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IDENTIFICATION OF THE REACTIVE (INHIBITORY) SITES OF CHYMOTRYPTIC INHIBITOR I FROM POTATOES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; chymotryptic inhibitor I; reactive (inhibitory) sites; chymotrypsin; trypsin.

Abstract—Chymotryptic Inhibitor I from potato tubers was subjected to limited hydrolysis with catalytic amounts of chymotrypsin and trypsin at pH 3. The fragments and peptides which resulted from the modifying cleavages at the reactive (inhibitory) sites were separated by gel filtration and high-voltage paper electrophoresis. Analyses of the fragments revealed that the major reactive site of the inhibitor for chymotrypsin was the $\frac{\text{Met}^{47}}{\text{Leu}}$—$\text{Asp}^{48}$ peptide bond, but some additional cleavage also occurred at the bond Phe^{57}—Asp^{58}. Two major and two minor sites of hydrolysis by trypsin were also identified.

INTRODUCTION

Chymotryptic Inhibitor I from potato tubers is a tetrameric protein which is a potent inhibitor of chymotrypsin and also possesses weaker activity towards trypsin [1]. Recent work in this laboratory has shown that the protein isolated from the variety 'Ulster Prince' is heterogeneous and is composed of at least 10 protomeric iso-inhibitors [2] as a result of amino acid substitutions at a number of positions in the sequence of 70 or 71 amino acid residues [3–6].

Ozawa and Laskowski [7] postulated that the proteinase inhibitors interacted with the proteinases via a specific reactive (inhibitory) peptide bond P_1—P_1', which is situated within a loop closed by a disulphide bridge, and is susceptible to partial hydrolysis when the inhibitor is incubated with catalytic amounts of the proteinase at low pH (2.5–4). Removal of the newly formed carboxyl-terminal residue by digestion with either carboxypeptidase A or B generally leads to inactivation of the inhibitors. This method has been used to identify the reactive sites of a large number of proteinase inhibitors from different sources [8, 9].

In an earlier communication [4] we described preliminary results which indicated that the major reactive site for chymotrypsin in Chymotryptic Inhibitor I was a Met—Asp bond. In this paper we show that the major reactive site is heterogeneous and is either Met^{47}—Asp^{48} or Leu^{47}—Asp^{48} and that prolonged incubation with chymotrypsin at pH 3 leads to the additional hydrolysis of the bond Phe^{57}—Asp^{58}. Similar treatment of the inhibitor with trypsin resulted in considerable hydrolysis at two major sites and much less cleavage at two other minor sites.

RESULTS AND DISCUSSION

During preliminary experiments it was observed that more than 95% of Inhibitor I (component D) was bound to Chymotrypsin-Sepharose 4B at pH 8 during affinity column chromatography. Although the applied material was quantitatively desorbed at pH 2 the specific inhibitory activity towards chymotrypsin was reduced by *ca* 25% indicating that some modification of the inhibitor might have occurred. Examination of the N-terminal amino acid(s) of the desorbed proteins revealed that low levels of a new residue (ASX) had been released during affinity chromatography, presumably as a result of partial catalytic cleavage at a chymotryptic-reactive site [7].

Incubation of Inhibitor I with catalytic amounts of chymotrypsin at pH 3 for 12 and 24 hr also led to the appearance of ASX as a new N-terminal amino acid, and a decrease in the inhibitory activity of the protein towards chymotrypsin. Furthermore treatment of samples of the modified inhibitor with carboxypeptidase A for 12 hr led to a further decrease in inhibitory activity.

Gel filtration on Biogel P-30 of the reduced and S-carboxy-methylated fragments in the mixtures after incubation with chymotrypsin for 12 and 24 hr yielded 3 major peaks (C1, C2 and C3). Peak C3 had no UV absorption and was therefore detected by TLC examination of small aliquots of each fraction for dansylated amino acids on polyamide sheets. Prolonged digestion (48 hr) with chymotrypsin produced two additional fragments of peptide material (C4 and C5) which were poorly resolved by gel-filtration and required further purification by high-voltage paper electrophoresis at pH 1.9.

Examination of the N- and C-terminal amino acid residues, and quantitative amino acid compositions of these fragments together with some amino acid sequence data obtained by the dansyl-Edman method (Table 1) revealed that chymotrypsin reacted specifically with the peptide bond 47—48 which is heterogeneous in this preparation of Inhibitor I and was either Met^{47}—Asp^{48} or Leu^{47}—Asp^{48} (see Fig. 1). Partial hydrolysis of this bond gave rise to fragments C2 and C3. A subsequent cleavage of the Phe^{57}—Asp^{58} bond in fragment C3 during prolonged incubations yielded peptides C4 and

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Table 1

Sample	N-terminal sequence from dansyl–Edman analyses	Amino acids released by Carboxypeptidase A	Identity of fragment (indicated by amino acid composition and other data)†
C1	Lys—Glx—Phe—Glx—	Val, Ala	1–71 (unmodified inhibitor I).
C2	Lys—Glx—Phe—Glx— Phe	Val, Thr, Leu, Met	1–47
C3	Asx—Tyr—Arg—Cys—Asx—Arg— Leu	Val, Ala	48–71
C4*	Asx—Asx—Ile—Leu—Gly— Phe	Val, Ala	58–71
C5*	Asx—Tyr—Arg—Cys—Asx—Arg—Val— Leu	Leu, Phe	48–57
T1	Lys Glu Gly Ile	Val, Ala, Leu, Lys	Mixture of unmodified 1–71 and various fragments linked by —S—S— bonds
T1a	Lys Glu Phe Gly Ile Ile	Val, Ala	Mixture (1–71) (unmodified) (24–71) probably also (1–53)
T1b	Gly—Ile—Ile—Glx—	None	24–53
T1c	Lys—Glx—Phe—Glx—	Leu, Ala, Lys	1–23
T2	Val—Arg—Leu—Phe—Asx—	Val, Ala	54–71
T3	Leu—Phe—Asx—Asx—	Val, Ala	56–71
T4	Gly—Ile—Ile—Glx—Lys	—	24–28
T5	Val—Arg	—	54–55

* Peptides further purified by high voltage paper electrophoresis at pH 1.9. The electrophoretic mobilities of C4 and C5 relative to dansyl–Arginine (1.00) were 0.43 and 0.75 respectively † See Figure 1 for numbering of amino acid residues.

C5. There was however no evidence during this investigation of any hydrolysis of Phe^{57}—Asp^{58} prior to cleavage of $\frac{Leu^{47}}{Met}$—Asp^{48}.

These results clarify our earlier report [4] that the reactive site of Inhibitor I was the Met^{60}—Asp^{61} bond

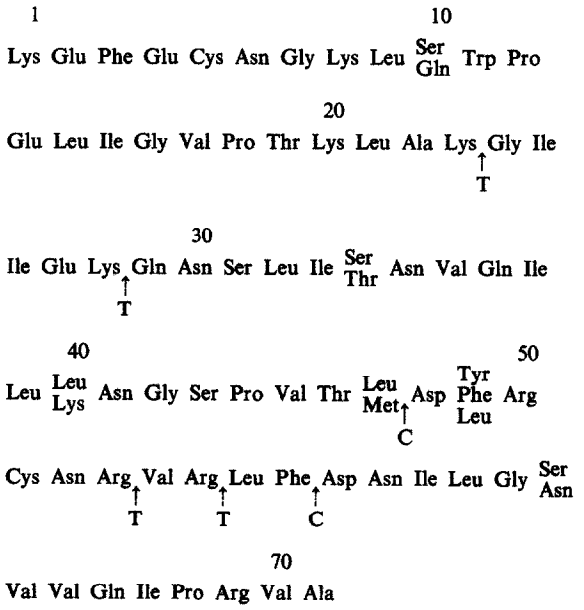

Fig. 1. Primary structure of chymotryptic Inhibitor I (component D) from potatoes to show the location of the reactive sites for chymotrypsin (C) and the sites of hydrolysis by trypsin (T) at pH 3. ↑ Major site; ↑ minor site.

and the report by Kiyohara *et al.* [10] of a Leu^{56}—Asp^{57} reactive bond in the same protein from potatoes of the Japanese variety 'Danshaku-Imo'. Both reports were based on an estimated MW of 9300–9500 (84 or 85 amino acids) for Inhibitor I. We have subsequently shown that a section of 14 amino acid residues were mistakenly inserted in the published amino acid sequence and that the correct MW of Inhibitor I based on sequence analysis [5] and physical data [6] is *ca* 8000 (70 or 71 residues). The only other known Met—Asp reactive site is a proteinase inhibitor is that reported in the protein from the dog sub-mandibulary gland [11].

Incubation of Inhibitor I with trypsin for 24 hr at pH 3 resulted in the complete abolition of inhibitory activity towards this enzyme. Chromatography of the trypsin-modified protein on Biogel P-30 yielded 5 peaks (T1–T5). Peak T1 was found to be a mixture of unmodified protein and several large modified fragments linked by disulphide bonds. When the material in peak T1 was reduced, S-carboxymethylated and rechromatographed on Biogel P-30 it was separated into 3 fractions (T1a, T1b and T1c). Table 1 shows the results of analyses carried out on these fragments. It is clear that peak T1a still contained some large modified polypeptides in addition to the unmodified protein, however consideration of the structures of the other fragments enabled the sites of cleavage by trypsin to be deduced as shown in Fig. 1.

The two major sites of tryptic hydrolysis were Lys^{23}—Gly^{24} and Arg^{53}—Val^{54}. The former bond lies within a loop closed by a disulphide bridge and therefore obeys one of the tentative rules drawn up for the reactive sites of proteinase inhibitors [12], whereas the latter bond does not. Peptides T3, T4 and T5 were only found

in low yield indicating the minor nature of the additional cleavages which occurred at Lys^{28}—Gln^{29} and Arg^{55}—Leu^{56}.

It remains to be seen whether either of the major specific sites of hydrolysis by trypsin can be regarded as true reactive sites in view of the rather weak and somewhat variable anti-trypsin activity of Inhibitor I [1, 13, 14]. Also Kowalski and Laskowski [15] have pointed out the need in certain cases for a more rigorous proof that the hydrolyzed bond is at the reactive site of the inhibitor. However the results of this investigation suggest that Inhibitor I from potatoes may be regarded as a 'double-headed' inhibitor having different binding sites for chymotrypsin and trypsin.

EXPERIMENTAL

Purification of chymotryptic inhibitor I. Inhibitor I was purified from potato tubers (*Solanum tuberosum* L. var. 'Ulster Prince') by the method of ref. [13] with the modifications described in ref. [3]. The material used was the last peak (component D) to elute when Inhibitor I was subjected to ion-exchange column chromatography on SP-Sephadex (or SE-Cellulose) in 8M urea as described previously [13]. This component D has recently been shown to contain two very similar iso-inhibitors (variants 9 and 10) having pIs of 8.6 and 8.9 respectively [2].

Affinity chromatography of inhibitor I on chymotrypsin-Sepharose-4B. Chymotrypsin was coupled to CNBr-activated Sepharose 4B by the method of ref. [16]. Inhibitor I (15 mg) was dissolved in 0.2 M triethanolamine adjusted to pH 8 with 11 M HCl and applied to a column (1.2 × 15 cm) of Chymotrypsin-Sepharose 4B equilibrated in the same buffer. The column was washed with buffer at pH 8 until the A at 280 nm returned to zero (*ca* 20 ml). The bound inhibitor I was then desorbed by eluting the column with 0.2 M KCl at pH 2.

Analyses. Amino acid sequences of fragments and peptides were determined by the dansyl–Edman procedure and by digestion with carboxypeptidase A [3]. Amino acid analyses were obtained using a Locarte amino acid analyser.

Limited hydrolysis of Inhibitor I with chymotrypsin or trypsin. Portions of Inhibitor I (40 mg of component D) were dissolved in 10 ml of 0.2 M HOAc containing 0.04 M $CaCl_2$ and the solns adjusted to pH 3 with 17 M HOAc. To these solns 1 mg of chymotrypsin or trypsin dissolved in the same buffer was added and the mixtures incubated at 30° for periods of 12, 24 and 47 hr The reaction mixtures were then freeze-dried.

Separation of modified fragments and peptides. (a) After chymotrypsin digest. Modified fragments and peptides resulting from catalytic cleavage with chymotrypsin were reduced and S-carboxymethylated as described in ref. [17]. The reagents were removed by gel filtration on a column (1.6 × 40 cm) of Sephadex G-10 equilibrated in and eluted with 10% HOAc. After freeze-drying the material was dissolved in a small vol. (< 1.5 ml) of 70% HCO_2H and applied to a column (1 × 180 cm) of Biogel P-30 equilibrated and eluted with 70% HCO_2H. The presence of proteins and peptides in the eluate was determined by measuring the A at 280 nm and by TLC examination of small aliquots of each fraction for dansylated N-terminal amino acids on polyamide sheets [3]. Certain peptides (C4 and C5) were also further purified by high-voltage paper electrophoresis at pH 1.9. (b) After trypsin. Peptides and fragments resulting from catalytic cleavage with trypsin were subjected to an initial gel filtration on a column (1 × 180 cm) of Biogel P-30 in 70% HCO_2H without prior reduction and S-carboxymethylation. The material in the first major peak to elute (T1) was then freeze-dried, reduced and S-carboxymethylated before being subjected to chromatography on a column of Biogel P-30.

Assay of Inhibitory Activities. Inhibitory activity of native and modified Inhibitor I against chymotrypsin was determined by measuring the hydrolysis of L-tyrosine ethyl ester [18] at pH 8 using a Radiometer pH titration/autoburette assembly. The inhibition of trypsin was measured spectrophotometrically by the haemoglobin method [1].

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