THE EFFECT OF UREA ON THE INHIBITION OF TRYPSIN BY SOYBEAN TRYPSIN INHIBITOR

by

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In 1947 KUNITZ[1] published a paper on the inhibition of trypsin by crystalline soybean trypsin inhibitor. He found the reaction between trypsin and soy inhibitor to be stoichiometric, irreversible, and practically instantaneous within a wide range of pH. These observations have since been confirmed by other research workers *e.g.* MCLAREN[2].

KUNITZ[1] stated that the irreversibility of the reaction between soy inhibitor and trypsin is independent of the method for measuring the proteolytic activity of trypsin. He suggested that soy inhibitor should be used as a standard for the determination of trypsin activity because this inhibitor is a stable protein and because it can be obtained highly purified in a crystalline state.

In a study of the trypsin-inhibiting activity of blood serum the present writer[3] checked the activity of trypsin with soy inhibitor in experiments using hemoglobin in urea as a substrate and found the reaction between soy inhibitor and trypsin to be reversible at pH 7.6. It could not be decided whether the inhibition was competitive or noncompetitive. The dissociation constant was, however, fairly small ($2.2 \cdot 10^{-9}\ M$ assuming noncompetitive inhibition), and it thus seemed possible that the dissociation might be missed at the relatively high concentrations of trypsin and inhibitor generally used in studies employing casein as a substrate.

On investigation of the reaction between trypsin and soy inhibitor in low concentrations using casein as a substrate and prolonged digestion time no dissociation of the soy inhibitor trypsin compound was detectable.

This suggested that urea might cause dissociation of the soy inhibitor trypsin complex, an assumption confirmed in experiments using as a substrate a casein solution containing urea.

METHODS AND MATERIALS

Trypsin. (Worthington Bioch., Freehold N.J.) 2 × crystallized.

Soybean trypsin inhibitor. (Worthington) 5 × crystallized.

Estimation of the concentration of trypsin and inhibitor. Stock solutions of trypsin and of soybean trypsin inhibitor were prepared in 0.0025 *N* HCl, and the protein concentration was determined by measuring the optical density at 280 mμ. KUNITZ[1] factors were used to convert optical densities to mg protein/ml (0.585 for trypsin and 1.10 for soy inhibitor).

Buffer. Dilution of trypsin and inhibitor were made in a buffer containing 5.9 g of the sodium salt of diethylbarbituric acid, 3.9 g sodium acetate, $3H_2O$, and 66.7 ml 50% calcium chloride per liter. The pH was adjusted to 7.6 with HCl.

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Substrates

A. *Casein without addition of urea.* 1 g casein was suspended in 100 ml of a buffer containing 5.9 g of the sodium salt of diethylbarbituric acid, 3.9 g sodium acetate, $3H_2O$, and 50 g of the di-sodium salt of ethylenediaminetetraacetic acid per liter. The pH was adjusted to 8.6* with sodium hydroxide. The suspension was heated for 15 minutes in a boiling water bath thus bringing about solution of the casein.

B. *Casein with addition of urea.* This substrate is identical with the former but for the addition of 40 g urea to 100 ml substrate.

Estimation of trypsin activity

This was done by a modified version of the casein digestion method of KUNITZ[1]. One ml samples of solutions containing varying amounts of trypsin were added to 3 ml substrate, and three hours later 5 ml 0.33 *N* trichloroacetic acid was added. After centrifugation the concentration of the split products in each tube was determined by measuring the optical density at 280 mμ of the supernatant. When the substrate contained urea the tubes were shaken and allowed to stand over night before centrifugation in order to obtain a clear supernatant. Reference curves for the determination of trypsin activity in the inhibition experiment were obtained by plotting E_{280} for each substrate against micrograms of trypsin.

Temperature. All experiments were performed at 25° C ± 0.1° C. All solutions were allowed to stand in the water bath for 10 minutes to attain temperature equilibrium before being mixed.

EXPERIMENTAL

Inhibition of trypsin by soy inhibitor using casein without urea as a substrate. Varying amounts of soy inhibitor were added to a given amount of trypsin and all mixtures were made up to one and the same volume with buffer. Triplicate determinations were made of the trypsin activity of 1 ml samples of these mixtures. The amounts of trypsin inhibited were estimated by comparing the trypsin activity of samples containing known amounts of inhibitor and samples free of inhibitor. The amounts of trypsin inhibited were plotted against micrograms of soy inhibitor (Fig. 1).

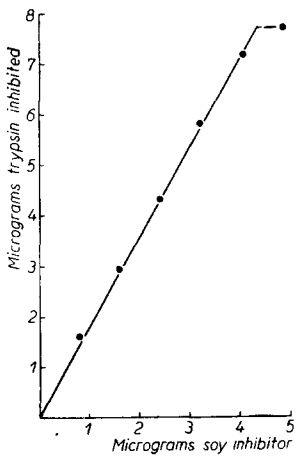

Fig. 1. *Irreversible* inhibition of trypsin by soybean trypsin inhibitor. Substrate: casein *without* addition of *urea*. Abscissa: Micrograms soy inhibitor per ml enzyme inhibitor mixture. Ordinate: Micrograms trypsin inhibited per ml enzyme inhibitor mixture. Total amount of of trypsin 7.8 micrograms.

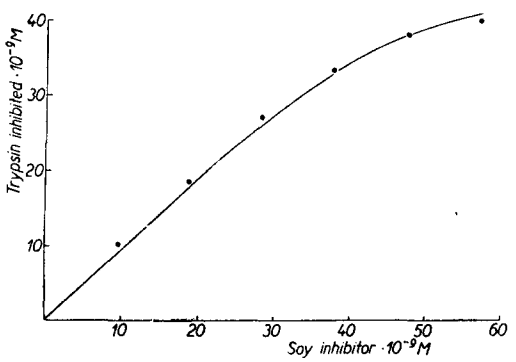

Fig. 2. *Reversible* inhibition of trypsin by soybean trypsin inhibitor. Substrate: casein *with* addition of *urea*. Abscissa: Molar concentration of soy inhibitor in digestion mixture. Ordinate: Molar concentration of inhibited trypsin in digestion mixture. Total concentration of trypsin in digestion mixture $46.6 \cdot 10^{-9}$ *M*.

* Ethylenediaminetetraacetic acid is added in order to avoid precipitation of casein by calcium contained in the buffer and stabilizing the trypsin. The final pH of the digestion mixture was 7.6.

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Inhibition of trypsin by soy inhibitor using casein containing urea as a substrate. But for the addition of urea and a slightly lower concentration of trypsin, this experiment was performed in the same way as that described above. The results are given in Fig. 2.

RESULTS AND DISCUSSION

Fig. 1 shows that—in the absence of urea—no dissociation of the soy inhibitor trypsin complex is detectable, and that 1 microgram of soy inhibitor inhibits 1.83 micrograms of trypsin. According to KUNITZ[1], 1 microgram of soy inhibitor inhibits about 1.0 microgram of pure trypsin. The trypsin used in the present experiment might have had a lower specific activity than that used by KUNITZ, which might explain this discrepancy. This difference will, however, not impair the validity of the conclusions suggested by the present experiments.

Assuming that 1 mol of trypsin reacts with 1 mol of soy inhibitor the hypothetical molecular weight of trypsin in the present experiments will be 1.83 times greater than that of soy inhibitor. In experiments using hemoglobin substrate[3] a molecular weight of 20,600 was selected for soy inhibitor. The molecular weight of trypsin will then be 38.000. Using these values in the experiments with hemoglobin substrate the dissociation constant for the soy inhibitor trypsin complex in the presence of urea was found to be $2.2 \cdot 10^{-9}$ *M*. The solid curve in Fig. 2 is based on these values for molecular weights and dissociation constant. The points represent the values found in the present experiment with casein containing urea as a substrate. It is evident that there is a good agreement between the theoretical and the observed values.

GROB[4], in a study of the inhibition of different proteolytic enzymes by different inhibitors including trypsin and soy bean inhibitor, stated that the results obtained with casein (without urea) and with hemoglobin (with and without urea) were essentially the same, but he gave no data about the experiments with hemoglobin. However, the present experiment clearly shows the dissociating effect of urea on the soy inhibitor trypsin complex.

ANSON's[5] well-known hemoglobin method is rapid and accurate, but if used for studies on the kinetics of the inhibition of proteolytic enzymes, it should be borne in mind that the urea present in the substrate can make an otherwise irreversible or at least practically irreversible reaction reversible.

This effect of urea is of interest *e.g.* in connection with the recent studies of GREEN AND WORK[6]. They reported that the inhibition of trypsin by pancreatic trypsin inhibitor is reversible. As they used a modification of ANSON's hemoglobin method and as the dissociation constant they gave for this complex was still less than that found in the present experiment with trypsin and soy inhibitor, it is still possible that the reaction between trypsin and pancreatic trypsin inhibitor may be irreversible in the absence of urea.

SUMMARY

The soybean inhibitor trypsin complex dissociates at pH 7.6 in the presence of urea. In experiments using a casein substrate containing urea in the same concentration as in ANSON's hemoglobin method the dissociation constant was found to be about $2.2 \cdot 10^{-9}$ *M*.

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RÉSUMÉ

Le complexe trypsine-inhibiteur du soja se dissocie à pH 7.6 en présence d'urée. Dans des expériences utilisant comme substrat de la caséine renfermant la même concentration d'urée que l'hémoglobine dans la méthode d'ANSON, la constante de dissociation trouvée est d'environ $2.2 \cdot 10^{-9} M$.

ZUSAMMENFASSUNG

Der Sojabohneninhibitor- Trypsinkomplex dissoziiert bei pH 7.6 in Gegenwart von Harnstoff. In Experimenten, in denen Casein als Substrat verwendet wurde, das Harnstoff in den gleichen Konzentrationen enthielt wie bei ANSON's Hämoglobinmethode wurde eine Dissoziationskonstante von etwa $2.2 \cdot 10^{-7} M$ gefunden.

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