CRYSTAL STRUCTURE OF THE SERINE PROTEASE DOMAIN OF PROPHENOLOXIDASE ACTIVATING FACTOR-I*

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Running Title: Crystal structure of PPAF-I, an easter homologue

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A family of serine proteases (SPs) mediates the proteolytic cascades of embryonic development and immune response in invertebrates. These proteases, called easter-type SPs, consist of clip and chymotrypsin-like SP domains. The SP domain of easter-type proteases differs from those of typical SPs in its primary structure. Herein, we report the first crystal structure of the SP domain of easter-type proteases, presented as that of prophenoloxidase activating factor (PPAF) – I in zymogen form. This structure reveals several important structural features including a bound calcium ion, an additional loop with a unique disulfide linkage, a canyon-like deep active site, and an exposed activation loop. We subsequently show the role of the bound calcium and the proteolytic susceptibility of the activation loop, which occurs in a clip domain-independent manner. Based on biochemical study in the presence of heparin, we suggest that PPAF-III, highly homologous to PPAF-I, contains a surface patch that is responsible for enhancing the catalytic activity through interaction with a nonsubstrate region of a target protein. These results provide insights into an activation mechanism of easter-type proteases in proteolytic cascades, in comparison with the well-studied blood coagulation enzymes in mammals.

The serine proteases (SPs) in extracellular signaling cascades activate each other in a highly ordered manner with some restricted specificity for substrates. The blood coagulation cascade in mammals employs SPs such as coagulation factor Xa and thrombin to amplify the signal from tissue damage (1). This cascade is tightly regulated both to prevent any loss of blood and to avoid inappropriate clots. In invertebrates, diverse biological processes including embryonic development and immune responses are also driven by SP cascades, many of which are accomplished by clip-domain SPs.
containing one or two clip domains at the N-terminus of the SP domain (2). To date, 37 clip-domain SPs have been identified in the Drosophila genome database, many of whose physiological functions have been genetically and biochemically studied (3-5).

Three clip-domain SPs were cloned and identified in the large beetle Holotrichia diomphalia (6-8). The three SPs, called prophenoloxidase (proPO) activating factor (PPAF) -I, -II, and –III, are involved in the prophenoloxidase activating cascade, a major defense system of the insect, where activated phenoloxidase produces melanin around injury sites or invading microorganisms in the hemolymph (insect blood) (9,10). While PPAF-I and –III belong to catalytic group of clip-domain SPs, PPAF-II belongs to non-catalytic group of clip-domain SP because of replacement of the active site serine residue by glycine (7). Recognition of aberrant surface and microbial cell wall components leads to activation of PPAF-I and –III by a SP cascade in insect hemolymph. The activated PPAF-I cleaves proPO into a smaller but inactive PO, and PPAF-III cleaves catalytically-defective PPAF-II. We previously demonstrated that the cleaved PPAF-II molecules by PPAF-III form a dodecameric oligomer and is able to recruit PO molecules forming a PO/PPAF-II complex that can exhibit a strong PO activity (11). We also determined the crystal structure of PPAF – II (11), revealing that the clip domain plays a role by interacting with a protein through its central cleft and is tethered to the SP domain of the protein.

Many members of catalytic group of the clip-domain SP are called easter-type proteases, because they are structurally related to the well-characterized Drosophila easter that cleaves the growth factor-like Spatzle in the Toll signaling pathway (12). The clip domain of easter-type proteases was reported to play an essential role in rapid activation of the protease, although the clip domain is not tethered to the SP domain, which is unlike the non-catalytic group of the clip-domain SPs (11). The SP domain of easter-type proteases is believed to share its general architecture and catalytic mechanism with those of trypsin, and participates in signal amplification by cleaving and activating downstream easter-type SPs or effector proteins. The SP domains of easter-type SPs are distinguished from other SPs including non-catalytic clip-domain SPs by comparison of amino acid sequences. For instance, a short insertion loop containing two cysteine residues is present only in easter-type proteases (Fig. 1). However, the structure of an easter-type protease has not been determined.

To understand the relationship between structure and function of easter-type SPs, we determined the crystal structure of the SP domain of PPAF-I from the large beetle H. diomphalia. Structure-based biochemical analyses provided insights into the roles of the structural features of easter-type proteases in zymogen activation.

**Experimental Procedures**
Overexpression and purification of proteins -
Overexpression and purification of recombinant full-length PPAF-I and PPAF-I SP domain (88-365) in the baculovirus expression system (Invitrogen) were previously reported (11). Mutant PPAF-I protein was constructed using the QuickChange Mutagenesis Kit (Stratagene, USA) and purified according to the same protocol used for wild-type PPAF-I (13).

Crystallization and structure determination -
Crystallization, data collection and phasing of the PPAF-I SP domain were reported (14). In brief, the crystals were obtained by the hanging-drop vapor-diffusion method at 14 °C in a precipitation solution containing 0.15 M lithium sulfate, 30% polyethylene glycol 4000, and 0.1 M Tris-HCl (pH 8.0). An x-ray diffraction data set was collected using the synchrotron radiation from the beamline 4A at Pohang Accelerator Laboratory (Korea), and processed using the program HKL2000 (15). The crystals contained one molecule of the PPAF-I SP domain in the asymmetric unit. Initial phases were determined by the molecular replacement package AMoRe (16) using the coordinates of the catalytic domain of kallikrein (PDB code 2ANY) as a search model. The diffraction data were used for many rounds of model building and refinement using the programs O (17) and CNS (18). REFMAC5 was used at the final step of refinement (19). Crystallographic data statistics are summarized in Table I. Structures were superimposed and analyzed out using the function "fitting two molecules" in the program MOLREP (19), and structure presentations were generated using MOLSCRIPT (20) and PYMOL (21).

Activation of PPAF-I by lysyl endopeptidase -
To activate the full-length PPAF-I or the SP domain of PPAF-I, 5 µg of each protein was incubated at 30 °C for 1 hour in 20 µl of 50 mM Tris (pH 8.0) buffer containing 5 mM CaCl$_2$ and 0.2 µg of lysyl endopeptidase.

PPAF-I and –III activity assay using a peptide substrate -
The reaction mixture containing activated PPAF-I or PPAF-III was added to the final 100 µl of 50 mM Tris (pH 8.0) buffer containing 2 mM peptide substrate, Boc-Phe-Ser-Arg-MCA and either 1 mM CaCl$_2$ or 10 mM EDTA. After 10 min, the reaction was stopped by adding 800 µl of 17% acetic acid followed by an incubation for 10 min. The proteolytic activity of each sample was measured as fluorescence intensity with excitation and emission wavelengths of 380 and 460 nm, respectively.

Heparin effect on the activity of PPAF-III -
To examine the effect of heparin on the activity of PPAF-III, heparin (up to 7.7 mg/ml) was added in the reaction mixture. To measure the proteolytic activity of activated PPAF-III on PPAF-II, 0.5 µg/ml PPAF-II was used instead of the peptide substrate in the presence (7.7 mg/ml) or absence of heparin. An aliquot of 20 µl of the reaction mixture was taken at given times (0, 30 min, 60 min) and immediately boiled for denaturing gel electrophoretic analysis.
RESULTS

Structure determination and overall structure of the SP domain of PPAF-I.

Based on a boundary between the clip and SP domains determined by a limited proteolysis experiment, the SP domain of PPAF-I (88 – 365) was produced in the baculovirus/insect cell system and was purified to homogeneity in a zymogen form (11). The purified zymogen protein was confirmed not to exhibit catalytic activity. The zymogen protein was crystallized, and a high-quality diffraction data set was collected from a single flash-cooled crystal (14). The crystal structure was determined by the molecular replacement method, and the final 1.7 Å resolution model contains residues 90 – 365 with an N-glycosylated residue, one calcium ion, and 128 water molecules (Fig 2A).

The SP domain of PPAF-I has the conserved core structure of the chymotrypsin fold with the catalytic residues Ser315 (c195; “c” for the chymotrypsinogen numbering), His155 (c57) and Asp220 (c102) (Fig 2A). The catalytic triad, including its hydrogen bonding network, is already prearranged in the structure, in agreement with similar observation with prethrombin2 (a zymogen form of thrombin) (22) and chymotrypsinogen (23). In the active serine proteases, three highly conserved residues (c193 – c195) of the oxyanion hole shows an unusual backbone conformation of a ‘C’-shaped sharp turn, in which all the backbone amine groups point toward the position where the negative charge developing during the transition state. However, this zymogen structure of PPAF-I shows that corresponding residues (313-315; c193 – c195) is in a stretched backbone conformation, in which each backbone amine group points dispersly, similar to that of prethrombin2 (22). Furthermore, Asp309 (c189), the primary determinant of the S1 substrate specificity, is flipped out toward the solvent in the crystal structure, which suggests inactive conformation (Fig 2A).

The crystal structure reveals several distinctive features compared with the structures of chymotrypsin(ogen)-like SPs. The activation loop and the unique additional loop (75-loop) protrude from the globular core structure of the SP domain, and are located close to each other (Fig. 2B). In addition, the loops around the active-site cleft display a large deviation from those of any other proteases and their zymogen forms whose structures are available (Fig. 2B).

Calcium-binding loop (70-loop)

A three-dimensional modeling study on Drosophila easter predicted that a calcium ion was anchored by Glu193 (c70) and Glu203 (c80) in the 70-loop of easter (24). The crystal structure of the PPAF-I SP domain showed a complete feature of the calcium ion bound in 70-loop, which is remote from the active-site cleft. In terms of calcium coordination, PPAF-I is different from other calcium-containing SPs including trypsin and PPAF-II (Fig. 1 and 3A). The calcium ion of PPAF-I is hepta-
coordinated with pseudo-octahedral geometry involving the carboxylates of Glu175 (c70) and Asp183 (c78), the carbonyl oxygens of Asn177 (c72) and Thr180 (c75), plus two water molecules (Fig. 3A). In particular, Glu175 (c70) and Asp183 (c78) are strictly conserved among easter-type proteases, suggesting the presence of a calcium cage in this family of proteases.

The bound calcium ion in easter has been suggested to play a critical role in its biological function (24). Mutation of Glu183 (c70) in easter that abrogates the calcium binding resulted in complete loss of easter activity in Drosophila development when the mRNAs were injected into embryos (24). Here, we show that the calcium ion is necessary for the enzymatic activity of PPAF-I using a highly purified protein and a calcium-chelating agent. The activated PPAF-I exhibited a significantly decreased enzymatic activity on the chromogenic peptide substrates following addition of EDTA to the reaction buffer (Fig. 3B). Subsequently, we observed that the proteolytic susceptibility of PPAF-I substantially increased when the bound calcium ion was removed from the protein by EDTA (Fig. 3C). These results suggest that the calcium ion stabilizes the overall structure of the SP domain leading to enhanced activity, although the calcium ion is not directly involved in the active site, as is the case with trypsin (25) and coagulation factor Xa (26).

A highly protruding activation loop

The activation loop of PPAF-I is highly extended out from the body of the SP domain in the crystal structure, although this region does not show distinct insertion in its primary structure, compared with chymotrypsinogen (Fig 1, 2A and 2B). Interestingly, the corresponding loop of PPAF-II extends out from the core structure as observed in PPAF-I (Fig. 2B), but this results from interaction with the clip domain (11). The cleavage sequence that is neighboring residues in the scissile bond of Lys109 (c15) - Ile110 (c16) is unusually far from the body of the SP domain. This unusual conformation of the activation loop is not a crystallographic artifact induced by crystal packing interactions since this region is exposed to the solvent channel in the crystal. The activation loop of PPAF-I is estimated to be very flexible considering the poorly defined electron density map (Fig. 4A) and high temperature factors in this region. Consistent with this, the activation loop of PPAF-I was preferentially cleaved by a protease lysyl endopeptidase that cleaves at lysine residues (Fig. 4B).

Previously, we presented evidence that the clip domain of PPAF-I is separable from the SP domain by a single proteolytic cleavage between the inter-domain linker (11). However, we could not exclude a possibility that the clip domain regulates the activation of the SP domain through a weak interaction between the clip domain and the activation loop. In the present work, we confirmed that the clip domain does not affect cleavage of the
activation loop of the SP domain by a limited proteolysis experiment using lysyl endopeptidase. The proteolytic susceptibility of the activation loop of the full-length PPAF-I was comparable to that of the PPAF-I SP domain lacking the clip domain (Fig. 4B and 4C).

**The unique additional loop (75-loop)**

The characteristic additional loop (75-loop) of easter-type SPs, which extends from the calcium-binding loop, consists of residues 184-196 including two unique cysteine residues (Fig. 5A). The crystal structure reveals that 75-loop includes a short hairpin structure (Fig. 5A) and is placed close to the activation loop at a distance of 4.6 Å (Fig. 5B). The short hairpin structure is stabilized by an inter-connection of the disulfide bond formed with the invariant cysteine residues (Cys184, Cys191) among the easter-type proteases. Owing to this inter-connection, this hairpin structure is anticipated to be present in all members of easter-type protease family despite the poor conservation of the flanking amino acids between the cysteine residues (Fig. 1). As noted above, 75-loop is located near the cleavage sequence for zymogen activation (Fig. 5B). This structural feature suggests that cleavage of the activation loop is resistant to proteases with deep active-site clefts, although it might be sensitive to proteases with shallow active-site clefts. A mutant PPAF-I, whose cleavage sequence was changed to a canonical sequence (Asp-Asp-Asp-Lys) for enteropeptidase substrates, was totally resistant to proteolysis by enteropeptidase (data not shown). Enteropeptidase, which is known to activate trypsinogen, has a deep active-site cleft (27). This led us to speculate that 75-loop might prevent access of the active site cleft of enteropeptidase to the activation loop of PPAF-I, for 75-loop is the nearest structural element to the activation loop (Fig. 5B).

**The canyon-like deep active-site cleft**

The structure of the PPAF-I SP domain defines a highly ordered and deep canyon-like active-site cleft. This canyon-like deep active site has been observed in thrombin, which is responsible for the high substrate specificity. The active-site cleft is lined with the surface loops composed of 30-loop (130-139), 60-loop (158-168) and 140-loop (263-269). Compared with amino acid sequences of trypsinogen and chymotrypsinogen, 30- and 60-loops of PPAF-I have insertions, which form longer β-strands and a short α-helix in the structure, respectively (Fig. 2A and 6A). The structure of 140-loop is well defined in the PPAF-I structure, while the corresponding loop is highly disordered in the mostzymogen form of SP structures. Remarkably, 60- and 140-loops are located over the catalytic triad, forming a narrow passage to the catalytic serine residue in the Cα trace of PPAF-I (Fig. 6A). Interestingly, the sidechains of Arg160 (c62) in 60-loop and Glu263 (c148) in 140-loop, which are also found in Drosophila easter and Spatzle processing enzyme (SPE) (5), may act as a
latch blocking the passage to the cleft, as shown in Fig. 6. A similar observation was reported in the zymogen structure of complement factor D (28), and this interaction might be involved with preventing accidental activity of the zymogen form.

**Surface patches enhancing the activity on a natural substrate**

Thrombin contains two exosites that are involved in recognizing and interacting with macromolecular substrates and inhibitors. Structural comparison of PPAF-I and thrombin reveals that PPAF-I contains a basic surface area between the active site cleft and the calcium binding loop, which is near the first exosite (exosite I) of thrombin (Fig. 7A). This region is composed of several basic residues, among which Arg172, Arg228, and Arg261 are exclusively conserved in easter-type SPs (Fig. 1). Given the availability of PPAF-III, showing 45% sequence identity to PPAF-I in the SP domains (Fig. 1), and its protein substrate PPAF-II, we checked whether the SP domain of PPAF-III contains such surface patches using heparin, an anionic glycosaminoglycan. We observed that the proteolytic activity of PPAF-III was severely impaired on its protein substrate PPAF-II, but not on the peptide substrate, in the presence of heparin (Fig. 7B and C). This result suggests that heparin may bind to a surface patch of PPAF-III, inhibiting an interaction with the protein substrate.

**DISCUSSION**

We determined the crystal structure of the SP domain of PPAF-I, revealing several structural features of easter-type proteases, such as the calcium coordination, the unique additional loop containing a disulfide bond, the deep active-site cleft, and the highly exposed activation loop. Subsequent biochemical studies demonstrated that the bound calcium ion provides structural integrity to the SP domain, leading to enhancement of the enzymatic activity. The exposed activation loop was preferentially cleaved by a non-specific protease in a clip-domain independent manner, indicating that the clip domain is not involved with the zymogen activation of its SP domain.

Comparison of the structure of the PPAF-I SP domain with thrombin identified basic surface elements in the PPAF-I SP domain, prompting us to carry out a biochemical study with PPAF-III in the presence of heparin, an anticoagulant against thrombin. Surprisingly, heparin inhibited the PPAF-III activity on the protein substrate, not on the peptide substrate, strongly suggesting that heparin interacts with the protein substrate competitively on a surface patch of PPAF-III which is not a part of active site cleft. This result offers further structural insight into the strategy of easter-type proteases to select their downstream protein substrate. We speculate that this surface patch of PPAF-III plays a similar role as the fibrinogen-recognition exosite (exosite I) of thrombin, providing high
substrate specificity. The interaction of the exosite I of thrombin with the central E region of fibrinogen is well-established, providing proper orientation of the catalytic triad for cleavage of fibrinogen fibrinopeptides (29,30). This nonsubstrate interaction of thrombin with fibrinogen promotes cleavage of fibrinopeptides, resulting in their conversion into fibrin. Analogous to thrombin, easter-type SPs might achieve their high specificity for recognition of their downstream proteins through their surface patches, together with their deep active-site clefts.

In conclusion, we presented the first three-dimensional structure of the SP domain of an easter-type protease with biochemical analyses on the bound calcium and the exposed activation loop. In addition, we presented evidence that an easter-type SP has a surface patch that plays a similar role as the fibrinogen recognition exosite of thrombin. These results provide insights into an activation mechanism of easter-type proteases in proteolytic cascades, in comparison with the well-studied blood coagulation pathway in mammals. The detailed structure of easter-type proteases will help to elucidate the molecular mechanism of substrate recognition and the specific zymogen activation in various biological processes.

REFERENCES

FOOTNOTES

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FIGURE LEGENDS

**Fig. 1.** Sequence alignment. Four easter-type SPs, snake, PPAF-II, thrombin, trypsin and chymotrypsin from top to bottom: Hd, *Holotrichia diomphalia*; Dm, *Drosophila melanogaster*; Hs, Homo sapiens; Bt, *Bos taurus*; chymo, chymotrypsine are shown. The alignment was conducted using CLUSTALW (31), and then adjusted based on the secondary structure and the conserved cysteine residues in yellow boxes. The cleavage sites for zymogen activation are indicated by arrows, and the catalytic triads (His, Asp, Ser) at the active sites are shown in red. The calcium-coordinating residues of easter-type SPs, PPAF-II and trypsin are indicated by green boxes. The disulfide linkages are shown in lines with a symbol (S-S), while the disulfide linkage that is absent in easter-type SPs is in a blue line. The regions where the large conformational changes are expected during the zymogen activation are shown in blue boxes. The regions whose structure shows a large deviation from those of typical SPs are shown in magenta boxes. The residues blocking the entrance of the active site in the easter-type proteases are indicated by red circles, and the primary determinant residues for the S1 binding pocket are indicated by a red star. Residue numbers of PPAF-I and chymotrypsin are shown above or below every ten amino acids.

**Fig. 2.** Overall structure of the SP domain of PPAF-I, shown in standard orientation (32). A. A ribbon representation of the PPAF-I SP domain structure. The SP domain is shown in cyan, except for several loop regions whose color scheme is as in Fig 1. Bound calcium ion is in green, and all the three disulfide bonds are drawn with sulfur atoms in yellow. The catalytic triad
residues (Ser-His-Asp) are shown in the ball-and-stick representation. Asn131 (c36) in 30-loop is N-linked glycosylated. B, Left, the superimposed Ca traces of the PPAF-I SP domain (cyan) and chymotrypsinogen (orange; PDB code 1CGI). Right, the superimposed Ca traces of the PPAF-I SP domain (cyan) and PPAF-II SP domain (grey; PDB code 2B9L).

**Fig. 3.** The calcium-binding loop (70-loop) and the function of the bound calcium. A, Electron density around the calcium-binding site. 2FoFc electron density map is contoured at 1.5 σ level. The bound calcium ion (Ca) is drawn as a ball, and hepta-coordinations are indicated by dotted lines. B, The bound calcium ion enhances the proteolytic activity of PPAF-I. Prior to the reaction, full-length PPAF-I protein (5 µg) was activated by lysyl endopeptidase (0.2 µg) in 20 mM Tris (pH 8.0) buffer 5 mM CaCl₂. To measure the enzymatic activity, 1 µl of the reaction mixture containing the activated PPAF-I protein was immediately added into 20 µl of 20 mM Tris (pH 8.0) buffer containing a chromogenic peptide substrate and 5 mM CaCl₂ or 10 mM EDTA. To remove lysyl endopeptidase activity used to activate the PPAF-I protein, all data were generated by subtracting the activity of lysyl endopeptidase in the reaction mixtures. We confirmed that the activity of lysyl endopeptidase was not affected by EDTA (data not shown). The PPAF-I SP domain protein showed the similar calcium effect as the full-length PPAF-I protein. The error bars represent standard deviations of three separate experiments (data not shown). C, SDS-PAGE gel showing degradation of PPAF-I by lysyl endopeptidase in the presence of CaCl₂ or EDTA. Digested mixtures containing 1 mg/ml full-length PPAF-I in 50 mM Tris (pH 8.0) and 10 µg/ml lysyl endopeptidase with 5 mM CaCl₂ (lanes 2, 4) or 10 mM EDTA (lanes 3, 5) were incubated at 30 °C for 30 min (lanes 2, 3) or 60 min (lanes 4, 5). Positions of molecular weight markers (kDa) are shown on the left, and the PPAF-I protein sample containing no lysyl endopeptidase is shown in lane 1.

**Fig. 4.** The flexible activation loop showing a high susceptibility to proteolysis. A, Electron density map around the activation loop. 2FoFc electron density map is contoured at 1.0 σ level. B, SDS-PAGE gel showing digestion of the full-length PPAF-I and the SP domain of PPAF-I with increasing incubation times. Positions of molecular weight markers (kDa) are displayed on the left, and the positions of digestion fragments are marked with arrows. The digested mixtures containing 20 µM of full-length PPAF-I or the SP domain of PPAF-I in 50 mM Tris (pH8.0), 5 mM CaCl₂ and 10 µg/ml lysyl endopeptidase were incubated at 30 °C. An aliquot of 20 µl of the reaction mixture was taken at given times (0, 15, 30, 60 min) and immediately boiled for SDS-PAGE analysis. The fragments from full-length PPAF-I and the SP domain of PPAF-I were denoted by “f” and “s” with the number, respectively. The fragments beginning with Ile110 are
denoted by “*”. C, Schematics of the full-length (FL; top) and the SP domain of PPAF-I (SP; bottom) presenting the region of the fragments shown in B. The cleavage sites are indicated by numbered circles, in which the numbers stand for the cleavage order. While the cleavage site ② (the activation site) was determined by the N-terminus of the corresponding fragments, the cleavage sites ① and ③ were estimated by the N-terminal and the apparent molecular weight of the fragments on the gel. Note that the band intensities between 3f*, 4f* and 3s*, 4s* are similar on the gel. The N-terminal amino acid sequence of the peptide fragments were determined by an automated gas-phase sequencer (Applied Biosystems).

**Fig. 5.** The unique additional loop (75-loop) and the activation loop. A, The calcium-binding loop (70-loop) and 75-loop are represented as Cα worms in green or magenta. The corresponding regions of trypsin and PPAF-II are shown as a Cα worm in yellow and grey, respectively. A calcium ion bound to PPAF-I or –II is represented as a green sphere, and that of trypsin is an orange sphere. B, Stereo representation of PPAF-I structure emphasizing 75-loop and the activation loop. For clarity, the cleavage sequence (106 – 110) in the activation loop and the flanking sequence between the two cysteine residues in 75-loop are shown in green. The distance between Cα positions of Ile110 and Gly186 are indicated.

**Fig. 6.** The canyon-like deep active site cleft. A, A side view of the active site from the right of the molecule in Fig. 2A. The coloring scheme is as in Fig 2A. The ionic interaction between Arg160 and Glu263 is shown as a dotted line. B, Surface representation of PPAF-I is shown with a Cα worm. The region and orientation of the molecule are similar to those in Fig. 4A. The surface representation was generated from a virtual mutant molecule (R160A/E263A), and it was perpendicularly clipped just before the catalytic serine residue for clarity. The distance between Arg160 and the Oγ of Ser315 is 11.1 Å as shown.

**Fig. 7.** The effect of heparin on PPAF-III activity. A, A view of surface representation of the PPAF-I SP domain, focusing on the area which corresponds to exosite II (Right) of thrombin. The surface is colored according to electrostatic potential (blue positive; red negative). Basic residues in the positively charged region are shown on the surface. B, Electrophoretic analysis of PPAF-III activity toward PPAF-II in the absence (lanes 1 – 4) and the presence (lanes 5 – 8) of heparin. An arrow indicates the cleaved product from PPAF-II. The incubation periods are shown below each lane. The numbers at the right show the molecular mass in kDa. C, PPAF-III activity using a chromogenic peptide substrate Boc-Phe-Ser-Arg-MCA in the presence or the absence of heparin. Heparin and hemolymph (HL) do not exhibit significant protease activity as
shown. The data points are averages of three independent experiments, with standard deviation indicated by the error bars.
Table 1. X-ray data collection and refinement statistics

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<tr>
<td>generously disallowed region</td>
<td>1.3%</td>
</tr>
<tr>
<td>disallowed region</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

¹ The numbers in parentheses are statistics for the highest resolution shell.
² R_{free} was calculated with 10% of the data.
Figure 1
Crystal structure of the serine protease domain of prophenoloxidase activating factor-I

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