The amino acid sequences around the disulfide bonds of soybean trypsin inhibitor[*]

J.R. Brown,[**] N. Lerman and Z. Bohak
Department of Biophysics,
The Weizmann Institute of Science,
Rehovoth, Israel

Received April 19, 1966

Soybean trypsin inhibitor (STI, Molecular weight 21,500, Wu and Scheraga, 1962) contains two disulfide bonds and no free sulfhydryl groups (Kunitz, 1947). The disulfide bonds can be reduced and then reoxidized to yield a protein with inhibitor activity (Steiner, 1965; Steiner et al., 1965; Lerman, 1965). As a part of a study of the reduction and reoxidation of the disulfide bonds we determined the sequence of the amino acids around the disulfide bridges in this protein. This was accomplished by digesting the inhibitor with pepsin, and identification of the cystine containing peptides by the diagonal mapping technique of Brown and Hartley (1963, 1966).

Soybean trypsin inhibitor was puchased from Worthington Biochemical Corporation. Preparation lot 5490, which yielded on chromatographic analysis according to Rackis et al. (1962) a single peak containing all activity and over 95% of total protein, was used without further purification. The inhibitor was digested with pepsin in 5% formic acid for 19 hours at 37°C (enzyme to substrate ratio 1:10). The digest was separated by paper electrophoresis at pH 6.5. Staining a narrow guide strip with cyanide-nitroprusside (Toennies and Kolb, 1951) revealed two cystine containing zones (A and B Fig. 1). A second guide strip was exposed to performic acid vapour and subjected to electrophoresis

[*] Taken in part from a dissertation to be submitted by N. Lerman to the Feinberg Graduate School of the Weizmann Institute of Science, in partial fulfilment of the requirements for the Ph. D. degree.

[**] Visiting Scientist from the MRC Laboratory of Molecular Biology, Cambridge, England, on a Fellowship from the National Institutes of Health, U.S. Public Health Service.

as described in legend for Fig. 1, whereupon the cysteic acid peptides moved off the diagonal line, containing all the other peptides. One cysteic acid peptide was obtained from zone A and four cysteic acid peptides were obtained from zone B (Fig. 1). The areas corresponding to zones A and B were cut out from

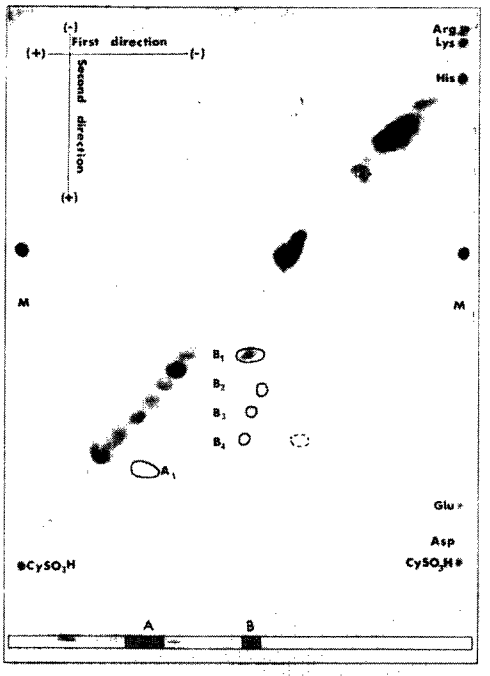
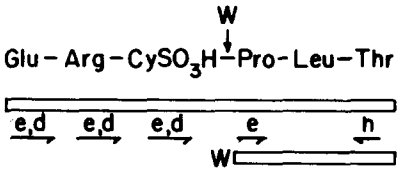


Fig. 1. Diagonal finger print of peptic peptides of STI. Peptides were separated by electrophoresis at pH 6.5 . A narrow strip was cut off the paper, oxidized in performic acid vapour, stitched to another sheet of paper and subjected again to electrophoresis at an angle of 90^0 to the first direction. Peptides appear now on a diagonal line; cysteic acid peptides only lie off the line. The barred strip at the bottom represents the location of cystine peptides after electrophoresis in the first direction (cyanide-nitroprusside stain, Toennies and Kolb, 1951).

the main part of the paper, exposed to performic acid vapours and the cysteic acid peptides were then isolated by high voltage paper electrophoresis.

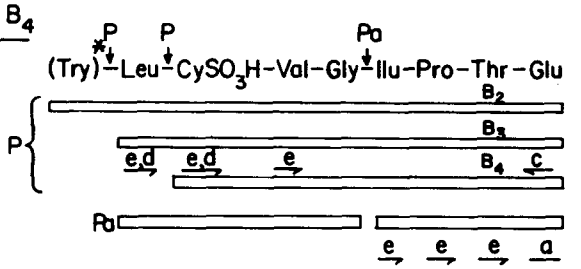
The sequences of the peptides arising from zone B were determined by the procedures indicated in Fig. 2. It was found that B_2, B_3 and B_4 are related; peptides B_3 and B_4 arising from B_2 by the partial cleavage of the Try-Leu and Leu-Cys bonds by pepsin. Of these three peptides, B_3 was obtained in the

Peptide B_1



W— Peptide isolated after cleavage by sodium in liquid ammonia (Wilchek et al., 1965)

Peptides B_2, B_3, B_4



P— Peptides isolated from zone B of the peptic digest of soybean trypsin inhibitor (see fig. 1)

Pa— Peptides isolated after cleavage of B_3 with papain

*The evidence for amino terminal tryptophan of peptide B_2 is based on the positive Erlich's reaction of zone B, the fluorescence of zone B and peptide B_2 and on the electrophoretic mobility of Peptide B_2

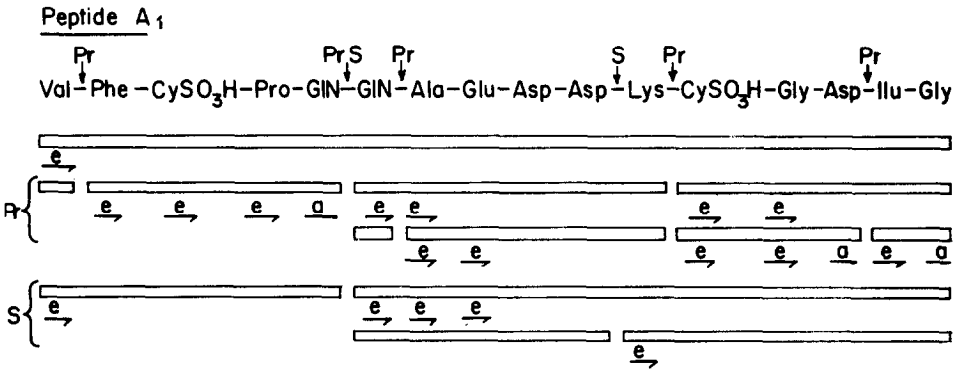
Fig. 2 Determination of the amino acid sequences of the cysteic acid peptides isolated from zone B.
e subtractive Edman ; d Edman - dansyl (Gray and Hartley, 1963) ;
a free amino acid after ultimate Edman cleavage ; c carboxypeptidase A ;
h hydrazinolysis.

highest yield and was, therefore, used for detailed study (Fig. 2). Peptide B_1 is different in composition and sequence from the other B peptides and is derived from the "mate" half cystine peptide. It seems that the group of peptides in zone B account completely for the part of the STI molecule containing one of the disulfide bonds. The sequence proposed for this part is:

H_2N - Glu - Arg - Cys - Pro - Leu - Thr - COOH
|
H_2N -(Try)- Leu - Cys - Val - Gly - Ilu - Pro - Thr - Glu - COOH

Peptide A_1 was found to contain sixteen amino acid residues of which two are cysteic acid. The procedures used for the determination of its sequence are shown in Fig. 3 . Peptide A_1 is readily cleaved by pronase, subtilisin and the alkaline proteinase of Aspergillus orisi (Subramanian and Kalnitsky, 1964). A

major split observed with all three enzymes occurs at the GlN-GlN bond. Pronase showed, in addition, a marked amino peptidase activity releasing valine from the original N-terminus and glutamine from the N-terminus of the split product (Fig. 3).



Pr— Peptides isolated after cleavage of A_1 with Pronase

S — Peptides isolated after cleavage of A_1 with Subtilisin

Fig.3 Determination of the amino acid sequence of the cysteic acid peptide isolated from zone A.
Pr = peptides isolated after cleavage of A_1 with pronase.
S = peptides isolated after cleavage of A_1 with subtilisin.
e = subtractive Edman ; a = free amino acid after ultimate Edman cleavage.

The occurrence of enzymatic cleavage between the two glutamine residues simplified considerably the location of the amides in the sequence. The first glutamine became the C-terminal residue of a short peptide, from which it was released after the third round of Edman degradation (Fig. 3). It was identified on the amino acid analyzer as a peak in the position of serine which disappeared on acid hydrolysis yielding glutamic acid. The position of the second glutamine was deduced from the examination of the products obtained on splitting peptide A_1 with pronase (Fig. 2). The assigned location for this glutamine was confirmed by allowing pronase to act on the purified peptide containing this glutamine which was obtained by subtilisin cleavage of A_1 (Fig. 3). One equivalent of glutamine, some alanine but no glutamic acid were released in this reaction.

It is pertinent to mention here that the Lys-$CySO_3H$ bond in the oxidized peptide A_1 was resistant to cleavage by trypsin under the conditions where the Arg-$CySO_3H$ bond of oxidized ribonuclease A is appreciably hydrolyzed (Hirs et al., 1956). This is probably due to the presence of the four dicarboxylic acid residues near the lysyl residue.

Peptide A_1 is the only cysteic acid peptide found in zone A and its sequence is completely different from that of any peptide from zone B. Peptide A_1 must therefore be derived from that part of the STI molecule which contains the second disulfide bond. Since two cysteic acid residues are found in this peptide it is apparent that one of the disulfide bonds of STI is a part of a small cyclic or loop structure, which is maintained on cleavage of the molecule with pepsin. The sequence proposed for this loop is:

$$\begin{array}{rl} & H_2N - Val - Phe - Cys - Pro - GlN - GlN - Ala \\ & \qquad\qquad\qquad\qquad\quad | \qquad\qquad\qquad\quad | \\ HOOC - Gly - Ilu - Asp - Gly - & Cys - Lys - Asp - Asp - Glu \end{array}$$

REFERENCES

1. Brown, J.R. and Hartley, B.S., Biochem. J. 89, 59P (1963).
2. Brown, J.R. and Hartley, B.S., Biochem. J. in press (1966).
3. Gray, W.R. and Hartley, B.S., Biochem. J. 89, 59P (1963).
4. Hirs, C.H.W., Stein, W.H. and Moore, S., J. Biol. Chem. 219, 623 (1956).
5. Kunitz, M., J. Gen. Physiol. 30, 291 (1949).
6. Lerman, N., unpublished results (1965).
7. Rackis, J.J., Sazame, H.A., Mann, R.K., Anderson, R.L. and Smith, A.K., Arch. Biochim. Biophys. 98, 471 (1962).
8. Subramanian, A.R. and Kalnitsky, G., Biochemistry, 3, 1861 (1964).
9. Steiner, R.F., Biochim. Biophys. Acta, 100, 111 (1965).
10. Steiner, R.F., De Lorenzo, F. and Anfinsen, C.B., J. Biol. Chem. 240, 4648 (1965).
11. Toennies, G. and Kolb, I.I., Anal. Chem. 23, 823 (1951).
12. Wilchek, M., Sarid, S. and Patchornik, A., Biochim. Biophys. Acta, 104, 616 (1965).
13. Wu, Y.V. and Scheraga, H.A., Biochemistry, 1, 698 (1962).