GUANIDINATION OF THE BOWMAN-BIRK SOYBEAN INHIBITOR: EVIDENCE FOR THE TRYPTIC HYDROLYSIS OF PEPTIDE BONDS INVOLVING HOMOARGININE*

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SUMMARY: Exposure of the Bowman-Birk soybean inhibitor (BBI) or its guanidinated, but fully active derivative to trypsin at pH 3.8 and subsequent treatment with carboxypeptidase B resulted in the release of approximately 1 residue of lysine from BBI and 0.4 residue of homoarginine from guanidinated BBI. C-terminal homoarginine residues were also produced when the guanidinated derivatives of fully reduced BBI or α-lactalbumin were digested with trypsin at pH 3.8 or 8.2. High voltage paper electrophoresis of the tryptic digest of these two proteins showed the presence of a greater number of Sakaguchi positive spots then could be accounted for solely on the basis of the cleavage of arginine bonds.

Based largely on reports by Weil and Telka (1) and Shields et al. (2) it is generally accepted that trypsin does not cleave bonds involving homoarginine (3). The site of reaction of trypsin with its natural inhibitors has been shown to be either a lysine or an arginine residue (4,5), and Ozawa and Laskowski (4) have postulated the splitting of a lys-x or arg-x in the inhibitor molecule to be a necessary reaction in the inhibition process. Thus, when Haynes and Feeney (6) found that some guanidinated "lysine inhibitors" were fully active towards trypsin, they concluded that, although homoarginine could still function as a binding site, peptide cleavage need not be an obligatory feature of the inhibition.

During the course of modification studies on BBI*** (7) we noted that trypsin did in fact split a homoarg-x bond in the active, guanidinated derivative of this inhibitor. The splitting of homoarginine bonds by trypsin was also observed with the guanidinated derivatives of fully reduced BBI and α-lactalbumin.

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*** Abbreviations used: BBI, Bowman-Birk soybean inhibitor; RCM-BBI, reduced, carboxamidomethyled BBI; CPB, carboxypeptidase B.

MATERIALS AND METHODS

BBI was purchased from Miles-Yeda, Ltd., and the fully reduced carboxamidomethyl derivative, RCM-BBI, was prepared according to the procedure of Cole (8) except that 6M guanidine·HCl was used as the denaturant and iodoacetamide as the alkylating agent. This derivative was completely devoid of trypsin inhibitor activity. Dr. Robert Jenness kindly provided the α-lactalbumin (B-variant) used in these studies.

Guanidination was performed by reacting equal volumes of a 1% solution of these proteins with an equal volume of 1M O-methylisourea sulfate at room temperature for 70 hrs. at pH 10.2. After terminating the reaction with an equal volume of 0.05M acetic acid, excess reagents were removed by dialysis and the protein lyophilized. The homoarginine, as well as the other basic amino acids of the guanidinated proteins, were determined on the short column of the amino acid analyzer (3). Molecular weights of 8000 and 14,400 were assumed for BBI (9) and α-lactalbumin (10) respectively.

Digestion with trypsin was carried out under acid (pH 3.8) or alkaline (pH 8.2) conditions. The protein (8 mg) was dissolved or suspended in 0.5 ml 0.05M $CaCl_2$, 3.8, or adjusted to pH 8.2 with 1M tris buffer of the same pH. Sufficient trypsin (TPCK-treated, Worthington), dissolved in an equal volume of 0.05M $CaCl_2$, was added to give a substrate: enzyme molar ratio of 70-100:1. Digestion was allowed to proceed at 30°C for 46 hrs. at pH 3.8 or for 3-4 hrs. at pH 8.2. The C-terminal residues produced by tryptic cleavage were determined by adding 0.2 mg (25 μl) of DFP-treated CPB (Worthington) to 0.2 ml aliquots of the digest. After 24 hrs. at room temperature the sample was diluted to 1 ml. with 0.2M citrate buffer, pH 2.2, and analyzed for basic amino acids on the short column of the analyzer. Corrections were made with blanks run in an identical fashion except for the omission of trypsin.

RESULTS

The effect of guanidination on the basic amino acids of BBI, RCM-BBI, and

Table I.

The basic amino acid composition of BBI, RCM-BBI, and α-lactalbumin before and after guanidination.

Protein	Amino Acid	Moles per mole	
		Unmodified	Guanidinated
BBI or RCM-BBI	Lys	5.14	0.16
	His	1.00	1.00
	Arg	2.10	2.00
	Homoarg	--	5.30
α-lactalbumin*	Lys	12	0.65
	His	3	3.00
	Arg	1	1.30
	Homoarg	--	11.60

* Composition of unmodified α-lactalbumin taken from (10).

α-lactalbumin is shown in Table I. It is evident that essentially all of the lysine residues of these proteins had been transformed into homoarginine. No change in the composition of the neutral and acid amino acids was observed.

The ability of BBI to inhibit trypsin was not impaired by guanidination (Fig. 1). Extrapolation of this curve to zero activity gives a molar combining ratio of approximately 1:1 for both the native and guanidinated BBI.

Data pertaining to the amino acids released by CPB from BBI and the guanidinated derivatives of BBI, RCM-BBI, and α-lactalbumin following treatment with trypsin are presented in Table II. When unmodified BBI was exposed to trypsin at pH 3.8, approximately 1 residue of lysine was released by CPB. Guanidinated BBI under the same conditions produced 0.4 residue of homoarginine. The cleavage of a homoarg-x bond could also be demonstrated when guanidinated RCM-BBI was digested with trypsin at pH 3.8 for 46 hrs. or pH 8.2 for 3 hrs.

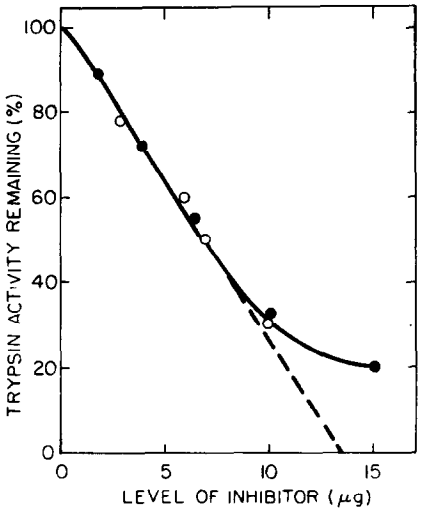

Fig. 1. Inhibitory effect of unmodified (O-O) and guanidinated (●-●) BBI towards 50 μg trypsin employing benzoyl-DL-arginine-p-nitroanilide as the substrate (11).

Table II.

Basic amino acids released by CPB from unmodified BBI and guanidinated derivatives of BBI, RCM-BBI, and α-lactalbumin after tryptic digestion.

Protein	Conditions for tryptic digestion		Amino acids released by CPB* moles per mole		
	pH	hrs.	Lys	Arg	Homoarg
BBI	3.8	46	0.92(5)	0(2)	0(0)
Guanidinated BBI	3.8	46	0(<1)	0(2)	0.41(5)
Guanidinated RCM-BBI	3.8	46	0(<1)	1.20(2)	0.98(5)
Guanidinated RCM-BBI	8.2	3	0(<1)	0.98(2)	0.82(5)
Guanidinated α-lactalbumin	8.2	4	0(<1)	0.65(1)	1.94(12)

* Total number of residues in protein are shown in parentheses and were taken from data in Table I.

In the case of α-lactalbumin an average of 2 such residues were apparently cleaved. This value, plus the close to one arginine residue released, represents a much lower degree of hydrolysis than might be expected for unmodified α-lactalbumin which has 13 peptide bonds susceptible to tryptic cleavage (1).

Further evidence for the tryptic cleavage of homoarg-x bonds was obtained by comparing the paper electrophoretic patterns of tryptic digests of RCM-BBI and α-lactalbumin before and after guanidination (Fig. 2). The total number of peptides were essentially the same in both the modified and unmodified proteins, and the number of Sakaguchi positive peptides in the tryptic digests of the guanidinated proteins are far greater than can be accounted for solely on the basis of the number of arginine residues which they contain.

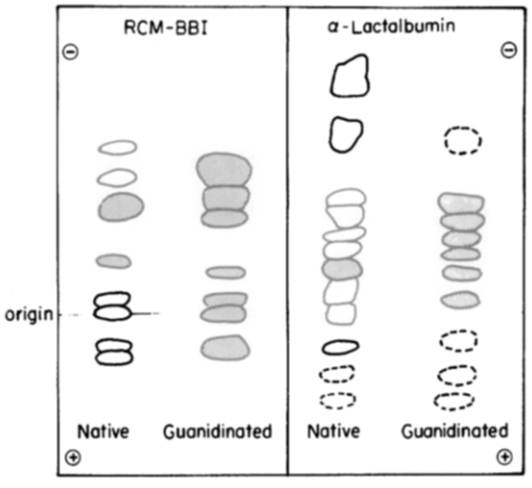

Fig. 2. High voltage paper electrophoresis of tryptic digests of native and guanidinated derivatives of RCM-BBI and α-lactalbumin (2000 volts, 60 min., pH 3.7). Peptides containing arginine and homoarginine were visualized by spraying with Sakaguchi reagent followed by dipping into acid ninhydrin solution (12). Sakaguchi positive peptides are cross-hatched.

DISCUSSION

Although peptide bonds containing homoarginine appear to be split more slowly by trypsin than those bearing the parent lysine residue, the present

study leaves little doubt that such a cleavage can occur. This apparent contradiction with earlier reports (1, 2) deserves comment. In the studies of Weil and Telka (1), the Van Slyke technique, which was used to measure the increase in free amino groups produced during the digestion of guanidinated α-lactalbumin by trypsin, may have lacked the sensitivity necessary to detect the slow splitting of peptide bonds. The studies of Shields et al. (2) involved a comparison of peptide maps of tryptic digests of native and guanidinated papain. The digest of the unmodified protein revealed 19 peptides, whereas 17 peptides were displayed by the modified protein despite the fact that 7 of the 8 lysine residues of papain had been converted to homoarginine, an observation which hardly supports the conclusion that no homoarginine bonds had been split by trypsin. The failure of Shields et al. (2) to observe the release of homoarginine when a tryptic digest of guanidinated papain was treated with CPB may be due to differences in experimental conditions from those employed here as well as to inherent differences in the relative susceptibility of certain peptide bonds to tryptic cleavage of papain compared to BBI.

Although trypsin has also been reported to be incapable of splitting benzoyl-L-homoargininamide (2), it is nevertheless of interest to note that two closely related substrates, benzoyl-L-canavaninamide (13) and hexyl-δ-guanidinovalerate (14) are slowly hydrolyzed by trypsin.

Our study confirms the earlier reports of Birk et al. (15) and Frattali and Steiner (16) regarding the tryptic modification of BBI under acid conditions and further identifies the reactive site as a lys-x bond.* The retention of antitryptic activity despite the replacement of lysine residues by homoarginine can now be considered compatible with the view that the site of reaction of a proteinase inhibitor is an amino acid residue whose peptide bond is susceptible to cleavage by that enzyme. Whether, however, this cleavage

* Birk has recently reported the same observation at the First International Research Conference on Proteinase Inhibitors, Munich, Germany, November 4-6, 1970.

does in fact constitute an obligatory step in the interaction of stoichiometric levels of enzyme and inhibitor at or near neutral pH still remains open to question (6).

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