KOR. JOUR. MICROBIOL December, 1992 p. 519~523 Vol. 30, No. 6

Purification and Properties of Glucoamylase from Yeast *Candida tsukubaensis*

Kim, Sang Moon*, Suk Bai, Hee Young Chung, Jong Chun Park Jin Jong Lee, Dong Ho Kim, Myung Hee Song and Soon Bai Chun

**Department of Spectacles and Optics, Kwang-ju Health Junior College, Kwang-ju, 506-701, Korea*
Department of microbiology, College of Natural Sciences, Chonnam National University, Kwang-ju, 500-757, Korea

The glucoamylase of *Candida tsukubaensis* was purified to homogeneity from culture filtrate by means of ultrafiltration, Sephacryl S-200 gel filtration and Sp-Sephadex C-50 chromatography. The purified enzyme was a glycoprotein with a molecular mass of approximately 50 kDa, which was a monomeric protein. Km values were 5.8 mg/ml for soluble starch and 0.04 mM for maltose. Glucoamylase also released only glucose from both pullulan and isomaltose. The analysis of amino acid composition revealed that the enzyme contained a high content of acidic and polar amino acids. In addition, Western blotting analysis indicates that *C. tsukubaensis* glucoamylase is resistant to glucose repression.

KEY WORDS □ *Candida tsukubaensis*, glucoamylase

Glucoamylase with a debranching activity plays an important role in the complete saccharification of starch. Many microorganisms producing glucoamylase with a debranching activity belong to either fungi or yeasts (4, 5, 19). Since amylolytic yeasts are closely related to *Saccharomyces cerevisiae*, they have been attracted as the donor of glucoamylase gene (9, 12, 13, 23). *Candida tsukubaensis* can produce extracellular glucoamylase with debranching activity (6). In preliminary experiment, we have found that *Candida tsukubaensis* glucoamylase synthesis is resistant to glucose unlike the corresponding enzymes reported from other sources (7, 8, 11, 20, 21). In this paper, we describe the purification of glucoamylase from *C. tsukubaensis* and its enzyme formation on glucose and starch.

MATERIALS AND METHODS

Organisms and chemicals

The yeast strain used was *Candida tsukubaensis* CBS 6389. Stock cultures were kept on plates containing 2% soluble starch (1). All chemicals and media ingradients were purchased from Sigma (St. Louis, MO) and Difco (Detroit, MI), respectively.

Enzyme preparation and purification

The yeast was grown in 400 ml of 0.67% yeast nitrogen base (YNB) containing 0.5% soluble starch (Merck, Darmstadt) or glucose at 30°C for 48 hr on a reciprocal shaker (120 rpm). After 24 or 48 hr of growth, the yeast cells were removed by centrifugation at 10,000×g for 30 min. The resulting supernatants were concentrated by using ultrafiltration as decribed by Bai *et al.* (1). The subsequent purification steps were also carried out according to the methods described by Bai *et al.* (1) except using Sp-sephadex C-50 cation exchange chromatography in final step instead of DEAE-Sephadex A-50 anion exchange chromatography.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in vertical slab gel (Hoeffer Scientific Instruments) by the method of Laemmli (16). Gel staining was carried out by using 0.25% Coomassie blue R-250.

Gel filtration

The relative molecular weight of native enzyme was estimated by HPLC system (Waters Associate Co.), and gel filtration column (60×0.75 cm, Biosil TSK-250, Bio-Rad) as described by Cho *et al.* (2).

Enzyme assay and protein determination

Gucoamylase activity was measured according to the procedures described by Bai *et al.* (1). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole glucose/min. Protein content was estimated by the method of Lowry *et al.* (18), with bovine serum albumin as a standard.

Determination of carbohydrate content

The content of carbohydrate in the purified enzyme was analyzed by the method of Dubois *et al.* (10), using 5% phenol solution and sulfuric acid.

pH optimum and stability

Optimal pH was determined by measuring the enzyme activity in the pH range of 2.0 to 8.0. The effect of pH on enzyme stability was measured over a range of 2.0 to 8.0 by using 50 mM McIlvaine buffer (pH 2.0~6.0) and 100 mM sodium phosphate buffer (pH 5.0~8.0). After incubation of the enzyme over the pH range of 2.0 to 8.0 for 24 hr at 20°C, the residual enzyme activity was measured under conditions already described.

Temperature optimum and stability

The temperature optimum was measured over a range of 20 to 70°C. Thermal stability was determined by the assay of residual enzyme activity after preincubation of the temperature range of 30 to 60°C for 30 min.

Determination of Km and Vmax

Km and Vmax were determined by Lineweaver-Burk plot (17) using soluble starch, dextrin, maltose, pullulan and isomaltose as substrates.

Amino acid composition

The purified enzyme was hydrolyzed with 6 N HCl for 24 hr at 110°C in sealed evacuated tubes. The hydrolysate was dried and derivatized with phenylisothiocyanate (PITC), and then analyzed with Pico-Tag column (3.9×150 mm) on HPLC. Cysteine and cystine were determined as cysteic acid after oxidizing with performic oxidation reagent.

Antiserum preparation and Western blotting

Antiserum to glucoamylase was prepared as described elsewhere (14). For Western blotting analysis, the culture supernatants were dialysed exhaustively against 75 mM disodium tartaric acid buffer (pH 5.5) and concentrated 20-fold by ultrafiltration and/or freeze dryer, respectively. Western blotting was conducted as described by Kubicek *et al.* (15), except using horse-radish peroxidase coupled secondary antibody.

RESULTS AND DISCUSSION

Enzyme purification

The glucoamylase of *Candida tsukubaensis* was purified to homogeneity from culture filtrate by means of ultrafiltration, gel filtration and cation exchange chromatography. Gel filtration on Sephacryl S-200 column was found to be essential for removing either low molecular weight proteins or dextin remained in ultrafiltration step. The purification of *C. tsukubaensis* glucoamylase is summarized in Table 1. Approximately 31-fold purification of glucoamylase was achieved with a yield of 7.0%. The enzyme purity at each purification step was analyzed by SDS-PAGE (Fig. 1). The active peaks after cation exchange chromatography gave a single protein band (Fig. 1, lane 3 and 4). The molecular weight was estimated to be approximately 50 kDa by SDS-PAGE, which was smaller than that (56 kDa) reported by De Mot *et al.* (6). The relative molecular weight (Mr) of the native enzyme was estimated to be approximately 40 kDa on a calibration of gel filtration, indicating that *C. tsukubaensis* glucoamylase was a monomeric polypeptide. The enzyme contained approximately 10 % carbohydrate by weight which was similar to glucoamylase from *Schwanniomyces occidentalis* (3).

Enzyme characterization

The highest enzyme activity was obtained in the acidic pH range of 4.0 to 5.5. At pH values lower than 3.0 and higher than 6.0, the enzyme activity was reduced. The enzyme remained stable over a wide range of pH, with >80% activity retained between 4.5 and 8.0. The optimal pH range and its stability were similar to those obtained from *Sch. alluvius* glucoamylase (22). The optimal temperature for the enzyme activity was 55°C, similar to other glucoamylases of yeast origin (50°C~60°C) (7, 20, 22). *Candida tsukubaensis* glucoamylase was, however, completely inacti-

Table 1. *Summary of purification of glucoamylase from C. tsukubaensis*

Purification step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Total activity (U)	Yield (%)	Purification (fold)
Culture supernatant	1,474	1.39	0.17	8.18	2,049	100	1
Ultrafiltrate	12	98.26	7.46	13.17	1,179	57.5	1.6
Sephacryl S-200 gel filtration	7	27.04	0.15	180.27	189	15.6	22.0
SP-Sephadex ion exchange chromatography	3.4	42.78	0.17	251.64	145	7.1	30.8

One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmole glucose/min from soluble starch.

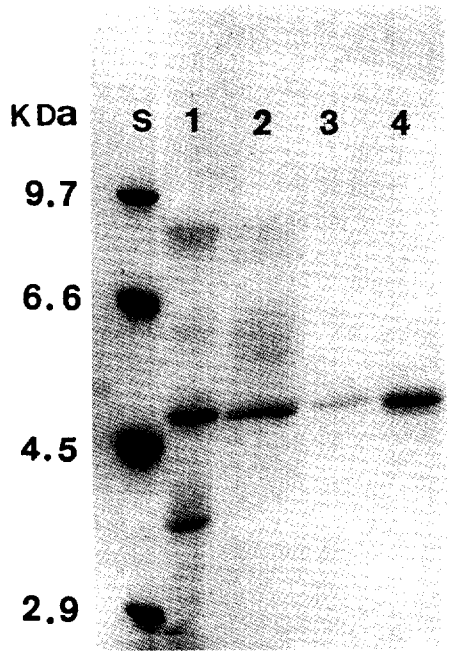

Fig. 1. *SDS-PAGE of glucoamylase. An acrylamide concentration of 10% was used, and gel was stained with 0.25% Coomassie blue R-250. Lanes: 1, culture filtrate (30 μg protein); 2, after Sephacryl S-200 gel filtration (30 μg protein); 3,4, after SP-Sephadex ion exchange chromatography (3, 20 μg protein; 4, 60 μg protein); S, standard protein.*

vated within 10 min at 60°C, and approximately 15% of initial enzyme activity remained after 30 min at 50°C. Therefore, *C. tsukubaensis* glucoamylase appears to be more sensitive to heat compared with the corresponding enzyme obtained from *Sch. alluvius* and *castellii* (21, 22). Kinetic parameters of the enzyme were determined using soluble starch, dextrin, maltose, pullulan and isomaltose (Table 2). The Km and Vmax value of the enzyme for soluble starch were estimated to be 5.8 mg/ml and 112.2 μM/mg/min, respectively. This result indicated that *C. tsukubaensis* glucoamylase had higher affinity toward soluble starch than other enzymes reported from *Lipomyces kononenkoae*, and *Sch. alluvius* and *castellii* (20-22) in which the corresponding values were 16.2, 12.7, and 10.3 mg/ml, respectively. The relative activities of the enzyme with various substrates were compared. The highest enzyme activity was obtained with soluble starch. Isomalose and pullulan were degraded, albeit in very slow, indicating that the enzyme was able to cleave α-1,6 glycosydic linkages. On the other hand, ρ-nitrophenyl-α-glucopyranoside (PNPG) was not hydrolysed at all. In this respect, *C. tsukubaensis* glucoamylase was similar to glucoamylase I of *Filobasidium capsuligenum* but different from glucoamylases from *Sch. castellii* and *L. kononenkoae* (1, 7, 20). The amino acid composition of enzyme was given in Table 3. The enzyme contained a high level of acidic and polar amino acids (except Cys and Tyr) and a low level of basic and nonpolar amino acids (except Ala, Val, Pro and Leu). The amino acid composition of the enzyme was, therefore, similar to that of *Sch. castellii* (1) in that both enzymes contained a high content of acidic and polar amino acids.

Table 2. *Km and Vmax values of various substrates for glucoamylase from C. tsukubaensis*

Substrates	Km	Vmax (μM/mg/ml)
Soluble starch	5.8 mg/ml	112.2
Dextrin	9.3 mg/ml	100.4
Pullulan	28.9 mg/ml	1.0
Maltose	0.04 mM	16.1
Isomaltose	0.15 mM	1.9

Table 3. *Amino acid composition of native glucoamylase from C. tsukubaensis*

Amino acid	Molar %
Asx.	11.0
Glx.	6.8
Ser.	10.0
Gly.	13.7
His.	1.5
Arg.	2.4
Thr.	7.5
Ala.	16.9
Pro.	5.0
Tyr.	1.7
Val.	6.5
Met.	0.9
Cys.	0.5
Ile.	2.1
Leu.	6.3
Phe.	3.6
Trp.	0.9
Lys.	2.8

Enzyme formation

When *C. tsukubaensis* was grown in the YNB supplemented with either 0.5% glucose or soluble starch as sole carbon source, the enzyme activity from the culture fluid of glucose-grown cell maintained the same level as that obtained from starch-grown cell (data not shown). The corresponding level was confirmed by SDS-PAGE and subsequent immunoblotting detection using antiserum raised against 50 kDa glucoamylase (Fig. 2), indicating that *C. tsukubaensis* gluco-

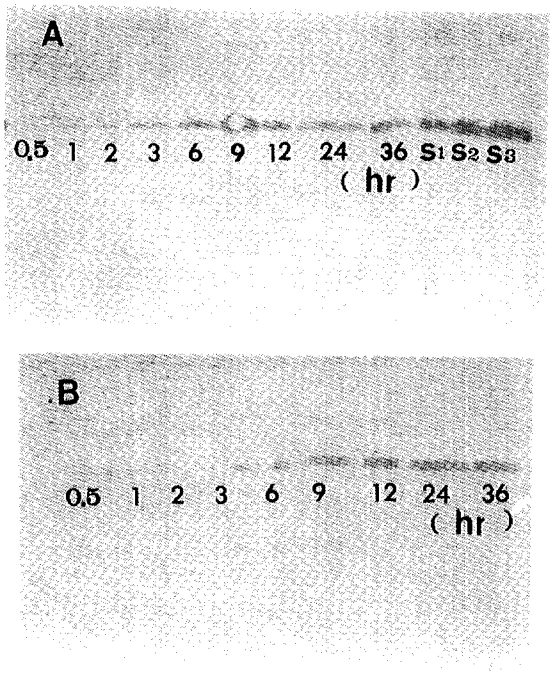

Fig. 2. *The production of glucoamylase during growth on glucose(A) or starch(B). C. tsukubaensis was grown on 0.5% glucose for 24 hr and used as starter culture for enzyme production. The washed cells were inoculated into YNB medium supplemented with 0.5% glucose or starch and grown up to 36 hr. The time course of glucoamylase formation was identified by SDS-PAGE and immunoblotting. S1, S2 and S3 are 10, 20, and 40 μg of purified glucoamylase protein. Each lane corresponds to culture time.*

amylase is not subject to a strong catabolite repression. This result is in contrast to glucoamylases from *L. kononenkoae* and *Sch. occidentalis* the formation of which was strongly repressed by glucose (8, 20, 21).

ACKNOWLEDGEMENT

This study was in part supported by a research grant (921-0400-010-1) from the Korean Science and Engineering Foundation to SBC.

REFERENCES

1. **Bai, S., J.C. Park, D.H. Kim, K.H. Kim and S.B. Chun,** 1991. Purification and properties of glucoamylase from *Schwanniomyces castellii. Kor. J. Microbiol.* **29**, 104-110.
2. **Cho, N.C., K.H. Kim, S.B. Chun and K.C. Chung,** 1990. Effect of cellobiose octaacetate, avicel and KC-flock on production of avicelase from *Penicillium verruculosum. Kor. J. Appl. Microbiol. Biotech.* **18**, 383-389.
3. **Deibel, M.R., R.R. Hiebsch and R.D. Klein,** 1988. Secreted amylolytic enzymes from *Schwanniomyces occidentalis:* Purification of FPLC and preliminary characterization. *Preparative Biochemistry.* **18**, 77-120.
4. **De Mot, R., K. Andries and H. Verachtert,** 1984a. Comparative study of starch degradation and amylase production by ascomycetous yeast species. *Syst. Appl. Microbiol.* **5**, 106-118.
5. **De Mot, R., M. Demeersman and H. Verachtert,** 1984b. Comparative study of starch degradation and amylase production by non-ascomycetous yeast species. *Syst. Appl. Microbiol.* **5**, 421-432.
6. **De Mot, R., E. Van Ouedendijck and H. Verachtert,** 1985. Purification and characterization of extracellular glucoamylase from the yeast *Candida tsukubaensis* CBS 6389. *Antonie van Leeuwenhoek.* **51**, 275-287.
7. **De Mot, R., H. Verachtert,** 1985. Purification and characterization of extracellular enzymes from the yeast *Filobasidium capsuligenum. Appl. Environ. Microbiol.* **50**, 1474-1482.
8. **Dowhanick, T.M., I. Russell, S.W. Scherer, G.G. Stewart and V.L. Seligy,** 1990. Expression and regulation of glucoamylase from the yeast *Schwanniomyces castellii. J. Bacteriol.* **172**, 2360-23 66.
9. **Dohmen, R.J., A.W.M. Strasser, U.M. Dahlems and C.P. Hollenberg,** 1990. Cloning of the *Schwanniomyces occidentalis* glucoamylase (GAM 1) and its expression in *Saccharomyces cerevisiae. Gene.* **95**, 111-121.
10. **Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith,** 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350-356.
11. **Erratt, J.A.,** 1980. Genetic, biochemical and technological studies of yeast strains capable of fermenting dextrin. Ph. D. Thesis. University of Western Ontario, London, Canada.
12. **Erratt, J.A. and A. Nasim,** 1986. Cloning and expression of a *Saccharomyces diastaticus* glucoamylase gene in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe. J. Bacteriol.* **166**, 484-490.
13. **Itoh, T., I. Ohtsuki, I. Yamashita and S. Fukui,** 1987. Nucleotide sequence of the glucoamylase gene GLU 1 in the yeast *Saccharomycopsis fibuligera. J. Bacteriol.* **169**, 4171-4176.
14. **Johnson, A. and R. Thorpe,** 1987. Immunochemistry in practice. Blackwell Scientific Publications, Oxford. 30-34.
15. **Kubicek, C.P., T. Panda, G. Schreferl-Kunar, F. Gruber and R. Messner.,** 1987. O-linked, but not N-linked, glycosylation in necessary for the secretion of endoglucanase I and II by *Trichderma reesei. Can. J. Microbiol.* **133**, 1481-1487.

16. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.*(London). **227**, 680-685.
17. **Lineweaver, H. and D. Burk,** 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-666.
18. **Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall,** 1951. Protein estimation with folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
19. **Manjunath, P., B.C. Shenoy and M.R.R. Rao,** 1983. Fungal glucoamylases. *J. Appl. Biochem.* **5**, 235-260.
20. **Spencer, M. and N. Van Uden,** 1979. Extracellular amylolytic system of the yeast *Lipomyces kononenkoae. European J. Appl. Microbiol. Biotechnol.* **6**, 241-250.
21. **Sills, A.M., M.E. Sauder and G.G. Stewart,** 1984. Isolation and charactrization of the amylolytic system of *Schwanniomyces castellii. J. Inst. Brew.* **90**, 311-314.
22. **Wilson, J.J. and W.M. Ingledew,** 1982. Isolation and characterization of *Schwanniomyces alluvius* amylolytic enzymes. *Appl. Environ. Microbiol.* **44**, 301-307.
23. **Yamashita, I., K. Suzuki and S. Fukui,** 1985. Nucleotide sequence of the extracellular glucoamylase gene STA 1 in the yeast *Saccharomyces diastaticus. J. Bacteriol.* **161**, 567-573.

(Received November 10, 1992)
(Accepted November 23, 1992)

초 록: *Candida tsukubaensis* Glucoamylase의 정제 및 특성
김상문* · 배 석 · 정희영 · 박종천 · 이진종 · 김동호 · 송명희 · 전순배 (*광주보건전문대학 안경광학과, 전남대학교 미생물학과)

Candida tsukubaensis glucoamylase를 배양상등액으로부터 ultrafiltration, Sephacryl S-200 gel filtration 그리고 SP-Sephadex C-50 cation chromatography를 통하여 정제하였다. 정제된 효소의 분자량은 약 50 KDa이었고 당단백질로 단량체이었다. Soluble starch와 maltose에 대한 Km값은 각각 5.8 mg/ml와 0.04 mM이었고 pullulan과 isomaltose 분해능을 가지고 있었다. 아미노산 조성분석 결과 본 효소는 산성과 극성 아미노산을 많이 함유하고 있었다. 또한 *C. tsukubensis* glucoamylase는 glucose repression을 받지 않았다.