Identification of transcription factors and their DNA binding sequences controlling acid protease production

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Research aims

Aspergillus oryzae is known as a fungus that produces many types of proteases in large quantities. However, the mechanism by which the fungi controls production of proteolytic enzymes remains unclear. The protease additives prepared from culture broths of *A. oryzae* contain many types of proteases, so it is difficult to make them specific for digestion of the proteins used in industry. We hypothesized that the reason for the difficulty in control of protease production was related to nitrogen metabolism in the fungi. Subsequently, we found that the nitrogen source in the culture broth affects the production of some proteases [1)]. In this study, we analyzed the transcriptional control of the acid protease gene in *A. oryzae.*

Methods

1) Exploration of the cis-element of the *A. oryzae pepO* gene and identification of the corresponding transcriptional factor.

   PepO is one of the major proteases in *A. oryzae*. The DNA sequences 2,024, 1473, 1400, 1300, 1200, 1100, 1000, and 500 bp upstream of *pepO* were ligated to the *amyB* gene and introduced into the *A. oryzae* triple amylase gene deletion mutant strain, Δ*amyABC*. The transformants were cultured and the amylase activities in the culture broth were assayed. It was observed that the transcription factor bound to the region -1,200 to -1,100 upstream of the *pepO* gene. Furthermore, an electrophoretic mobility shift assay (EMSA) was performed to determine the DNA binding sequence of the putative transcription factor; we identified a 20-bp sequence in the region that was correlated with binding.

2) Analysis of the *A. oryzae prtR* deletion mutant, Δ*prtR*.

   PrtT is found in *A. niger* and *A. fumigatus* and is known to comprehensively control the production of proteases. We identified the homologous gene, *prtR*, in *A. oryzae* and constructed the *A. oryzae* Δ*prtR* strain by displacing *prtR* with *ptrA* using the *A. oryzae* NS4 strain as a host. The deletion strain was cultivated in chemically defined medium containing casein or Glu and Met and then the acid protease activity was assayed with casein as a substrate. Additionally, the transcription level of *pepO* was estimated by semi-quantitative RT-PCR.

3) Control of acid protease expression with amino acids as a nitrogen source.

From the results above [2)] and our previous study, it was hypothesized that some amino acids in the medium could change the production of acid protease. *A. oryzae* was cultivated with sodium nitrate as the nitrogen source, and then the mycelia were transferred new medium containing each amino acid as a solo nitrogen source. After four hours, the transcription of acid protease genes was estimated by qPCR.

Results

1) As the result of a promoter assay using α-amylase, the transcription factor binding site was identified in the DNA region -1,200 to -1,100 bp upstream of the *pepO* gene. Then, the binding region was narrowed to 22 bp with EMSA. Two transcription factors were presumed to be candidates by their DNA binding motif. However, as the DNA-protein interaction was not inhibited by a 3-bp substitution in their motifs, we hypothesized that there might be unknown DNA binding proteins, other than the two prospective transcription factors.
2) The acid protease activity of the Δ*prtR* strain in the 36-h culture broth was drastically lower than that of the control strain, and the transcriptional analysis showed *pepO* was scarcely transcribed in the Δ*prtR* strain. The 48-h culture broth showed higher acid protease activity than that of the 36-h broth, but transcription of *pepO* was again barely observed. The change in acid protease activity was particular when cultivating in culture broth containing Glu. We hypothesized that the transcription of *pepO* was activated by prtR and that any acid protease other than PepO was expressed at 48 h instead of PepO. Furthermore, it was shown that Glu up-regulated the acid protease coming up at 48 h. However, since Met and Glu or sodium nitrite were necessary to cultivate the *prtR* deletion mutant, it would be difficult to clarify the reason for the change in activity, whether it was due to the addition of amino acid or the deletion of the gene. So, we constructed new prtR deletion mutants from *A. oryzae* RIB40, Δ*ligD* and Δ*pyrG*. We are currently analyzing these mutants.
3) It was shown that the translation of *pepO* was activated by Gln, Phe, Val, Leu, Ile, Met, His, and Lys, and that Glu, Asp and Arg suppressed the translation of *pepO*.

Conclusion

It was shown that the cis-element of the *pepO* gene, which consisted of less than 22 bp, was around 1,000 bp upstream of the gene. There were two known motif sequences that were recognized by transcription factors, but it was presumed that an unknown protein might bind to the region, so we have been searching for that protein. It was observed that the comprehensive transcriptional factor, *prtR*, in *A. oryzae* also activated the transcription of

pepO. We are analyzing the new Δ*prtR* strain in detail. Furthermore, it was shown that some amino acids up-regulated the transcription of *pepO*, while others suppressed its transcription. It is hypothesized that these results are related to each other, so we are trying to determine the mechanism of the control of acid protease production, including PepO.

References

1) Maeda H, Sakai D, Kobayashi T, Morita H, Okamoto A, Takeuchi M, Kusumoto K-I, Amano H, Ishida H, Yamagata Y (2016) Three extracellular dipeptidyl peptidases found in *Aspergillus oryzae* show varying substrate specificities. *Appl. Microbiol. Biotechnol.*, **100**: 4947-4958.