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Properties of enzymatically cleaved inhibitors of trypsin

The incubation of several trypsin inhibitors with trypsin in acidic solution can result in proteolytic cleavage of the inhibitor, with little effect on inhibitory activity[1-3] In the case of chicken ovomucoid and soybean trypsin inhibitor, subsequent removal of the new C-terminal residue with carboxypeptidase B results in almost complete loss of inhibitory activity. This has been cited as indicating that the trypsin-inhibitory reaction consists of a cleavage of one especially sensitive bond in the inhibitor by trypsin and of subsequent formation of a covalent bond between trypsin and the inhibitor[2,4] FINKENSTADT AND LASKOWSKI[5] have also made the novel observation that the particular peptide bond which is hydrolyzed by trypsin is resynthesized by the trypsin We have therefore reasoned that modification of the new α-amino group might result in loss of inhibitory activity To test this hypothesis, soybean trypsin inhibitor and chicken ovomucoid were treated with trypsin in acidic solution and the amino groups then reacted with trinitrobenzenesulfonic acid (TNBS)

The enzymes, TNBS, and inhibitors were preparations similar to those recently employed[3,6,7] Ethyl acetimidate was synthesized[8] Amidination was done by incubating the protein (5 mg/ml) in an aqueous solution containing 0 5 M ethyl acetimidate, 0 1 M borate, and 0 01 M ethylenediaminetetraacetic acid, at pH 9.5 for 5 h at 4° Determination of free amino groups, time-course modifications with TNBS, and assays for inhibitory activity were done as described previously[7] Enzymatic treatments with trypsin in acid solution and carboxypeptidase B at neutrality were carried out according to the method of FINKENSTADT AND LASKOWSKI (refs. 5 and 2, respectively)

TABLE I

EFFECTS OF TREATMENTS OF NATIVE AND AMIDINATED INHIBITORS WITH TRYPSIN AND CARBOXYPEPTIDASE B

Treatments with trypsin were in acidic solution and amidinations were with ethyl acetimidate as described in text

Inhibitor	*Without amidination*			*With prior amidination*		
	Amino groups per mole	*Inhibitory activity (%)**		*Amino groups per mole*	*Inhibitory activity (%)**	
		Before carboxypeptidase	*After carboxypeptidase*		*Before carboxypeptidase*	*After carboxypeptidase*
Chicken ovomucoid**	15 3	100	96	0 25	100	97
Chicken ovomucoid, trypsin treated	17 0	58	18	1 69	72	17
Soybean trypsin inhibitor**	11 8	100	98	0 90	100	102
Soybean trypsin inhibitor, trypsin treated	13 0	101	22	1 73	99	15

* All activities compared to native inhibitor, which was set at 100%

** These samples were controls in the sense that they were not treated with trypsin, but otherwise received identical treatments, including treatments with carboxypeptidase B

Abbreviation TNBS, trinitrobenzenesulfonic acid

Numbers of free amino groups and the trypsin-inhibitory activities in the various preparations are given in Table I The amino groups in chicken ovomucoid and soybean trypsin inhibitor increased by 1 7 and 1.2 per molecule, respectively, after treatment with trypsin at acidic pH. The inhibitory activity of soybean trypsin inhibitor was unaffected, but chicken ovomucoid lost approx 40% of its activity. When the trypsin-treated inhibitors were subsequently treated with carboxypeptidase B, their activities were reduced to about 20% that of the native inhibitors, in confirmation of previous reports[3,4]

The losses in inhibitory activities given in Fig 1 were obtained when the trypsin-treated inhibitors were reacted with TNBS in a time-course modification The rapid loss of inhibitory activity in soybean trypsin inhibitor was most likely due to modification of the new α-amino group since modification of amino groups in the native inhibitor has no effect[7,9] On the other hand, modification of trypsin-treated chicken ovomucoid with TNBS gave only a very slow loss of inhibitory activity which could not be correlated to the modification of an α-amino group To confirm that losses in activity were not due to the cumulative effect of modifying the ε-amino groups with

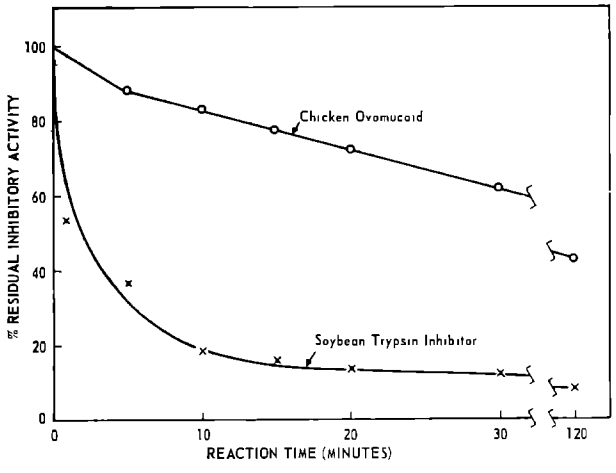

Fig 1 Effect of treatment with TNBS on the trypsin inhibitory activity of trypsin-treated inhibitors The inhibitory activities of the chicken ovomucoid and soybean trypsin inhibitor after trypsin treatment were 58% and 100%, respectively, of the activities of the native proteins

TNBS, the above series of reactions was also imposed on amidinated inhibitors Amidination had little effect on the number of new amino groups appearing or on the inhibitory activities after trypsin treatment (Table I). Reaction of the trypsin-treated amidinated inhibitors with TNBS in a time-course modification gave losses of activities very similar to those shown in Fig. 1.

From the results of this and other studies[2,5,10,11], it is now evident that naturally occurring inhibitors of proteolytic enzymes may differ in many ways Either an arginyl or lysyl residue is apparently required for the inhibitory activity against trypsin[7,10], but these residues may only serve as combining sites in some inhibitors, because some inhibitors form competitive complexes with catalytically inactive derivatives of the enzymes[11]. The intact bond of soybean trypsin inhibitor is not essential for activity,

but either modification of the new N-terminal amino acid or removal of the new C-terminal amino acid of trypsin-treated soybean trypsin inhibitor results in almost complete loss of inhibitory activity This is consistent with the hypothesis that the bond cleaved by trypsin is at the reactive site of the inhibitor With chicken ovomucoid, however, modification of the new amino groups in the trypsin-treated protein does not cause inactivation This shows that the resynthesis of a peptide bond is not required for inhibitory activity Nevertheless, the covalent bond postulated by FINKENSTADT AND LASKOWSKI[2] could still be formed from the free carboxyl group of the arginyl residue, although the amino group of the other residue from the original peptide bond was modified with TNBS The results could still be interpreted, however, on the basis of a non-covalent combination of the enzyme with the guanidino side chains of arginine. We are planning to characterize the residues modified with the TNBS in soybean trypsin inhibitor and chicken ovomucoid and to use this approach to identify the other amino acids in the reactive site

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