DIGESTION OF THE BASIC TRYPSIN INHIBITOR OF BOVINE PANCREAS BY THERMOLYSIN

by

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Received July 15, 1970

Summary. Extensive digestion of the basic trypsin inhibitor by thermolysin at 60-80° has been demonstrated by loss of inhibitory activity, increase in ninhydrin positive material and separation of the breakdown products by electrophoresis. Formation of the inhibitor-trypsin complex does not protect the inhibitor from digestion by thermolysin; both components are digested. Between 40° and 80° the inhibitor undergoes a reversible transition involving a slight conformational change; this may account for its susceptibility to thermolysin.

We are reporting the first successful enzymic digestion, without previous chemical modification, of the basic trypsin inhibitor* of bovine pancreas. This small protein (molecular weight 6,513, cross-linked by 3 disulfide bonds) is remarkably stable and is resistant to digestion by more than twenty enzymes previously tested (1,2). Digestion at 60-80° has now been accomplished with thermolysin, an enzyme from Bacillus Thermoproteolyticus Rokko (3,4).

MATERIALS

The inhibitor was the same as previously described (5). Thermolysin was purchased from Calbiochem. Bovine trypsin was a gift from Novo Industries.

RESULTS

Digestion of free inhibitor. Digestion of the inhibitor by thermolysin was followed by three methods: decrease in ability to inhibit trypsin, determined with benzoyl-DL-arginine-p-nitroanilide as substrate (6), increase in ninhydrin color (7) and separation of the breakdown products of the inhibitor by paper electrophoresis.

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* Also known as Kunitz inhibitor, or kallikrein inhibitor of bovine organs. Subsequently called "the inhibitor".

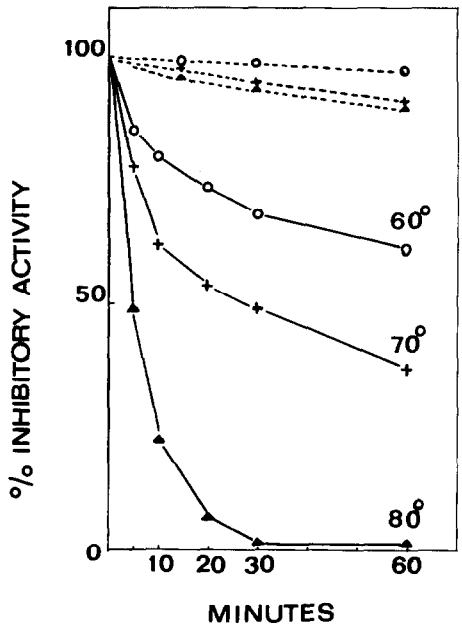

Figure 1. Loss of trypsin-inhibiting activity during incubation of free inhibitor with thermolysin. Digests (solid lines): 6 µg of inhibitor and 1.5 µg of thermolysin in 1 ml of 50 mM borate buffer, pH 8.0. Controls (dotted lines): 6 µg of inhibitor in the same buffer. After incubation for the times shown, the tubes were placed in an ice bath until assayed.

In Figure 1 the loss of trypsin-inhibiting activity in the digestion mixture is compared to the inhibitor without enzyme at the same temperature. In the presence of thermolysin at 80° there was almost complete loss of activity and at 60° there was still considerable loss. The high temperature alone had little effect on the activity. Control experiments showed that thermolysin did not interfere with the determination of trypsin-inhibiting activity and that the inhibitor did not affect the action of thermolysin on casein.

The increase in ninhydrin color during digestion of the inhibitor by thermolysin is shown in Figure 2. At 60°, the increase in ninhydrin color was slow and amounted to only one third of the increase at higher temperature. At 70° the rate of digestion increased markedly, and at 80° the maximum value, reached in 30 minutes, corresponded to 9 equivalents of leucine per mole of

TABLE I

DIGESTION OF INHIBITOR-TRYPSIN COMPLEX WITH THERMOLYSIN

70°, pH 8.0, complex 632 µg/ml, thermolysin 63.2 µg/ml

Incubation Time (min)	Complex		Complex + Thermolysin	
	Tryptic Activity(%)	Inhibitory Activity(%)	Tryptic Activity(%)	Inhibitory Activity(%)
0	100	100	100	100
10	95	92	73	88
20	90	90	68	82
30	84	91	63	74

spectrophotometer equipped with thermospacers and an adjustable water bath. A separate portion of the solution was incubated for each temperature and readings were made at 20 and 30 minutes at 293 nm (the wave length giving maximum change in absorbance). The control was a cold portion of the same solution placed in the cuvette holder only momentarily for the reading. After the readings, the solutions were rapidly cooled and kept at 4° overnight to test reversal. Figure 4 shows that there was a thermal change with a midpoint at 56°. The greater part of the change was reversible, even at 80°, in agreement with retention of most of the inhibiting activity. The increase in absorbance was very small, indicating a minimal conformational change.

DISCUSSION

During thermal denaturation the inhibitor is subject to a slight conformational change, which may be necessary for the start of digestion by thermolysin. At 70° and 80° digestion of the inhibitor by thermolysin is extensive

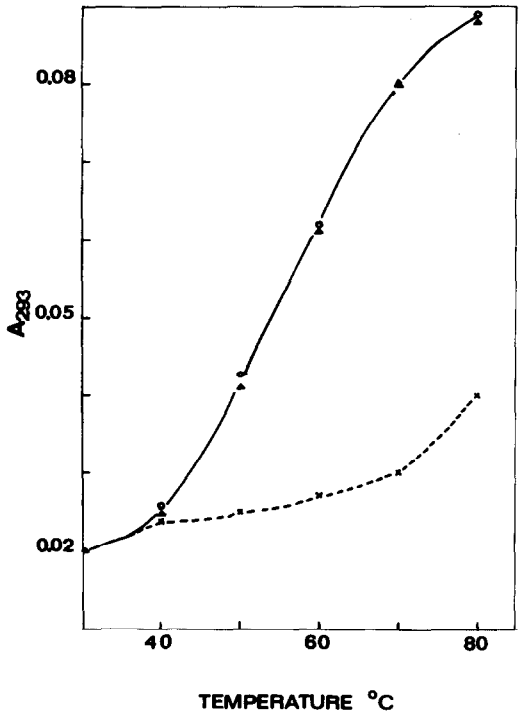

Figure 4. Change in absorbance at 293 nm with temperature. Inhibitor: 0.2 mM solution in 50 mM borate buffer, pH 8.0. For procedure, see text. Solid line: incubated for 20 (Δ) and 30 (o) minutes at each temperature. Dotted line: after reversal overnight at 4° (X).

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and is not prevented by formation of the inhibitor-trypsin complex. Thus, thermolysin is able to penetrate a highly cross-linked structure that other enzymes do not attack. This property of the enzyme should make it useful for structural studies of other resistant proteins (cf. 13).

This work was supported by grants from the National Science Foundation (GB-6032) and from the National Institutes of Health (AM-09826).

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