Chymotrypsin Inhibitor I from Potatoes: Reactivity with Mammalian, Plant, Bacterial, and Fungal Proteinases*

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ABSTRACT: The capability of chymotrypsin inhibitor I from potatoes to inhibit, or be digested by, proteinases from mammalian, plant, bacterial, and fungal origins was studied. Four enzymes besides α-chymotrypsin were inhibited; *Bacillus subtilis* proteinase, *Streptomyces griseus* proteinase, carboxypeptidase B, and trypsin. The bacterial and fungal proteinases, like chymotrypsin, were stoichiometrically inhibited. However, the bacterial proteinase was not as tightly bound to inhibitor as chymotrypsin and in competition experiments between the two enzymes chymotrypsin selectively complexed inhibitor. The fungal proteinase was demonstrated to be a mixture of at least two enzymes, of which only one reacted with inhibitor. Carboxypeptidase B was strongly inhibited in an apparently competitive manner.

Trypsin was inhibited in its proteolytic but not in its esterolytic activity, indicating that only large protein molecules and not small esters are sterically hindered by inhibitor I at the active center of this enzyme. Pepsin, carboxypeptidase A, papain, bromelin, ficin, and rennin were relatively unaffected. Of all the enzymes tested none hydrolyzed inhibitor I at neutral pH to the extent of destroying its inhibitory capacity toward chymotrypsin. Pepsin, at pH 2, however, rapidly destroyed inhibitory activity.

In 1962 a powerful inhibitor of chymotrypsin, protein in nature, was isolated in crude form from potatoes (*Solanum tuberosum*) (Ryan and Balls, 1962) and subsequently crystallized (Balls and Ryan, 1963). The crystallized inhibitor, now referred to as inhibitor I because of the crystallization from potatoes of another chymotrypsin inhibitor,[1] stoichiometrically inhibits chymotrypsin over a wide range of inhibitor and enzyme concentrations (Balls and Ryan, 1963). The reactivity of the crystalline inhibitors toward other enzymes has not been well characterized. It was found, however, that partially purified preparations inhibit bovine trypsin during the hydrolysis of protein substrates but not of esters (Ryan and Balls, 1962). The inability of inhibitor I to alter the proteolytic activity of bromelin, papain, and pepsin has also been noted (Balls and Ryan, 1962).

It is the purpose of this communication to describe the reactivity of crystallized inhibitor I with several mammalian, plant, bacterial, and fungal proteinases, with respect to its inhibitory capacity toward these enzymes and to its susceptibility to their proteolytic attack.

Experimental Procedure

Chymotrypsin inhibitor I was twice crystallized (2×) hexagons, prepared as previously described (Balls and Ryan, 1963). Bovine α-chymotrypsin (3×), bovine trypsin (2×), and pepsin (2×) were obtained from Worthington Biochemical Corp. Carboxypeptidase A (5×) and carbobenzoxyglycyl-L-phenylalanine[2] were purchased from Mann Biochemical Co. Porcine carboxypeptidase B, pepsin (2×), and *Streptomyces griseus* proteinase, Type IV, were obtained from Sigma Chemical Co. Ficin (2×), bromelin, hemoglobin substrate, BAEE, TEE, and TAME were obtained from Nutritional Biochemical Co. Crystalline *Bacillus subtilis* proteinase was purchased from Calbiochem. It is similar to the enzyme subtilisin (Guntelberg and Ottesen, 1954). ATEE and HA were also obtained from Calbiochem. Carnation nonfat dry milk was used in the milk clotting experiments.

Esterase activities were determined by a modified (Aldrich and Balls, 1958) titrimetric method (Schwert *et al.*, 1948). Using 2.0 ml of 0.01 M substrate (pH 6.3 for TEE, pH 7.9 for TAME, BAEE, and ATEE) the hydrogen ions liberated during the reactions were titrated at constant pH using 0.100 N NaOH and a Corning Model 12 pH meter with a Beckman micro single-probe electrode. The specific activities were calculated as micromoles of substrate hydrolyzed per minute per milligram of enzyme/2.0 ml. Hemoglobin digestion was determined spectrophotometrically by measuring at 280 mμ the 5% trichloroacetic acid soluble

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[1] The two inhibitor species differ in crystalline habit, composition, size, and reactivity.

[2] The following abbreviations are used: CGP, carbobenzoxyglycyl-L-phenylalanine; HA, hippuryl-L-arginine; BAEE, benzoyl-L-arginine ethyl ester; ATEE, acetyl-L-tyrosine ethyl ester; TEE, L-tyrosine ethyl ester; TAME, tosyl-L-arginine methyl ester.

peptides released at varying time intervals from 10 mg of hemoglobin in 2.0 ml of 0.05 M Tris–phosphate buffer, pH 7.35. Milk clotting was performed at 37° (Aldrich and Balls, 1958). Carboxypeptidase A activity was determined on the substrate CGP (Schwert and Neurath, 1950) and carboxypeptidase B activity on HA (Folk, 1956) both using ninhydrin (Moore and Stein, 1948). Inhibitor activity was determined in all cases using an aliquot of a mixture of inhibitor and enzyme preincubated at 25° for 2–10 min. The stability of the inhibitor in the presence of enzymes was determined by its ability to inhibit chymotrypsin in the TEE assay at pH 6.3.

Results

Effect of Inhibitor I on the Activities of Various Proteolytic Enzymes. The effect of inhibitor I on the milk clotting, esterase, and hemoglobin digesting activities of several highly purified proteolytic enzymes of animal, plant, bacterial, and fungal origins are shown in Table I.

TABLE I: Effect of Potato Inhibitor on Various Enzyme Activities.

Enzyme	Mg of Enzyme Inhibited/Mg of Inhibitor[a]		
	Milk Assay	Hemoglobin Assay	Ester Assay
Chymotrypsin	3.10	3.42	3.10[b]
Trypsin	...	0.30	0[c]
Carboxypeptidase B	...	...	Competitive inhibitor[d]
Carboxypeptidase A	...	...	0[e]
Pepsin	0	0	...
B. subtilis protease	4.55	2.70	4.40
S. griseus protease	...	1.75	1.38[f] 0[g]
Bromelin	0.10	0.10	...
Ficin	0.10	0.10	...
Papain	0.10	0.10	...

[a] Maximum inhibitions recorded. Inhibitions of chymotrypsin, *B. subtilis* protease, and the esterase activity of *S. griseus* protease were found to be stoichiometric. Other inhibitions varied with varying enzyme and inhibitor concentrations. [b] TEE. [c] TAME. [d] Hippuryl-L-arginine; see Figure 1. [e] Carbobenzoxyglycyl-L-phenylalanine. [f] ATEE. [g] BAEE and TAME.

Mammalian Enzymes. Of the mammalian enzymes tested only chymotrypsin was stoichiometrically inhibited. The kinetics of this inhibition have already been described (Balls and Ryan, 1963).

Carboxypeptidase B activity on HA was powerfully inhibited by inhibitor I. The cumulative data of two

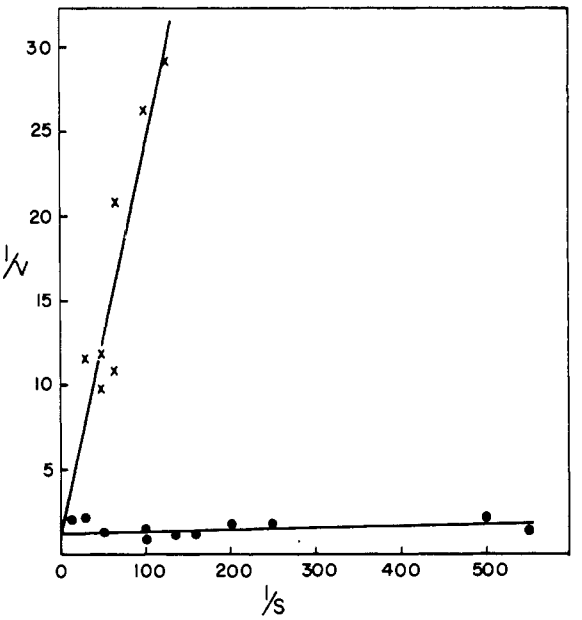

FIGURE 1: Apparent competitive inhibition of carboxypeptidase B by potato inhibitor I. Conditions, in 1.0 ml of total volume, 25°: 0.004 M Veronal buffer, pH 7.5, 0.1 M NaCl, and 7 μg of carboxypeptidase B with no inhibitor (●), and 200 μg of inhibitor (×). $1/v$ is the reciprocal of the increase in optical density of the ninhydrin assay at 570 mμ/5-min reaction time. $1/S$ is the reciprocal of the molarity of hippuryl-L-arginine.

experiments indicate that the inhibition is competitive. A constant inhibitor quantity of 200 μg and a constant enzyme quantity of 7 μg of carboxypeptidase B were incubated with varying concentrations of substrate. Figure 1 shows the data in a conventional reciprocal plot (Lineweaver and Burk, 1934). Since a K_i cannot be determined until the equivalent weight of inhibitor I has been established, a simple approximation of the order of magnitude of an apparent K_i under these conditions was made assuming the change in slope (Figure 1) is due to $(1 + I/K_i)$. Depending on whether the molecular weight or the equivalent weight of inhibitor (Balls and Ryan, 1963) is used for the calculation, the K_i falls into the range 10^{-8}–10^{-9} and thus ranks among the most potent competitive inhibitors known.

Trypsin hydrolysis of hemoglobin was measurably inhibited by inhibitor I but as reported previously (Ryan and Balls, 1962), using partially purified preparations, TAME was not inhibited. A fourfold weight excess of inhibitor I over trypsin did not inhibit the hydrolysis of TAME over a concentration range 0.01–0.001 M. This is to be compared with the 55% inhibition of hemoglobin hydrolysis by a smaller (3:1) inhibitor to trypsin ratio.

Inhibitor in 45-fold weight excess failed to inhibit pepsin at pH 2 or pH 6 in either the hemoglobin or the milk clotting assays. Carboxypeptidase A activity was

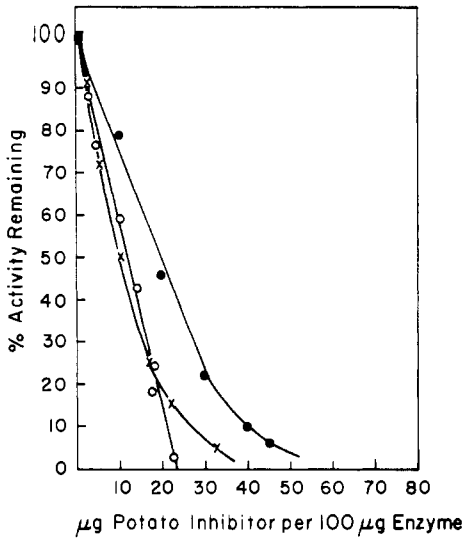

FIGURE 2: Inhibition of bacterial proteinase by potato inhibitor I. Milk clotting activity is designated as (×); hemoglobin digestion (●); and L-tyrosine ethyl ester hydrolysis (o); for conditions of assay see text.

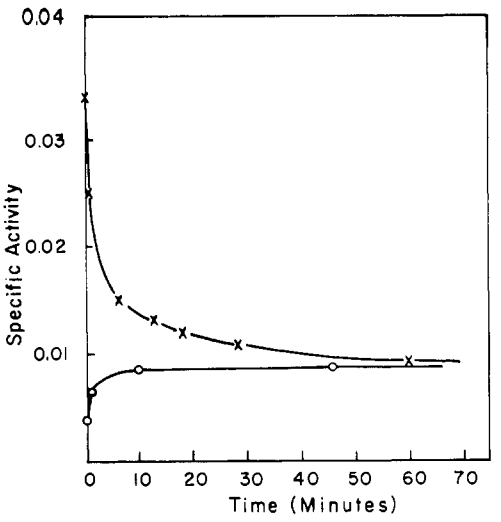

FIGURE 3: Competition of chymotrypsin and bacterial proteinase for potato inhibitor I. Upper curve: 360 μg of free chymotrypsin was added to complex of 390 μg of bacterial proteinase and 130 μg of inhibitor I. Lower curve: 390 μg of bacterial proteinase was added to complex of 360 μg of chymotrypsin and 130 μg of inhibitor I. Incubation mixtures were in 0.3 ml of 0.1 M sodium phosphate, pH 7.0. Activity was assayed using L-tyrosine ethyl ester.

also not inhibited, even by a 50-fold weight excess of inhibitor I.

Bacterial and Fungal Proteinases. The *B. subtilis* extracellular proteinase commonly known as subtilisin (Guntelberg and Ottesen, 1954) was potently inhibited by potato inhibitor I. Esterase activity was inhibited stoichiometrically even at high inhibitor to enzyme ratios (Figure 2), but the hemoglobin digestion and milk clotting activity were markedly less inhibited at the higher inhibitor concentrations. Chymotrypsin on the other hand is stoichiometrically inhibited at all inhibitor concentrations with all three substrates. Therefore, bacterial proteinase apparently did not bind inhibitor as tightly as chymotrypsin. A ninefold difference between the specific activities of chymotrypsin and bacterial proteinase on TEE (chymotrypsin being the most active) permitted the testing of the relative affinities of the two enzymes for inhibitor. A competition experiment was performed in which a bacterial proteinase–inhibitor complex was prepared from 390 μg of crystalline bacterial proteinase (specific activity 0.04) and 130 μg of inhibitor I (a 41 μg excess), and challenged with 360 μg of chymotrypsin (specific activity, 0.036). As shown in Figure 3 chymotrypsin did compete with bacterial proteinase for the inhibitor and a marked decrease in the esterolytic activity of the solution to 0.009 resulted (upper curve). If no competition had occurred the specific activity would have been 0.023. A similar experiment is also shown in Figure 3 (lower curve) in which the same quantities of enzymes and inhibitor are used and an inert chymotrypsin inhibitor complex is challenged with free bacterial proteinase. In this experiment competition also occurred and the specific activity of the solution increased. Using the combining weights given in Table I for chymotrypsin with inhibitor (3.1–1.0) and of bacterial proteinase with inhibitor (4.4–1.0) the compositions of the equilibrium mixtures were calculated. With 0.016 μequiv of inhibitor I, 0.012 μequiv of *B. subtilis* proteinase, and 0.014 μequiv of α-chymotrypsin present in the equilibrium mixture, all of the inhibitor has combined with 0.004 μequiv of bacterial proteinase and 0.012 μequiv of the chymotrypsin. The final mixture was independent of the starting conditions and demonstrated the greater affinity of chymotrypsin for inhibitor. This experiment also demonstrated that the sites on the inhibitor for binding chymotrypsin and bacterial proteinases are either identical or they are so close together that inhibition of both enzymes at once does not occur.

The fungal proteinase from *S. griseus* was inhibited in its proteolytic activity on hemoglobin and in its esterase activity on ATEE. Esterase activity on BAEE or TAME was not inhibited. Examination of this finding revealed that the fungal proteinase preparation used was a mixture of enzymes having differing specificities. Figure 4 shows a plot of the fungal proteinase activity remaining when titrated with potato inhibitor I using assay systems of ATEE, hemoglobin digestion, and BAEE or TAME. The inhibition of the ATEE activity is linear and resembles that observed with chymotrypsin or bacterial proteinase. The BAEE and TAME activities are essentially unaffected. The selective inhibition of at least one of the fungal proteinase enzymes but not the other(s) is also readily seen in the hemoglobin assay in which about 40% of the total proteinase activity is rapidly neutralized by a small quantity of

inhibitor I while 60% of the activity remains relatively unaffected in the presence of a much higher concentration of inhibitor.

Plant Enzymes. The three plant enzymes used were relatively unaffected in their milk clotting or hemoglobin digesting ability by as much as a six–eightfold weight excess of inhibitor. A slight but variable inhibition was recorded with all three enzymes but the maximum inhibition did not exceed the values given in Table I.

Effect of Various Proteolytic Enzymes on the Inhibitory Activity of Inhibitor I toward Chymotrypsin. Experiments to test the susceptibility of inhibitor I to various proteolytic enzymes were carried out under the conditions described in the experimental section and summarized in Table II. Only pepsin at pH 2 was able to digest the inhibitor to the extent of destroying its inhibitory activity toward chymotrypsin. The inhibitor is stable at pH 2 in the absence of pepsin. No further experiments were conducted to determine whether the inhibitor had been altered other than in its inhibitory activity toward chymotrypsin.

TABLE II: Effect of Proteolytic Enzymes on Inhibitory Activity.

Enzyme	Wt Ratio of Inhibitor to Enzyme	pH	Temp (°C)	Incubation Time (min)	Inhibitor Activity Remaining[a,b] (%)
Papain	0.26	7.3	37	80	100
Bromelin	0.21	7.3	37	150	100
Ficin	2.50	7.3	37	150	100
S. griseus proteinase	0.15	7.3	29	125	100[c]
Trypsin	0.44	8.0	25	100	100
Carboxypeptidase A	3.50	7.3	25	100	100
Carboxypeptidase B	0.25	7.3	26	92	100
Pepsin	0.5	6.2	38	300	100
Pepsin	1.0	2.1	28	12	50
	Inhibitor alone	2.1	38	Stable in acid	

[a] For details of assay see Experimental Section. [b] Enzyme activity was determined at the end of the incubation times. Papain lost 71% activity, bromelin 40%, ficin 12%, and trypsin, *S. griseus* proteinase, pepsin, and carboxypeptidase A and B <10%. [c] Calculated from inhibitor activity after the initial reaction of inhibitor with *S. griseus* proteinase.

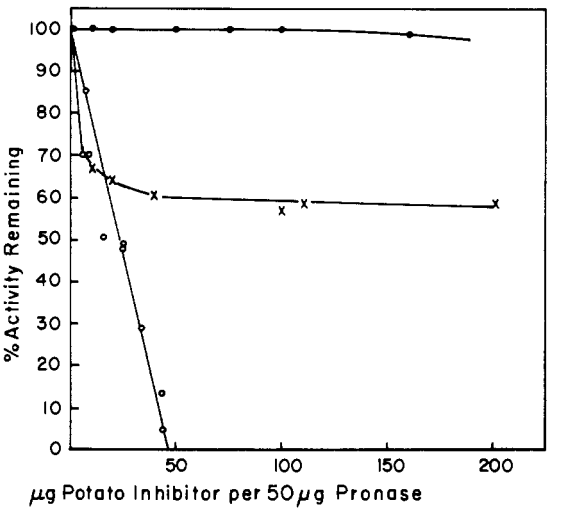

FIGURE 4: Inhibition of *S. griseus* proteinase by potato inhibitor I. L-Tyrosine ethyl ester hydrolysis is designated as (●); benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester hydrolysis (o); and hemoglobin digestion (×); for assay conditions see text.

Discussion

Of 11 proteolytic enzymes tested five were strongly inhibited by potato inhibitor I. Of the five, the inhibition of chymotrypsin, bacterial proteinase, and fungal proteinase was stoichiometric. It is probably more than a coincidence that these three enzymes, having similar substrate specificities, were similarly inhibited. Chymotrypsin had a greater affinity for inhibitor than bacterial proteinase (Figure 3) and this may reflect the differences in primary structures at the binding sites of these two enzymes. Fungal proteinase affinity for inhibitor was not compared to chymotrypsin or bacterial proteinase, nor was its stoichiometry calculated, since only TEE hydrolysis and not BAEE or TAME hydrolysis (Figure 4) was inhibited by inhibitor I. The unequal inhibition of two substrates indicates that *S. griseus* proteinase is a mixture of two or more enzymes having different specificities. It has been shown recently that commercial pronase contains several enzyme components (Hiramatsu and Ouchi, 1963), and some of these have been partially purified (Wahlby *et al.*, 1965). Of interest is the fact that only the enzyme(s) having chymotrypsin-like specificities were inhibited by inhibitor I.

The enzymes that were not stoichiometrically inhibited by inhibitor I, *i.e.*, carboxypeptidase B and trypsin, are specific for substrates containing basic amino acids (Neurath, 1963; Desnuelle, 1963). Carboxypeptidase B was inhibited in an apparently competitive manner. The magnitude of the inhibition places the inhibitor among the most potent carboxypeptidase

inhibitors known, regardless of the mode of inhibition. Inhibitor I is the first highly purified naturally occurring protein known to inhibit carboxypeptidase B and may have future value in controlling the activity of this enzyme. Trypsin was inhibited, but as reported in our earlier investigations using partially purified preparations, and confirmed here using crystalline preparations, only proteolysis and not esterolysis was affected. The nature of this inhibition was not competitive and remains undetermined. The present data suggest that a reversible combination of trypsin with inhibitor I occurs at a locus somewhat displaced from the catalytic center of the enzyme and only the approach of large protein molecules and not of small esters is hindered sterically, resulting in the inhibition of only proteolysis.

None of the plant enzymes tested were appreciably inhibited, nor were the mammalian enzymes, carboxypeptidase A, or pepsin. A crude rennin preparation was also unaffected in its ability to clot milk at pH 6.4.

The unusual resistance of inhibitor I toward proteolytic digestion at neutral pH is a property shared by many, if not all, naturally occurring protease inhibitors and reflects the inaccessibility of the peptide bonds in the inhibitor molecule. Pepsin however easily digests the inhibitor at pH 2 indicating a pH-dependent conformational change. A study (Kassel and Laskowski, 1956) of the effect of pepsin on six naturally occurring trypsin inhibitors revealed wide differences in their resistance to this enzyme. Of the six inhibitors only three (blood, Kazal's, and ovomucoid) were readily hydrolyzed, even at pH 1.5. The ease of digestion of potato inhibitor I by pepsin near the normal pH of the stomach (*i.e.*, pH 2) probably accounts for the lack of adverse physiological effects upon eating raw potatoes. Although inhibitor I is quite stable to high temperatures when purified, it is quickly destroyed in the intact potato by heating (C. A. Ryan, in preparation).

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