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BINDING OF THE RECOMBINANT PROTEINASE INHIBITOR EGLIN *c*, OF THE SOYBEAN BOWMAN-BIRK PROTEINASE INHIBITOR AND OF ITS CHYMOTRYPSIN AND TRYPSIN INHIBITING FRAGMENTS TO Leu-PROTEINASE, THE LEUCINE SPECIFIC SERINE PROTEINASE FROM SPINACH (*Spinacia oleracea* L.) LEAVES: THERMODYNAMIC STUDY

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The effect of pH and temperature on the apparent association equilibrium constant (K_a) for the binding of the recombinant proteinase inhibitor eglin *c* (eglin *c*), of the soybean Bowman-Birk proteinase inhibitor (BBI) and of its chymotrypsin and trypsin inhibiting fragments (F-C and F-T, respetively) to Leu-proteinase, the leucine specific serine proteinase from spinach (*Spinacia oleracea* L.) leaves, has been investigated. On lowering the pH from 9.5 to 4.5, values of K_a (at 21°C) for complex formation decrease thus reflecting the acidic pK-shift of the hystidyl catalytic residue from ~6.9, in the free Leu-proteinase, to ~5.1, in the enzyme: inhibitor adducts. At pH 8.0, values of the apparent thermodynamic parameters for the proteinase:inhibitor complex formation are: Leu-proteinase:eglin *c* - $K_a = 2.2 \times 10^{11}$ M^{-1}, $\delta G° = -64$ kJ/mol, $\delta H° = +5.9$ kJ/mol, and $\delta S° = +240$ J/molK; Leu-proteinase:BBI $- K_a = 3.2 \times 10^{10}$ M^{-1}, $\delta G° = -59$ kJ/mol, $\delta H° = +8.8$ kJ/mol, and $\delta S° = +230$ J/molK; and Leu-proteinase:F-C $- K_a = 1.1 \times 10^{6}$ M^{-1}, $\delta G° = -34$ kJ/mol, $\delta H° = +18$ J/mol, and $\delta S° = +180$ J/molK (values of K_a, $\delta G°$ and $\delta S°$ were obtained at 21.0°C; values of $\delta H°$ were temperature

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-independent over the range explored, i.e. between 10.0°C and 40.0°C). F-T does not inhibit Leu-proteinase up to an inhibitor concentration of 1.0×10^{-3} M, suggesting that the upper limit of K_a is 1×10^2 M^{-1}. Considering the known molecular models, the observed binding behaviour of eglin *c*, BBI, F-C and F-T to Leu-proteinase has been related to the inferred stereochemistry of the enzyme/inhibitor contact region.

KEY WORDS: Leu-proteinase, recombinant proteinase inhibitor eglin *c*, soybean Bowman-Birk proteinase inhibitor, chymotrypsin inhibiting fragment (of the soybean Bowman-Birk proteinase inhibitor), trypsin inhibiting fragment (of the soybean Bowman-Birk proteinase inhibitor), proteinase:inhibitor complex formation, thermodynamics (of proteinase:inhibitor complex formation), pH and temperature effects (on proteinase:inhibitor complex formation).

ABBREVIATIONS: Leu-proteinase, leucine specific serine proteinase from spinach leaves; eglin *c*, recombinant proteinase inhibitor eglin *c*; BBI, soybean Bowman-Birk proteinase inhibitor; F-C, chymotrypsin inhibiting fragment of the soybean Bowman-Birk proteinase inhibitor; F-T, trypsin inhibiting fragment of the soybean Bowman-Birk proteinase inhibitor.

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INTRODUCTION

In the course of solubilization and purification of fusicoccin binding sites present in the microsomal fractions of spinach (*Spinacia oleracea* L.) leaves,[1] some endogenous hydrolases, responsible for the poor stability of the receptors, were identified.[2–5] Among them, a serine proteinase displaying a remarkable specificity for the leucyl residue (hereafter Leu-proteinase) was purified and partially characterized.[3–5] Leu-proteinase shows a molecular weight of 60,000 ± 3,000 daltons, an isoelectric point of 4.8 ± 0.1, and a relative electrophoretic mobility of 0.58 ± 0.03; the enzyme activity is characterized by a bell-shaped profile with an optimum pH value around 4.5.[3–5] According to the Leu-proteinase primary specificity,[3–5] *N*-α-benzoyl-L-leucine *p*-nitroanilide and *N*-α-carbobenzoxy-L-leucine *p*-nitrophenyl ester show the most favourable catalytic parameters; next, only *N*-α-tosyl-L-leucine chloromethyl ketone strongly inhibits the enzyme action.[3–5] Moreover, Kazal- and Kunitz-type proteinase inhibitors, all containing an arginyl or a lysyl residue at their P_1 position, do not inhibit at all the Leu-proteinase activity.[3,4]

The unique primary specificity of this serine proteinase towards the leucyl residue,[2–5] together with the role in spinach protein metabolism suggested for this enzyme,[3] prompted our interest in a detailed thermodynamic investigation on the binding of the recombinant proteinase inhibitor eglin *c* (hereafter eglin *c*), of the soybean Bowman-Birk proteinase inhibitor (hereafter BBI) and its chymotrypsin and trypsin inhibiting fragments (hereafter F-C and F-T, respectively) to Leu-proteinase. As expected on the basis of the enzyme primary specificity,[3–5] eglin *c*, BBI and F-C, all containing a leucyl residue at their P_1 position, strongly inhibit Leu-proteinase; on the other hand, F-T, containing a lysyl residue at the P_1 position does not affect at all the enzyme activity. These results have been analyzed in parallel with the molecular, catalytic and inhibitor binding properties of related serine proteinase/macromolecular inhibitor systems.[6–9] For the purpose of comparison amongst enzymes endowed with similar specificities, thermodynamics of human cathepsin G:eglin *c* complex formation, as well as of BBI, F-C and F-T binding to bovine α-chymotrypsin were also determined.

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MATERIALS AND METHODS

Materials

Leu-proteinase has been purified from the soluble fraction of spinach leaves as detailed previously.[4] Human cathepsin G was isolated from leukocyte granule extracts as described elsewhere.[10] Bovine α-chymotrypsin, treated with *N*-α-tosyl-L-lysine chloromethyl ketone in order to abolish tryptic activity, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Eglin *c* was kindly provided by Ciba-Geigy AG (Basel, CH). BBI was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and further purified as detailed previously.[9–11] F-C and F-T were prepared by treating BBI with cyanogen bromide and then pepsin as detailed elsewhere.[8,9] *N*-α-carbobenzoxy-L-leucine *p*-nitrophenyl ester, *N*-α-benzoyl-L-tyrosine ethyl ester and *N*-α-acetyl-L-tyrosine ethyl ester were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, FRG). All chemicals were of analytical grade and used without further purification. The characterization of Leu-proteinase, human cathepsin G, bovine α-chymotrypsin, eglin *c*, BBI, F-C, F-T, *N*-α-carbobenzoxy-L-leucine *p*-nitrophenyl ester, *N*-α-benzoyl-L-tyrosine ethyl ester and *N*-α-acetyl-L-tyrosine ethyl ester has been reported elsewhere.[3–13]

Determination of the Apparent Enzyme:Inhibitor Association Equilibrium Constant (K_a)

Values of the apparent association equilibrium constant (K_a) for the proteinase:inhibitor complex formation were determined, between pH 4.5 and 9.5 (I = 0.1 M), and between 10.0°C and 40.0°C, by the evaluation of the inhibitory effect of eglin *c*, BBI, F-C and/or F-T on the hydrolysis of *N*-α-carbobenzoxy-L-leucine *p*-nitrophenyl ester, of *N*-α-benzoyl-L-tyrosine ethyl ester and *N*-α-acetyl-L-tyrosine ethyl ester catalyzed by Leu-proteinase,[3–5] human cathepsin G[6] and bovine α-chymotrypsin,[8] respectively. The detailed biochemical procedures have been published previously.[3–6,8] An average error value of ± 8% was ascribed to K_a values, as the standard deviation.[13]

RESULTS AND DISCUSSION

Under all the experimental conditions, eglin *c*, BBI and F-C binding to Leu-proteinase, eglin *c* association to human cathepsin G, as well as bovine α-chymotrypsin:BBI and :F-C complex formation conform to a simple equilibrium as indicated by the Hill coefficient (n) always being equal to 1.00 ± 0.02. Moreover, K_a values were always independent of the enzyme concentration, and were in excellent agreement with published data.[6–9]

The pH-dependent change in affinity of eglin *c*, BBI and F-C binding to Leu-proteinase, shown in Figure 1, is strictly reminescent of those obtained for the binding of eglin *c*, BBI and/or F-C to human leukocyte elastase, bovine α-chymotrypsin, and subtilisin Carlsberg[8,13] and therefore may be described in identical terms. Thus, the decrease in the K_a values for eglin *c*, BBI and F-C binding to Leu-proteinase, lowering the pH from 9.5 to 4.5, may be interpreted as reflecting the acidic pK-shift of the hystidyl catalytic residue, upon inhibitor binding, from $pK_{UNL} \sim 6.9$ in the free enzyme, to $pK_{LIG} \sim 5.1$ in the proteinase:inhibitor complexes. Values of pK_{UNL} (see

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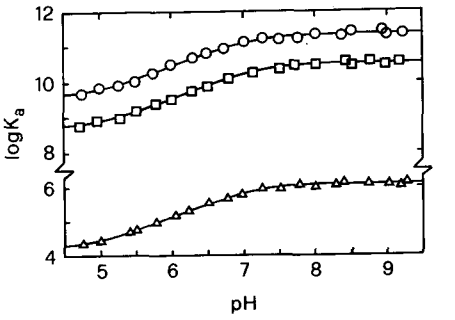

FIGURE 1 pH-dependence of the apparent association equilibrium constant (K_a; M^{-1}) for eglin *c* (○), BBI (□) and F-C (△) binding to Leu-proteinase, at 21.0°C. The unbroken lines were generated from the following equation:[13]

$$\log K_a = C - \log\{([H^+] + K_{UNL})/([H^+] + K_{LIG})\} - \log(K_{LIG}/K_{UNL})$$

where C is a constant that corresponds to the alkaline asymptote of $\log K_a$, and pK_{UNL} and pK_{LIG} are the pK values of the proton dissociation equilibrium constants for the inhibitor-free (K_{UNL}) and the inhibitor-bound (K_{LIG}) Leu-proteinase, respectively. Unbroken lines, generated with the following sets of parameters: eglin *c* — C = 11.4, pK_{UNL} = 6.9 and pK_{LIG} = 5.1; BBI — C = 10.5, pK_{UNL} = 7.0 and pK_{LIG} = 5.2; and F-C — C = 6.1, pK_{UNL} = 6.9 and pK_{LIG} = 5.0, were obtained with an iterative non-linear least-squares curve fitting procedure, which also allowed to ascribe an average error value of ± 12% to K_{UNL} and K_{LIG} values, as the standard deviation.[13] The pH profile was explored using the following buffers: acetate (pH 4.5 to 6.0); phosphate (pH 6.0 to 8.5); and borate/glycine (pH 8.5 to 9.5); all at I = 0.1 M (sodium salts). According to Aducci *et al.*,[3-5] no specific ion effects were found using different buffers with overlapping pH values.

Figure 1) appear to be closely related to those calculated from the pH dependence of kinetic parameters for the Leu-proteinase catalyzed hydrolysis of natural and synthetic substrates.[3-5]

Values of the apparent thermodynamic parameters for eglin *c*, BBI and/or F-C binding to Leu-proteinase, human cathepsin G, human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg, obtained at pH 8.0, are given in Table I. The inspection of Table I allows some relevant considerations. (*i*) As expected on the basis of the enzyme primary specificity,[3-5] the affinity of eglin *c*, BBI and F-C (all containing a leucyl residue at their P_1 position)[7,9] for Leu-proteinase is higher than that reported for inhibitor binding to human cathepsin G, human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg. Next, no inhibitory effect of F-T on the catalytic properties of Leu-proteinase and bovine α-chymotrypsin[8,9] has been observed. (*ii*) According to the literature,[8,9] the lower affinity of F-C for Leu-proteinase and bovine α-chymotrypsin, as compared to that of intact BBI may be related to the reduced conformational stability of the isolated chymotrypsin inhibiting fragment of BBI. (*iii*) The $\delta S°$ and $\delta H°$ values indicate that the complex formation is an entropy-driven process.[14] (*iv*) The positive values of $\delta S°$ could reflect the removal of the proteinase- and/or inhibitor-bound water molecules during complexation.[14]

As a whole, the thermodynamic data here reported indicate that Leu-proteinase binds eglin *c*, BBI and F-C with the highest affinity known for these inhibitors relative to serine proteinases acting on non-cationic substrates. Next, the complex formation

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TABLE I

Values of ther apparent thermodynamic parameters for the binding of eglin *c*, BBI and/or F-C to Leu-proteinase, human cathepsin G, human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg (pH 8.0, phosphate buffer, $I = 0.1$ M)

Complex	K_a^a (M^{-1})	δG^{oa} (kJ/mol)	δH^{ob} (kJ/mol)	δS^{oa} (J/molK)
Leu-proteinase:eglin c^c	2.2×10^{11}	− 64	+ 5.9	+ 240
Human cathepsin G:eglin c^c	7.0×10^{9}	− 55	+ 7.9	+ 210
Human leukocyte elastase:eglin c^d	1.0×10^{10}	− 56	+ 7.5	+ 220
Bovine α-chymotrypsin:eglin c^d	5.0×10^{9}	− 54	+ 8.4	+ 210
Subtilisin Carlsberg:eglin c^d	6.6×10^{9}	− 55	+ 8.4	+ 220
Leu-proteinase:BBI[c]	3.2×10^{10}	− 59	+ 8.8	+ 230
Bovine α-chymotrypsin:BBI[c]	1.6×10^{9}	− 51	+ 12	+ 210
Leu-proteinase:F-C[c]	1.1×10^{6}	− 34	+ 18	+ 180
Bovine α-chymotrypsin:F-C[c]	9.3×10^{4}	− 32	+ 19	+ 170
Leu-proteinase:F-T[c,e]	$< 1 \times 10^{2}$	> − 11	—	—
Bovine α-chymotrypsin:F-T[c,e]	$< 1 \times 10^{2}$	> − 11	—	—

[a]Values of K_a, $\delta G°$ and $\delta S°$ were obtained at 21.0°C (1 cal = 4.184 J). Average error values of ± 8% (for K_a and $\delta G°$) and ± 12% (for $\delta S°$) were evaluated as the standard deviation.[13]

[b]Values of $\delta H°$ were obtained from the linear dependence of $\log K_a$ on 1/T: the temperature ranged between 10.0°C and 40.0°C (1 cal = 4.184 J). An average error value of ± 12% was ascribed to $\delta H°$ values as the standard deviation.

[13c]Present study.

[d]From Ascenzi *et al.*[13]

[e]F-T does not inhibit at all the Leu-proteinase and the bovine α-chymotrypsin activity up to an inhibitor concentration of 1.0×10^{-3} M. Indeed, values of K_a and $\delta G°$ should be taken as maximal and minimal estimates, respectively. Values of $\delta H°$ and $\delta S°$ were not evaluated.

seems to be essentially independent of the nature of the amino acid residues at positions P_n-P_2 and P_1'-P_n' of the non-homologous eglin *c*, and BBI proteinase inhibitors.[7,9] Thus, in view of its unique primary specificity for the leucyl residue, Leu-proteinase (*i*) may be of relevant interest for its potential biotechnological applications, and (*ii*) should be taken as a valuable target-enzyme to investigate the association of macromolecular inhibitors, containing a leucyl residue at their P_1 position, to serine (pro)enzymes acting on non-cationic substrates.

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