Chemical modification of *Vibrio vulnificus* metalloprotease with activated polyethylene glycol

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Abstract: *Vibrio vulnificus*, an opportunistic human pathogen causing septicemia, produces a metalloprotease which is suspected to be a virulence determinant, but which is labile in vivo due to inactivation by α-macroglobulin. To obtain a derivative which is stable in vivo, the metalloprotease was modified with activated monomethoxy polyethylene glycol. The modified protease retained full activity to a peptide substrate and 10-20% activity to protein substrates, and was resistant to entrapment by α-macroglobulin because of the increased molecular size (approx. 90 kDa). These findings suggest that the modified protease is stable in vivo and may be used to investigate the pathological actions of the protease in the bloodstream.

Key words: Bacterial metalloprotease; Polyethylene glycol; Chemical modification; *Vibrio vulnificus*

Introduction

*Vibrio vulnificus* is an etiologic agent of wound infection and septicemia in humans, and the infections are characterized by edema and ulcer action of the skin [1-4]. The septicemia due to this vibrio is an opportunistic infection occurring in individuals with underlying diseases of the liver or hemochromatosis [1-4]. We have previously purified a metalloprotease (VVP) from *V. vulnificus* culture supernatant [5], and reported on its vascular permeability enhancing activity through exocytotic histamine release from mast cells [6,7] and/or activation of the Hageman factor-plasma kallikrein-kinin system [8,9]. Therefore, VVP is considered to be largely responsible for the development of local skin lesions [10]. However, the role of the protease in systemic infections remains to be clarified because the enzyme is rapidly removed from circulation in vivo.

Recently, we showed that the plasma α-macroglobulin (α M) was the inactivator of VVP in plasma and caused the immediate disappearance of injected VVP [11,12]. The α M inactivates many proteases, including bacterial metalloproteases, at a molar ratio of 1:1 and acts as a host defense factor against exogenous proteases [13-15]. Target proteases are inactivated by physical entrapment by the α M, and the protease-α M complex is rapidly removed by receptor-mediated endocytosis on hepatocytes, fibroblasts and macrophages [15,16]. Therefore, α M cannot inactivate large molecules because of steric hindrance.
The chemical modification of VVP to a large molecule may be useful to assess in order to understand the pathological role of VVP produced during *V. vulnificus* systemic infections. To achieve this, we modified VVP with activated monomethoxy polyethylene glycol (PEG) which had been widely used for modification of the enzyme [17], and some properties of modified VVP were compared with the native enzyme.

**Materials and Methods**

**Substances**

PEG (5 kDa), azocasein and hide powder azure were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine plasma α M preparation was obtained from Boehringer-Mannheim (FRG), and the amount of active α M in the preparation was determined with trypsin (Type 1, Sigma) according to the method of Swenson and Howard [18]. Carbobenzyl-glycyl-phenylalanine amide (Z-Gly-Phe-NH₂), and phosphoramidon were purchased from Peptide Institute Inc. (Minoh, Osaka, Japan).

VVP (45 kDa) was purified from *V. vulnificus* strain L-180 as described previously [5] with minor modifications [10]. Rabbit IgG antibody against native VVP was prepared as reported previously [12].

**Modification of VVP with PEG**

VVP (6 μM) was incubated with PEG₁ (600 μM) in 100 mM borate buffer (pH 8.5) at 4°C for 24 h, and the reaction mixture was filtered through a YM 30 membrane to remove free PEG₁. The preparation thus obtained was used as the modified VVP (PEG₁-VVP) preparation. The concentration of PEG₁-VVP was determined by the Lowry method [19] with bovine serum albumin as the standard, and the free amino groups were assayed by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [20].

**Physicochemical and immunological properties of PEG₁-VVP**

The pH stability was tested with GTA buffer (pH 4–10) [5]. Protease solutions, in buffers of pH 4–10 were incubated at 4°C for 24 h, and the residual peptidase activity was measured as described below [3,4]. To test heat stability, the protease (33 nM) was heated at 60°C for 0–60 min and the residual peptidase activity was measured.

Antigenicity of the PEG₁-VVP was examined by the single radial immunodiffusion (SRID) test and enzyme-linked immunosorbent assay (ELISA). SRID was carried out by the method of Mancini et al. [21]. Briefly, 50-μl samples were added to wells of an agar gel containing diluted IgG antibody against native VVP, incubated for 24 h at 37°C, and the diameters of the immunoprecipitation zones were measured. ELISA was performed as described previously [12].

**Enzymatic assays**

To test the peptidase activity toward Z-Gly-Phe-NH₂, protease (33 nM) and substrate (2.0 mM) were incubated at 37°C for 30 min in 300 μl of 20 mM borate buffer; the reaction was terminated by addition of 700 μl of ice-cold water, and the extent of hydrolysis of the substrate was measured by the TNBS method [20]. To test the effect of phosphoramidon, VVP (33 nM) was treated with an appropriate concentration of phosphoramidon at 37°C for 10 min and the residual peptidase activity was measured; the Kᵢ value was then calculated from the Dixon plot. For neutralization tests, 20 pmol of protease in 300 μl of 20 mM borate buffer (pH 8.5) was incubated with the rabbit anti-VVP IgG (100 μl) at 37°C for 10 min, and then the residual peptidase activity was measured.

The proteinase activity toward azocasein was measured as noted previously [5]. One unit of activity was defined as the amount of the enzyme which hydrolysed 1 μg of azocasein at 37°C in 1 min [5]. Inactivation of the VVP by α M was studied using both purified bovine α M and human plasma (crude human α M preparation) [11]. The protease was incubated with an equimolar amount of α M at 30°C for 10 min, and the residual caseinolytic activity was assayed.

The activity toward hide powder azure was assayed by the method of Horvat et al. [22] with the following modification: protease and hide powder azure (2 mg) were mixed in 200 μl of 50 mM Tris-HCl buffer (pH 8.0), and incubated at 30°C for 30 min with gyratory agitation (180 cycles/min). Thereafter, the reaction was termi-
nated by addition of 700 μl of the ice-cold buffer, and the absorbance of the supernatant obtained by centrifugation (1000 × g for 5 min) was measured at 595 nm. One unit of activity was defined as the amount of enzyme producing a change in A_{595} of 0.01 at 30°C in 30 min.

Results and Discussion

Physicochemical and immunological properties of PEG₁-VVP

On incubation of VVP with PEG₁, 80% of the total amino groups were modified. Since VVP reportedly possesses 11 amino groups [5], it appeared that nine molecules of PEG₁ were bound per molecule of VVP such that the molecular mass of the modified VVP (PEG₁-VVP) was approximately 90 kDa. Although PEG₁-VVP was eluted from a Superose 6 HR 10/30 column (Pharmacia LBK, Uppsala, Sweden) as a single peak with an apparent molecular mass of 680 kDa, this modified VVP passed through a Diaflo XM 300 membrane (molecular mass cut-off = 300 kDa). Therefore, the behavior of PEG₁-VVP in gel filtration was considered to be different from that of a globular protein as PEG₁ is a long-chain molecule.

PEG₁-VVP was stable at acidic pH but labile to heat treatment. Thus, the peptidase activity of modified enzyme was completely lost by heating at 60°C for 60 min, but 30% of the activity remained after incubation at pH 4.0 for 24 h. However, the activity of native enzyme was partially retained after heat treatment but completely abolished at the acidic pH. These observed differences between native and modified VVPs may be attributed to the increased hydrophobicity of the modified enzyme.

Some proteins modified with PEG₁ lose their antigenicity [23]; thus, the antigenicity of PEG₁-VVP was also examined. The results of ELISA showed that the antigenicity of PEG₁-VVP was reduced to 30% of that of native VVP. Furthermore, the modified VVP formed a faint precipitation zone in SRID, indicating that some of the epitopes were maintained in PEG₁-VVP.

Enzymatic behavior of PEG₁-VVP

Table 1

<table>
<thead>
<tr>
<th>Protease</th>
<th>pH 7.0</th>
<th>pH 8.5</th>
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</thead>
<tbody>
<tr>
<td>Kₘ (mM)</td>
<td>kₜₐₜ (s⁻¹)</td>
<td>Kₘ (mM)</td>
</tr>
<tr>
<td>Native protease</td>
<td>5.3</td>
<td>19.8</td>
</tr>
<tr>
<td>Modified protease</td>
<td>5.3</td>
<td>20.8</td>
</tr>
</tbody>
</table>

* The extent of hydrolysis of Z-Gly-Phe-NH₂ upon incubation with protease at 37°C (0.5–4.0 mM) for 30 min was measured by the TNBS method; Kₘ and kₜₐₜ values were determined by Lineweaver-Burk plots (n = 2).

Table 2

<table>
<thead>
<tr>
<th>Protease</th>
<th>Kᵢ value (μM) at</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
</tr>
<tr>
<td>Native protease</td>
<td>0.8</td>
</tr>
<tr>
<td>Modified protease</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Proteases were preincubated with phosphoramidon at 37°C for 10 min, and the residual peptidase activity was measured. Kᵢ value was estimated by Dixon plots (n = 2).
complex and associate with the active center of PEG-VVP.

As shown in Table 3, the proteinase activity of PEG-VVP toward casein and hide powder was reduced to 20% and 10% of those of native VVP, respectively, suggesting the inhibition of macro-molecule substrate–enzyme interaction due to the steric hindrance. On the other hand, in the presence of equimolar amounts of bovine or human α M, the proteinase activity of native VVP toward casein was drastically reduced by the α M-entrapment, but that of PEG-VVP was not reduced. This result denotes that modified VVP is sufficiently large to resist the entrapping action of plasma α M.

It has been reported that plasma α M inactivates many proteases by physical entrapment of the protease molecule [13–15] and that the α M-protease complex is rapidly excluded by receptor-mediated endocytosis [15,16]. Therefore, VVP injected into the blood stream of healthy animals is thought to be inactivated by α M and removed immediately. However, because plasma α M levels in patients with severe septicemia are greatly reduced [24,25], VVP action may be long-lasting in patients suffering from V. vulnificus septicemia. Also, the invading vibrio could continuously elaborate VVP which would decrease the level of plasma α M. Thus, studied with the α M-resistant VVP derivative may allow a more realistic assessment of the role of VVP in systemic V. vulnificus infections.

Table 3

<table>
<thead>
<tr>
<th>Protease Addition</th>
<th>Proteinase activities toward</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Casein (n = 3) b</td>
</tr>
<tr>
<td></td>
<td>(U/pmol)</td>
</tr>
<tr>
<td>Native protease</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Bovine α M</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Human plasma</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Modified protease</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Bovine α M</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Human plasma</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

a Proteinase activity was determined as described in the text.
b Results are the means ± SD.
c Not tested.

References