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IDENTIFICATION OF THE CHYMOTRYPSIN-REACTIVE SITE OF THE BOWMAN–BIRK SOYBEAN INHIBITOR

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SUMMARY

End-group analysis of the Bowman–Birk inhibitor which had been exposed to catalytic levels of chymotrypsin (EC 3.4.4.5) at pH 3.8 indicated the cleavage of a Leu–Ser bond. Performic acid oxidation of the chymotrypsin-modified inhibitor yielded two fragments which could be separated by gel filtration on Sephadex G-25. From the end groups and amino acid composition of these two fragments, the chymotrypsin-reactive site was identified as Leu-48 and Ser-49. As in the case of the trypsin-reactive site, the sequence of amino acids in the vicinity of the chymotrypsin-reactive site of the Bowman–Birk inhibitor bears a high degree of homology with the corresponding site of the lima bean inhibitor.

INTRODUCTION

The interaction of trypsin (EC 3.4.4.4) with its naturally occurring inhibitors is believed to involve a unique lysine or arginine residue in the inhibitor molecule[1–7]. Although the splitting of a Lys–X or Arg–X bond can be usually demonstrated under acid conditions with catalytic levels of trypsin[1,3,4], whether this cleavage does in fact constitute an obligatory step in the interaction of stoichiometric levels of enzyme and inhibitor at or near neutral pH has been challenged[8,28]. In addition to an inhibitor of trypsin, some of these inhibitors are also capable of inhibiting chymotrypsin (EC 3.4.4.5) at a site which is independent of the trypsin-sensitive site. Examples of these so-called "double-headed" inhibitors are turkey ovomucoid[9], the lima bean inhibitor[6,10], and the Bowman–Birk soybean inhibitor[11,12].

In a previous paper[13] the trypsin-sensitive site of the Bowman–Birk soybean inhibitor was identified as lysine-17, and the sequence of amino acids surrounding this residue was found to bear a striking resemblance to the proposed antitrypsin site of the lima bean inhibitor[14,15]. This paper reports the identification of the chymo-

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trypsin-sensitive site of the Bowman–Birk soybean inhibitor. Again a degree of homology with the corresponding site of the lima bean inhibitor was apparent.

MATERIALS AND METHODS

Analogous to studies leading to the identification of the trypsin-reactive site of natural inhibitors[1–7], the chymotrypsin-reactive site of Bowman–Birk soybean inhibitor is presumed to involve the peptide bond which is split by catalytic amounts of chymotrypsin under acid conditions. Accordingly, 3 μmoles (24 mg) Bowman–Birk soybean inhibitor, prepared as previously described[13], were dissolved in 1.5 ml 0.05 M calcium acetate, pH 3.8, followed by the addition of 0.03 μmole (0.7 mg) chymotrypsin (crystallized 3 times, Worthington). This solution was allowed to stand at 30° for 48 h and then lyophilized. This preparation will hereafter be referred to as Bowman–Birk soybean inhibitor (chymotrypsin). Oxidative cleavage of the disulfide bonds of Bowman–Birk soybean inhibitor (chymotrypsin) was performed by oxidation with performic acid[16], and the resulting fragments were separated by chromatography on Sephadex G-25 using 0.2 M acetic acid for development of the column.

The details pertaining to the techniques used for end-group and amino acid analyses are described in a previous paper[13]. N-terminal sequences were determined by subtractive Edman degradation[17].

RESULTS

The results of the end-group analyses of Bowman–Birk soybean inhibitor and Bowman–Birk soybean inhibitor (chymotrypsin) are summarized in Table I. In addition to aspartic acid, which is derived from the amino-terminal asparagine residue of Bowman–Birk soybean inhibitor[18], the dansylation of Bowman–Birk soybean inhibitor (chymotrypsin) disclosed the presence of an N-terminal serine residue. The action of carboxypeptidase A (peptidyl-L-lysine hydrolase, EC 3.4.2.1) on Bowman–Birk soybean inhibitor (chymotrypsin) resulted in the release of 0.74 mole leucine per mole of protein, whereas only traces of glutamine were produced from intact Bowman–Birk soybean inhibitor. These results indicate quite clearly that a Leu–Ser bond had been split by the action of chymotrypsin on Bowman–Birk soybean inhibitor under the conditions specified here. Both Bowman–Birk soybean inhibitor and Bowman–Birk soybean inhibitor (chymotrypsin) proved to be quite resistant to the action of leucine aminopeptidase or aminopeptidase M (L-leucylpeptide hydrolases EC 3.4.1.1); the difficulty with which Bowman–Birk soybean inhibitor is attacked by exopeptidases had been noted previously[30]. The failure of these enzymes to release serine from Bowman–Birk soybean inhibitor (chymotrypsin) may be attributed to the proximity of a proline residue to the newly exposed serine group (see below).

When an oxidized preparation of Bowman–Birk soybean inhibitor (chymotrypsin) was subjected to gel filtration on Sephadex G-25, two components could be separated as shown in Fig. 1. These two fragments were characterized with respect to end groups (Table I) and amino acid composition (Table II). The larger of these two fragments, Fragment I, was composed of 48 amino acid residues and possessed the N-terminal asparagine residue of the original molecule. Digestion of Fragment I with

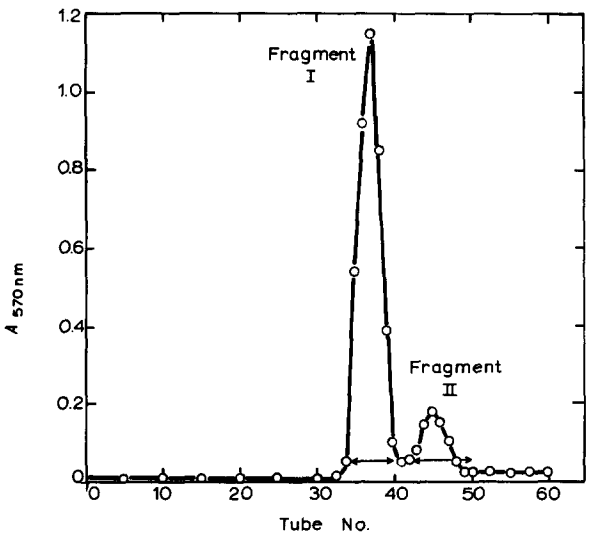

Fig. 1. Gel filtration of oxidized Bowman–Birk soybean inhibitor(chymotrypsin). A solution of 25 mg of oxidized Bowman–Birk soybean inhibitor(chymotrypsin) in 1.5 ml 3 M acetic acid was applied to a column (1 cm × 100 cm) of Sephadex G-25 (fine) and eluted with 0.2 M acetic acid. Flow rate was 9.6 ml/h and each tube contained 2.4 ml. Effluent was monitored by subjecting aliquots of each tube to alkaline hydrolysis followed by reaction with ninhydrin using the automated technique described by WALL[19].

TABLE I

END GROUPS OF BOWMAN–BIRK SOYBEAN INHIBITOR, BOWMAN–BIRK SOYBEAN INHIBITOR(CHYMOTRYPSIN), AND FRAGMENTS OF OXIDIZED BOWMAN–BIRK SOYBEAN INHIBITOR(CHYMOTRYPSIN)

Method used	*Bowman–Birk soybean inhibitor*	*Bowman–Birk soybean inhibitor (chymotrypsin)*	*Fragment from oxidized Bowman–Birk soybean inhibitor(chymotrypsin)*	
			I (large)	*II (small)*
N-terminus				
Dansylation	Asx	Asx, Ser	Asx	Ser
Leucine aminopeptidase	None	None	None	None
Aminopeptidase M	None	None	None	None
Edman	Asx–Asx–Cys–*	—	—	Ser–Tyr–Pro–
C-terminus				
Carboxypeptidase A**	Gln(0.05)	Leu(0.74), Gln(0.14)	Leu(0.74), Ala(0.74), *Plus* small amounts of $CysSO_3H$, Ile, Ser	None

* Unpublished data (M. A. JARVIS AND I. E. LIENER).

** The extent to which amino acids were released at the end of 1 h at 37° is shown in parentheses as moles per mole protein or peptide.

TABLE II

AMINO ACID COMPOSITION OF FRAGMENTS FROM OXIDIZED BOWMAN–BIRK SOYBEAN INHIBITOR(CHYMOTRYPSIN)

Amino acid	*Oxidized Bowman–Birk soybean inhibitor*	*Fragments from oxidized Bowman–Birk soybean inhibitor(chymotrypsin)*	
		I (large)	*II (small)*
Lysine	4.9(5)	3.7(4)	1.2(1)
Histidine	0.9(1)	1.0(1)	0.0(0)
Arginine	2.2(2)	2.2(2)	0.0(0)
Aspartic acid	11.7(12)	8.1(8)	4.2(4)
Threonine	2.1(2)	1.3(1)	0.8(1)
Serine	8.5(8)*	6.8(7)	1.5(1)*
Glutamic acid	6.9(7)	3.9(4)	3.2(3)
Proline	5.7(6)	4.2(4)	2.1(2)
Glycine	0.0(0)	0.0(0)	0.0(0)
Alanine	4.2(4)	3.2(3)	1.0(1)
Cysteic acid	13.7(14)	9.9(10)	3.7(4)
Valine	1.0(1)	0.1(0)	0.9(1)
Methionine sulfone	1.0(1)	1.0(1)	0.0(0)
Isoleucine	2.0(2)	1.0(1)	0.8(1)
Leucine	2.2(2)	1.8(2)	0.0(0)
Tyrosine**	1.5(2)	0.1(0)	1.4(2)
Phenylalanine	1.8(2)	0.2(0)	1.6(2)
Total number of residues	71	48	23

* There is some uncertainty as to whether there are 8 or 9 serine residues in Bowman–Birk soybean inhibitor[18]. The lower value has been tentatively adopted here in order to permit comparison with data reported earlier[13].

** Tyrosine values are lower than the expected 2 residues/mole[13] presumably due to the formation of halotyrosine derivatives by performic acid oxidation[16].

carboxypeptidase A resulted in the release of equivalent amounts of leucine and alanine followed by small amounts of cysteic acid, isoleucine, and serine. In view of the action of carboxypeptidase A in Bowman–Birk soybean inhibitor (chymotrypsin), leucine was judged to be the C-terminal residue of Fragment I and alanine the penultimate residue. Fragment II, the small fragment, was composed of 23 amino acids, and serine was established as its N-terminal residue on the basis of dansylation. Since Fragment II must be obviously derived from the C-terminal region of Bowman–Birk soybean inhibitor, its resistance to the action of carboxypeptidase A was not unexpected[13]. Three steps of the Edman degradation indicated a sequence of Ser–Tyr–Pro–. A heptapeptide with the sequence Ser–Tyr–Pro–Ala–Gln–Cys–Phe has been isolated from a chymotryptic digest of carboxamidomethylated Bowman–Birk soybean inhibitor (unpublished data). This sequence would indicate that this peptide is most likely derived from the N-terminal region of Fragment II. The proximity of the proline residue to the N-terminal serine residue would explain why serine was not released from Bowman–Birk soybean inhibitor (chymotrypsin) or Fragment II by leucine aminopeptidase or aminopeptidase M.

DISCUSSION

From the evidence presented it may be concluded that the chymotrypsin-

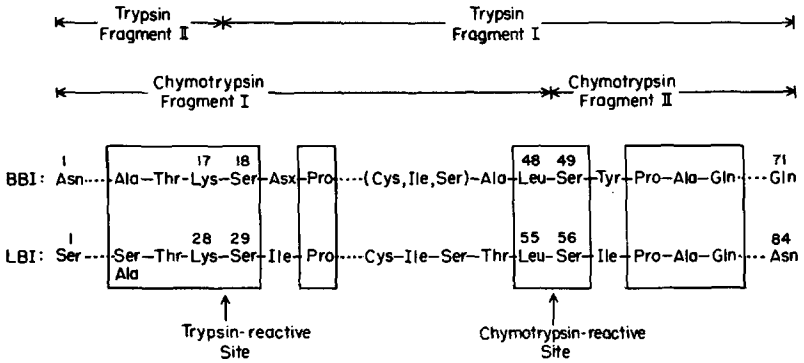

Fig. 2. Comparison of amino acid sequences around the trypsin-reactive and chymotrypsin-reactive sites of Bowman–Birk inhibitor (BBI) and the lima bean inhibitor (LBI). Sequence of trypsin-reactive site of BBI is taken from SEIDL AND LIENER[13], and sequence shown for LBI is taken from TAN AND STEVENS[15]. Segments of identical sequences in both proteins are enclosed in boxes. Deleted sequences of the molecules are denoted by dotted lines.

sensitive site of Bowman–Birk soybean inhibitor is a Leu–Ser bond which is located between Positions 48 and 49 in the primary structure of the molecule. Our present knowledge concerning the sequence of amino acids surrounding this particular bond is depicted in Fig. 2. Also included in this figure is the sequence of amino acids in the immediate vicinity of the trypsin-sensitive site of Bowman–Birk soybean inhibitor taken from a previous paper[13]. The sequences around the corresponding sites of the lima bean inhibitor, as reported by TAN AND STEVENS[15], are shown for comparison. The high degree of structural homology between the trypsin-reactive sites of Bowman-Birk soybean inhibitor and the lima bean inhibitor noted earlier[13] appears to be true of their chymotrypsin-reactive sites as well. To what extent this homology may extend to other regions of the molecule must await the outcome of sequence studies on Bowman–Birk soybean inhibitor now in progress.

Despite the intensive study which the interaction of trypsin and its natural inhibitors has received (see ref. 20 for review of the literature), the precise manner in which these macromolecules combine to form an inactive complex has not been fully clarified. Trypsin which has been inactivated by chemical modification of its active site is no longer capable of combining with various inhibitors[21–23], and the enzyme is protected from reacting with active site-directed reagents when complexed with such inhibitors[24]. Thus the combination of trypsin with its natural inhibitors seems to involve the participation of its active site in much the same way as the interaction of trypsin with a substrate for which it is specific. IMHOFF AND KEIL-DLOUHA[25] claim that the inhibition of trypsin by pancreatic inhibitor involves an electrostatic interaction between Asp-177, the amino acid residue believed to be responsible for the characteristic substrate specificity of trypsin[26,27], and Lys-15 of the inhibitor. In an analogous fashion the inhibition of trypsin by Bowman–Birk soybean inhibitor could involve Asp-177 of trypsin and Lys-17 of Bowman–Birk soybean inhibitor.

On the other hand, examples of chymotrypsin inhibition by natural inhibitors are less numerous, and the mechanism, of their interaction has received much less attention. In addition to Bowman–Birk soybean inhibitor and the lima bean inhibitor where the chymotrypsin-reactive site has been identified as a Leu–Ser bond, UY AND

FEENEY[28] have recently reported a Leu–Gly bond to be the site of cleavage of turkey and penguin ovomucoids by chymotrypsin.

A clue to the possible mode of interaction of chymotrypsin with Bowman–Birk soybean inhibitor is provided by an examination of the amino acid composition of Fragment II which contains Residues 49–71 derived from the C-terminal region of the inhibitor molecule. This portion of the molecule contains 6 hydrophobic residues (phenylalanine, tyrosine, leucine, isoleucine, and valine) out of the 9 which are present in the entire molecule. If one includes Leu-48 and the isoleucine residue which is near it (see Fig. 2), 8 of the 9 hydrophobic residues of Bowman–Birk soybean inhibitor are located in the C-terminal region making up about one-third of the total number of residues of the molecule. It is tempting to speculate that this region of the molecules, by virtue of its high concentration of hydrophobic residues, is favorably endowed to serve as a locus of binding with the specificity site of chymotrypsin which is buried in a hydrophobic environment[29].

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While this work was in progress BIRK AND GERTLER[30] reported that a Leu-Ser bond was the chymotrypsin reactive site of the Bowman-Birk inhibitor although experimental details were lacking.

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REFERENCES

1 K. OZAWA AND M. LASKOWSKI, JR., *J. Biol. Chem.*, 241 (1966) 3955.
2 L. F. KRESS AND M. LASKOWSKI, SR., *J. Biol. Chem.*, 243 (1968) 3548.
3 M. RIGBI AND L. J. GREENE, *J. Biol. Chem.*, 243 (1968) 5457.
4 K. HOCHSTRASSER AND E. WERLE, *Z. Physiol. Chem.*, 350 (1969) 249.
5 H. FRITZ, E. FINK, M. GEBHARDT, K. HOCHSTRASSER AND E. WERLE, *Z. Physiol. Chem.*, 350 (1969) 933.
6 J. KRAHN AND F. C. STEVENS, *Biochemistry*, 9 (1970) 2466.
7 D. S. SEIDL AND I. E. LIENER, *Biochem. Biophys. Res. Commun.*, 42 (1971) 1101.
8 R. HAYNES AND R. E. FEENEY, *Biochemistry*, 7 (1968) 2879.
9 F. C. STEVENS AND R. E. FEENEY, *Biochemistry*, 2 (1963) 1346.
10 R. HAYNES AND R. E. FEENEY, *J. Biol. Chem.*, 242 (1967) 5378.
11 Y. BIRK, A. GERTLER AND S. KHALEF, *Biochim. Biophys. Acta*, 147 (1967) 402.
12 V. FRATTALI AND R. F. STEINER, *Biochem. Biophys. Res. Commun.*, 34 (1969) 480.
13 D. S. SEIDL AND I. E. LIENER, *Biochim. Biophys. Acta*, 251 (1971) 83.
14 C. G. L. TAN AND F. C. STEVENS, *Eur. J. Biochem.*, 18 (1971) 503.
15 C. G. L. TAN AND F. C. STEVENS, *Eur. J. Biochem.*, 18 (1971) 515.
16 C. H. W. HIRS, in C. H. W. HIRS, *Methods Enzymol.*, 11 (1967) 197.
17 W. T. SHEARER, R. A. BRADSHAW, F. R. N. GURD AND T. PETERS, JR., *J. Biol. Chem.*, 242 (1967) 5451.
18 B. KASSELL, in G. E. PERLMANN AND L. LORAND, *Methods Enzymol.*, 19 (1970) 859.
19 R. A. WALL, *Anal. Biochem.*, 35 (1970) 203.
20 I. E. LIENER AND M. L. KAKADE, in I. E. LIENER, *Toxic Constituents of Plant Foodstuffs*, Academic Press, New York, 1969, p. 8.
21 N. M. GREEN, *J. Biol. Chem.*, 205 (1953) 535.

22 E. F. ESTERMAN AND A. D. MCLAREN, *Photochem. Photobiol.*, 1 (1962) 109.
23 G. FEINSTEIN AND R. E. FEENEY, *J. Biol. Chem.*, 241 (1966) 5183.
24 J. PUDLES AND D. BACHELLERIE, *Arch. Biochem. Biophys.*, 128 (1968) 133.
25 J.-M. IMHOFF AND V. KEIL-DLOUHA, *FEBS Lett.*, 12 (1971) 345.
26 R. L. SMITH AND E. SHAW, *J. Biol. Chem.*, 244 (1969) 4704.
27 B. S. HARTLEY, *Phil. Trans. Roy. Soc. London Ser. B*, 257 (1970) 77.
28 R. L. UY AND R. E. FEENEY, *Fed. Proc.*, 30 (1971) 1130.
29 D. M. BLOW, J. J. BIRKTOFT AND B. S. HARTLEY, *Nature*, 221 (1969) 337.
30 Y. BIRK AND A. GERTLER, in H. FRITZ AND H. TSCHESCHE, *Proceedings of the International Research Conference on Proteinase Inhibitions*, Walter de Gruyter, Berlin, 1971, p. 142.