IDENTIFICATION OF THE REACTIVE SITES IN TWO HOMOLOGOUS SERINE PROTEINASE INHIBITORS ISOLATED FROM BARLEY

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Chymotrypsin inhibitors CI-1 and CI-2 from barley were subjected to limited hydrolysis by subtilisin Carlsberg, subtilisin Novo and chymotrypsin. Analysis of CI-2 derived peptides by amino acid sequencing identified peptide bond, Met⁵⁹-Glu⁶⁰, as the reactive site towards these three proteinases. In contrast, inhibitor CI-1 showed a single reactive site (Leu⁵⁹-Asp⁶⁰) towards chymotrypsin and two reactive sites (Met³⁰-Ser³¹ and Leu⁵⁹-Asp⁶⁰) towards either of the subtilisins.

1. INTRODUCTION

The high lysine content of Hiproly barley originates mainly from its lysine-rich, water soluble albumin fraction (4,8,11,13). Attempts to identify the proteins responsible for the high lysine content of the Hiproly albumin fraction led to the isolation and characterization of a lysine-rich protein of molecular weight 9,100 which was originally named SP II albumin after the chromatographic fraction from which it was obtained (7,8). This protein was later demonstrated to be

an inhibitor of chymotrypsin and subtilisin (17). It is therefore desirable to rename the protein, chymotrypsin inhibitor 2 (CI-2), in accordance with this activity (17).

In addition to CI-2, Hiproly endosperm contains another chymotrypsin inhibitor named CI-1 (6). It has been shown that the two barley inhibitors and an inhibitor from leach (*Hirudo medicinalis*) display extended amino acid sequence homology with potato inhibitor I (16) and thus they belong to the "potato inhibitor I family" (17).

Abbreviations: CI-1 and CI-2 = chymotrypsin inhibitor 1 and 2 from barley, GPNA = glutaryl-L-phenylalanine-p-nitroanilide, Polybrene = hexadimethrine bromide, PTH = phenylthiohydantoin, SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis, THEED = N, N, N', N'-tetrakis (2-hydroxyethyl) ethylenediamine.

It has been shown that the inhibitory action in the majority of specific serine proteinase inhibitors are localized to a specific reactive (inhibitory) peptide bond situated within a loop closed by a disulfide bridge (15). Such a loop is not, however, present in the two barley inhibitors. The purpose of the present communication is to identify the reactive sites of these inhibitors and to compare them with reactive sites found in other members of the "potato inhibitor I family".

2. MATERIALS AND METHODS

2.1. Materials

Hordeum vulgare cv. Hiproly was grown at Carlsberg Plant Breeding's farm "Hyldagergård" in the summer of 1979. Subtilisin Novo, subtilisin Carlsberg and porcine chymotrypsin were gifts from Novo Industri, Copenhagen. All reagents and solvents used with the sequencer have been described previously (18). Glutaryl-L-phenylalanine-p-nitroanilide (GPNA) was from Sigma, St Louis, USA. Sephadex G 75 Superfine was from Pharmacia Fine Chemicals, Uppsala, Sweden and Biogel P-10 was a product of Calbiochem AG, Switzerland. DEAE- and CM-celluloses, types DE-52 and CM-52, were from Whatman, Maidstone, England. Unless otherwise specified, all other chemicals were of analytical grade and were used without further purification.

2.2. Methods

2.2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The analytical slab gel electrophoretic system was a 1 mm thick 7.5-15% gradient of polyacrylamide using a discontinuous alkaline buffer system (3,14). All samples were prepared as described in (3).

2.2.2. Chymotrypsin inhibitor assay

Inhibitors of chymotrypsin were assayed at 37°C with GPNA as substrate (2,5). A 0.1 ml sample of inhibitor was preincubated for 10 min with 0.15 ml 0.001 M-HCl containing 0.8 mg · ml⁻¹ of chymotrypsin. 2.5 ml substrate solution (0.4 mg GPNA · ml⁻¹, 0.02 M-CaCl₂, 0.05 M-Tris-HCl, pH 7.6) were then added and incubation continued for another 10 min. The reaction was stopped by addition of 1.0 ml 20% acetic acid

in methanol and the absorbance measured at 410 nm. The inhibitor content of the samples was diluted to give an inhibition in the range of 20-70%. One inhibitor unit was defined as the amount of inhibitor required to inactivate 1 mg of chymotrypsin.

2.2.3. Isolation of chymotrypsin inhibitor CI-1

Isolation of chymotrypsin inhibitor CI-1 was achieved by a modification of the method of MIKOLA and SOULINNA (12). Lyophilized water-soluble albumin prepared as described earlier (7) was dissolved in elution buffer (0.005 M-2-mercaptoethanol, 0.010 M-Tris-HCl, pH 8.0). The sample was applied to a column (2.6 × 21 cm) of DEAE-cellulose equilibrated with elution buffer and subsequently eluted with a linear NaCl gradient (0.0-0.12 M) in the same buffer. 14 ml fractions were collected at a flowrate of 95 ml · h⁻¹. The CI-1 containing fractions were dialyzed against elution buffer (0.005 M-2-mercaptoethanol, 0.02 M-acetate, pH 4.5). The sample was then applied to a column (1.6 × 8 cm) of CM-cellulose equilibrated with the same buffer and subsequently eluted with a gradient of NaCl (0.0-0.5 M). 1.7 ml fractions were collected at a flow rate of 120 ml · h⁻¹. The CI-1 containing fractions were concentrated by evaporation and applied to a column (1.6 × 88 cm) of Sephadex G 75 pre-equilibrated with the same buffer. 2.1 ml fractions were collected at a flowrate of 2 ml · h⁻¹ and the CI-1 containing fractions were dialyzed and lyophilized. The purity of the isolated inhibitor CI-1 was controlled by activity measurements (section 2.2.2.) and by SDS-PAGE (section 2.2.1.) which showed that the preparation only contained one band.

2.2.4. Proteolytic cleavage of chymotrypsin inhibitor CI-1 and CI-2

Cleavage of inhibitor CI-1 and CI-2 with a catalytic amount of either chymotrypsin, subtilisin Carlsberg or subtilisin Novo were performed at low pH as recommended by OZAWA and LASKOWSKI (15). The following pH values were chosen in the present study to give the highest yield of cleaved inhibitor. Inhibitor CI-1 and CI-2 were cleaved at pH 3.0 (0.1 M-glycine-HCl buffer) by chymotrypsin whereas CI-1 were cleaved at pH 3.5 (0.1 M-ammonium acetate buffer) by subtili-

sin Carlsberg and subtilisin Novo. Inhibitor CI-2 was likewise cleaved by subtilisin Carlsberg and subtilisin Novo at pH 4.5 in the same buffer. The peptides produced in this way were separated by gel filtration on a Biogel P10 column (0.9×120 cm) equilibrated with 30% acetic acid. Fractions of 1 ml were collected with a flow-rate of $1.4 \text{ ml} \cdot \text{h}^{-1}$. Peptides were located by their absorption at 280 nm, by SDS-PAGE, and by the arginine spot test described by YAMADA and ITANO (20).

Alternative cleavage of inhibitor CI-2 by subtilisin Carlsberg and subtilisin Novo was obtained as follows. One mg of inhibitor and two mg of enzyme was dissolved in 0.7 ml 0.05 M-Tris-HCl buffer, pH 8.0 (17). The inhibitor-enzyme complex was isolated by gel filtration on a Sephadex G 75 column (1.6×88 cm) equilibrated with 0.1 M- NH_4HCO_3 . 2.1 ml fractions were collected at a flow-rate of $2 \text{ ml} \cdot \text{h}^{-1}$. The appropriate fractions were pooled and lyophilized. The lyophilized enzyme-inhibitor complex was thereafter redissolved in 30% acetic acid. This procedure liberated a mixture of subtilisin, intact inhibitor and inhibitor cleaved at the reactive site. The peptides were separated by gel filtration as described for inhibitors cleaved at low pH. The two elution profiles displayed the same characteristics, the profile obtained for the subtilisin Carlsberg CI-2 complex is presented in Figure 1.

Figure 1. Separation of peptides derived from the denatured inhibitor CI-2-subtilisin Carlsberg complex on Biogel P10 in 30% acetic acid.

Bars indicate the identified peptides. A. Subtilisin Carlsberg. B. Peptides derived from CI-2 with N-terminals starting at: Asn¹¹, Gly¹³ and Ala¹⁴. C. peptides derived from CI-2 with N-terminals starting at Glu⁶⁰.

2.2.5. Amino acid sequence determinations

For the determination of the amino acid sequences, an automatic liquid phase sequencer, Beckmann, model 890 C was used with programme 122974 supplied by this firm. However, 0.1 M-N,N,N',N'-tektrakis(2-hydroxyethyl)-etylendi-amine (THEED) was used instead of 1.0 M-Quadrol, and ethylacetate contained 15% propanol as recommended by BEGG and MORGAN (1). In all cases hexadimetrine (Polybrene) was added to the cup as described by TARR et al. (19). Identification of PTH-amino acids was done by high pressure liquid chromatography and thin layer chromatography as described earlier (18).

3. RESULTS AND DISCUSSION

Inhibitor CI-2 forms a very stable complex with either of the subtilisins near neutral pH (17). Due to the very tight binding of CI-2 to the subtilisins it was anticipated that some of the inhibitor present in the complex was cleaved at the susceptible bond of the reactive site. Advantage was taken of the fact that the subtilisins denature quickly at low pH, and that suspending the subtilisin-inhibitor complex in solution at acid pH liberates the inhibitor as a mixture of intact inhibitor and inhibitor cleaved at the reactive site. Amino acid sequencing of the peptides isolated by gel filtration of the denatured complexes between CI-2 and subtilisin Carlsberg or subtilisin Novo, respectively, showed that inhibitor CI-2 in part had been cleaved at peptide bond Met⁵⁹-Glu⁶⁰ (Figure 2). These results are in agreement with the effects of serine proteinases on their inhibitors as described by LASKOWSKI and KATO (10) although neither CI-1 nor CI-2 is stabilized by disulfide bridges. In this respect they are unique among the proteinase inhibitors isolated from higher plants (17).

OZAWA and LASKOWSKI (15) have showed that partial hydrolysis of a specific reactive (inhibitory) peptide bond in trypsin inhibitors could be obtained by incubation of the inhibitors at low pH with a catalytic amount of their cognate proteinase. Amino acid sequence analysis of peptides obtained by incubation of CI-2 at low pH with catalytic amounts of either chymotrypsin, subtilisin Carlsberg or subtilisin Novo respectively identified the reactive site of CI-2 as peptide bond Met⁵⁹-Glu⁶⁰. The reactive site identified in

Figure 2. Primary structure of barley chymotrypsin inhibitor CI-1 and CI-2.

Active sites towards the three proteinases are indicated by arrows. Abbreviations: CHY = chymotrypsin, SC = subtilisin Carlsberg; SN = subtilisin Novo.

this way is identical to the reactive site obtained from the denatured complex between CI-2 and either subtilisin Carlsberg or subtilisin Novo as described above.

When CI-1 was subjected to cleavage at low pH by catalytic amounts of either of the three proteinases, two cleavage sites were obtained. The two subtilisins cleaved CI-1 at peptide bonds Met³⁰-Ser³¹ and Leu⁵⁹-Asp⁶⁰ with approximately the same efficiency but chymotrypsin cleaved CI-1 only at peptide bond Leu⁵⁹-Asp⁶⁰. In addition a minor, presumably non-specific, cleavage site was obtained at Glu³³-Lys³⁴ by subtilisin Novo. From the amino acid sequence homologies between the two barley inhibitors (CI-1 and CI-2), leech inhibitor and potato inhibitor I it was inferred that the reactive site of inhibitor CI-1 and CI-2 were localized to peptide bond Leu⁵⁹-Asp⁶⁰ and Met⁵⁹-Glu⁶⁰, respectively (16). However, the second reactive site of CI-1 (Met³⁰-Ser³¹) was quite unexpected from the amino acid sequence homology in this region compared to that of CI-2 and the other members of the potato inhibitor I "family". MIKOLA and SOULINA (12) studied the activity of inhibitor CI-1 towards chymotrypsin and microbial serine proteinases. Their study indicated that the inhibitor CI-1 forms a 1:1 complex with chymotrypsin and a 2:1 complex with subtilisin. These data are in good agreement with the cleavage sites obtained in the present study suggesting that CI-1 is "single-headed" towards chymotrypsin (Leu⁵⁹-Asp⁶⁰) but "double-headed" towards either of the subtilisins (Met³⁰-Ser³¹ and Leu⁵⁹-Asp⁶⁰).

Inhibitor CI-1 was formerly isolated and se-

quenced with a "ragged" N-terminus starting at Tyr⁷ or Glu⁹ (16) using the numbering of the present study (Figure 2). However, the prominent N-terminal residue in the present study was Lys⁶ together with small amounts of longer sequences, as seen from the occurrence of the single Tyr in positions later than expected. Unfortunately, the sequence data were too mixed to enable a clear determination of the N-terminal sequence, but the data indicate the presence of nine more residues. In the present study residues No. 19 and 60 were determined to be Ser and Asp instead of Gly and Asn residues as previously found (16).

The different N-terminals observed in the present study were also found in the case of inhibitor CI-2 which was originally isolated together with three smaller, immunologically identical peptides (7). Amino acid sequencing and in vitro protein synthesis demonstrated that the small peptides were produced by proteolytic cleavage of inhibitor CI-2 (9,18), and that the release of 11 to 17 amino acid residues from the N-terminal did not affect the inhibitor activity of CI-2 (17). This finding is also in agreement with the observation that when inhibitor CI-2 with a blocked N-terminal residue was used to form the complex with the subtilisins, the resulting N-terminal peptide always displayed the known "ragged" form. It is probable that during complex formation excess subtilisin has cleaved various peptide bonds in the N-terminal sequence of the inhibitor. The presence of several Pro and Gly residues in this part of the molecule suggest a "random coil" structure which would make attack by proteolytic enzymes

easier. Such a mixture of a proteolytically degraded inhibitor gives a distinct chromatographic pattern (2) which should not be interpreted as iso-inhibitor patterns (7,9,18). CI-1 was sequenced with a "ragged" N-terminus (16) which might have been produced by endogenous proteinases during purification. This conclusion is supported by the fact that a different isolation procedure used in the present study suggested the presence of additional 10 amino acids.

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