Cloning and Expression of the Alkaline Proteinase Gene from *Pseudomonas aeruginosa* IFO 3455

YUSAKU ATSUMI,1 SHUNJI YAMAMOTO,2 KAZUYUKI MORIHARA,1,2 JUN FUKUSHIMA,1 HIROAKI TAKEUCHI,1 NOBUYUKI MIZUKI,1 SUSUMU KAWAMOTO,1 AND KENJI OKUDA1*

Department of Bacteriology, Yokohama City University School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 226,1 and Kyoto Research Laboratories, Toho Pharmaceutical Industry Co., 7-4, Chikatake, Shohryoji, Nagaokakyo-shi, Kyoto 617,2 Japan

Received 12 October 1988/Accepted 22 May 1989

The alkaline proteinase gene from *Pseudomonas aeruginosa* IFO 3455 was cloned and expressed in *Escherichia coli*.

The roles of several extracellular products have been established or implicated in the pathogenicity of *Pseudomonas aeruginosa*. These include proteases, phospholipase, hemolysin, exotoxin A, and exoenzyme S (6, 8). Protease pathogenicity is explained by an aggressin activity (7), whereby the virulence of non-protease-producing strains is increased by the administration of a minute amount of protease to an experimental animal infected with *P. aeruginosa*. *P. aeruginosa* can produce two or three proteases (5), one of which is alkaline proteinase. This enzyme was crystallized from fermentation broth of strain PM and from that of IFO 3080. Alkaline proteinase can be regarded as a metalloproteinase, since it is inactivated by the addition of o-phenanthroline (7, 8). In the present study we were able to clone the alkaline proteinase gene; the characterization of the cloned gene, as well as its restriction map, is also given.

*P. aeruginosa* IFO 3455 and IFO 3080 were obtained from the Institute for Fermentation, Osaka, Japan. Crystal alkaline proteinase of *P. aeruginosa* IFO 3080 was obtained from Nagase Biochemical Co., Kyoto, Japan. Anti-alkaline proteinase antibody was produced in rabbits by injecting 0.4 mg of alkaline proteinase, which had been 80% inactivated by formalin, per animal. The anti-alkaline proteinase antibody was detectable at 32°C by Ouchterlony assay.

As a first step, we constructed the SphI-digested genomic libraries from *P. aeruginosa* IFO 3455. The libraries were screened with rabbit anti-alkaline proteinase antibody and then treated with peroxidase-labeled anti-rabbit immunoglobulin antibody. By using this procedure, several clones were obtained out of about 6,000 possible clones. However, when the antibody assays were redone, only one clone ([Escherichia coli HB101(pAP101)] reacted repeatedly. This pAP101 plasmid contained a fragment of *P. aeruginosa* DNA of approximately 5.3 kilobases (kb). From this clone, we constructed the subclone pAPDS2 by unidirectional digestion with exonuclease III (2, 3, 9); this subclone possessed both the antigenicity and enzyme activity of alkaline proteinase. Figure 1 shows the restriction enzyme map of the cloned gene.

The alkaline proteinase activity of each clone was studied. *E. coli* HB101(pAP101), *E. coli* HB101(pAPDS2), *E. coli* HB101(pUC18), and parental *E. coli* HB101 were cultured for 24 h at 37°C in L broth. The bacteria were washed, and these suspensions were sonicated and then ultracentrifuged at 42,000 × g for 10 min. The supernatant fractions were assayed to determine enzyme activity as the soluble fraction (Table 1). The precipitants were solubilized with 8 M urea. Solubilized samples of 42,000 × g precipitants were again ultracentrifuged and dialyzed against 10 mM Tris buffer (pH 8.0). Only these supernatants were used as the bacterial precipitate fractions. Alkaline proteinase activity was determined by a modification of the method of Long et al. (4). Briefly, 1 ml of each sample and 2 ml of 1% azocasein (Sigma Chemical Co.) were mixed and incubated at 37°C. When the inhibition test was performed, 5 μl of antiserum was added to this system. After 10 min, 1 ml of 10% trichloroacetic acid was added. After samples were passed through a Teflon paper filter, A440 was measured to estimate proteinase activity.

High alkaline proteinase activity was observed for both pAP101 (5.3-kb fragment) and pAPDS2 (2.3-kb fragment) clones (Table 1). In addition, this alkaline proteinase activity was inhibited by anti-alkaline proteinase antibody (99% inhibition of alkaline proteinase activity).

To confirm the clone, an Ouchterlony assay was done. The sonicated bacterial samples were solubilized with 10% sodium dodecyl sulfate in 0.15 M phosphate-buffered saline. Then, a solubilized bacterial sample or an equivalent volume of the purified alkaline proteinase was reacted with anti-alkaline proteinase antibody. A 10% sodium dodecyl sulfate-solubilized bacterial sample (Fig. 2, well C) or chemically purified alkaline proteinase (well B) was allowed to react with rabbit anti-alkaline proteinase antibody (well Ab). Interestingly, these two precipitin lines fused completely, which showed that the *E. coli* antigen is not missing any antigenic sites present on the *P. aeruginosa* antigen. In addition, this antibody did not react with any solubilized component of control *E. coli* HB101(pUC18) (Fig. 2).

To further verify the molecular size of this gene product,
TABLE 1. Proteinase activities of sonicated or solubilized bacterial fractions

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Proteinase activitya of:</th>
<th>Supernatant</th>
<th>Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli(pAP101)</td>
<td></td>
<td>0.085</td>
<td>1.422</td>
</tr>
<tr>
<td>E. coli(pAPDS2)</td>
<td></td>
<td>0.070</td>
<td>1.344</td>
</tr>
<tr>
<td>E. coli(pUC18)</td>
<td></td>
<td>0.046</td>
<td>0.050</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>NT</td>
<td>0.043</td>
</tr>
</tbody>
</table>

a The activities of azocasein cleavage by alkaline proteinase were measured by A440 (4). NT, Not tested.

10% sodium dodecyl sulfate-solubilized bacterial samples or control samples were electrophoresed and stained with Coomassie blue (Fig. 3A) (9). Chemically purified alkaline proteinase showed a band at about 49.5 kilodaltons (kDa) (Fig. 3A, lane 1). Some proteins produced bands at around the same position (Fig. 3A, lanes 2 and 3). However, we were not able to discern the alkaline proteinase band as a separate entity (Fig. 3A). In addition, Western blotting (immunoblotting) was done by previously described methods (1, 9). Electrophoresed and blot-transferred samples were treated with rabbit anti-alkaline proteinase antibody. Only antibody that reacted with alkaline proteinase was detected with peroxidase-labeled anti-rabbit immunoglobulin antibody. Control alkaline proteinase showed a major band of about 49.5 kDa and a minor one of about 45 kDa (Fig. 3B, lane 1). We were able to observe a slightly larger band of alkaline proteinase only in E. coli(pAP101) (Fig. 3B, lane 3).

In the present study, we cloned the structural gene of alkaline proteinase from P. aeruginosa. Alkaline proteinase activities of both pAP101 (5.3-kb fragment) and pAPDS2 (2.3-kb fragment) clones were at almost the same level, which suggests that the complete structural gene may reside in the 2.3-kb DNA fragment. We were not able to obtain a clone capable of secreting alkaline proteinase. The gene for secretion might be lacking in these DNA fragments, or this might reflect a difference between P. aeruginosa and E. coli.

Western blotting produced two bands of purified alkaline proteinase (Fig. 3B). A weak but significant line was observable (Fig. 3B, lane 1). The antibody used in the present study may be directed mainly to the low-molecular-size protein fragment of purified proteinase or to its minor component. The molecular size of the product of pAP101 (about 51 kDa [Fig. 3B, lane 3]) is somewhat larger than purified alkaline proteinase (about 49.5 kDa). This product might be a fused protein with β-galactosidase or a precursor protein of mature proteinase. In another case, the chemically purified alkaline proteinase might be cleaved into two smaller fragments. The identity of purified alkaline proteinase and the cloned gene product was also observed by Ouchterlony analysis (Fig. 2). The immunodiffusion results strongly suggest that the antigen-antibody components of the two samples are identical.

We thank K. Chiba for secretarial assistance.

LITERATURE CITED


