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MICROBIAL ENZYMES FOR THE PRODUCTION OF GLUCOSAMINE AND N-ACETYLGLUCOSAMINE FROM CHITINOUS BIOMASS

Yeon Jin Choi and Yong Chul Shin
Division of Life Sciences and Genetic Engineering Institute, Gyeongsang National University, Chinju 660-701, Korea. E-mail: ycshin@nongae.gsnu.ac.kr

1. Research Background

Chitin, a major component of shells of crab, shrimp, krill, and other crustacean animals, is an important biomass used for versatile applications in food and pharmaceutical industries. Among them, D-glucosamine (DGA) is one of the most promising substances produced from chitinous materials. Nowadays, it is used worldwide as a medicine and food supplement for curing the osteoarthritis. In addtion, DGA has anti-inflammatory, cardio-protective, and liver-protective activities in animals (1, 2, 3).

DGA is commercially produced from chitin by acid hydrolysis with 12N HCl at 80℃. This severe conditions prompt the breakdown of the glycosidic linkages and simultaneous deacetylation of N-acetylglucosamine (NAG) residue of chitin into DGA. This process yields about 5% of impurities by weight basis, which requires complicated purification steps for DGA production. Generally, DGA with 99% purity can be obtained by repeated recrystallization of acid hydrolysate. The final yield of DGA is rather low by about 40~60% (4). D-glucosamine · HCl or D-glucosamine · sulfate form is sold as a medicine or health-care aid. The price of purified DGA · HCl is about 4,500¥/kg in Japan.

In human body, DGA is converted into glucosamine-6-phosphate by glucosamine synthase, which is subsequently converted into NAG through some biosynthetic steps. When NAG and DGA are supplemented in a diet, incorporation of NAG into mucosal cells is much faster than DGA. NAG constitutes an major sugar component of glycoproteins or mucopolysaccharides that are found in tendon, cartilage, gastrointestinal mucosal layer, and other cementing materials between cells. Especially, it has been known that NAG has curing activity for ulcerative gasterointestinal tract (5). These results indicate that NAG has higher value than DGA in its biological activity in human body. Today, NAG is commercially produced by acid hydrolysis of chitin with 6~12N HCl at 60~80℃ (Fig. 1). However, acid hydrolysis of chitin produces both NAG, a monomeric unit of chitin and DGA, a deacetylated product of NAG, which needs purification of NAG from DGA and acetylation of remaining DGA with acetic

anhydride.

Conclusively, conventional processes using high concentration of HCl at high temperature have several disadvantages for the production of DGA and NAG, such as handling risks using concentrated HCl, erosion of equipments, impurities production, requirements of sophisticated purification steps, high energy consumption, and low recovery yield. Enzymatic process would be an alternative method to overcome these problems (Fig. 2). For a past decade, we have investigated some kinds of enzymes for the production of DGA and NAG from chitosan and chitin. Here, we presented some properties of the enzymes and possible enzymatic processes for DGA and NAG production were discussed.

2. Exo-chitosanase and Enzymatic Production of DGA

A fungal strain GM31 was isolated from a soil as an exo-chitosanase producer. The strain GM31 was identified as a fungal species which has been used for producing food-grade enzymes. This fungus secreted exo-chitosanase (GM31-ECS) into culture medium only in the presence of chitosan. At fifth day of cultivation, it produced 10~20U/ml of GM31-ECS. GM31-ECS was purified to a homogeneity from the culture supernatant. The molecular weight of GM31-ECS was estimated as about 110kDa by SDS-PAGE. Its optimum temperature and pH were 50℃ and pH 4~5, respectively. It was stable for 4 days at 40℃. The stable pH range of the enzyme was 3.0 to 7.0. It produced DGA as a sole end-product from chitosan and chitosan oligosaccharides (Fig. 3). About 70~80% of DGA by weight basis was obtained from chitosan by an enzymatic process: 40℃, pH 5.0, 7U GM31-ECS/g chitosan, 2 days. Two-steps process using bacterial endo-chitosanase (first step) and GM31-ECS (second step) was much more efficient for DGA production than single step process using GM31-ECS.

3. N-Acetyl-β-D-Glucosaminidase (NagA) and Enzymatic Production of NAG

In order to produce NAG from chitin, combined action of endo-chitinase and exo-chitinase (N-Acetyl-β-D-Glucosaminidase) was prerequisite for a complete and efficient hydrolysis. Previously, we isolated *Serratia liquefaciens* GM1403 and *Cellulomonas* sp. GM13 as strong endo-chitinase producers. Endo-chitinases of strains GM1403 and GM13 were purified and characterized. (6, 7, 8, 9). These endo-chitinases predominantly released dimeric unit [$(NAG)_2$] of N-acetylglucosamine from chitin (10). Even though culture supernatant of strain GM1403 had N-Acetyl-β-D-Glucosaminidase (NagA) activity, NagA activity was too low to produce NAG from chitin. For the efficient production of NAG from

chitin, supplementation of NagA to chitinolytic enzyme mixture was necessary. During the gene cloning of endo-chitinase from strain GM13, unexpectedly, a gene encoding NagA was cloned. NagA turned out to be an intracellular enzyme of strain GM13. Its molecular weight was 54kDa on SDS-PAGE gel. Its optimum temperature and pH were 55℃ and 6～7, respectively. NagA showed the highest activity toward $(NAG)_2$ and also showed a high activities for chitin oligosaccharides, about 70～95% based on $(NAG)_2$ activity. It hydrolyzed polymeric chitin but did not hydrolyze chitosan and chitosan oligosaccharides at all. By the combined action of endo-chitinase and NagA, NAG was produced from swollen chitin with a yield of about 80%.

4. Conclusions and Perspectives

In this study, some kinds of chitinolytic and chitosanolytic enzymes were characterized and enzymatic processes for the production of DGA and NAG from chitinous materials were investigated for the substitution of conventional acid hydrolysis process.

Exo-chitosanase of a fungal strain GM31 produced DGA as a sole end-product from chitosan by an exo-cleavage mode of action. Supplementation of a bacterial endo-chitosanase from strain GM44 increased productivity of DGA in exo-chitosanase-mediated hydrolysis of chitosan. Sequential two-step hydrolysis of chitosan with endo- and exo-chitosanase was proved to be feasible for DGA production. Yield of DGA by enzymatic process was about 70-80%. Because commercially available chitosans have about 5-10% acetylated NAG residues in their backbones and our exo-chitosanase could not remove terminal NAG in from chitosan, addition of N-acetyl-β-D-glucosaminidase (NagA) would increase the recovery yield of DGA by removing the NAG residues from chitosan.

Two bacterial isolates, *S. liquefaciens* GM1403 and *Cellulomonas* sp. GM13 showed strong endo-chitinase activities. These endo-chitinases predominantly produced $(NAG)_2$ from chitin. Intracellular NagA of strain GM13 produced NAG as a sole end-product from both chitin oligosaccharides and polymeric chitin. The combined action of endo-chitinase and NagA efficiently hydrolyzed swollen chitin and gave about 70-85% yield of NAG. Because commercial chitins have about 5-10% deacetylated DGA residues in their backbones and our NagA could not remove terminal DGA in chitin, addition of fungal exo-chitosanase would increase the recovery yield of NAG by removing the DGA residues from chitin.

DGA and NAG are now produced from chitin by a severe acid hydrolysis (6-12N HCl, 60-80℃). This harsh condition evoked serious problems such as

impurity production, requirement of sophisticated purification steps, and waste-water treatment. Enzymatic process will be an alternative method of choice when considering disadvantages of acid hydrolysis. This study showed that enzymatic processes would be feasible. However, much further researches should be undertaken to realize industrial production of DGA and NAG from chitinous materials: increase in enzyme productivities, re-cycling of enzymes, enzymatic deacetylation of NAG into DGA, development of efficient pre-treatment methods of insoluble chitin, and so on. We expect that the enzymatic processes will replace the present acid hydrolysis in a near furture.

5. References

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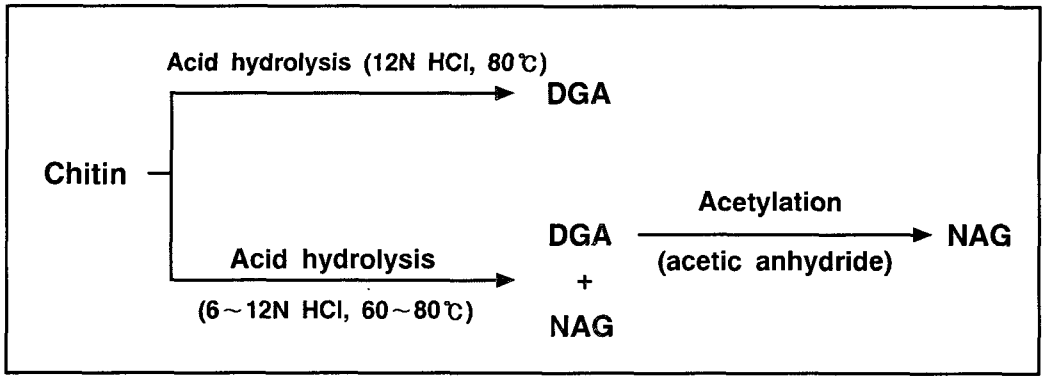

Fig. 1. Production of D-glucosamine (DGA) and N-acetyl-β-D-glucosamine (NAG) by conventional acid hydrolysis processes.

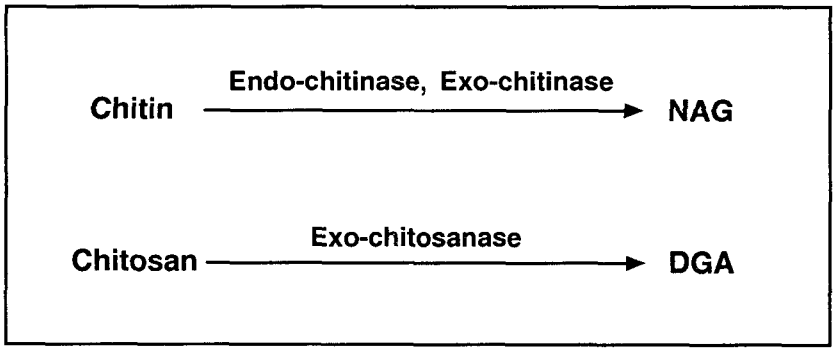

Fig. 2. Production of D-glucosamine (DGA) and N-acetyl-β-D-glucosamine (NAG) by new enzymatic processes.

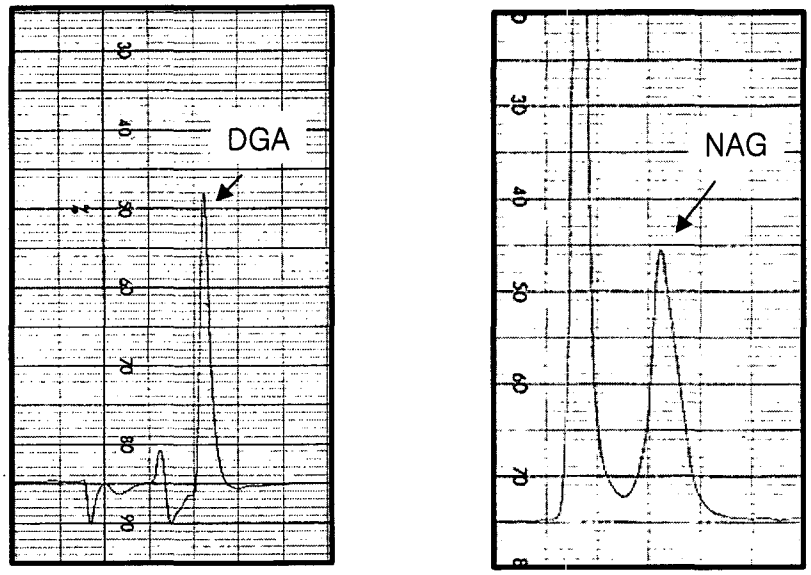

Fig. 3. HPLC chromatograms of D-glucosamine (A) and N-acetyl-β-D-glucosamine (B) produced by enzymatic processes.