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Short Paper

Proteolytic Cleavage of Various Human Serum Proteinase Inhibitors by *Candida albicans* Aspartic Proteinase

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Abstract

The secreted *Candida albicans* aspartic proteinase (SAP) is presumed to be one of the putative *Candida* virulence factors, while serum proteinase inhibitors depend on host defense mechanisms. We examined the interaction between SAP and serum proteinase inhibitors, such as C1-inhibitor, α2 plasmin inhibitor, and antithrombin Ⅲ. SAP progressively inactivated plasmin inhibitory activity of C1-inhibitor and α2 plasmin inhibitor. It also inactivated thrombin inhibitory activity of antithrombin Ⅲ. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, these host proteinase inhibitors were progressively degraded by SAP during prolonged incubation.

These results suggest that SAP induces an imbalance of complements, coagulation and fibrinolytic systems through the degradation and inactivation of these host proteinase inhibitors, and that SAP may play an important role in the development of *Candida albicans* infections.

Introduction

Candida albicans is a common pathogenic fungus in severely immunocompromised hosts [1-3]. Many studies have reported that secreted *Candida albicans* aspartic proteinase (SAP) has many biological functions, and is a possible virulence factor in *Candida albicans* infection through its action on host proteins [4-7]. SAP appears to be secreted in the local site and/or released into the circulation and deep organs. *Candida albicans* is able to adjust environmental pH to an acidic milieu for its growth by obtaining nutrients through digestion by SAP [8]. SAP is known to have broad substrate specificity, degrading keratin [9], collagen [10], immunoglobulins [11], albumin [12], laminn [12], fibronectin [12] and intestinal mucin [13]. We also reported that SAP degrades cystatin A [14] and the precursor form of endothelin 1 (big endothelin-1) [15], which are contained in human epidermis. On the other hand, serum proteinase inhibitors participate in regulating cascade systems, such as complement, coagulation and fibrinolytic mechanisms. They play important roles in modulating the activity of the proteinase cascade systems involved in these mechanisms [16]. C1-inhibitor (C1-inh) is the main inhibitor of C1r, C1s, kallikrein and factor XIIa. α2-plasmin inhibitor (α2-PI) inactivates plasmin, kallikrein, factor Xa and thrombin. AT-Ⅲ is an inhibitor of the coagulation system such as thrombin, factor IXa and factor Xa. The imbalance of complements, blood coagulation and fibrinolytic systems in infection may be important for the prognosis of the infected patients,

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because the imbalance of these systems may result in disseminate intravascular coagulation (DIC), organ failure and/or sepsis [17]. Hesselvik *et al.* [18] and Phillipe *et al.* [19] reported that decreased AT-Ⅲ and protein C were seen in patients with infectous diseases and sepsis. In 1975, Kaminishi *et al.* [20] also reported that two serum proteinase inhibitors, α1-proteinase inhibitor and α-2 macroglobulin, are degraded by SAP. However, no studies on the relationship between SAP and other serum proteinase inhibitors have been reported. Therefore, we examined the interaction between SAP and three main serum proteinase inhibitors, C1-inh, α2-PI and AT-Ⅲ.

Materials and Methods

1. Proteinases and proteinase inhibitor

Plasmin (0.03 casein unit/ml), a gift from Dr. H. Sumi, was dissolved in distilled water. Thrombin (0.2 NIH unit/ml) was obtained from Green Cross Co., LTD, Osaka, Japan and was dissolved in distilled water. C1-inh, AT-Ⅲ and α2-PI were obtained from Sigma Chemical Co.,St. Louis, MO, USA, and they were diluted in distilled water. Synthetic substrates were obtained from Kabi Vitrum AB, Sweden. H-D-valyl-leucyl-lysyl-p-nitroanilide was used for plasmin activity. H-D-phenylalanyl-pipeconyl-arginine-p-nitroanilide was used for thrombin activity. The concentration of proteins was determined by the method of Lowry *et al.* [21] with bovine serum albumin as a standard.

2. Secreted *Candida albicans* aspartic protinase

Secretion of SAP was incubated by *Candida albicans* IFO 1060 in yeast cabon base containing bovine serum albumin as reported previously [14, 15]. SAP actitvity was assayed with 1.25% (w/v) bovine hemoglobin as the substrate by modifying the method of Anson [22]. SAP was purified from media according to the method of Tsushima *et al.* [14, 15] by DE-52 ion exchange chromatography. The purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3. Plasmin inhibitory activity of α2-PI or C1-inh

Twentyfive μl of α2-PI (24μg/ml) or C1-inh (20μg/ml) was incubated with 25μl of 10 mM sodium acetate buffer, pH 4.5 or SAP (0.01μg) in 10 mM sodium acetate buffer, pH 4.5 for various time intervals. The SAP activity was then inhibited by the addition of 5μl of 25 mM Tris HCl buffer, pH 8.0. The mixture was incubated with 25μl of plasmin (0.0025 casein unit/ml) for 10 min. Then, 25μl of 3 mM of H-D-valyl-leucyl-lysyl-p-nitroanilide was added and the change in absorbance at 405 nm after 30 min was assayed. The results were expressed as the residual inhibitory activity compared to the initial inhibitor as percentages.

4. Thrombin inhibitory activity of AT-Ⅲ

Twentyfive μl of AT-Ⅲ (176μg/ml) was incubated for various time intervals with 2μl of 10 mM sodium acetate buffer at pH 4.5 or SAP (0.03μg). Then 5μl of 25 mM Tris HCl buffer at pH 8.0 containing 7.5 mM EDTA and heparin (3,000 units/ml) was added. After 25μl of thrombin (1 unit/ml) was added for 10 min, the residual thrombin activity was measured using synthetic substrate (2mM H-D-phenylalanyl-pipeconyl-arginine-p-nitroanilide). The results were expressed as the residual inhibitory activity compared to the initial inhibitor as percentages.

5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Serum proteinase inhibitors (25μg) were incubated with buffer (0.01 M sodium acetate buffer, pH 4.5) or with SAP in a final volume of 50μl. The reaction was stopped at various time intervals by adding of 50μl sample buffer (2% SDS, 5% 2 mercaptoethanol, 10% glycerol) at 100℃ for 2 min. Ten μl of the treated sample was applied in each well. SDS-PAGE was performed as described by Laemmli [23] on 5-20% gradient gels. After electrophoresis, the gel was stained with Coomasie brilliant blue R-250. Molecular weight markers were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Results and Discussion

SAP isoenzymes are encoded by ten different genes [24]. In this study, we used an enzyme as a SAP which was already purified from the *Candida albicans* strain IFO-1060 [14, 15]. The purified SAP was homogeneous after SDS-PAGE, and only one protein band of molecular mass 42 kDa with Coomasie brilliant blue stain was observed. The enzyme in this study may be SAP 2, because the dominant proteinases secreted from yeast forms of most strains are SAP 2 [25].

The ability of α2-PI and C1-inh to inhibit the amidolytic activity on plasmin progressively decreased by incubation with purified SAP (Fig. 1). No loss of plasmin inhibitory activity was detected when the inhibitors were incubated without SAP (Fig. 1). The activity of AT-III to inhibit the amidolytic activity of thrombin also progressively decreased by incubation with SAP (Fig. 2). In these experiments (Fig. 1, 2), inhibitory activities after 60 min incubation had decreased to 80-100% in the presence of SAP, whereas no loss of activities in the controls was observed.

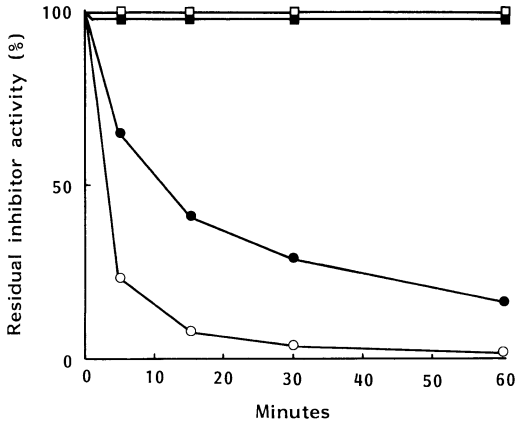

Fig. 1 Inactivation of α2-PI and C1-inh by SAP
α2-PI (filled) and C1-inh (open) were incubated with buffer (■, □) or SAP (●, ○) for various time intervals, respectively. Then these inhibitors were incubated with plasmin for 10 min followed by the additon of synthetic substrate, and the changes in absorbance at 405 nm were assayed by spectrophotometry.

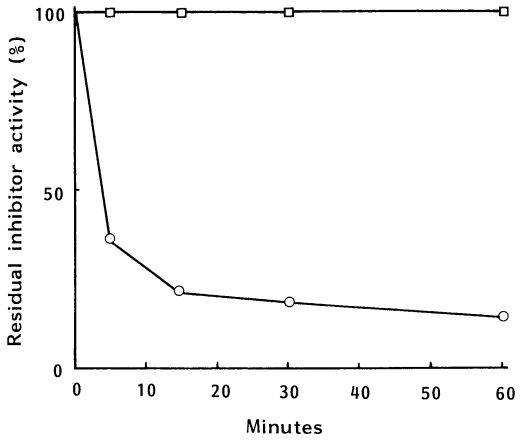

Fig. 2 Inactivation of AT-III by SAP
AT-III was incubated with buffer (□) or SAP (○) for various time intervals. Then SAP was incubated by the addition of 25 mM Tris-HCl buffer, pH 8.0, containing 7.5 mM EDTA and heparin. The mixture was incubated with thrombin for 10 min, and the residual thrombin activity measured by the addition of synthetic substrate at 405 nm.

Proteolytic degradation patterns of proteinase inhibitors were evaluated by SDS-PAGE. Electrophoretic analysis indicated that intact MW 105 kD C1-inh was rapidly converted to several lower molecular mass identifiable fragments (MW; 14-96 kDa) by incubation with SAP (Fig. 3). After 60 min incubation with SAP,the intact C1-inh band (MW; 105 kDa) almost disappeared and low molecular mass fragments appeared. It was assumed that the loss of inhibitory activity of C1-inh was correlated with the disappearance of the 105 kDa intact inhibitor. As shown in Fig. 4, SAP cleaved α2-PI into three identifiable fragments (MW; 56,000, 40,000 and 11,000). However, AT-Ⅲ was easily cleaved into various low molecular mass

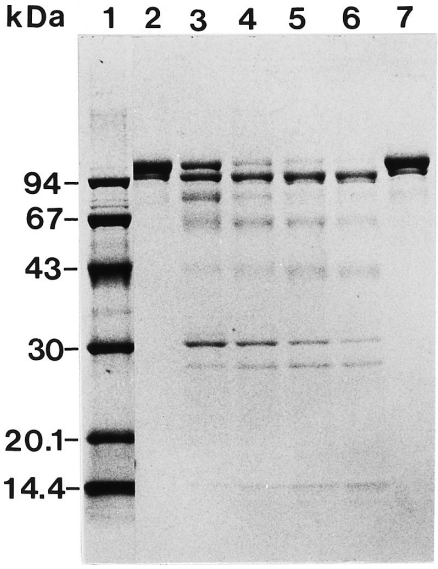

Fig. 3 Electrophoretic analysis of the effect of SAP on the C1-inh
Intact C1-inh (35μg) was incubated with SAP (0.2μg) at 37°C for 0, 5, 10, 20 and 30 min in lanes, 2, 3, 4, 5 and 6, respecively. Lane 1 shows standard marker proteins. Lane 7 shows intact C1-inh incubated for 30 min without SAP. The kDa lane was shown in position of molecular mass markers as kDa.

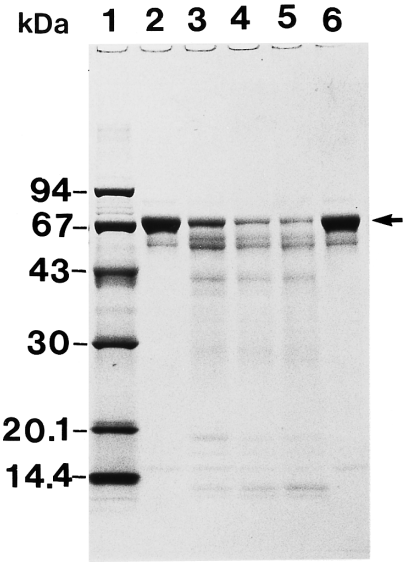

Fig. 4 Electrophoretic analysis of the effect of SAP on α2-PI
Intact α2-PI (42μg) was incubated with SAP (0.3μg) at 37°C for 0, 10, 30 and 60 min, as shown in lanes, 2, 3, 4 and 5, respectively. Lane 1 shows standard marker proteins. Lane 6 reveales intact α2-PI incubated for 60 min without SAP. The kDa lane was shown in position of molecular mass as kDa.

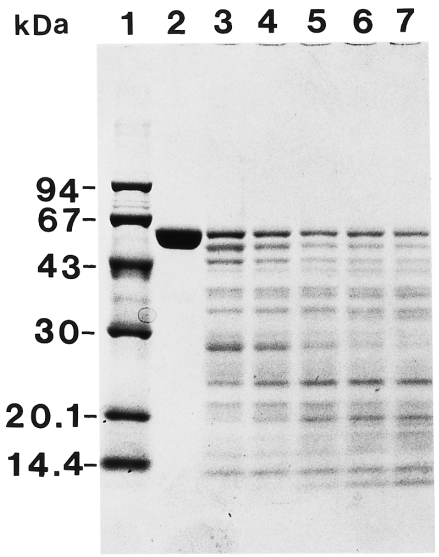

Fig. 5 Electrophoretic analysis of the effect of SAP on AT-III
Lane 1 shows standard marker proteins. Lanes 2 through 7 are 28μg of intact AT-III incubated with 0.2μg of SAP at 37°C for 0, 5, 10, 20, 30 and 60 min, respectively. The kDa lane was shown in position of molecular mass as kDa.

fragments by SAP randomly (Fig. 5).

Serum proteinase inhibitors such as C1-inh, α2-PI and AT-III are involved in critical regulation of complement systems, clotting systems and plasma kallikrein system [16]. They have important functions against host defense mechanisms. Fallon *et al.* [7] reported that SAP plays an essential role in progression of disseminated *Candida albicans* infection. Ruchel [26] reported that a patient with *Candida* septicemia exihibited acronecrosis. He speculated that acronecrosis occured with activation of the coagulatoin system due to factor X activation by SAP. Kaminishi *et al.* [27] also reported that clotting factors (XII, X, prothrombin) were converted to active forms by SAP. We showed here for the first time that SAP is a potent inhibitor of C1-inh, α2-PI and AT-III by proteolytic degradation, which are involved in complements, coagulation and fibrinolytic systems. Degradation patterns of these proteinase inhibitors by SAP were differed from, and broader than, those reported for other proteinases [28-32]. SAP showed broad degradation fragments against these serum proteinase inhibitors. These intact serum proteinase inhibitors were degraded into smaller modified inactive fragments. In these *Candida albicans* infectious lesions, SAP may alter their microenvironment by relative deficiencies of C1-inh, α2-PI and AT-III, then it may degrade and inactivate these serum proteinase inhibitors. In the absence of these inhibitors, the activated serine proteinases of the coagulation, fibrinolysis, and kallikrein-kinin pathways will be unopposed, thereby potentiating the local and/or systemic infectious reaction. SAP may reduce host defense functions and aggravate infectious diseases in immunocompromised hosts. Additional data concerning these proteinase inhibitors in patients with *Candida* infection are needed to confirm our results.

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