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Production of N-acetyl-D-glucosamine from Chitin Using Crude Enzyme Preparations in Large Scale

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Abstract

Glucosamine and *N*-acetyl-D-glucosamine (NAG) have attracted much attention owing to its therapeutic activity in osteoarthritis. NAG is more suitable than glucosamine for oral administration because of its sweet taste. Recently we have reported possibility of production of NAG from chitin directly by use of crude enzymes from *Aeromonas hydrophila* H-2330 or commercially available cellulase preparation from *Trichoderma viride*. The present work describes the effective production of NAG from chitin using crude enzymes derived from *T. viride*. These crude enzymes contained both N-acetylhexosaminidase and chitinase. Chitin flakes (250g) from squid pen (β-chitin) were digested by the crude enzyme derived from *T. viride* and NAG was precipitated from concentrated solution of the hydrolyzates by addition of ethanol. The yield of purified NAG was 107g after drying.

Introduction

Glucosamine is a monosaccharide, which exists as a component of proteoglycan in cartilage, skin, and connective tissue. Recently glucosamine has attracted much attention owing to its therapeutic activity in osteoarthritis and been evaluated as a food supplement. Although hydrochloride or sulfate salt of glucosamine is already commercialized for this disease, it is not suitable for oral administration owing to its bitter taste nor applied for cooked foods due to instability on heating. On the other hand, *N*-acetyl-D-glucosamine (NAG) has been a focusing material for the improvement of osteoarthritis as well as glucosamine, because NAG is also a component of proteoglycan and a part of glucosamine is transformed to NAG by metabolism. Moreover, NAG will be able to apply for oral administration because of its sweet taste and stability on heating. At the mean time, NAG is produced by acid (conc. HCl) hydrolysis of chitin, which exists in crab shell (β-chitin) or squid pen (β-chitin). This procedure, however, has some problems such as low yield (below 65%) and acidic wastes by use of conc. HCl, etc. *N*-acetylation of glucosamine is also possible to produce NAG. This product, however, is not approved as a natural type material owing to its chemical modification process. Although effective refinement for the production of NAG is required, there is no report on such work.

Recently we have reported the production of NAG from chitin by use of crude enzymes originated from *Trichoderma viride* or *Aeromonas hydrophila* H-2330 and obtained NAG in good yield (Sashiwa *et al.*, 2003; Sashiwa *et al.*, 2002; and Sukwattanasinitt *et al.*, 2002). The present article describes the detailed study on the production of NAG in a large scale using the crude enzyme originated from *Trichoderma viride*.

Experimental

Materials

β-Chitin and β-chitin flakes were supplied from Koyo Chemical Co., Ltd. (Osaka, Japan). Crude enzyme solutions from *Trichoderma viride* and *Aeromonas hydrophila* H-2330 were prepared in Rakuto Kasei Industrial Co., Ltd. (Shiga, Japan) and Kyoto Institute of Technology (Kyoto, Japan), respectively.

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Measurements

HPLC analysis was performed on a Tosoh LC-8020 apparatus (column, Shodex Asahipak NH2P-50; rt; CH_3CN/H_2O = 7/3; flow rate = 1.0 mL/min; injection, 0.02 mL; detection, UV at 210 nm).

General methods for hydrolysis

Typical procedure in a small scale is as follows: chitin was suspended in 10 mL of 0.1 M acetate buffer (pH=4.0). To a suspension, enzyme solution was added and the mixture was shaken. After the prescribed time, a part of the reaction mixture (10-100 μL) was taken out, diluted with H_2O (0.4-0.49 mL) and CH_3CN (1.0 mL), filtered, and analyzed to measure the amount of NAG in the reaction mixture by HPLC. The amount of NAG was estimated from the calibration curve of standard NAG.

In a large scale production chitin (250 g) was sterilized with 70% aq. ethanol and diluted with acetate buffer solution and enzyme solution derived from culture broth of *T. viride* to prepare reaction mixture of 2.3 L with ethanol concentration of 11% and pH 4. The mixture was stirred at 30°C. After the prescribed time, a part of reaction mixture was taken out and analyzed to measure the amount of NAG by HPLC. After 10 days the mixture was centrifuged to remove residual chitin and supernatant was filtered through a ultrafiltration membrane to remove enzyme. The filtrate was concentrated to 150 mL at 50°C by an evaporator. This solution was mixed with ethanol (1.5 L) and left in a refrigerator 4°C. The solids precipitated from the solution were collected, washed with ethanol, and then dried.

Results and Discussion

We cultured *T. viride* in 20L medium using a 50 L jar fermentor with β-chitin powders for induction. Before the main culture we pre-cultured it twice in 50 mL and 500 mL media, respectively. After removing cells we concentrated the culture broth by ultrafiltration to obtain 8 L preparation. The activity of endo-chitinase was 250 mU/mL. Total activity was 2000 U. Table 1 shows that β-chitin was easily digested by the crude enzyme solution derived from *T. viride* with high activity. During the reaction time of 6 days NAG was produced in the yield of 50% when the concentration of chitin was 10%. The production rate was almost same when the concentration was different from each other. We selected the strain of *A. hydrophila* H-2330 which had the endo-chitinase activity more than 100 mU/mL in pre-culture (100 mL medium) under shaking. This strain was cultured in 20 L mediun using a 30 L jar fermentor. The crude enzyme preparation was separated from the culture broth by salting out with ammonium sulfate (80% saturated) and dissolved in McIlvaine buffer (ph 7, 640 mL). The endochitinase activity of this preparation was 2152 mU/mL. Total activity was 1377 U.

Table 1. Hydrolysis of β-chitin by crude enzyme derived from *T. viride* in a small scale

Substrate concentration=10%	1 day	2 days	4 days	6 day
Concentration of NAG (mg/mL)	19.1	34.8	41.4	50.4
Yield (%)	19.1	34.8	41.4	50.4
Production rate of NAG (mg/mL/ 1mg pro/1day)	36.0	32.8	19.5	15.8
Substrate concentration = 15%				
Concentration of NAG (mg/mL)	22.0	29.7	35.4	47.9
Yield (%)	14.7	19.8	23.6	31.9
Production rate of NAG (mg/mL/ 1mg pro/1day)	41.5	28.0	16.7	15.1
Substrate concentration= 20%				
Concentration of NAG (mg/mL)	–	27.9	36.5	43.9
Yield (%)	–	14.0	18.3	22.0
Production rate of NAG (mg/mL/ 1mg pro/1day)	–	26.0	17.0	13.6

Figure 1 shows the effect of ethanol concentration on the production of NAG. Ethanol has some sterilization effect on microbe growth in the reaction mixture because microbes digest NAG in some extent. Without ethanol NAG was digested by microbes after production in the reaction system. But addition of ethanol in 10% suppressed the digestion. The production of NAG decreased with an increase of the concentration of ethanol (data are not shown). The production decreased remarkably when the concentration of ethanal was increased from 10% to 20%. The optimum concentration of ethanol for producing NAG in high yield and suppressing digestion was around 10%.

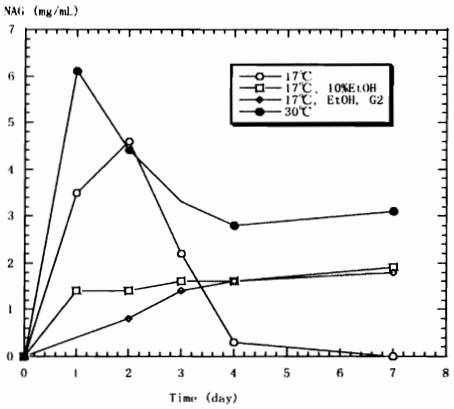

Figure 1. The effect of addition of ethanol in the reaction mixture on production of NAG from β-chitin; G1 means NAG; G2 means NAG-NAG (diacetyl chitobiose)

In the next experiment large amount of β-chitin (250g) was hydrolyzed after the crude enzyme solution from *T. viride* was ultrafiltrated. Table 2 shows the results of the hydrolysis. NAG was produced in the yield of 47% after 10 days reaction. After precipitation by addition of ethanol and drying we obtained purified NAG of 107 g. The mixing ratio of NAG solution to ethanol was one to ten. The purity of NAG was more than 95%, which was analyzed by HPLC. In the case of *A. hydrophila* H-2330, the total activity was 1377 U, so efficient digestion would be expected as same as in the case of *T. viride*.

Table 2. Hydrolysis of β-chitin by crude enzyme derived from *T. viride* in a large scale

	1 day	2 days	4 days	10 day
Concentration of NAG (mg/mL)	17.5	27.7	45.2	52.5
Amount of NAG (g)	39.4	62.3	102.0	118.0
Yield (%)	15.8	25.0	40.7	47.3
Production rate of NAG (mg/mL/ 1mg pro/1day)	37.2	29.5	24.0	14.0

Conclusions

In conclusion the method and condition for the large scale production of crude enzyme preparation and NAG from β-chitin was developed.

References

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