Purification and Properties of an S-PI(Pepstatin Ac)-insensitive Carboxyl Proteinase from a Xanthomonas sp. Bacterium

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A S-PI(Pepstatin Ac)-insensitive carboxyl proteinase was found in culture filtrate of a Xanthomonas sp. bacterium. The carboxyl proteinase was highly purified and about 100 mg of the enzyme was obtained from 60 l of culture filtrate, with a recovery of 25%. The optimum condition for the action of the purified enzyme toward casein was approx. pH 2.7 and its activity was not inhibited by any of such carboxyl proteinase inhibitors as Pepstatin, S-PI, and DAN but EPNP inhibited it. Such behavior of the enzyme against inhibitors resembles that of Pseudomonas sp. carboxyl proteinase, the first found from a bacterium. Some differences were observed, however, in their properties such as optimum pH, isoelectric point, and amino acid composition.

Most carboxyl proteinases from animals and microbes are inactivated by DAN,1) EPNP,2) Pepstatin,3) and S-PI (Pepstatin Ac).4,5) With porcine pepsin, DAN and EPNP have been shown to modify Asp-215 and Asp-32, respectively, both of which constitute the active site. The same results have been observed with other carboxyl proteinases.5) Since the essential catalytic site involves carboxyl groups of aspartic acid residues, carboxyl proteinases are now called aspartic proteinases.6)

Scytalidium lignicolum ATCC 24568, isolated by us in 1972, produces carboxyl proteinases A-1, A-2, B, and C, all of which are insensitive to such carboxyl proteinase inhibitors as DAN, EPNP, Pepstatin, and S-PI, except that carboxyl proteinase B is sensitive to EPNP.7~11) These carboxyl proteinases are unique in such properties as substrate specificity,12~15) nature of active site,7) and complete amino acid sequence (carboxyl proteinase B)16) in addition to the behavior against inhibitors. It was found that the catalytic site of carboxyl proteinase B does not involve aspartic acid residues, unlike other carboxyl proteinases, but glutamic acid residues.17) Such carboxyl proteinases having properties similar to those of Scytalidium enzymes are widely distributed among Basidiomycetes, especially in edible mushrooms.18~20) Recently, we found that a gram-negative Pseudomonas species produces a Pepstatin- and DAN-insensitive carboxyl proteinase having properties similar to those of Scytalidium enzymes.

Abbreviations: S-PI, Streptomyces-pepsin inhibitor; Pepstatin Ac, N-acetyl pepstatin; DAN, diazoacetyl-DL-norleucine methylster; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; PCMB, p-chloromercuribenzoate; PCMPS, p-chloromercuribenzenesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

*1 S-PI(acetyl-valyl-valy-4-amino-3-hydroxy-6-methylheptanoyl-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid)22) has the same structure as Pepstatin Ac.23)
proteinase. This is the first demonstration of a carboxyl proteinase in a bacterium.\textsuperscript{21}

We tried to isolate other bacteria producing Pepstatin- and DAN-insensitive carboxyl proteinases and succeeded in obtaining a strain, No. T-22, from soil. The strain belongs to the genus \textit{Xanthomonas}. In this paper, the purification and characterization of the purified carboxyl proteinase are described.

\textbf{MATERIALS AND METHODS}

1) \textit{S-PI, DAN, and EPNP}. Crystalline \textit{S-PI} (acetyl-valyl-valyl-4-amino-3-hydroxy-6-methylheptanoyl-analyl-4-amino-3-hydroxy-6-methylheptanoic acid) was prepared by the method of Murao and Satoi.\textsuperscript{4} \textit{S-PI} has the same structure as Pepstatin Ac.\textsuperscript{23} \textit{DAN} and \textit{EPNP} were purchased from the Sigma Chemical Co., and Eastman Kodak, respectively.

2) \textit{Isolation and identification of the bacterium}. A gram-negative bacterium, designated tentatively as strain No. T-22, was isolated from a soil sample. The isolate was taxonomically characterized by the conventional methods\textsuperscript{24} and the chemotaxonomic methods.\textsuperscript{25} The strain was maintained on an agar medium of potato-glucose (200 g of potato and 10 g of glucose per liter of water at pH 5.0).

3) \textit{Culture conditions}. Strain No. T-22 was aerobically cultured in a 30-l jar fermentor containing 20 l of medium (3\% glucose, 0.5\% malt extract, and 0.5\% yeast extract, pH 6.0). The culture conditions were as follows: agitation at 300 rpm; aeration at 20 l/min; temperature of 30°C. The carboxyl proteinase began to accumulate approx. 10 hr after the beginning of cultivation and its amount in the culture fluid increased almost linearly with time, to reach a maximum at 21 ~25 hr. The culture fluid at 25 hr (pH 4.8) was used for the preparation of the carboxyl proteinase.

4) \textit{Assay of proteinase activity}. Proteinase activity was assayed at pH 2.7 and 37°C for 30 min with casein as the substrate. One unit of enzyme activity was defined as the quantity of enzyme that liberates 1 \(\mu\)g of tyrosine per ml of reaction mixture per minute.\textsuperscript{10}

5) \textit{Protein measurement}. Protein was measured from the absorbance at 280 nm by the factor, \(E_{1%}^{1\text{cm}} = 11.7\).

6) \textit{Isoelectric focusing}. Electrofocusing was done on an LKB column (110 ml) containing 1\% carrier ampholyte with pH from 3.5 ~5.0.

7) \textit{Amino acid analysis}. Amino acid composition and free thiol content were assayed by the methods reported previously.\textsuperscript{21}

8) \textit{Polyacrylamide gel electrophoresis}. Polyacrylamide gel electrophoresis was done using Jovin's buffer system (pH 5.5 and 7\% gel).\textsuperscript{26} Sodium dodecyl sulfate gel electrophoresis was done as described by Weber and Osborn, using 7.5\% gels.\textsuperscript{27} Protein bands were stained with Coomassie brilliant blue. The reference proteins used for the molecular weight measurement were bovine serum albumin (68,000), \(\gamma\)-globulin (50,000), egg albumin (43,000), alcohol dehydrogenase (41,000), \(\alpha\)-chymotrypsinogen (25,700), \(\gamma\)-globulin (L) (23,500), and cytochrome c (11700).

9) \textit{Carbohydrate analysis}. The presence of carbohydrate was investigated by the phenol–sulfuric acid method.\textsuperscript{28}

10) \textit{Purification of carboxyl proteinase}.

\textbf{Step 1}. Preparation of culture filtrate of strain, No. T-22. After cultivation of strain No. T-22 for 24 hr, the pH of the culture fluid was adjusted to 4.8 with glacial acetic acid, and it was then centrifuged at 6,000 rpm for 10 min.

\textbf{Step 2}. Ammonium sulfate precipitation and acetone fractionation. The precipitate at 80\% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} saturation was prepared and dissolved in 50 mM sodium acetate buffer, pH 4.8. The resulting precipitate was removed by centrifugation at 8,000 rpm for 10 min. To the

![FIG. 1. DEAE-Cellulose Column Chromatography of Xanthomonas Carboxyl Proteinase.](image-url)
S-PI-Insensitive Carboxyl Proteinase from Xanthomonas sp.

Fig. 2. Sephadex G-75 Gel Filtration of Xanthomonas Carboxyl Proteinase. Carboxyl proteinase from DEAE-Cellulose (total act. = 1.9 x 10^5, specific act. = 1,400) was put on a column (2.5 x 93.5 cm) equilibrated with 50 mM sodium acetate buffer, pH 4.8. The column was developed with the same buffer at a flow rate of 10 ml per hr. Fractions of 10 ml were collected.

-■■-, absorbance at 280 nm; ○○○○, proteinase activity; ××××, specific activity.

supernatant, cold acetone was again added to give a concentration of about 75 vol%. The precipitate collected by centrifugation was dissolved in buffer 1, and the solution was exhaustively dialysed against the same buffer.

Step 3. DEAE-Cellulose column chromatography. The dialysed solution was then put on a DEAE-Cellulose column (5 x 40 cm) equilibrated with buffer 1. The column was first washed with 2 vols. of buffer 1, then eluted with a linear gradient of 0~1 M NaCl in the same buffer. As shown in Fig. 1, carboxyl proteinase was eluted as a single active peak. The active fractions were collected and dialyzed against the same conditions.

Step 4. Sephadex G-75 column chromatography. The enzyme solution obtained from DEAE-Cellulose column chromatography was concentrated by an evaporator at 40°C and filtered on Sephadex G-75 gel (2.5 x 93.5 cm; flow rate, 10 ml/hr with buffer 1). Active fractions were collected. At this step, the carboxyl proteinase was eluted as a single peak of protein having a specific activity of 1470 (PU/absorbance at 280 nm) as shown in Fig. 2. Approx. 100 mg of purified carboxyl proteinase was obtained from 60 l of culture filtrate.

RESULTS

1. Identification of the strain T-22

Strain T-22 was a gram-negative, polar-flagellated rod. The color of the colony was yellow on nutrient agar. It showed positive reaction to catalase and oxidase tests. Acid was produced from glucose aerobically. Since glucose was not used as a sole carbon source, strain T-22 was supposed to require some growth factor. It had ubiquinone with eight isoprene units. The predominant components of the cellular fatty acids are iso-C17:0, iso-C15:0, anteiso-C15:0, and iso-C17:1.

2. Purification of S-PI (Pepstatin Ac)-insensitive carboxyl proteinase

The purification is summarized in Table I. The purified enzyme represents about 2900-fold purification over the original culture filtrate, with a 25% recovery (1.6 mg/ml culture filtrate). The purified enzyme displayed a single band when about 30 μg protein were electrophoresed at pH 5.5 (Fig. 3), or at pH 7.0 with SDS after reduction and denaturation.

One of the cause of the increase of total activity after acetone fractionation is the removal of some gummy compounds in the crude enzyme solution. Such gummy compounds disturb the enzyme assay.

3. Physicochemical properties of carboxyl proteinase

a) Extinction coefficient. The ultraviolet absorption spectrum of the enzyme was measured in 0.05 M sodium acetate buffer, pH 4.8. The E°₁₀⁰ at 280 nm was 11.7.

b) Molecular weight. The molecular weight was 40,000 by Andrew’s gel filtration method, and 41,000 by SDS-polyacrylamide gel electrophoresis.

c) Carbohydrate contents. Less than 0.05% carbohydrate was detected as glucose by the phenol–H₂SO₄ method.

d) Amino acid composition. The results of amino acid analysis are shown in Table II, with those for the Pseudomonas sp. carboxyl proteinase. Of particular interest is the absence of methionine residues. The sulfhydryl content was assayed with the Ellman reagent, 5,5'-dithiobis(2-nitrobenzoic acid); less than 0.03 μmol thiol group/molecule was present. The absence of free thiol groups and presence
Table I. Summary of Purification of Xanthomonas Carboxyl Proteinase

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Total Protein ($A_{280} \times 10^{-3}$)</th>
<th>Total Activity (PU $\times 10^{-3}$)</th>
<th>Specific act. (PU/$A_{280}$)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>60,000</td>
<td>986</td>
<td>493</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>10,000</td>
<td>—</td>
<td>298</td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>1,700</td>
<td>—</td>
<td>372</td>
<td>—</td>
<td>76</td>
</tr>
<tr>
<td>DEAE-Cellulose eluate</td>
<td>270</td>
<td>0.134</td>
<td>187</td>
<td>1,400</td>
<td>38</td>
</tr>
<tr>
<td>Sephadex G-75 eluate</td>
<td>110</td>
<td>0.084</td>
<td>124</td>
<td>1,470</td>
<td>25</td>
</tr>
</tbody>
</table>

Fig. 3. Polyacrylamide Gel Electrophoresis of Purified Xanthomonas Carboxyl Proteinase.
Electrophoresis was done at pH 5.5 for 3 hr on a 7.5% polyacrylamide gel column (1 mA/tube). Protein (about 30 μg) was stained with Coomassie brilliant blue.

of 2 mol half-cystine/molecule indicated the presence of one disulfide bridge/mol carboxyl proteinase.

e) Isoelectric point. Isoelectric focusing (on a column containing carrier ampholyte at pH 3.5–5.0) showed that the pl value of the enzyme was 4.0.

4. Enzymatic properties of carboxyl proteinase

a) Effects of pH on activity and stability. The carboxyl proteinase showed similar activities on acid-denatured hemoglobin and on casein, and its optimal pH for both substrates was 2.7 (Fig. 4a). The enzyme was stable at 3.5–5.3 when stored 37°C for 20 hr (Fig. 4b).

b) Effects of temperature on activity and stability. The reaction was done at various temperature at pH 2.7 for 15 min. The temperature optimum was at around 55°C. The
**Fig. 4.** Effects of pH on Activity and Stability of *Xanthomonas sp.* Carboxyl Proteinase.

(a) (b)

**Table III. Effects of Various Proteinase Inhibitor on *Xanthomonas sp.* Carboxyl Proteinase**

*Xanthomonas sp.* carboxyl proteinase (13 nM) was incubated at 37°C for 10 min with various compounds at pH 4.8 (for PCMB and PCMPS; pH 7.5), and then the remaining activities were assayed at pH 2.7 with casein as substrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.91</td>
<td>100</td>
</tr>
<tr>
<td>S-SI</td>
<td>0.08</td>
<td>100</td>
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<tr>
<td>Elastatinal</td>
<td>0.17</td>
<td>100</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.09</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>38</td>
</tr>
<tr>
<td>PCMPS</td>
<td>0.91</td>
<td>100</td>
</tr>
<tr>
<td>E-64</td>
<td>0.24</td>
<td>100</td>
</tr>
<tr>
<td>Thiolstatin</td>
<td>0.52</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.91</td>
<td>100</td>
</tr>
<tr>
<td>Talopeptin</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>MAPI-α</td>
<td>0.09</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>63</td>
</tr>
<tr>
<td>MAPI-β</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>0.09</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>16</td>
</tr>
<tr>
<td>Antipain</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.21</td>
<td>100</td>
</tr>
</tbody>
</table>

b) pH stability. 3.5 ml of carboxyl proteinase were incubated at each pH for 20 hr at 37°C, and the remaining activities were assayed. McIlvaine buffer was used (50 mM Na₂HPO₄/10 mM citrate).

c) Effects of metal ions on activity. The effects of 1 mM solutions of various metal ions (HgCl₂, CuCl₂, ZnCl₂, NiCl₂, MnCl₂, MgCl₂, CaCl₂, and CoCl₂) were tested. None of them inhibited the enzyme.

d) Effects of various proteinase inhibitors on enzyme activity. The enzyme was incubated at 37°C for 10 min with various compounds at pH 4.8 (for PCMB and PCMPS; pH 7.5), and then the remaining activities were assayed at pH 2.7 with casein as substrate. No inhibition was observed with PMSF, EDTA, or PCMPS at 0.91 mM (Table III). PCMB slightly inhibited the enzyme: to inactivate 38% of the total activity, a 70 molar excess of PCMB was required.

Microbial proteinase inhibitors were also tested: S-SI (*Streptomyces subtilisin inhibitor*)²³ and Elastatinal³⁰ [serine proteinase inhibitor], Talopeptin (M-KI)³¹ [metal proteinase inhibitor], Thiolstatin³² and E-64³³ [thiol proteinase inhibitor], MAPI (microbial alkaline proteinase inhibitor),³⁴ Chymostatin,³⁵ Antipain³⁶ and Leupeptin³⁷ [serine-and thiol-proteinase inhibitor]. Among them, Chymostatin and MAPI-α showed inhibition to the enzyme. However, the inhibition was so weak that large amount of inhibitors were required for inactivation.

e) Effects of carboxyl proteinase inhibitor on enzyme activity. As shown in Fig. 5a, the carboxyl proteinase was not inhibited by S-PI²²,³⁸ even at a 400-fold molar excess. In contrast, pepsin was completely inactivated by equimolar S-PI. Chemical modification was done with DAN¹¹ and EPNP.² The results are shown in Fig. 5b and 5c. The carboxyl proteinase was not inactivated by DAN at pH 4.8 even at 55-fold excess of reagent. The same experiments were also done at pH 4.0 or 5.5. No inhibition was observed. The carboxyl
Fig. 5. Inhibition of *Xanthomonas* Carboxyl Proteinase with S-PI (Pepstatin Ac), DAN and EPNP.

a) S-PI. A half ml of carboxyl proteinase dissolved in 50 mM sodium acetate buffer, pH 4.8 (66.6 nM) was mixed with 50 μl S-PI (1 mM, 5 mM or 10 mM in methanol). After incubation at 37°C for 10 min, the remaining activities were assayed at pH 2.7 with casein as substrate.

b) DAN. Three ml of carboxyl proteinase dissolved in 0.1 M sodium acetate buffer, pH 4.8 (22 nM) were mixed with 30 μl CuSO4 (98 μM) and 30 μl DAN (120 μM) [enzyme:Cu2+:DAN=1:45:55]. A control was run without DAN. At the times indicated, samples were taken for testing the remaining activity. The same experiments were also done at pH 4.0 or 5.5. No inhibition was observed.

c) EPNP. A half ml of carboxyl proteinase dissolved in 50 mM sodium acetate buffer (13.3 μM) were incubated in the presence of 5.4 mg EPNP at 25°C with stirring. A control was run without EPNP. At the time indicated, samples were taken for the remaining activities.

O-O, the *Xanthomonas* sp. carboxyl proteinase; ●-●, porcine pepsin.

proteinase was inactivated by EPNP, and 1 mol EPNP/molecule enzyme was incorporated with concomitant loss of its activity.

**DISCUSSION**

We isolated a gram-negative bacterium which produces a carboxyl proteinase in the culture filtrate. Taxonomic characteristics of the bacterium shown in Results agree with those of the genus *Xanthomonas*.39,40) Thus, the bacterium belongs to the genus *Xanthomonas*. Interestingly, the highly purified enzyme was insensitive to Pepstatin,2) S-PI,4) and DAN,1) specific inhibitors of carboxyl proteinases, but it was sensitive to EPNP.2)

As Pepstatin- and DAN-insensitive carboxyl proteinases, carboxyl proteinases A-1, A-2, B and C of *Scytalidium lignicolum*,7,8,10,11) carboxyl proteinase A of *Aspergillus niger* var. *macrosporus*,41) and carboxyl proteinases of *Lentinus edodes*,19) *Ganoderma lucidum*20) and *Irpex lacteus*22) (Basidiomycetes) have been reported and characterized. In addition to these enzymes, we isolated a Pepstatin- and DAN-insensitive carboxyl proteinase produced by a *Pseudomonas* sp. last year.21)

The properties of the carboxyl proteinase from *Xanthomonas* sp. were compared with those of other Pepstatin- and DAN-insensitive enzymes (Table IV).

The *Xanthomonas* carboxyl proteinase resembles *Pseudomonas* carboxyl proteinase21) and *S. lignicolum* carboxyl proteinase B7,10,13) in its sensitivity to inhibitors, but, some differences were observed in other properties. The *Xanthomonas* carboxyl proteinase differs from *Pseudomonas* carboxyl proteinase in optimum pH, isoelectric point, and amino acid composition (Table II, especially in Asp, Thr, Glu, Ala, Leu, and Tyr contents). It differs from *S. lignicolum* carboxyl proteinase B in optimum pH, molecular weight, isoelectric point, etc.

Thus, the carboxyl proteinase of *Xanthomonas* sp. in this work differs substantially from the Pepstatin- and DAN-insensitive carboxyl proteinases reported so far.

Microbial carboxyl proteinases have been found mostly in fungi (yeast); none in bacteria33) until we reported one in *Pseudomonas* sp.21) Therefore, the finding of another Pepstatin- and DAN-insensitive carboxyl proteinase from *Xanthomonas* sp. is of great value for further studies on carboxyl proteinases.

Some structural studies are now under way to find whether the catalytic residues of the enzyme are glutamic acid as are those of *S. lignicolum* carboxyl proteinase B.17)

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**REFERENCES**

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### Table IV. Some Properties of S-PI (Pepstatin Ac)-insensitive Carboxyl Proteinases

<table>
<thead>
<tr>
<th>Properties</th>
<th>Xanthomonas No. T-22</th>
<th>Pseudomonas No. 101&lt;sup&gt;21&lt;/sup&gt;</th>
<th>S. lignicolum</th>
<th>Lentinus edodes&lt;sup&gt;19&lt;/sup&gt;</th>
<th>Ganoderma lucidum&lt;sup&gt;20&lt;/sup&gt;</th>
<th>Aspergillus niger A&lt;sup&gt;41&lt;/sup&gt;</th>
<th>Irpex lacteus&lt;sup&gt;42&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>2.7</td>
<td>3.0</td>
<td>3.0 ~ 3.3</td>
<td>2.2</td>
<td>2.0</td>
<td>2.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>41,000</td>
<td>43,000</td>
<td>40,000</td>
<td>22,000</td>
<td>406,000</td>
<td>40,000</td>
<td>36,000</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.0</td>
<td>3.2</td>
<td>3.6 (3.8)</td>
<td>3.2</td>
<td>2.4</td>
<td>4.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Amino acid composition</td>
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<td>yes</td>
<td>yes</td>
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<td>yes</td>
<td>yes</td>
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<tr>
<td>Met</td>
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<td>none</td>
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<td>His</td>
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<td>10</td>
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<tr>
<td>Carbohydrate content</td>
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<td>10</td>
<td>0</td>
<td>30</td>
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<td>0</td>
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<tr>
<td>Inhibition with</td>
<td>Pepstatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-PI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>DAN</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>EPNP</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<sup>19</sup> = not determined

<sup>41</sup> = not determined

<sup>42</sup> = not determined

<sup>21</sup> = not determined