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CHYMOTRYPSIN INHIBITOR FROM POTATOES: INTERACTION WITH TARGET ENZYMES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; protease inhibitor; protease; chymotrypsin; subtilisin.

Abstract—The interactions of chymotrypsin, subtilisin and trypsin with a low MW proteinase inhibitor from potatoes were investigated. The K_i value calculated for the binding of inhibitor to chymotrypsin was $1.6 \pm 0.9 \times 10^{-10}$M, while the second-order rate constant for association was 6×10^5 M^{-1}/sec. Although binding was not observed to chymotrypsin which had been treated with diisopropyl fluorophosphate or with L-tosylamide-2-phenylethyl chloromethyl ketone, the 3-methylhistidine-57 derivative bound inhibitor with a K_i value of 9.6×10^{-9} M. The inhibitor also exhibited a tight association with subtilisin ($K_i < 4 \times 10^{-9}$ M). In contrast, little inhibition of trypsin was observed, and this was believed to be due to low levels of a contaminant in our preparations. No evidence for reactive site cleavage was observed after incubation of the inhibitor with catalytic amounts of chymotrypsin or subtilisin at acid pH.

INTRODUCTION

Numerous proteinase inhibitors have been isolated from potatoes and partially characterized (for review, see [1]). Recently a polypeptide (MW *ca* 5400), which is a potent inhibitor of chymotrypsin and also affects trypsin, was described [2]. The partial amino acid sequence of this inhibitor suggested that it may be homologous to a carboxypeptidase inhibitor which is also found in potatoes [2]. An evolutionary relationship was clearly demonstrated between the polypeptide chymotrypsin inhibitor and proteinase inhibitor II (MW *ca* 20 000) [3, 4].

This report presents an investigation of the kinetics and the thermodynamics of the interactions of the low MW inhibitor with target enzymes. In addition, the binding of inhibitor to catalytically-inactive derivatives is given to afford a comparison of the mechanism of action of this inhibitor with those of well-characterized serine proteinase inhibitors [5] as well as that of the carboxypeptidase inhibitor [6, 7].

RESULTS

Interaction with chymotrypsin and its derivatives

The dissociation constant, K_i, for the inhibitor with respect to chymotrypsin was estimated by the method of Green and Work [8]. After incubation of inhibitor and enzyme for a period of time to produce maximum inhibition, substrate was added to initiate the reaction. The K_i value estimated from the amount of free enzyme and inhibitor present at equivalence (Fig. 1) was $1.6 \pm 0.9 \times 10^{-10}$ M. No correction for competition by substrate was necessary, since the half-time for the first-order dissociation of the complex is large compared with time required to perform each assay (see below).

The second-order rate constant for the association of chymotrypsin and inhibitor, k_{on}, was determined

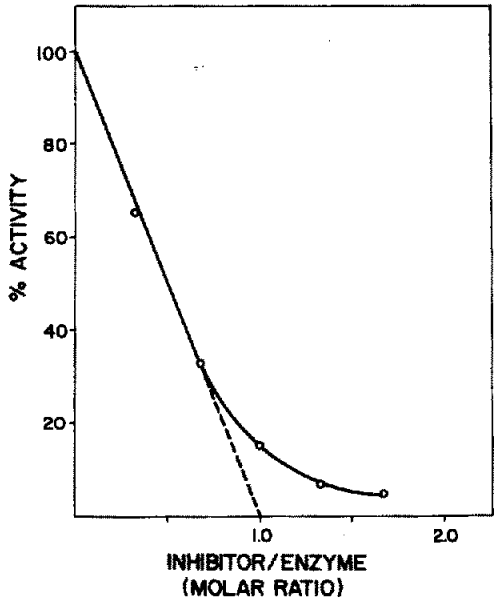

Fig. 1. Titration of chymotrypsin with the inhibitor. Chymotrypsin (final concn = 2 nM) was allowed to associate with inhibitor for 2 hr at 25° in 2.8 ml of 11 mM $CaCl_2$, 32 mM Tris–HCl (pH 8) containing 13% MeOH (v/v). Assay was initiated by the addition of 200 μl of a solution of carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester (final concn = 40 μM) in acetone. Observed rates were corrected for the spontaneous hydrolysis of substrate.

from second-order plots shown in Fig. 2. The k_{on} for the association calculated from the slope of the line was 5.8×10^5 M^{-1}/sec. The first-order rate constant for the dissociation of enzyme and inhibitor, calculated from the K_i value and k_{on}, was 9.2×10^{-5} sec^{-1}. This corresponds to a half-life of *ca* 2 hr.

Methylchymotrypsin, diisopropylphosphoryl-chymotrypsin, and chymotrypsin which had been treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone were prepared and tested for their ability to bind to the inhibitor. After the chymotrypsin derivative and the inhibitor were allowed to associate, free inhibitor was then back-titrated with unmodified enzyme (Fig. 3). For methylchymotrypsin a linear relationship was observed between free chymotrypsin in the assay solution and derivative present, suggesting a tight association. In contrast, the other derivatives did not appreciably bind to inhibitor even when present in large molar excess (Fig. 3).

An estimate of the strength of binding of methylchymotrypsin to inhibitor relative to that of unmodified enzyme was obtained by equilibrium competition experiments (Fig. 4). Chymotrypsin, an equimolar concentration of inhibitor, and varying amounts of methylchymotrypsin were incubated and assays were performed to monitor the approach to equilibrium. The ratio of association constants as calculated from

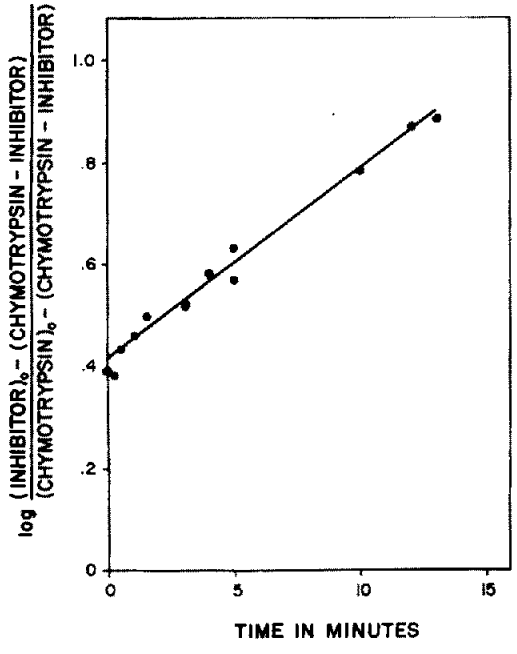

Fig. 2. Second-order kinetics of the association of chymotrypsin and the inhibitor. Chymotrypsin (1.8 nM) and inhibitor (4.4 nM) were allowed to associate from between 0 and 60 min at 25° in 2.8 ml of buffer comprised of 13% MeOH (v/v), 11 mM $CaCl_2$, 32 mM Tris–HCl (pH 8). Assay was initiated by the addition of 200 μl of carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester in acetone (final concn = 40 μM). Because the substrate hydrolyses spontaneously under these conditions, the observed rates were corrected (~25% at the lowest observed activities) for hydrolysis in the absence of enzyme.

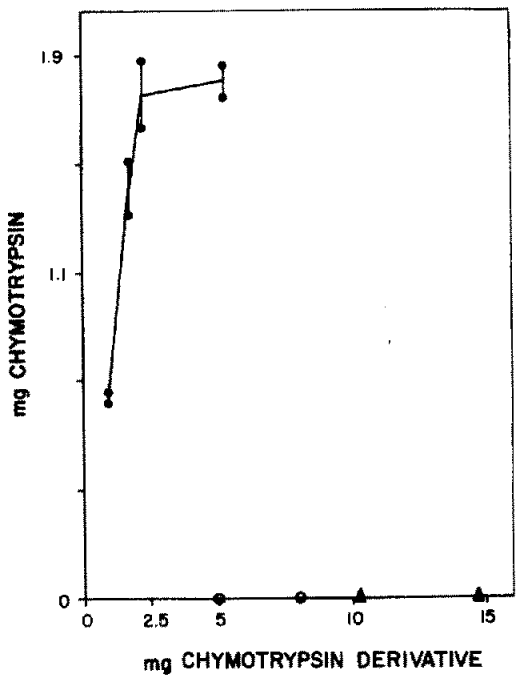

Fig. 3. Interaction of the inhibitor with catalytically impaired derivatives of chymotrypsin. Various amounts of the methyl- (●), diisopropylphosphoryl- (△), and TPCK- (○) derivatives were incubated with 0.41 μg inhibitor for 5 min in a solution containing 32 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.8). Chymotrypsin (1.9 μg, final concn = 140 nM) was then added, and, after 2 min, chymotrypsin activity was measured with benzoyl-L-tyrosine ethyl ester as substrate.

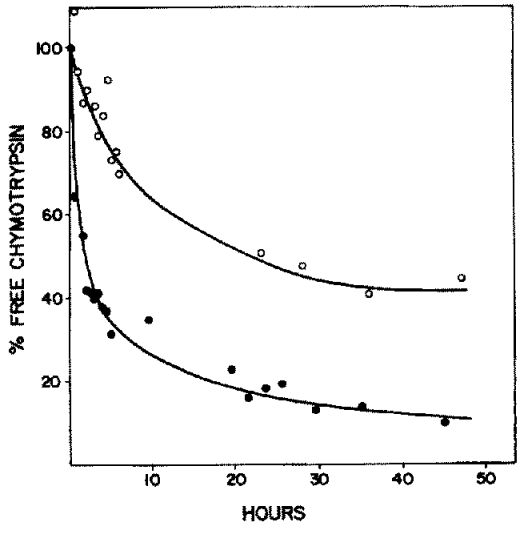

Fig. 4. Equilibrium competition between methylchymotrypsin and unmodified enzyme. Inhibitor (1 μM) and an equimolar amount of methylchymotrypsin (○) or a five-fold excess of methylchymotrypsin (●) were incubated for 1.5 hr in 11 mM $CaCl_2$, 32 mM Tris–HCl (pH 8) containing 13% MeOH (v/v). Chymotrypsin was then added to give a concentration of 1 μM, and aliquots were removed at various times and assayed with benzoyl-L-tyrosine ethyl ester as substrate.

equation 1 indicated that chymotrypsin bound inhibitor 60±20 times as tightly as did methylchymotrypsin. Thus, the dissociation constant for the methylchymotrypsin–inhibitor complex was *ca* 9.6×10^{-9} M.

$$\frac{K_{assn,Cht}}{K_{assn,MeCht}}=\frac{[MeCht][Cht\text{–}I]}{[Cht][MeCht\text{–}I]} \quad (1)$$

Interaction with subtilisin

The titration curve of subtilisin Carlsberg (3.5×10^{-7} M) with the inhibitor according to the method of Green and Work [8] was linear. Thus, no free inhibitor could be detected at the equivalence point and estimation of a K_i value from these data could not be accomplished. When a rather large experimental error (10%) was allowed in the estimation of free inhibitor at equivalence, a maximum K_i value of 4×10^{-9} M was calculated.

Interaction with trypsin

Preparations of the chymotrypsin inhibitor also weakly affected trypsin (Fig. 5A). A virtually identical titration curve was observed when the methylchymotrypsin–inhibitor complex was used to inhibit trypsin, suggesting that either: (a) the inhibitor possessed separate, non-overlapping binding sites for chymotrypsin and trypsin; or (b) the inhibition of trypsin was due to a contaminant in the preparations. A ternary complex was neither observed when trypsin, chymotrypsin and the inhibitor were cochromatographed on Sephadex G-100 nor when mixtures of these molecules were exposed to a crosslinking agent, dimethyl suberimidate [9], and then subjected to SDS-gel electrophoresis [10]. Thus, trypsin would probably interact very weakly, if at all, with pure chymotrypsin inhibitor.

Reactive sites

All attempts to identify the reactive site(s) for chymotrypsin, subtilisin and trypsin using enzyme-catalysed cleavage at low pH as developed by Laskowski *et al.* [5] were unsuccessful.

DISCUSSION

The chymotrypsin inhibitor described in this study and the carboxypeptidase inhibitor from potatoes are believed to be homologous proteins even though they are directed toward unrelated enzyme classes [2]. Thus, the mechanism of inhibition of the chymotrypsin inhibitor as described in this report and data available on that of the carboxypeptidase inhibitor provide a means of studying the evolution of structure and function in this system. The following observations suggest that the chymotrypsin inhibitor behaves very much like a typical inhibitor of the serine proteases and afford a means of comparison with properties of carboxypeptidase inhibitor. (1) It exhibits a high degree of specificity with chymotrypsin as compared with trypsin. In contrast, the carboxypeptidase inhibitor binds to carboxypeptidase A only slightly better than to carboxypeptidase B [11]. (12) The second-order rate constant for the association of enzyme and inhibitor (6×10^5 M^{-1}/sec) is within the range normally observed for the reaction of serine proteinases with

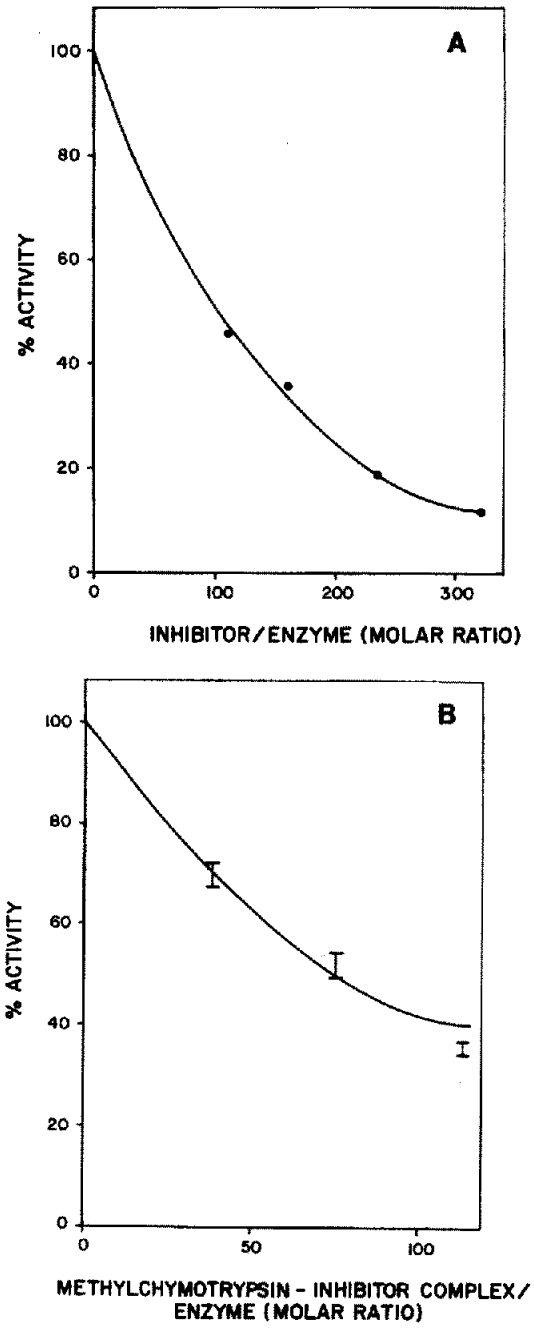

Fig. 5. Titration of trypsin with free inhibitor and with the inhibitor–methylchymotrypsin complex. (A) Trypsin (140 nM was incubated with inhibitor at 25° in 50 mM $CaCl_2$, 50 mM Tris–HCl (pH 8.1). After incubation for 15 min, trypsin activity was determined with tosylarginine methyl ester as substrate. (B) Prior to the addition of trypsin (final concn = 150 nM), the inhibitor was allowed to associate with a 1.33 molar excess of methylchymotrypsin.

their inhibitors [5]. Since this rate is somewhat lower than that expected for a strictly diffusion controlled process, the formation of one or more transitory intermediates has been suggested as rate-limiting for the conversion to a stable complex [5]. (3) The binding of the chymotrypsin inhibitor to enzyme derivatives with modified active site structures is similar to the pattern

observed with other inhibitors of serine proteinases. Relatively strong binding is possible when a small substituent (e.g. methyl group on histidine 57) is introduced while larger groups block access to the enzyme's substrate binding pocket and prevent or greatly weaken protease inhibitor binding [12]. In contrast, modification of carboxypeptidase A by treatment with the affinity label, *N*-bromoacetyl-*N*-methyl-L-phenylalanine, which presumably occupies the binding pocket, has little effect on the strength of binding of the carboxypeptidase inhibitor [6].

Although the evidence for homology between the chymotrypsin and carboxypeptidase inhibitors is somewhat weak, there is no doubt that the polypeptide chymotrypsin inhibitor and potato inhibitor II [3, 4], a dimer of subunit MW 10 500, are homologous proteins [13]. The many subunit forms of inhibitor II have been separated into two classes called IIa and IIb, which preferentially affect trypsin and chymotrypsin, respectively [3, 14]. Iwasaki has shown that both IIa [15] and IIb [16] exhibit selective cleavage at their reactive sites (Lys-Ser) under conditions specified by Laskowski, Jr. [5]. Thus, it was somewhat surprising that the inhibitor described in these studies is refractory to reactive site cleavage. The molecular basis for this difference is obscure at this time; however, it should be noted that the Lys-Ser sequence which constitutes the reactive sites of IIa and IIb [15, 16] is absent in our inhibitor [13].

EXPERIMENTAL

Materials. Polypeptide chymotrypsin inhibitor was prepared from Russet Burbank potatoes as described previously [1]. α-Chymotrypsin was purchased from Sigma Chemical Co. (60 units/mg) and from Worthington Biochemical Co. (58 units/mg). Methylchymotrypsin, prepared as described in ref. [17] as modified by in ref. [12], exhibited less than 2% of the sp. act. of unmodified enzyme. Diisopropyl [18] and tosylphenylalanyl chloromethyl ketone (TPCK) derivatives [19] of chymotrypsin contained 0.1 and 1% active enzyme, respectively. Subtilisin Carlsberg (18 units/mg) and trypsin (260 units/mg) were purchased from Sigma Chemical Co. *N*-Benzoyl-L-tyrosine ethyl ester, *N*-α-*p*-tosyl-L-arginine methyl ester hydrochloride, L-1-tosylamide-2-phenylethyl chloromethyl ketone, *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester, and diisopropyl fluorophosphate were purchased from Sigma Chemical Co. and methyl-*p*-nitrobenzene sulfonate was from Pierce Chemical Co.

Protein solns were prepared by dissolving lyophilized protein in 1 mM HCl. Protein concns were estimated using the following $A_{280}^{1\%}$ values: chymotrypsin and its derivatives, 20.4 [20]; subtilisin Carlsberg, 9.6 [21]; trypsin, 14.3 [22]; and the inhibitor, 10.5 calculated assuming $M_r = 5400$ [1], four tyrosine residues per molecule [2], and $E_{278} = 1758\ M^{-1}\ cm$ for tyrosine [23]. Concns of enzyme and inhibitor solns as estimated by agreed to within 5% with values obtained by enzyme assay and amino acid analysis of acid hydrolysates.

Enzyme assays. Chymotrypsin was routinely assayed spectrophotometrically at 25° using *N*-benzoyl-L-tyrosine ethyl ester as substrate [24]. When a more sensitive assay for chymotrypsin was required, *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester was employed as substrate [25]. Trypsin and subtilisin activities were measured by monitoring the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester [24] at 25°.

Reactive sites. Identification of reactive sites of inhibitor was attempted by the method of ref. [5]. Inhibitor was incubated at 25° with 2–10% (mol/mol) chymotrypsin, subtilisin, or trypsin in 0.01 M $CaCl_2$, 0.1 M NaOAc at pH values between 3 and 6. After incubation for up to 19 days, reactive site cleavage was assessed by electrophoresis in 15% polyacrylamide gels at pH 3.2 [26] and 9.7 [27]. The dansyl procedure [28] was also used to identify new amino-terminal groups produced during cleavage. Dansylated inhibitor was hydrolysed for 16 hr at 110° in 6 M HCl. Hydrolysates were dried, extracted with EtOAc satd with H_2O, and the dansyl amino acids were identified by TLC on polyamide plates [29].

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