Expression and characterization of recombinant alkaline protease from *Aspergillus sojae* in yeast *Pichia pastoris*

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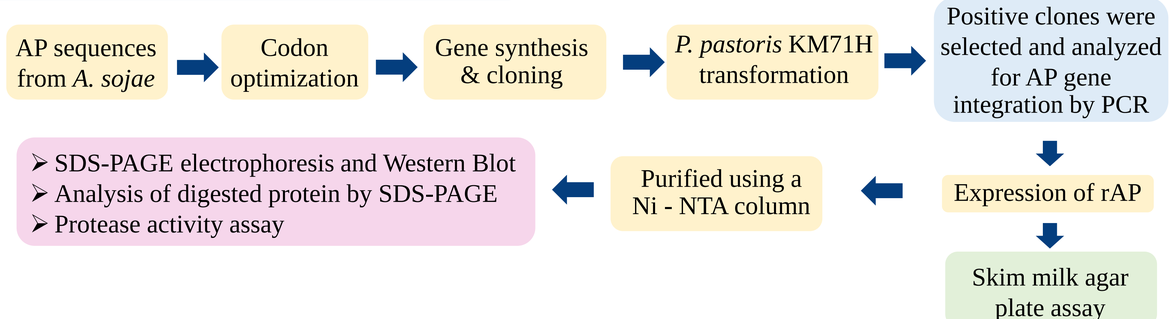
ABSTRACT

Alkaline protease (AP) is a group of enzymes that breaks peptide bonds within polypeptide chain under alkaline conditions. It has been extensively used in many industries in particularly food and detergent industries. In this study, we produced a recombinant alkaline protease (rAP) via cloning *AP* gene from an alkalophilic fungus *Aspergillus sojae* into pPIC9K expression vector and introduced into the yeast *Pichia pastoris* KM71. The rAP was successfully expressed and secreted to culture medium. The resultant rAP was composed of 389 amino acids with a molecular weight of approximately 35 kDa. Two positive yeast clones, rAP-3 and rAP-4, that were highly resistant to Geneticin antibiotic were selected and further analyzed. The crude protein yields were 370 and 344.6 mg/L in clone 3 and 4 respectively. Protease screening assay of rAP from both clones, via skim milk agar plate, showed positive results. Folin-phenol method using casein as a substrate revealed that the enzyme activity of rAP-3 and rAP-4 were 2.966 and 3.923 U respectively, these were higher the commercial alkaline proteases from *Bacillus subtilis* whose enzyme activity was 2.026 U. SDS-PAGE analysis showed that rAP-3 and rAP-4 completely digested protein casein and protein from cell line lysate. This result suggests that rAP might have broad range of substrate specificity. In summary, we successfully expressed *AP* gene from *A. sojae* in *P. pastoris* in the form of secreted enzyme in high quantity and with satisfactory activity. This established platform could be used in various applications in the future.

STUDY DESIGN AND METHODS



RESULTS

Cloning of the AP gene from A. sojae into pPIC9K vector

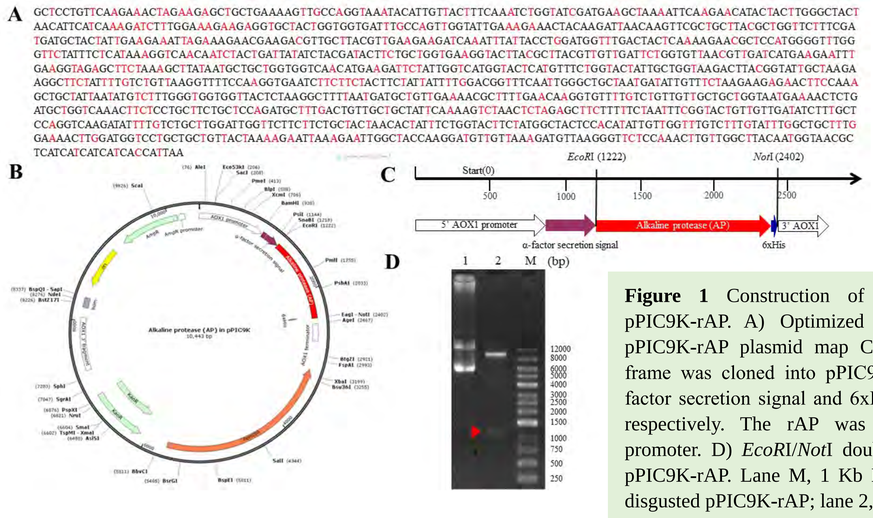


Figure 1 Construction of the expression vector pPIC9K-rAP. A) Optimized AP gene sequence B) pPIC9K-rAP plasmid map C) The AP open reading frame was cloned into pPIC9K vector flanked by α-factor secretion signal and 6xHis tag, at 5′ and 3′ end, respectively. The rAP was downstream of AOX1 promoter. D) *EcoRI*/*NotI* double enzyme digestion of pPIC9K-rAP. Lane M, 1 Kb DNA ladder; lane 1, un-disgusted pPIC9K-rAP; lane 2, digested pPIC9K-rAP.

The optimized sequence of AP gene from A. sojae **(Fig. 1A)** was successfully synthesized and cloned into the vector pPIC9K, after α-factor sequence, at EcoRI and NotI sites and designated as pPIC9K-rAP **(figure 1B and 1C)**. To confirm the present of AP gene insert, the purified pPIC9K-rAP was digested with EcoRI and NotI. The agarose gel electrophoresis revealed two bands at approximately 9,000 and 1,100 bp which were close to the actual size of empty plasmid (9,256 bp) and AP gene (1,187 bp) respectively **(Fig. 1D)**. The pPIC9K-rAP was further confirmed by DNA sequencing (data not shown).

Yeast transformation and screening for positive transformants

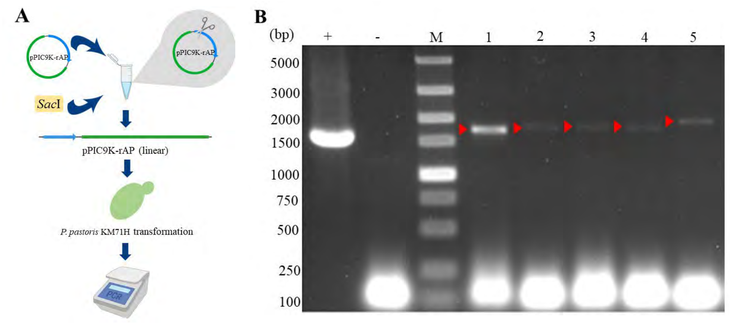


Figure 2 Identification of positive transformant yeast clones by PCR. A) An illustration for yeast transformation and screening via PCR analysis. B) Results of PCR analysis. Lane M, DNA ladder; lane +, Positive control; lane -, Negative control; lane 1-5, PCR results of transformed yeast clone 1-5.

The pPIC9K-rAP was linearized via *Sac*I, and then transformed into *P. pastoris* KM71H. The G418-resistant clones were selected for confirmation of rAP gene integration via PCR **(Fig. 2A)**. The results showed that all of the 5 G418-resistant clones were positive as observed by the presence of PCR product at approximately 1,500 bp indicating that the AP gene was successfully integrated into the *P. pastoris* genome **(Fig. 2B)**.

ACKNOWLEDGEMMENTS

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Expression of rAP

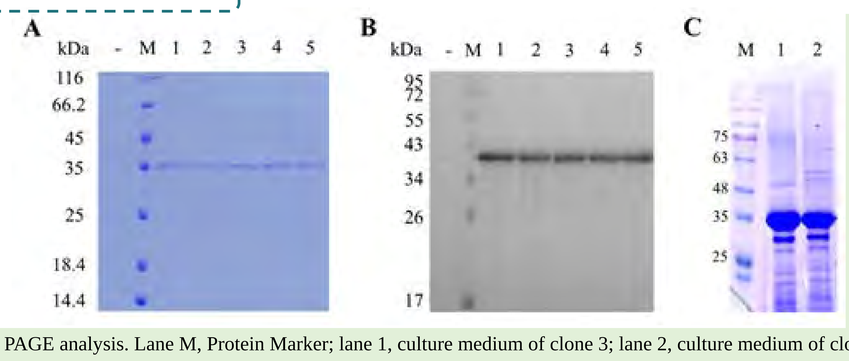

Figure 3 SDS-PAGE and Western Blot analysis of rAP. A) rAP protein samples were analyzed by 10% SDS-PAGE and B) separated proteins were further analyzed by Western Blot. Lane M, Protein Marker; lane -, Un-induced sample; lane 1-5, Induced samples. C) SDS-PAGE analysis of TCA-precipitated protein samples. 200 µL of cell-free supernatant was precipitated using TCA and was subjected to SDS-PAGE analysis. Lane M, Protein Marker; lane 1, culture medium of clone 3; lane 2, culture medium of clone 4.

The PCR positive *P. pastoris* transformants were cultured and induced via methanol every 24 h. After 7 days of induction, the cell-free supernatant was harvested via centrifugation. The rAP was precipitated via salting out method using ammonium sulfate and removed the remaining salt via dialysis. The crude rAP was then purified via Nickel-affinity chromatography column. Analysis of rAP protein by SDS-PAGE (**Fig. 3A**) and Western Blot (**Fig. 3B**) revealed a single protein band at approximately 35 kDa which was similar to the calculated molecular weight of rAP. Similar results were observed in all 5 clones, no protein band was observed in un-induced sample. Clone 3 and 4 had a higher growth rate in G418 antibiotic-containing growth medium when compared with clone 1, 2 and 5 (data not show) suggesting higher gene copy number. Therefore, clone 3 and 4 were selected for enzyme production and further analyses. rAP was highly expressed by clone 3 and 4 with the crude protein yield of 370.0 and 344.6 mg/L respectively. SDS-PAGE analysis of TCA-precipitated protein samples were shown in **Fig. 3C.**

Determination of rAP activity

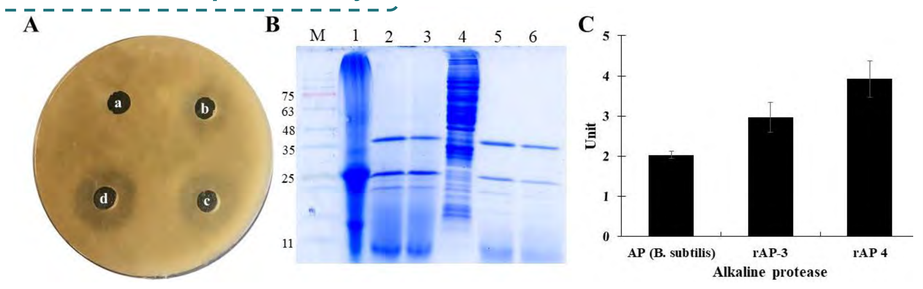

Figure 4 Alkaline protease activity assays. A) Protease activity of rAP was assessed by skim milk agar plate. (a) cell-free supernatant from non-transformed yeast cell culture (negative control), (b) purified rAP 10 µg, (c) cell-free supernatant from *P. pastoris* KM71H pPIC9K-rAP clone 3 and (d) clone 4 culture. B) non-digested and digested proteins analyzed by SDS–PAGE, lane M, protein marker; lane 1, non-digested casein; lane 2, casein digested with rAP-3; lane 3, casein digested with rAP-4; lane 4, non-digested cell line lysate; lane 5, cell line lysate digested with rAP-3; lane 6, cell line lysate digested with rAP-4. C) Alkaline protease assay via Folin-phenol method using casein as substrate.

In order to screen for protease activity, skim milk agar plate assay was performed. As shown in **Fig. 4A**, cell-free supernatant from *P. pastoris* KM71H pPIC9K-rAP clone 3 and clone 4 showed clear zone, indicating positive for protease activity. Similar result was observed with purified rAP. On the other hand, the cultured medium of non-transformed *P. pastoris* did not show a clear zone, indicating the absence of protease activity. SDS-PAGE results showed that, after 10 min of incubation, rAP completely digested both casein and human cell line lysate as compared with non-digested control **(Fig. 4B)**. Alkaline protease activity assay by Folin-phenol method, using casein as a substrate, showed that the enzyme activity of rAP-3 and rAP-4 was 2.966 and 3.923 U, respectively. The activity of rAP from both clones was higher than that of the commercial alkaline proteases from B. subtilis, which was 2.026 U **(Fig. 4C)**.

DISCUSSION AND CONCLUSION

Aspergillus sojae is known to produce wide variety of proteases including extracellular AP, it has been widely used in food industry for decades. In this study, we utilized DNA technology in order to express and produce rAP in *P. pastoris* KM71H. The resultant protein was approximately 35 kDa, which is similar to a report from previous study [1]. Skim milk agar plate assay was performed for screening of protease activity. Our results showed that rAP clone 3 and clone 4 were positive for protease activity as shown by the presence of clear zones. It seems that rAP-4 may have stronger protease activity than rAP-3 as shown by wider clear zone. The rAP was tested for the digestion of casein and total protein from human cell line, we found that rAP-3 and rAP-4 were able to digest both protein samples, suggesting a broad substrate specificity. This is consistent with the previous study demonstrating that alkaline proteases from *Aspergillus spp*. have a wide range of substrates such as soy isolate protein, BSA and glutin [1, 2].

To measure activity of rAP, we utilized Folin-phenol method using casein as a substrate. Although this method is a common test for many proteases, however, we performed the test at 40 °C and the pH 10.0 which is optimal condition for alkaline proteases. This incubation condition was reported previously with AP from *B. subtilis* [3] and rAP from *Aspergillus oryzae* [4]. In the present study, we successfully produced rAP in *P. pastoris* with satisfactory quantity and activity. Our recombinant yeast clones can be used in various applications and subjected to production on a larger scale in the future.

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