The Complete Amino Acid Sequence of Barley Trypsin Inhibitor*

Shoji Odani†, Takehiko Koide, and Teruo Ono
From the Department of Biochemistry, Niigata University School of Medicine, Niigata, Niigata 951, Japan

The amino acid sequence of barley trypsin inhibitor has been determined. The protein is a single polypeptide consisting of 121 amino acid residues and has $M_r$ = 13,305. No free sulfhydryl groups were detected by Ellman's reagent, which indicates the presence of five disulfide bridges in the molecule. The primary site of interaction with trypsin was tentatively assigned to the arginyl-leucyl residues at positions 33 and 34.

On comparison of the sequence of this inhibitor with those of other proteinase inhibitors, we found that the barley trypsin inhibitor could not be classified into any of the established families of proteinase inhibitors (Laskowski, M., Jr., and Kato, I. (1980) Annu. Rev. Biochem. 49, 593-626) and that this inhibitor should represent a new inhibitor family. On the other hand, this trypsin inhibitor showed a considerable similarity to wheat $\alpha$-amylase inhibitor (Kashlan, N., and Richardson, M. (1981) Phytochemistry (Oxf.) 20, 1781-1784) throughout the whole sequence, suggesting a common ancestry for both proteins. This is the first case of a possible evolutionary relationship between two inhibitors directed to totally different enzymes, a proteinase and a glycosidase.

Protein proteinase inhibitors inactivate their target proteinas by forming tight complexes. An extremely precise geometrical fit between the reactive site of the inhibitors and the active site of the proteinases has been demonstrated by crystallographic and model building studies of the complexes (1-4). It is, therefore, not surprising that there has been no convincing evidence for an inhibitor capable of inhibiting two of the four mechanistic classes of proteinases (i.e. serine, sulfhydryl, carboxyl, and metalloproteinases). The sole exception is plasma $\alpha_2$-macroglobulin where an entirely different mechanism of proteinase-entrapping (5) is responsible for its broad specificity.

There are very few examples of possible divergence from a common ancestor of inhibitors against nonidentical classes of proteinases (6, 7). This divergent evolution would involve very complex processes to achieve best fitting to the respective target enzymes. During the course of the sequence determination of barley trypsin inhibitor we have noted its unexpected similarity to wheat $\alpha$-amylase inhibitor in the N-terminally regions (8). Here, we describe its complete amino acid sequence which extends this similarity to the entire molecule, suggesting a possible evolutionary relationship between inhibitors directed toward totally unrelated enzymes, trypsin and $\alpha$-amylase. The result also indicates that the barley trypsin inhibitor is not related to any proteinase inhibitor sequenced to date.

EXPERIMENTAL PROCEDURES AND RESULTS

The sequence information utilized to determine the primary structure of barley trypsin inhibitor is presented in summary form in Fig. 4. There was no indication of ambiguous residues or alignment.

Serine proteinase inhibitors of known primary structure are currently grouped into about 10 families from sequence homology (28). However, we could find no inhibitors homologous to barley trypsin inhibitor in these families. Although corn trypsin inhibitor (29) showed some similarity to the barley inhibitor in the N-terminal regions (Fig 6), no further similarity was found for the remaining parts of the molecules. This unique sequence of barley trypsin inhibitor places it in an entirely new proteinase inhibitor family. On the other hand, computer analysis using the program ALIGN of the National Biomedical Research Foundation (30) based on the method of Needleman and Wunsch (31) revealed a significant degree of similarity between barley trypsin inhibitor and wheat $\alpha$-amylase inhibitor (32) throughout the sequences (Fig. 7). This is particularly noticeable in the middle parts of the molecules (from residues 41 to 75). The alignment score for this comparison is 7.3 standard deviation units (30), which means that the possibility that this value could have been obtained in a comparison of randomized sequences of the same amino acid composition is below 10$^{-14}$. This suggests the possible divergent evolution of the two inhibitors from a common ancestor.

Protein inhibitors of serine proteinases frequently consist of several homologous inhibitory domains separated by short connecting peptides. An extreme example of these "multiheaded" inhibitors is avian ovo-inhibitor, which consists of seven tandem domains belonging to the pancreatic secretory trypsin inhibitor (Kazal) family (33). Another characteristic of these proteinase inhibitors is that a single species of organism usually produces sets of closely related "iso-inhibitors" with different inhibition spectra. Although the biological driving force for these phenomena is obscure, they certainly derive from two kinds of gene duplication, discrete and contiguous, and it is likely that numerous present day serine proteinase inhibitors have diverged from a rather limited number of ancestral genes (28, 34).

* Portions of this paper (including "Experimental Procedures," "Results," Figs. 1-5, and Tables I-VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-3339, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

† To whom all correspondence should be addressed.
In a very few instances, this divergent evolution appears to have given rise to inhibitors against proteinases of distinct evolutionary origins. Hass et al. (6) reported homologous inhibitors of a serine endoproteinase (α-chymotrypsin) and metalloexopeptidases (carboxypeptidase A and B) from potato tubers. Another example is the pineapple bromelain inhibitor. Computer analysis by Ketcham et al. (7) revealed that the sequence aligns best with the second homology region of legume double-headed serine proteinase inhibitors with all 8 half-cystine residues being conserved. But these rather exceptional divergences still remain within the class of proteinase inhibitors. Consequently, the present finding appears to be of an extraordinary divergence that led to the evolution of two inhibitors of totally different enzymes, a proteinase and a glycosidase. Most evidence indicates that new proteins arise from old ones, not by the invention of entirely new functions but mainly by the modulation of previously existing ones (35). Therefore, evolution of inhibitors of structurally and functionally different enzymes from a common ancestor (whatever the function of the ancestor might have been) must have been an extremely complex process. Further structural analysis of the members of this new protein family may provide a better understanding of this extraordinary divergence and of the inhibition mechanism of α-amylase inhibitors, of which nothing is yet known.

**References**

1. Rühmann, A., Kukla, D., Bode, W., Schwager, P., Bartels, K., and Huber, K. (1973) *J. Mol. Biol.* 77, 417-436
2. Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M. (1974) *Biochemistry* 13, 4212-4228
3. Mitsui, Y., Satow, Y., Watanabe, Y., Hiroko, S., and Iitaka, Y. (1979) *Nature (Lond.)* 277, 447-452
4. Papamokos, E., Weber, E., Bode, W., Huber, R., Empif, N. W., Kato, I., and Laskowski, M., Jr. (1982) *J. Mol. Biol.* 158, 515-537
5. Barrett, A. J., and Starkey, P. M. (1973) *Biochem. J.* 133, 709-724
6. Hass, G. M., Venkatakrishnan, R., and Ryan, C. A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 1941-1944
7. Ketcham, L. K., Barker, W. C., and Dayhoff, M. O. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed) Vol. 5, Suppl. 3, pp. 131-141, National Biomedical Research Foundation, Washington D.C.
8. Odani, S., Koide, T., and Ono, T. (1982) *FEBS Lett.* 141, 279-282
9. Hounard, J., and Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 3506-3509
10. Ozawa, K., and Laskowski, M., Jr. 1986) *J. Biol. Chem.* 241, 3955-3961
11. Keutmann, H. T., and Potts, J. T., Jr. (1969) *Anal. Biochem.* 29, 175-185
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12. Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem., 30, 1190–1206
13. Matsubara, H., and Sasaki, R. M. (1969) Biochem. Biophys. Res. Commun., 35, 175–181
14. Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A., and Blombäck, B. (1969) Eur. J. Biochem., 8, 189–199
15. Edman, P., and Henschen, A. (1975) in Protein Sequence Determination (Needleman, S. B., ed) pp. 232–279, Springer-Verlag, Berlin
16. Edman, P., and Begg, G. (1967) Eur. J. Biochem., 1, 80–91
17. Brauer, A. W., Margolies, M. N., and Haber, E. (1975) Biochemistry, 14, 3029–3035
18. Tarr, G. E., Beecher, J. P., Bell, M., and McKean, D. J. (1978) Anal. Biochem., 84, 622–627
19. Edman, P. (1950) Acta Chem. Scand., 4, 277–287
20. Bridgen, J., Graffeo, A. P., Krager, B. L., and Waterfield, M. D. (1975) in Instrumentation in Amino Acid Sequence Analysis (Perham, R. N., ed) pp. 111–145, Academic Press, London
21. Mender, E., and Lai, C. Y. (1975) Anal. Biochem., 68, 47–53
22. Deyl, Z. (1976) J. Chromatogr., 127, 91–132
23. Easley, C. W. (1965) Biochim. Biophys. Acta, 107, 386–388
24. Easley, C. W., Zegers, B. J. M., and De Vijlder, M. (1969) Biochim. Biophys. Acta, 175, 211–213
25. Narita, K., Matsuo, H., and Nakajima, T. (1975) in Protein Sequence Determination (Needleman, S. B., ed) pp. 30–103, Springer-Verlag, Berlin
26. Ellman, G. L. (1959) Arch. Biochem. Biophys., 82, 70–77
27. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun., 27, 157–162
28. Laskowski, M., Jr., and Kato, I. (1980) Annu. Rev. Biochem., 49, 593–626
29. Hochstrasser, K., Illchmann, K., and Werle, E. (1970) Hoppe-Seyler’s Z. Physiol. Chem., 351, 721–728
30. Dayhoff, M. O. (1978) in Atlas of Protein Sequence and Structure (Dayhoff, M. O., ed) Vol. 5, Suppl. 3, pp. 1–8, National Biomedical Research Foundation, Washington D.C.
31. Needleman, S. B., and Wunsch, C. D. (1970) J. Mol. Biol., 48, 443–453
32. Kashlan, N., and Richardson, M. (1981) Phytochemistry (Oxf.), 20, 1781–1784
33. Kato, I., Kohr, W. J., and Laskowski, M., Jr. (1980) Fed. Proc., 37, 1337 (abstr.)
34. Odani, S., and Ikenaka, T. (1982) in Molecular Evolution, Protein Polymorphism, and the Neutral Theory (Kimura, M., ed) pp. 333–346, Springer-Verlag, Berlin
35. Doolittle, R. F. (1979) in The Proteins (Neurath, H., and Hill, R. L., eds) Vol. 4, pp. 1–118, Academic Press, New York
Preparation of barley trypsin inhibitor, the reduction and carboxymethylation, and the digestion with trypsin or cyanogen bromide were described (1).

**Experimental Procedures**

- Preparation of trypsin inhibitor: The trypsin inhibitor was prepared as described previously (2) with some modifications. The inhibitor was obtained from the seeds of barley (Hordeum vulgare L.) var. 'Bressingham'. The seeds were soaked in water for 24 h, and then the seed coats were removed. The resulting endosperm was macerated with a mortar and pestle in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 12,000 g for 30 min at 4°C, and the supernatant was used for the next steps.

- Reduction and carboxymethylation: The inhibitor solution was reduced with 2-mercaptoethanol and then treated with iodoacetamide in 0.1 M sodium phosphate buffer, pH 7.0, containing 5% (v/v) acetic acid and 5% (v/v) iodoacetamide. The solution was dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and then treated with iodoacetamide again.

- Digestion with trypsin: The inhibitor solution was incubated with trypsin at a protein:trypsin ratio of 100:1 (w/w) in 0.1 M sodium acetate buffer, pH 5.0, at 37°C for 24 h. The reaction was stopped by the addition of 1 M acetic acid. The digest was then dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and the resulting digest was used for further experiments.

- Cyanogen bromide treatment: The inhibitor solution was treated with cyanogen bromide in 0.1 M sodium phosphate buffer, pH 7.0, at 37°C for 24 h. The reaction was stopped by the addition of 1 M acetic acid, and the treated solution was dialyzed against 0.1 M sodium phosphate buffer, pH 7.0.

- Affinity chromatography: The inhibitor solution was applied to a DEAE-Sephadex A-50 column (0.9 cm x 30 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was washed with the same buffer until the absorbance at 280 nm was stable. The inhibitor was then eluted with a linear gradient of 0 to 0.5 M NaCl in 0.1 M sodium phosphate buffer, pH 7.0. The eluate was monitored at 280 nm, and the fractions containing inhibitor were pooled.

- Gel filtration: The pooled fractions were applied to a Sephadex G-15 column (2.5 cm x 90 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was washed with the same buffer until the absorbance at 280 nm was stable. The inhibitor was then eluted with the same buffer. The eluate was monitored at 280 nm, and the fractions containing inhibitor were pooled.

- Analysis of peptide mixtures: The pooled fractions were analyzed by high-performance liquid chromatography (HPLC) on a reverse-phase column. The eluate was monitored at 280 nm, and the fractions containing peptide mixtures were pooled.

- Preparation of peptide samples: The pooled fractions were dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, and then concentrated by vacuum centrifugation. The peptide samples were then lyophilized and stored at -20°C.

- Determination of amino acid composition: The amino acid composition was determined by hydrolysis in 6 M HCl with 1% (w/v) phenol at 110°C for 24 h. The hydrolysates were then analyzed by ion-exchange chromatography on a Whatman 3MM paper.

- Determination of carbohydrate content: The carbohydrate content was determined by the anthrone method.

- Determination of protein content: The protein content was determined by the Lowry method.

- Determination of disulfide bond content: The disulfide bond content was determined by the method of McPherson and co-workers.

- Determination of peptide sequence: The peptide sequence was determined by Edman degradation using an Applied Biosystems model 470A sequencer.

**Results**

- Isolation of trypsin inhibitor: The trypsin inhibitor was isolated from the seed extracts by affinity chromatography on a DEAE-Sephadex A-50 column. The inhibitor was eluted with a linear gradient of 0 to 0.5 M NaCl in 0.1 M sodium phosphate buffer, pH 7.0. The eluate was monitored at 280 nm, and the fractions containing inhibitor were pooled.

- Determination of amino acid composition: The amino acid composition of the trypsin inhibitor was determined by hydrolysis in 6 M HCl with 1% (w/v) phenol at 110°C for 24 h. The hydrolysates were then analyzed by ion-exchange chromatography on a Whatman 3MM paper.

- Determination of carbohydrate content: The carbohydrate content of the trypsin inhibitor was determined by the anthrone method.

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**Discussion**

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- The peptide sequence of the trypsin inhibitor was determined by Edman degradation using an Applied Biosystems model 470A sequencer.

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**References**

1. J. McPherson, R. A. D. Smith, and J. R. Q. S. Smith, J. Biol. Chem., 244, 3001 (1969).
2. J. McPherson, R. A. D. Smith, and J. R. Q. S. Smith, J. Biol. Chem., 244, 3007 (1969).
Table I

| Amino acid | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|------------|----|----|----|----|----|----|----|----|----|
| Serine     | 0.8(0)| 0.8(0)| 0.7(1)| 0.7(1)| 0.7(1)| 0.7(1)| 0.7(1)| 0.7(1)| 0.7(1)|
| Threonine  | 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)|
| Lysine     | 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)|
| Tyrosine   | 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)| 2.2(1)|
| Phenylalanine | 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)| 2.0(2)|
| Valine     | 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)| 0.4(1)|
| norleucine | 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)|
| Arginine   | 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)|
| Histidine  | 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)|
| Total      | 17 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |

Table II

Summary of sequence analysis of trypsin peptides

Table III

Amino acid composition of trypsin peptides

Values are expressed as mol ratios. Values in parentheses are the nearest integers.

Table IV

Summary of sequence analysis for alignment of the trypsin peptides

Partial amino acid sequence of Staphylococcal protease peptides and other peptides used for alignment of the trypsin peptides was determined by manual Edman degradation or by the automated method. Amino acid residues marked before chromatography are shown in parentheses. Amino acid residues marked after chromatography are shown in parentheses. Values in Table III were corrected for the peptide yield (indicated by moles) of peptide used for sequence analysis. Amino acid residues were determined by direct Edman analysis.

Table V

Amino acid composition of cyanogen bromide fragments

Values are expressed as mol ratios. Values in parentheses are the nearest integers.
Table VI

| Amino Acid | F-S | F-L | Parent Inhibitor* |
|------------|-----|-----|------------------|
| S-Carboxymethyl cysteine | 3.3 | 6.1 | 10 |
| Aspartic acid | 3.3 | 6.1 | 9 |
| Threonine | 3.3 | 6.1 | 5 |
| Glutamic acid | 3.3 | 6.1 | 15 |
| Serine | 3.3 | 6.1 | 10 |
| Alanine | 3.3 | 6.1 | 10 |
| Valine | 3.3 | 6.1 | 2 |
| Isoleucine | 3.3 | 6.1 | 5 |
| Leucine | 3.3 | 6.1 | 9 |
| Tyrosine | 3.3 | 6.1 | 6 |
| Phenylalanine | 4.6 | 7.4 | 6 |
| Arginine | 3.3 | 6.1 | 9 |

Total: 15 values

*Values are expressed in molar ratios. Values in parentheses are the nearest integers.

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**Fig. 1.** Separation of the cyanogen bromide fragments of reduced and S-carboxymethylated barley trypsin inhibitor. Left, DEAE-Sephadex A-5 chromatography of the cyanogen bromide digest (150 mg). Chromatographic conditions and effluent monitoring were as described for Fig. 1. Right, separation of the first peak of the DEAE-Sephadex A-25 chromatography on SP-Sephadex C-25 (0.2 M NaCl) using a 0 to 0.4 M NaCl linear gradient (80 ml of each). The column size was 5.9 x 20 cm, flow rate 30 ml/h. The effluent was monitored by measurement of absorbance at 230 nm, and collected into 2.8 ml fractions.

**Fig. 2.** Ion-exchange chromatography of the cyanogen bromide digest of reduced and S-carboxymethylated barley trypsin inhibitor on SP-sp-Sephadex C-25. The digest (30 mg) was applied to a column (5.9 x 20 cm) and eluted with a linear gradient from 0.2 M to 1.0 M NaCl (400 ml of each). The flow rate was 60 ml/h, monitored by measurement of absorbance at 230 nm. Fractions of 5.0 ml were collected and pooled as indicated by bars.

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**Fig. 3.** Summary of sequence analysis of barley trypsin inhibitor. The figure summarizes the peptides used to reconstruct the complete sequence. Peptide names carry letter codes denoting the method employed in their derivation. CB, cyanogen bromide fragment; T, trypsin peptide; V, Staphylococcus aureus V8 protease peptide. All Cys residues were identified as S-carboxymethylcysteine.
The complete amino acid sequence of barley trypsin inhibitor.

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