various time intervals by two or three different methods.

Determination of Trypsin-Inhibiting Activity—Most observations were made by the spectrophotometric method of Kunitz (2) with casein as substrate, except that the casein solution was made in 0.1 M borate buffer, pH 8.0, and Ca was added to give a final concentration of 0.01 M in the digestion mixture. The use of casein as a substrate for enzyme determinations has been criticized (17); the results reported in this paper, however, were in agreement with the other methods.

The second method used was the spectrophotometric hemoglobin digestion method of Anson (7) as modified by Green and Work (18), except that the phosphate buffer of the hemoglobin solution was replaced with an equimolar amount of boric acid, HCl was used to adjust the solution to pH 7.5, and the final concentration of Ca in the digest was 0.01 M. We did

1 We wish to thank Dr. L. A. Kazal for the sample of his inhibitor from pancreas. Soy bean inhibitor was purchased from the Worthington Biochemical Corporation, and the other inhibitors were prepared in this laboratory. We are grateful to Mr. R. J. Peanasky for the blood plasma inhibitor and to Miss Feng Chi Wu for the ovomucoid.

2 It was first established that soy bean inhibitor was digested with an equal velocity in 0.1 and 0.01 per cent pepsin.
not find dissociation of the trypsin inhibitor complex in the presence of urea, as reported by Jacobsson (19), but our urea concentration was only half of his, and the digestion time only 5 minutes.

Third, the manometric method of Parks and Plaut (20), originally described for chymotrypsin, was modified and applied to the determination of trypsin activity with TAMe as substrate. The results with our three preparations of trypsin are shown in Fig. 1. As in the original method, after tipping of the enzyme solution, the first 3 minutes were disregarded and the readings from 3 to 15 minutes were recorded. The rate of evolution of CO₂ was constant for at least 15 minutes. Within the range of concentrations from 0.7 to 2.5 γ per ml., the curve approaches a straight line. The upward trend of the curve may be caused either by retention of CO₂ or by inactivation of trypsin. Both of these would affect the lower part of the curve more markedly. The choice of 0.27 M substrate³ is jus-

³ The rather large quantities of TAMe used may be recovered by evaporation of the combined solutions used for manometric analysis, acidification, and extraction of the substrate and its hydrolysis product from the salts and trypsin with warm methyl alcohol, and reesterification according to Bergmann et al. (16).
tified by the results of Fig. 2, which shows that at this region small variations in substrate concentration will not affect the results.

Benzoyl-L-arginine esters may be substituted for TAMe. With benzoyl-L-arginine methyl ester, the optimal substrate concentration was established as 0.067 M, and the rate of CO₂ liberation was about one-fifth of that with TAMe.

Green (21) has shown that ester substrates displace certain protein inhibitors from their complexes with trypsin. However, with pancreatic, colostrum, and blood plasma inhibitors, no significant displacement was found, and therefore this method was successfully applied to these three inhibitors. In agreement with Green, who found that ovomucoid and soy bean inhibitor were displaced by benzoyl-arginine ethyl ester, it was found that TAMe also displaced these inhibitors and in addition displaced Kazal's inhibitor.

RESULTS AND DISCUSSION

The relative resistance of the inhibitors to peptic digestion is presented in Table I and in Fig. 3. The time required for 50 per cent inactivation of different inhibitors varied from less than 30 seconds to more than 3 weeks (Table I). In the case of blood plasma inhibitor, the inactivation is so rapid in acid alone that no claim can be made regarding the accelerating action of pepsin. It is therefore omitted from Fig. 3. Kazal's inhibitor and ovomucoid, known from their methods of preparation to be stable in acid, were rapidly digested by pepsin, indicating that instability in acid is not a prerequisite for peptic digestion.

The soy bean inhibitor was digested at a slower rate than the previous three inhibitors. Our conditions differed somewhat from those of Kunitz (2), and no direct comparison of values seems warranted; however, our results are in general agreement with his. The rate of inactivation of soy bean inhibitor by acid alone, at pH 1.5, at 37° was also measured (see the dotted curve, Fig. 3). This curve represents the lower limit of the rate of denaturation, since some reversal of denaturation occurred in the slightly alkaline solutions during the time required for activity determination. The actual rate of denaturation may therefore be almost equal to the rate of digestion with pepsin. In agreement with Kunitz (3), it was found that reversal of denaturation after short exposure to acid alone was nearly complete. With peptic digests, no reversal whatsoever occurred, even after 72 hours at pH 7.6.

Colostrum inhibitor (Fig. 3) and particularly pancreatic inhibitor were considerably more stable than the others tested. Pancreatic inhibitor is not indicated in Fig. 3 because no digestion, detectable by inactivation, occurred. Failure of digestion cannot be ascribed to inactivation of pepsin, which, after 1 week of exposure, retained 40 per cent of its original
activity, and after 3 weeks still retained 21 per cent. This inhibitor also showed no inactivation in 0.1 per cent pepsin. The possibility of protection of pancreatic inhibitor by complex formation with pepsin, or inhibition

**Table I**

*Loss of Activity of Trypsin Inhibitors on Treatment with Pepsin and with Acid*

Pepsin, 0.01 per cent, pH 1.5, 37-38°. Acid alone, 0.032 x HCl (pH 1.5), 37-38°. Inhibitor concentrations between 7.5 and 35 γ per ml. (see Fig. 3).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Time required for 50 per cent digestion by pepsin</th>
<th>Effect of acid alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>&lt;30 sec.</td>
<td>Inactivated in &lt;30 sec.</td>
</tr>
<tr>
<td>Kazal’s</td>
<td>5 min.</td>
<td>Fully active after 40 min.</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>13 min.</td>
<td>&quot; &quot; &quot; 2 hrs.</td>
</tr>
<tr>
<td>Soy bean</td>
<td>44 &quot;</td>
<td>50% denaturation in 1 hr. (reversible)</td>
</tr>
<tr>
<td>Colostrum</td>
<td>183 min.</td>
<td>Stable in acid (12)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>&gt;3 wks.</td>
<td>&quot; &quot; &quot; (3)</td>
</tr>
</tbody>
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The figures in parentheses represent bibliographic references.

![Graph](http://www.jbc.org)

*Fig. 3.* Digestion of four trypsin inhibitors by pepsin at 37°. Per cent digestion is plotted against time. Final concentrations, inhibitor equivalent to about 50 γ per ml. of trypsin, pepsin 0.01 per cent, HCl 0.032 n, pH 1.5. Inhibitors, KAZ, Kazal’s; OVO, ovomucoid; SOY, soy bean; and COL, colostrum. Methods of determination, O, casein, •, hemoglobin, ○, manometric; dash line on the soy bean chart represents inactivation by acid alone.

of pepsin by the inhibitor, was also excluded by an experiment in which no inhibition of pepsin was found with as much as 5-fold excess by weight of inhibitor over pepsin.

To demonstrate actual digestion by pepsin, and not merely inactivation, paper chromatograms in a butanol-acetic acid system were made of the digests with two inhibitors, ovomucoid and soy bean. The results are
presented in Fig. 4. With soy bean inhibitor, a large number of small breakdown products were formed, whereas with ovomucoid large products were formed which moved very slowly.

It appears, therefore, that inactivation may occur by two distinct mechanisms. In the case of soy bean inhibitor, peptic digestion was preceded by acid denaturation, and proteolytic breakdown of the denatured inhibitor was extensive. With ovomucoid and Kazal's inhibitor, no denaturation by acid was observed; inactivation of the ovomucoid by pepsin was accompanied by proteolysis limited to a small number of peptide bonds.

![Fig. 4. Paper chromatography of peptic digests of inhibitors in a system: butanol (40)-acetic acid (10)-water (50), Whatman No. 1 paper, development with ninhydrin. A: soy bean inhibitor digested 5 hours at 37° with 0.01 per cent pepsin at pH 1.5 (Line 1), exposed to acid alone at pH 1.5 (Line 2); 93 γ samples chromatographed 20 hours. B: ovomucoid digested 24 hours at 37° with 0.01 per cent pepsin at pH 1.5 (Line 1), exposed to acid alone at pH 1.5 (Line 2); 375 γ samples chromatographed 72 hours; parallel autolysates of 0.01 per cent pepsin showed no spots.

Incidentally in our experiments we obtained some data on the ratio in which trypsin combines with the various inhibitors. With the exception of soy bean and ovomucoid, which agreed with earlier data, the values found were somewhat higher than those previously reported (1). By the casein, hemoglobin, and manometric methods, respectively, 1 γ of pancreatic inhibitor was equivalent to 2.32, 2.34, and 2.36 γ of trypsin, and 1 γ of colostrum inhibitor was equivalent to 2.83, 2.65, and 2.61 γ of trypsin. This discrepancy in values is partly caused by the use of a different optical factor for trypsin (0.670 instead of 0.585). It may also be influenced, as Green and Work (18) have pointed out, by the relative differences in activity of different batches of inhibitor and trypsin. On the other hand, when the activity of the same solution of inhibitor and trypsin was determined with two or three different substrates, the inhibition ratios agreed surprisingly well, in view of the differences in values previously reported for different substrates.

4 The values previously reported for pancreatic inhibitor with three substrates were casein 2.07 (13), hemoglobin 2.5 (18), and benzoyl-L-arginine ethyl ester 3.7 γ (21) of trypsin inhibited by 1 γ of inhibitor. For colostrum inhibitor, the ratio of trypsin to inhibitor was 2.3 with casein (12) (compare Laskowski and Laskowski (1) for other inhibitors).
From the coincidental appearance of immune globulins and trypsin inhibitor in cow's colostrum, it has been postulated (12) that the inhibitor may be a part of the mechanism involved in transmitting immune globulins from a mother to a new-born. Recently, this hypothesis was tested on swine by Barrack, Matrone, and Osborne (22). They used an amorphous preparation of trypsin inhibitor (origin not specified) obtained from Armour and Company. They found that feeding swine γ-globulin through a stomach tube did not result in the appearance of γ-globulin in the circulating blood of the new-born pig, regardless of whether it was administered with or without the inhibitor. The appearance of γ-globulin was observed after feeding normal colostrum. In view of our results, the negative conclusion of Barrack et al. (22) appears to require reevaluation.

Our thanks are due to Miss Feng Chi Wu for valuable suggestions, and to Dr. M. Privat de Garilhe for help in chromatography experiments.

SUMMARY

1. The manometric method of Parks and Plaut, originally described for the determination of chymotrypsin activity, has been applied to the determination of trypsin.

2. Naturally occurring trypsin inhibitors differ markedly in their resistance to pepsin. In the order of increasing stability, the six which we have studied are blood plasma inhibitor, Kazal's inhibitor from pancreas, ovo-mucoid, soy bean inhibitor, bovine colostrum inhibitor, and the inhibitor of Kunitz and Northrop from pancreas.

3. These findings suggest that only pepsin-resistant inhibitors are likely to produce physiological effects when administered orally to animals.

BIBLIOGRAPHY


6 Dr. M. A. Schooley of Armour and Company kindly informed us that the inhibitor was prepared from soy bean.
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