**[Noda Institute for Scientific Research GRANT]**
**2006 Young Investigator Research Grant**
**Outline of Research Result**

# Application of Salt-tolerant Glutaminase from *Aspergillus oryzae* to Soy Sauce Fermentation

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## Research aims

Glutaminase is one of the most important enzymes in soy sauce fermentation, since it hydrolyzes L-glutamine to produce L-glutamic acid, which is a highly savory amino acid.[1] However, glutaminases of *A. oryzae* that have been reported, are markedly inhibited by the high salt concentrations present in the soy sauce fermentation process.[2] A salt-tolerant glutaminase was shown to exhibit high ability in the production of L-glutamic acid in a model reaction of soy sauce fermentation.[3] Thus, salt-tolerant glutaminase is suitable for application in soy sauce fermentation. We have found a novel gene from *A. oryzae* RIB40 encoding a glutaminase (AoGls) that shows a higher salt-tolerance than the other known glutaminases from *A. oryzae*.[4] In this research, effects of growth conditions on expression of *A. oryzae* glutaminases, including AoGls, were investigated for the final goal of applying the glutaminases to soy sauce fermentation.

## Methods

*A. oryzae* RIB40 was obtained from National Research Institute of Brewing. The strain was cultured at 30°C in a wheat bran medium (wheat bran moistened with the same weight of potassium phosphate buffer (pH 7.2)), a malt extract medium (1% malt extract and 0.1% yeast extract), or LB medium (1% peptone, 0.5% yeast extract, and 1% sodium chloride). Hydrochloric acid or sodium hydroxide was used for pH adjustment for the media.

The wheat bran culture was extracted with twice its weight of 10 mM tris(hydroxymethyl)aminomethane (Tris, pH 9.0) for over night at 4°C and then filtered through gauze. The filtrate was centrifuged and used as the extracellular glutaminase. The mycelia were harvested from the malt extract or LB agar medium that was covered with cellophane to separate them from the debris. The mycelia were frozen by liquid nitrogen and disrupted with the same weight of sea sand and 10 mM Tris (pH 9.0) in a mill, followed by centrifugation. The supernatant was used as the intracellular glutaminase.

Glutaminase activity was assayed by determining the formation of L-glutamic acid with L-glutamate dehydrogenase as previously described.[5] The reaction mixture contained 100 mM Tris (pH7.5) and 30 mM L-glutamine. The reaction was carried out at 30°C for 10 min and stopped by boiling for 3 min followed by centrifugation. The supernatant was added to the reaction mixture for L-glutamic acid determination. One unit of glutaminase was defined as the amount of enzyme which catalyze the formation of 1 mol of L-glutamic acid per minute. The protein concentration was determined by BCA protein assay kit (PIERCE).

An expression of AoGls was determined by immunoblot analysis. Anti AoGls antibodies were prepared by immunization of a rabbit with purified histidine tagged AoGls that was overproduced in *E. coli.* Protein samples were subjected to SDS-PAGE and the proteins in the gel were transferred onto PVDF membrane. The anti-AoGls antibodies were used as a primary antibody and a horseradish peroxidase-conjugated anti-rabbit IgG (Immunstar GAR, Biorad) as a secondary antibody, and an Immunstar GAR-HRP detection kit (Biorad) and a Hyperfilm ECL (GE Healthcare Bioscience) were used for the antibody detection.

## Results

1) Extracellular glutaminase

The extracellular enzyme fraction prepared from the wheat bran culture showed a little activity (5 mU/mg). This activity decreased to 76% after an incubation at 4°C for 24 h even at pH 9.0, at which extracellular enzymes produced by *A. oryzae* were found to be more stable than at pH 5.0 or pH 7.0. However, partially purified glutaminase by Q-Sepharose and Butyl-Toyopearl was stable for 2 weeks at 4°C. These results suggest that the extracellular glutaminases tend to be digested by protease from *A. oryzae* RIB40. No glutaminase activity was detected in the extracellular enzyme fractions prepared from *A. oryzae* RIB40 cultured in the malt extract nor LB liquid medium at pH 5, 7, or 9. An addition of 50 mM L-glutamine to those liquid media did not increase the glutaminase activity of the extracellular enzyme fractions. Instability of the extracelluar glutaminases may be one of the reasons why no glutaminase activity was detected in the extracellular enzyme fractions from the liquid cultures.

2) Intracellular glutaminase

The intracellular enzyme fraction showed slightly higher glutaminase activity than the extracellular enzyme fraction did. The intracellular enzyme fraction from LB medium culture showed higher glutaminase activity than that from the malt extract agar culture (less than 1 mU/mg). The high-

est glutaminase activity (16 mU/mg) was obtained from LB agar culture (pH 9.0). AoGls was not detected from 30 g of the intracellular enzymes from LB agar culture (pH 9.0) by immunoblot analyses with the anti-AoGls antibody. Since the immunoblot analysis could detected 10 ng of AoGls and its specific activity is 730 U/mg,[6] the major glutaminase responsible for the intracellular activity may not be AoGls. For the application of the salt-tolerant glutaminase AoGls to soy sauce fermentation, further investigation is necessary to increase AoGls production, though there is a possibility that the anti-AoGls antibody used in our experiments is unable to react with AoGls from *A. oryzae* RIB40.

## Conclusion

The culture conditions for *A. oryzae* RIB40 to show an extracellular or an intracellular glutaminase activity were investigated. The culture conditions for production of the salt-tolerant glutaminase AoGls were also examined by immunoblot analysis. The extracellular glutaminase activity was obtained from the wheat bran culture, though the activity was not obtained from the malt extract or LB liquid culture. The highest activity (16 mU/mg) was obtained from the intracellular enzyme fraction prepared from LB agar culture (pH 9.0). AoGls was not detected from *A. oryzae* cells by immunoblot analysis under the conditions used. Further investigation is necessary to increase the production of AoGls.

## References


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