

Limited proteolysis of C1-inhibitor by chymotrypsin-like proteinases

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Limited proteolysis of C1-inhibitor was observed with human skin chymase, human cathepsin G, and bovine chymotrypsin. In each case, the inhibitor was degraded to one major product migrating slightly faster than the native inhibitor in an SDS-polyacrylamide gel. The inhibitory activity of C1-inhibitor against human plasma kallikrein was not altered by the modification with chymase. Edman degradation of the proteolyzed inhibitor revealed two sequences in a 1:1 ratio: NPNATSSSQ, the N-terminus of native C1-inhibitor, and VEPILVSSL. This second sequence showed that the Phe³³-Val³⁴ bond was hydrolyzed. Our results provide another example of the susceptibility of the N-terminal region of C1-inhibitor to proteolytic cleavage.

C1-inhibitor; Limited proteolysis; Chymotrypsin-like proteinase; N-terminal sequence

1. INTRODUCTION

C1-inhibitor inhibits human plasma kallikrein [1,2], human plasmin [2], factor XIa [3], factor XIIa [3,4], human thrombin [2], and C1s and C1r [5,6] forming an SDS-stable complex that is typical for the reaction of proteinases with members of the serine proteinase inhibitor superfamily (serpins). The reactive center of C1-inhibitor and of other serpins is located near the C-terminal [7] in a stressed loop [8]. Proteinases not inhibited by a particular serpin can cleave within that loop resulting in a conformational change [9] and in a loss of inhibitory activity [8]. In the case of C1-inhibitor, the N-terminal region is also sensitive to proteolysis [10,11]. Human leukocyte elastase and snake venom from *Bitis arietans* cleave initially in the N-terminal region without a loss of inhibitory activity and then near the reactive site [11]. The snake venom from *Bungarus fasciatus* and *Crotalus atrox* α -protease, on the other hand, cleave only at the N-terminal region [10,11]. Here we investigate the interaction of C1-inhibitor with human skin chymase. Although the inhibitor has 6 potential cleavage sites between P'2 and P'13 (Schechter and Berger notation [12]), we show in this paper that C1-inhibitor was only hydrolyzed in the N-terminal region. Furthermore, we present evidence that chymotrypsin and cathepsin G reacted with the C1-inhibitor in a similar way.

2. MATERIALS AND METHODS

Human skin chymase was isolated from mast cells as described

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[13,14]. Cathepsin G was purified according to published methods [15–17]. Bovine chymotrypsin was purchased from Sigma and human plasma kallikrein was obtained from Calbiochem. Human C1-inhibitor was purified according to Harrison [18]. All substrates for the proteinases as well as all other chemicals were purchased from Sigma.

Chymase and chymotrypsin activities were determined in 0.45 M Tris-HCl, pH 8.0, containing 1.8 M NaCl and 9% dimethylsulfoxide. The concentration of the substrate Suc-Ala-Ala-Pro-Phe-pNA was 1 mM. Human plasma kallikrein activity was measured in 0.1 M Tris-HCl buffer, pH 8.3, containing 0.005% Triton X-100. The concentration of the substrate Bz-Pro-Phe-Arg-pNA was 0.15 mM. All assays were performed in 1 ml cuvettes at room temperature.

The concentration of chymotrypsin was determined with the active site titrant methylumbelliferyl *p*-trimethylammonium cinnamate chloride [19,20] on a fluorometer that was calibrated with a trypsin solution using methylumbelliferyl *p*-guanidinobenzoate [20]. The concentration of a freshly made trypsin solution was determined with nitrophenyl *p*-guanidinobenzoate [21]. Trypsin and chymotrypsin were used to titrate a lima bean trypsin inhibitor solution, which in turn was used to titrate human skin chymase. The concentration of cathepsin G was determined using its k_{cat}/K_M [22], the human plasma kallikrein concentration was calculated by using the weight of the lyophilized enzyme (1 U = 1/15 mg according to the manufacturer) and a molecular mass of 98 kDa [23], and the C1-inhibitor concentration was estimated using $A_{280}^{1\%} = 3.6$ [18]. SDS-PAGE (10% acrylamide, 0.37% *N,N'*-methylene-bis-acrylamide) was performed according to Laemmli [24] using a Bio-Rad Mini-Protean II dual slab cell. N-Terminal sequence analyses were performed at the protein chemistry facility at the Wistar Institute in Philadelphia.

3. RESULTS AND DISCUSSION

3.1. Interaction of C1-inhibitor with human skin chymase

C1-inhibitor was incubated with human skin chymase for 1 h at different inhibitor/enzyme ratios (mol/mol) ranging from I/E = 17 to I/E = 560 and the reaction was then analyzed on an SDS-polyacrylamide gel. A

complete conversion of the C1-inhibitor (96 kDa) to a lower molecular mass (80 kDa) appeared (fig.1). The lowest amount of chymase able to convert more than 90% of C1-inhibitor was observed at a molar I/E ratio of 170 (fig.1, lane 4). Lowering the amount of enzyme to a ratio of I/E = 560 (fig.1, lane 5), 40% of the proteolyzed C1-inhibitor was still obtained. The limited proteolysis of the inhibitor could be prevented when chymase was preincubated either with phenylmethylsulfonylfluoride or with chymostatin (not shown).

3.2. Interaction of C1-inhibitor with other chymotrypsin-like proteinases

To see whether the limited proteolysis of the C1-inhibitor is a common feature of chymotrypsin-like enzymes, we incubated the inhibitor with human cathepsin G and bovine chymotrypsin. Both proteinases were able to proteolyze C1-inhibitor and the same SDS-polyacrylamide gel band pattern as with chymase was obtained (fig.2, lanes 1–5). For these two proteinases, limited proteolysis was completed in 1 h only at a molar I/E ratio of 17 (fig.2, lanes 2 and 4). With a lesser amount of enzyme (I/E = 70) about 50% of the inhibitor was proteolyzed in this time (fig.2, lanes 3 and 5). A plausible reason for the slower proteolysis of C1-inhibitor by human cathepsin G and bovine chymotrypsin was the presence of α_1 -antichymotrypsin, which is a better inhibitor for cathepsin G and chymotrypsin than for chymase [25]. α_1 -Antichymotrypsin was a very small impurity in our C1-inhibitor preparation. It can

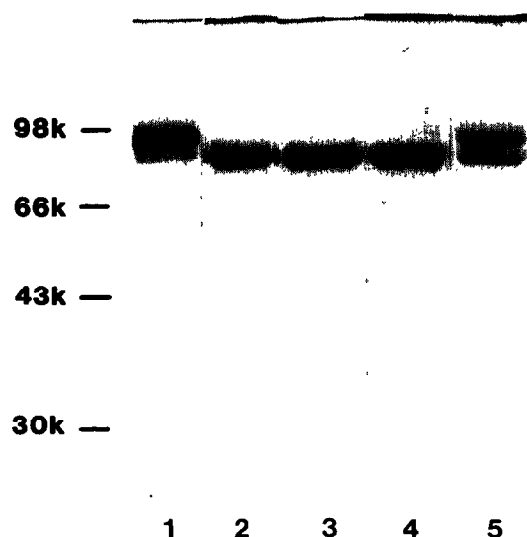


Fig.1. 10% SDS-polyacrylamide electrophoresis under reduced conditions of C1-inhibitor incubated for 1 h with chymase in 50 mM Tris-buffer, containing 0.5 M NaCl, pH 8.0, at different molar I/E ratios: no enzyme (lane 1), I/E = 17 (lane 2), I/E = 70 (lane 3), I/E = 170 (lane 4), and I/E = 560 (lane 5). The reaction was stopped with phenylmethylsulfonylfluoride. Each lane contained 7 μ g inhibitor. Molecular mass standards: phosphorylase b (98 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). A trace of degraded C1-inhibitor was already present in the starting material.

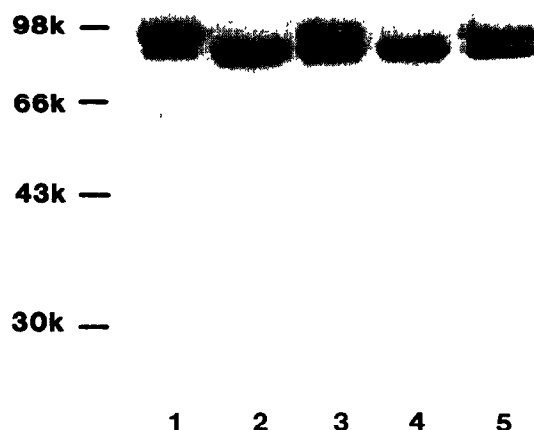


Fig.2. Incubation of C1-inhibitor with cathepsin G (lanes 2 and 3) and chymotrypsin (lanes 4 and 5). The experimental conditions were the same as in fig.1. Inhibitor/enzyme ratios: no enzyme (lane 1), I/E = 17 (lane 2 and 4) and I/E = 70 (lanes 3 and 5).

be seen as a weak band on the SDS-polyacrylamide gels (molecular mass 68 kDa) and was also detected by Western blot analysis and by chymotrypsin inhibition in an activity assay (data not shown).

3.3. Characterization of the product of limited proteolysis of C1-inhibitor

The product of the reaction of C1-inhibitor with human skin chymase was characterized by its inhibition of human plasma kallikrein and by amino terminal sequence analysis. C1-inhibitor incubated with human

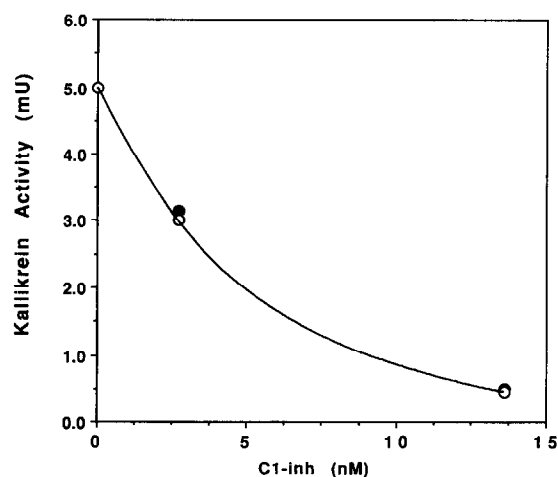


Fig.3. Human plasma kallikrein inhibition by native (●) and proteolyzed (○) C1-inhibitor. y-axis = residual kallikrein activity in mU; x-axis = inhibitor concentration. 100 μ l human plasma kallikrein (1 U reconstituted in 5 ml 0.1 Tris-HCl buffer, pH 8.3, containing 0.005% Triton X-100) and C1-inhibitor were preincubated for 30 min at room temperature. 900 μ l buffer were added and the activity was measured with Bz-Pro-Phe-Arg-pNA as substrate. The kallikrein concentration in the assay was 13.6 nM and the measured activity was 5 mU (ϵ for *p*-nitroaniline = 8800 M⁻¹ · cm⁻¹).

chymase for up to 20 h at room temperature did not change the kallikrein inhibition compared to the control experiment without enzyme (fig.3), indicating a cleavage away from the reactive center. N-Terminal sequence analysis was consistent with the above observation and confirmed that C1-inhibitor was not cleaved near the reactive center. Ten Edman degradation cycles were performed for the native and for the proteolyzed inhibitor. C1-inhibitor not treated with chymase gave the native sequence NPNATSSSSQ, whereas the limited proteolyzed inhibitor revealed in a 1:1 ratio a second sequence VEPILVSSL. Based on the published C1-inhibitor sequence [8] the starting valine of that second sequence could be assigned to Val-34. The amino acid in position 33 is a Phe and cleavage of the Phe-33-Val-34 bond in C1-inhibitor by human skin chymase is consistent with specificity of the enzyme [14].

Our results with C1-inhibitor and results reported by other groups [10,11] show that the N-terminal region of C1-inhibitor is susceptible to limited proteolysis and that the first 40 amino acid residues are not involved in the inhibitory activity. Other examples of N-terminal regions of proteinase inhibitors that are unimportant for inhibitory activity come from studies of oryzacystatin from rice [26], the inter- α -trypsin inhibitor from human serum [27,28], and the ovomucoid third domains, which often differ in the length of the 'connecting peptide fragment' [29].

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