INTERACTION OF TRYPSIN AND CHYMOTRYPSIN WITH A SOYBEAN PROTEINASE INHIBITOR[1]

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Several protein inhibitors of trypsin possess a particular Arg-X or Lys-X bond that is susceptible to the action of the enzyme (1-3). The Kunitz soybean trypsin inhibitor, for example, has an accessible Arg-Ile bond that can be cleaved and reformed under certain conditions (1,4). The Bowman-Birk inhibitor, a soybean protein distinct from the Kunitz inhibitor, is almost equally active against trypsin and α-chymotrypsin (5,6). Birk, Gertler and Khalef reported that a catalytic amount of trypsin at acid pH may generate a new lysine or arginine carboxyl terminal in the inhibitor without affecting inhibiting capacity (7). On the other hand, it was found that similar treatment with chymotrypsin resulted in a modified inhibitor with substantially reduced chymotrypsin-inhibiting capacity.

As a result of an investigation along the lines given by Birk, Gertler and Khalef (7), we have obtained additional evidence that requires reinterpretation of the inhibiting capacities of trypsin-modified and of chymotrypsin modified inhibitor. Moreover, an insight into the possible mechanism of action of chymotrypsin on the inhibitor has been gained.

MATERIALS AND METHODS

Crystalline trypsin and chromatographically pure α-chymotrypsin were the same as those used previously (8). Trypsin treated with p-toluene-

[1] The opinions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department. From the Bureau of Medicine and Surgery, Navy Department, Research Task MR005.06.0005.

sulfonyl-L-phenylalanyl chloromethane (PTCK-trypsin) was obtained from Calbiochem. Inhibitor was purchased from Miles Laboratories and purified electrophoretically according to a published procedure (9). With some alterations that will be specified, enzyme assays, analytical-scale polyacrylamide gel electrophoresis, and the gel staining technique were conducted according to protocols already given (6,8).

Chymotrypsin-modified inhibitor, I_c, was prepared by reacting inhibitor, 5 mg/ml in 0.05 M $CaCl_2$ at pH 4.0, with 1 mole % of enzyme for 24 hours at room temperature. Trypsin-modified inhibitor, I_t, was prepared similarly using PTCK-trypsin but allowed to react an additional 24 hours at 4°. Since almost identical procedures were followed to ascertain the activities of I_c and I_t, a generalized format will be presented. After reaction, a portion of the modified inhibitor solution was diluted with ice cold 0.05 M KH_2PO_4, pH 8.0. Immediately after dilution, an aliquot of this solution and an aliquot of a stock enzyme solution, either α-chymotrypsin or crystalline trypsin, were added to a volume of cold phosphate buffer at a time designated as t_o. The final concentration of inhibitor was 10.5 µg/ml in either case. The final concentrations of chymotrypsin and of trypsin, uncorrected for inactive enzyme, were 26.4 and 22.4 µg/ml, respectively. At various times after t_o, 100 µl aliquots of enzyme-inhibitor solution were withdrawn and added to 2.0 ml of an appropriate synthetic substrate, either p-toluenesulfonyl-L-arginine methyl ester or N-acetyl-L-tyrosine ethyl ester, for assay. Parallel experiments, exeluding the catalytic amounts of trypsin and chymotrypsin, served as native inhibitor controls.

Two other sets of control experiments, one with I_t and the other with I_c, were conducted for reasons that will be discussed in the following section. For these, solutions of modified inhibitors, which had been diluted with cold phospate buffer immediately prior to time t_o, served as inhibitor sources. It is important to emphasize that these solutions still retain a catalytic amount of enzyme. The sequence of events was as follows: at var-

ious times after dilution with phosphate buffer, aliquots of modified inhibitors were combined with aliquots of stock enzyme solution, and the residual enzymic activity for each enzyme-inhibitor mixture was determined 5 minutes after combination. In all other respects, conditions for these assays were the same as those mentioned in the preceeding paragraph. All kinetic data were obtained with the use of a Gilford model 2000 recording spectrophotometer.

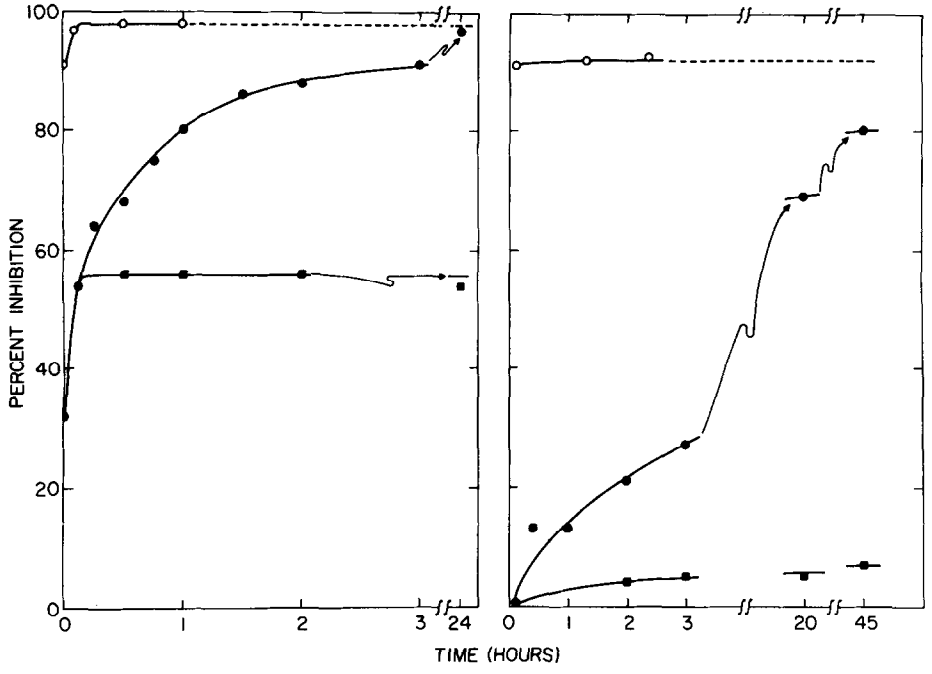

Fig. 1 Inhibiting capacities of native and modified forms of Bowman-Birk inhibitor. In the left illustration, the enzyme and modified inhibitor are trypsin and trypsin-modified inhibitor. In the right illustration, the enzyme and modified inhibitor are chymotrypsin and chymotrypsin-modified inhibitor. Percent inhibition is calculated from the residual enzymic activity in enzyme-inhibitor solutions relative to total activity in the absence of inhibitor. Percent inhibition of enzyme by modified inhibitor (●) and by native inhibitor (○) exposed to enzyme at pH 8, 0-4°, for the length of time indicated on the abscissa. Percent inhibition of enzyme by modified inhibitor (■) maintained in the presence of a catalytic amount of enzyme at pH 8, 0-4°, combined with an assayable amount of enzyme at the time indicated on the abscissa, then assayed 5 minutes after combination. Calculated mole ratios of inhibitor (native or modified):active trypsin and inhibitor (native or modified):chymotrypsin are 2.7:1 and 1.24:1, respectively. The trypsin preparation used in this study was previously determined to contain 52% active trypsin (8). Enzyme concentrations under assay conditions were either 0.55 µg active trypsin/ml or 1.26 µg chymotrypsin/ml. An inhibitor molecular weight of 8,000 was assumed (6).

RESULTS AND DISCUSSION

Inhibiting capacities of I_c, I_t, and native inhibitor are illustrated in Fig. 1 (upper and middle curves in both illustrations). Compared to native inhibitor, it is evident that, initially, I_t is not as efficient against trypsin, and I_c is completely ineffective against chymotrypsin. However, the inhibiting capacities of both modified forms increase with time of exposure to enzyme at pH 8 and eventually approach that of the native form. In the absence of inhibitor, neither trypsin nor chymotrypsin loses appreciable activity upon standing at pH 8 under the given conditions. To show that the regeneration of inhibiting capacity requires the presence of more than a catalytic amount of enzyme, the control experiments with modified inhibitors are included in Fig. 1 (the consistently lowest curves in both illustrations). These results indicate that exposure for any length of time to only a catalytic amount of enzyme at pH 8 has virtually no regenerative effect on the inhibiting capacity of modified inhibitor. The elevated, constant level of inhibiting capacity is due solely to the 5 minute exposure of individual samples to a near-equimolar amount of enzyme.

The question then arose as to the nature of this recovery of inhibiting capacity. To explore the possibility of a transformation of the modified form back to a native state, the following experiments were conducted with the knowledge that inhibitor is soluble in 60% ethanol at pH 8 whereas both enzymes are practically insoluble. Twenty-three mg of chymotrypsin were combined with 10 mg of I_c in a total volume of 2 ml, the pH was adjusted to 8 and the solution was stored at 4° for 24 hours. A solution of 30 mg crystalline trypsin plus 10 mg I_t was also prepared and so treated; however, since this protein mixture was incompletely soluble at pH 8, the pH was adjusted to 9.5 and maintained for an additional 2 hours. Calculated mole ratios of inhibitor:enzyme, uncorrected for the presence of inactive enzyme (8), are 1:1 and 1.4:1 for trypsin and chymotrypsin, respectively. Again, since both mixtures were handled similarly from this point on, a generalized procedure

will be presented. To the enzyme-modified inhibitor solution was added 3 ml of absolute ethanol, the apparent pH was adjusted to 3 with 1 M HCl (at this pH all protein components are soluble) then rapidly adjusted to 8 by addition of 3 M KOH. The suspended protein precipitate was centrifuged and the supernatant was removed by decantation. A double volume of acetone was then added to the ethanol supernatant and the resulting precipitate was centrifuged, separated from the acetone-ethanol supernatant by decantation, dissolved in 1 ml of water, and lyophilized. The alcohol precipitate was washed with ethanol, suspended in 2 ml of water, dissolved by adjusting the pH to 2.5, and lyophilized.

The above fractions, alcohol-soluble and alcohol-insoluble, along with enzyme, inhibitor, and enzyme-inhibitor controls were subjected to polyacrylamide gel electrophoresis (Fig. 2). These results indicate that the transformation of native inhibitor to either of the modified forms in the presence of a catalytic amount of enzyme at acid pH approaches completion in 48 hours. From the areas under a densitometer trace of sample 6 in Fig. 2 (left), the composition of the sample is estimated to be 20% native and 80% modified inhibitor. Conversion to I_t is thus incomplete under these conditions, as is suggested by the finite inhibiting capacity retained at zero time (Fig. 1). Digests of inhibitor with a catalytic amount of either trypsin or chymotryptsin yield only one major, ninhydrin-positive component after high voltage paper electrophoresis at pH 4.4[2]. Hence, it is likely that I_t and I_c are compositionally the same as native inhibitor. Although it is not apparent in Fig. 2, the mobilities of I_t and I_c are virtually indistinguishable. When both sets of samples 5, 6, and 7 in Fig. 2 were analyzed by disc gel electrophoresis in a cationic medium (running pH = 2.7) migration patterns analogous to those in Fig. 2 were observed, suggesting that I_t and I_c contain the same number of charge groups[2]. Therefore, it is plausible that the modifications caused by trypsin and chymotrypsin, although different, are of a similar type. This would be the case if, for example, modification by either

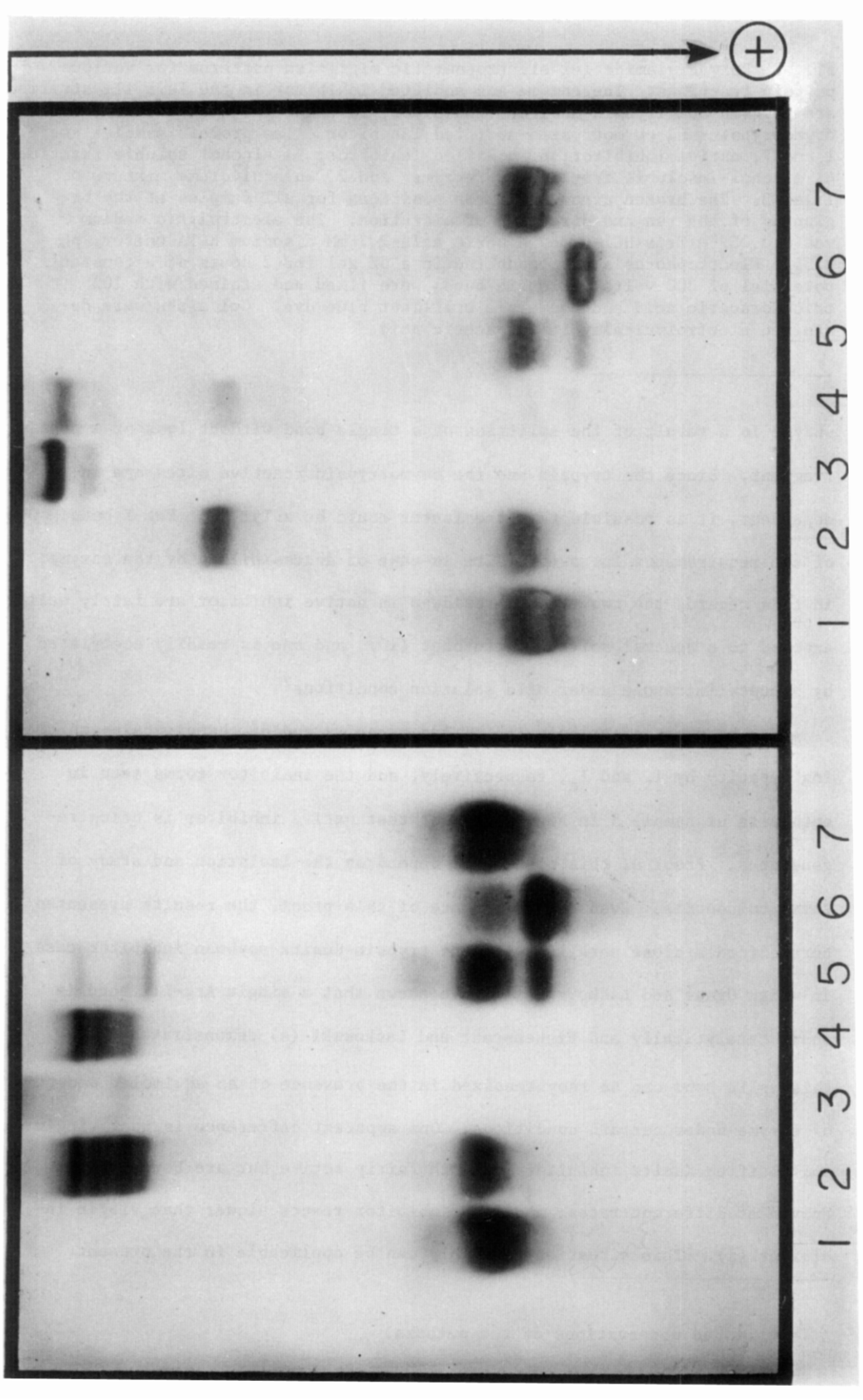
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Fig. 2 Polyacrylamide gel electrophoretic migration patterns for various protein fractions. The enzyme and modified inhibitor in the left illustration are trypsin and trypsin-modified inhibitor; in the right illustration, chymotrypsin and chymotrypsin-modified inhibitor. The protein samples are: 1 and 7, native inhibitor; 6, modified inhibitor; 5, alcohol-soluble fraction; 4, alcohol-insoluble fraction; 3, enzyme; and 2, an equivolume mixture of 1 and 3. The broken arrow indicates positions for all samples at the beginning of the run and direction of migration. The electrolytic medium was a 0.089 M Tris·HCl-0.089 M boric acid-2.7 mM disodium EDTA buffer, pH 8.35. Electrophoresis was conducted in a 5% gel for 2 hours at a constant potential of 300 volts. Protein bands were fixed and stained with 10% trichloracetic acid and Coomassie brilliant blue dye. Gel slabs were destained electrolytically in 15% acetic acid.

enzyme is a result of the splitting of a single bond without loss of a peptide fragment. Since the trypsin and the chymotrypsin reactive sites are independent, it is possible that the latter could be a Tyr-X or Phe-X bond. One of the requirements for such a site is ease of accessibility by the enzyme; in this regard, the two tyrosyl residues in native inhibitor are fairly well exposed to a neutral solvent perturbant (10), and one is readily acetylated by N-acetylimidazole under mild solution conditions[2].

The recovery of trypsin-inhibiting capacity and of chymotrypsin-inhibiting capacity by I_t and I_c, respectively, and the inhibitor forms seen in both sets of sample 5 in Fig. 2 suggest that native inhibitor is being regenerated. Proof of this point will depend on the isolation and study of these components. Even in the absence of this proof, the results presented here afford a close parallel with the trypsin-Kunitz soybean inhibitor case in which Ozawa and Laskowski (1) have shown that a single Arg-Ile bond is split catalytically and Finkenstädt and Laskowski (4) demonstrated that this split bond can be resynthesized in the presence of an equimolar amount of enzyme under certain conditions. One apparent difference is that virgin and modified Kunitz inhibitor are both fairly active but are bound by enzyme at different rates; modified inhibitor reacts slower than virgin inhibitor (1). This situation certainly can be applicable in the present

[2] Unpublished observations of the authors.

study; however, because of the big difference in the reaction rates between native inhibitor and I_c for combination with chymotrypsin, we have considered I_c as an inactive form.

In summary, two distinguishable modified forms of the Bowman-Birk inhibitor are produced at acid pH with catalytic amounts of trypsin and of chymotrypsin. In both cases, conversion to the modified form approaches completion within 48 hours. Both modified forms are inefficient inhibitors, however, inhibiting capacity comparable to that of native inhibitor is restored upon prolonged exposure to a near-equimolar amount of enzyme. After such exposure, both modified forms exhibit native inhibitor characteristics.

SUMMARY

The Bowman-Birk inhibitor is an 8,000 molecular weight soybean protein which inhibits tryptic and α-chymotryptic activity. Two distinguishable forms, trypsin-modified and chymotrypsin-modified inhibitor, are produced at acid pH with catalytic amounts of enzyme. The modification caused by chymotrypsin could be the cleavage of a Tyr-X or Phe-X bond without release of a peptide fragment from the inhibitor. In either case, conversion to the modified form is at least 80% complete within 48 hours. Both modified forms are inefficient inhibitors. However, inhibiting capacity comparable to that of native inhibitor is restored upon prolonged exposure at pH 8 to a near-stoichiometric amount of enzyme. After such exposure, both modified forms exhibit native inhibitor characteristics.

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