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# Purification and Characterization of Two Extracellular Glucoamylase Isozymes from *Lipomyces kononenkoae* CBS 5608 Mutant

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**Abstract:** Two forms of glucoamylase (GI and GII) from starch-grown *Lipomyces kononenkoae* CBS 5608 mutant were purified to apparent homogeneity by means of ultrafiltration, Sephacryl S-200 gel filtration and DEAE Sephadex A-50 chromatography. The apparent molecular weight was calculated as ca. 150 kDa for GI and ca. 128 kDa for GII, respectively. Both enzymes were glycoproteins with isoelectric points of 5.6 (GI) and 5.4 (GII). They had a pH optimun of 4.5 and were stable from pH 5 to 8. The temperature optimum for both enzymes was 60℃, but they were rapidly inactivated above 70℃. The $K_m$ values toward starch were estimated to be 6.57 mg per ml for GI and 4.52 mg per ml for GII, and the $V_{max}$ values were 16.28 μM per mg for GI and 32.25 μM per mg for GII, respectively. The $K_m$ and $V_{max}$ values of GII for α- or β-cyclodextrin were estimated to be 0.15 mg per ml and 2.0 mg per ml, respectively ($K_m$) and 1.02 μM per mg or 1.02 μM per mg, respectively ($V_{max}$). Neither enzyme exhibited pullulanase activity but they released only glucose from starch or cyclodextrin. Amino acid analysis indicated that both glucoamylases were enriched in proline and acid amino acids. Glucoamylase GII strongly cross-reacted with a monoclonal antibody raised against GI enzymes, and the two enzymes shared very similar amino acid composition. Western blot analysis indicated that *L. kononenkoae* CBS 5608 mutant produced two forms of glucoamylase on starch, and that synthesis of them was subject to glucose repression.
**Key words:** characterization, formation, glucoamylases, *Lipomyces kononenkoae*.

There are numerous yeasts that are capable of degrading starch as their sole carbon and energy source. These yeasts have been investigated as promising microorganisms for the conversion of starch to single cell protein or ethanol, and recently as favorable candidates as donors of genes encoding amylolytic enzymes, mainly because of their close relationship to *S. cerevisiae*. Among starch assimilating yeasts, *Lipomyces kononenkoae* converts over 50% of sarch into single cell protein and thus has shown promise for the industrial conversion of starch into biomass (Spencer-Martin, 1977). This yeast produces an extracellular amylolytic system consisting of α-amylase, glucoamylase, isoamylase, and cyclodextrinase(Spencer-Martin and Van Uden, 1979; Spencer-Martin, 1984). Of these enzymes, some biochemical properties of glucoamylase have been investigated (Spencer-Martin and Van Uden, 1979). In a preliminary experiment, we found that two forms of glucoamylase with different molecular masses were secreted into the culture supernatant by *L. kononenkoae* CBS 5608 mutant grown on starch. Many glucoamylases have been purified from fungi (Jensen *et al.*, 1987; Fagerstrom *et al.*, 1990; Gosh *et al.*, 1990; Gosh *et al.*, 1991; Orsin *et al.*, 1993) and yeasts (DeMot and Verachtert, 1985, 1987; Deibel *et al.*, 1988; Kim *et al.*, 1992). Most fungal glucoamylases exist in multiform varying in size (Pazur *et al.*, 1971; Sevensson *et al.*, 1982; Fagerstrom *et al.*, 1990; Yamshita *et al.*, 1989).

In this paper, as part of an effort to clone glucoamylase genes, we purified two forms of glucoamylase from *Lipomyces kononenkoae* CBS 5608 mutant. The enzyme synthesis in starch medium by this yeast was also examined using a monoclonal antibody raised against a purified glucoamylase.

## Materials and Methods

### Organisms and reagents

In this experiment, *Lipomyces kononenkoae* CBS 6509 mutant, showing much less production of extracellular polysaccharide but higher enzyme activities

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than the wild type cell, was used. It was isolated from the wild type of *Lipomyces kononenkoae* CBS 5608 by treatment of $2\times10^7$m*l* cells with 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine. All reagents and media ingredients were purchased from Sigma Chemical Co. (St. Louis, USA) or Difco Co. (Detroit, USA).

**Enzyme purification**

The yeast cells were grown in 1L YNB (0.67% yeast nirogen base) supplemented with 0.5% (w/v) soluble starch at 30℃ for 60~72 h in a reciprocal shaking incubator (120 rpm). After growth, the yeast cells were removed by centrifugation at 1000×g for 30 min. Purification of enzyme from the resulting supernatant was performed as described by Kim *et al.*, (1992). For time course pattern of enzyme formation, the yeast was grown in YNB (yeast nitrogen base, Difco Co.) supplemented with 0.5% (w/v) starch or glucose at 30℃ for 72 h. The culture supernatant sampled periodically was concentrated 20-fold by ultrafiltration/or freeze-dryer. The resulting concentrates were used for Western blot analysis or enzyme assay.

**Estimation of molecular weight and isoelectric point**

The molecular weight of the enzymes was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. SDS-PAGE was performed using a vertical slab gel (Hoeffer Scientific Instruments, USA) at 10% concentration by the method of Laemmli (1970). The gel filtration was carried out using a HPLC system (Waters Associate Co., Miford, USA) with a gel filtration column (60 mm×0.75 mm, Biosil TSK-250, Bio-Rad) as described by Cho *et al.* (1990). The molecular weight of the enzyme was estimated by comparing the mobility of the enzyme protein or the elution volume of the protein peak with those of the standard proteins.

Isoelectric focusing was carried out in 5% acrylamide gel with pH range 3.5~10.0, on a Hoeffer SE600 (Hoeffer Scientific Instruments). After equilibrium focusing the gel was stained for proteins using Coomassie Brilliant Blue R-250 (Sigma). After termination of focusing, pI was estimated by pH measurement of ampholite eluted from gels corresponding to glucoamylase.

**Enzyme assay and protein determination**

p-Nitrophenyl-α-D-glucose was used as substrate for glucoamylase assay because this compound was not degraded by *L. kononenkoae* α-amylase. Glucoamylase and cyclodextrinase activity were measured using soluble starch or β-cyclodextrin (Sigma) as substrates according to the procedures described by Kim *et al.* (1992) One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mole glucose per min. Protein content was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

**Optimal pH and temperature**

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 0.05 M McIlvine buffer (pH 2.0~8.0) or 0.1 M sodium phosphate buffer (pH 5.0~6.0). After incubation of the enzyme solution over the pH range of 2.0 to 8.0 for 24 h at 30℃, the residual enzyme activity was measured under the conditions described above.

The temperature optimum was determined over a range of 20 to 70℃. Thermal stability was determined by measuring residual enzyme activity after preincubation of the reaction mixtures at temperatures between 30 and 70℃ for 30 min.

**Determination of $K_m$ and $V_{max}$**

$K_m$ and $V_{max}$ were determined by Lineweaver-Burk plot (1934) using soluble stach, α-, or β-cyclodextrin as substrates.

**Amino acid composition and carbohydrate content analysis**

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

The content of carbohydrate in the purified enzyme was estimated by the method of Dubois *et. al.* (1956) using 5% (w/v) phenol solution and sufuric acid.

**Thin layer chromatography of hydrolysis products**

The enzyme action on soluble starch, pullulan, α- or β-cyclodextrin was monitored by thin layer chromatography at several incubation times. Reaction mixtures contained 50 mM citrate phosphate buffer (pH 4.5) and 0.5% substrates. The reaction was initiated by 1 ug of purified enzymes. The samples were incubated at 60℃. The hydrolysate (4 or 8 μl) sampled at each reaction time point was spotted on a precoated silica gel plate and separated using n-butanol : ethanol : water (5 : 3 : 2) as the mobile-phase system. Chromatography was developed using a silver nitrate-sodium hydroxide solution.

**Monoclonal antibody preparation and Western blotting**

For monoclonal antibody preparation, purified glucoamylase I (1 μg/50 μl phosphate buffered saline) was injected directly into the spleen of four week old Balb/c mice, obtained from the animal breeding facility of G.E.G. Boostings were done 2 times with the ELISA method. The mice were sacrified, and cell fusion and hybrid cloning were performed as described by Lee *et al.* (1989). For Western blot analysis, the culture supernatants were dialysed exhaustively against 75 mM disodium tartaric acid buffer (pH 5.5) and concentrated by ultrafiltration and/or freeze dryer. Western blotting was conducted as described by Kubicek *et al.* (1987).

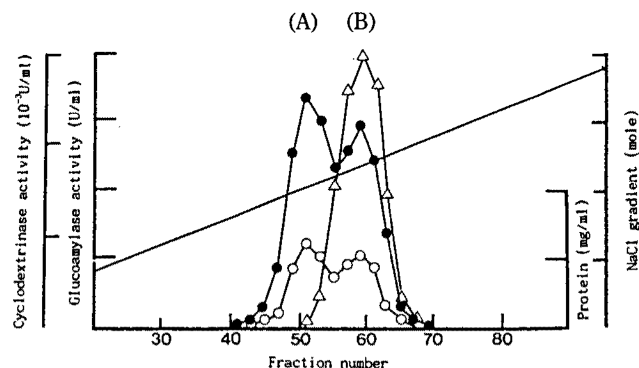


**Fig. 1.** Separation of glucoamylase I and II by DEAE-Sephadex A-50 anion exchange chromatography. The flow rate was maintained 0.1 ml/min. Symbols: -●- : glucoamylase activity, -△- : cyclodextrinase activity.

## Results and Discussion

**Enzyme purification**

Two forms of glucoamylase (G I and G II) were purified to homogeneity from the culture fluid of *Lipomyces kononenkoae* CBS 5608 mutant by ultrafiltration, gel filtration and anion exchange chromatography. The purification procedure is summarized in Table 1. The concentrated enzyme fraction from the Sephacryl S-20 column (3.5 ml) was dialyzed against 100 volumes of 50 mM sodium phosphate buffer (pH 6.3 ) overnight at 4℃. The dialyzed enzyme fraction (4 ml) was applied to a DEAE-Sephadex A-50 column (flow rate, 0.1 ml/min.; 60 by 2.6 cm) that had been equilibrated with 50 mM sodium phosphate buffer (pH 6.3). The enzyme was eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer. Two peaks were separated in this purification step, and they were designated peak A and peak B, respectively (Fig. 1). Peak A fractions (41~52) had only glucoamylase activity, whereas peak B fractions (56~69) exhibited both glucoamylase and cyclodextrinase activity. G I and G II were purified 15- or 25-fold, respectively, and the specific activity was 38 U/mg of protein for the former and 51 U/mg of protein for the latter. The active fraction recovered from peak A (GI) or peak B (GII) showed one protein band on SDS-polyacrylamide gel (Fig. 2, lane 3, 4). Purified G I had a relative molecular weight of about 150 KDa by SDS-PAGE or 230 KDa by gel filtration (HPLC using Biosil-TSK-250 (data not shown), and a carbohydrate content estimated as 7.5% using glucose as the standard. This enzyme could be a dimeric glycoprotein since Mr estimates using agarose or dextran-based media, such as Ultragel and Sehpacryl, were significantly lower than those given by SDS-PAGE (DeMot and Verachtert, 1987). On the other hand, G II had a molecular weight of about 128 KDa by SDS-PAGE, or 140 KDa by gel filtration (data not shown), and its carbohydrate content was estimated as 6.5%. This result indicates that purified G II could be a monomeric glycoprotein. After deglycosylation of both enzymes by Endo H treatment (Fig. 3), their molecular weights, estimated by SDS-PAGE, were about 135 and 116 KDa, respectively. These values were close to the molecular weights calculated from carbohydrate content and amino acid composition (ca. 140 for G I and ca. 120 for G II). These molecular weights were large compared with the values of most glucoamylases from bacteria (Sha and Zeikus, 1989), fungi (Saha and Zeikus, 1989; Forgaty and Kelly, 1980) and yeasts (Forgaty and Kelly, 1980; Saha and Zeikus, 1989), which lie between 50 and 90 KDa, but lower than the Mr of glucoamylase from *Saccharomyces diastaticus* (300 kDa) with an extremely high content of carbohydrates (Modena *et al.*, 1986). Spencer-Martin and Van Uden (1979) partially purified

**Table 1.** Purification scheme of glucoamylase I and II of *Lipomyces kononenkoae* CBS 5608 mutant

| Step | Volume (ml) | Protein (mg) | Units (U) | Specific activity (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| Crude | 950 | 175.75 | 361,07 | 2.05 | 100 | 1 |
| Ultrafiltrate | 7.5 | 36.25 | 285,05 | 7.80 | 78.97 | 3.8 |
| Sephacryl S-200 | 3.5 | 4.51 | 117,56 | 26.03 | 32.55 | 12.69 |
| DEAE-Sephadex A-50 | | | | | | |
| G I | 1.9 | 0.83 | 31.54 | 38.00 | 8.7 | 15.38 |
| G II | 2.0 | 1.49 | 75.49 | 50.64 | 14.02 | 24.70 |

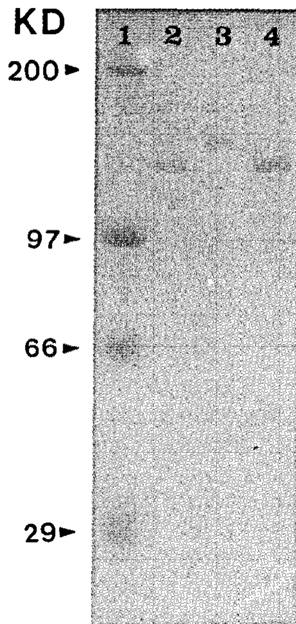


**Fig. 2.** SDS-polyacrylamide gel electrophoresis of glucoamylase I and II purified from *L. kononenkoae* CBS 5608 mutant. Lanes: 1, molecular size markers; 2, crude enzyme; 3, glucoamylase I (GI); 4, glucoamylase II (G II).

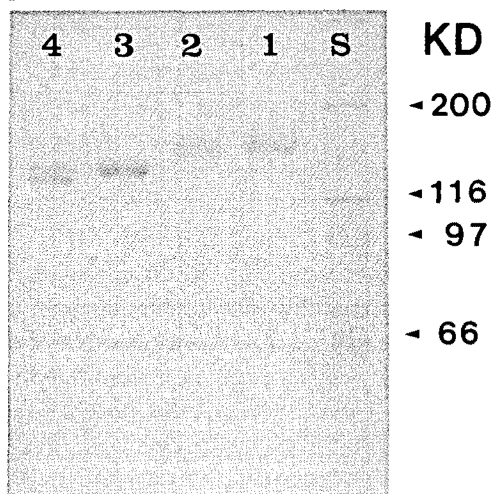


**Fig. 3.** SDS-polyacrylamide gel electrophoresis of glucoamylase I and II before and after digestion with endoglycosidase H. Lanes: 1, G I before digestion; 2, G I after digestion; 3, G II befor digestion; 4, G II after digestion; S, Molecular size markers

glucoamylase from *L. kononenkoae* IGC4052 and estimated its molecular weight as 81.5 KDa using a Sephadex G-100 column. A discrepancy was observed between the 81.5 kDa reported by them and the 230 kDa or 150 kDa obtained in this study by a Biosil-TSK-250 column (Bio-Rad). Such a discrepancy could be in part due to the difference in the affinities of each of the gel materials to the enzyme proteins. A similar observation has been reported by DeMot and Verachtert (1987), who have observed significant differences

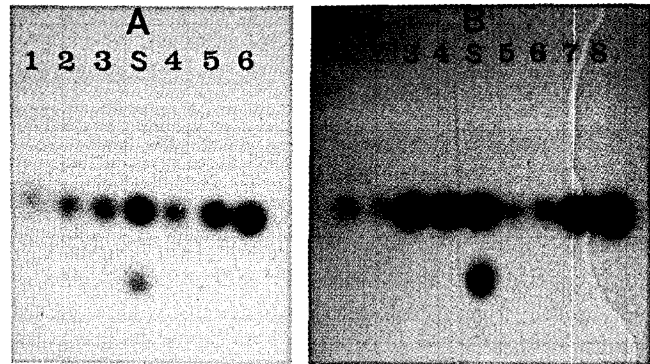


**Fig. 4.** Thin layer chromatography of starch (panel A), pullulan and dextrin (panel B) hycrolyzing products by glucoamylase from *L. kononenkoae* CBS 5608 mutant. Experimental details were described in Materials and Methods. Lanes in panel A, 1 (12 h), 2 (24 h), and 3 (36 h) are G I plus starch; 4 (12 h), 5 (24 h), and 6 (36 h) are GII plus starch. S; standard glucose and maltose. Lanes in panel B, 1 (12 h) and 2 (24 h) are G I plus pullulan; 3 (12 h) and 4 (24 h) are G I plus dextrin; 5 (12 h) and 6 (24 h) are G II plus pullulan; 7 (12 h) and 8 (24 h) are G II plus dextrin.

in the molecular weight of *Candida anarctica* glucoamylase when estimated by gel filtration using a Sephadex G-75 or Bio-Gel P-100.

**Characterization of enzyme properties**

Both glucoamylases were stable between pH 4.0 and 6.5, and showed optimal activity at pH 4.5 (data not shown), and the optimal temperature for their activity was 60℃, and stability was between 50 and 60℃ (data not shown). These results were consistent with those reported by Spencer-Martin and van Uden (1979), and Spencer-Martin (1984). The optimal pH range and stability was close to the values obtained from *Schwanniomyces allubius* glucoamylase (Wilson and Ingledew, 1982), and the temperature optimum and thermal stability for enzyme activity was similar to other glucoamylases of yeast origin (Wilson and Ingledew, 1982; DeMot and Verachtert, 1987; Kim *et al.*, 1992). Both enzymes released only glucose from starch or dextrins but did not hydrolyze pullulan (Fig. 4). The faint spots of glucose in lane 1, 2, 5, and 6 in panel B of Fig. 4 were due to the presence of a trace amount of glucose in pullulan samples (Hwa Sung co., Tokyo, Japan). The $K_m$ values on starch were estimated as 6.57 mg/ml for G I and 4.52 mg/ml for G II, and the $V_{max}$ values as 16.28 μM/mg for the former and 32.25 μM/mg for the latter, respectively. The $K_m$ value was different from the corresponding values obtained from *L. kononenkoae* IGC 4052 by Spencer-Martin and van Uden (1979). They reported the $K_m$ to be 16.2 mg/ml for glucoamylase, which is approximately 2-fold higher than the values obtained here from *L. kononenkoae*

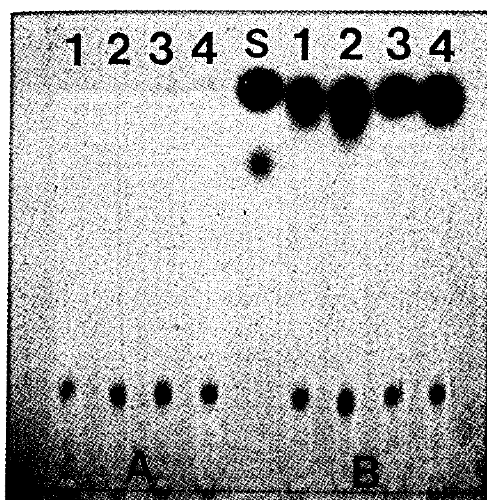


**Fig. 5.** Thin layer chromatography of α-cyclodextrin and β-cyclodextrin hydrolysing products by glucoamylase from *L. kononenkoae* CBS 5608 mutant. Reaction and detection were carried out by the same method as described in Fig. 6. Lanes in panel A : 1, 2; G I plus α-cyclodextrin. 3, 4; G I plus β-cyclodextrin. Lanes in panel B : 1, 2; G II plus α-cyclodextrin 3, 4; G II plus β-cyclodextrin. S; standard glucose and matltose.

CBS 5608. mutant. G II had a $K_m$ of 0.15 mg/ml and a $V_{max}$ of 1.02 M/mg on β-cyclodextrin, a $K_m$ of 2.02 mg/ml and a $V_{max}$ of 12.7 μM/mg on β-cyclodextrin, respectively, indicating that G II from *L. kononenkoae* CBS 5608 mutant was more efficient in hydrolyzing α- or β-cyclodextrin than was the cyclodextrinase from *L. kononenkoae* IGC 4052 (Spencer-Martin, 1984) (Fig. 5). Furthermore, the $K_m$ value (4.5) of G II with soluble starch was slightly lower than the 5.4 obtained from *C. anarctica* glcoamylase, the $K_m$ value of which has been known to be lower than that for other glucoamylases of yeast origin (DeMot and Verachtert, 1987). However, the $V_{max}$ value of 32 μM/ml was lower than the 112 μM/ml obtained from *C. tsukubaensis* glucoamylase (Kim *et al.*, 1992), but higher than the values of glucoamylases from other yeast origin (Wilson and Ingledew, 1982; DeMot and Verachtert, 1985, 1987; Saha and Zeikus, 1989).

Amino acid composition analysis indicates that both enzymes were enriched in proline and acidic amino acids, and that they had very similar molar ratios in their amino acid residues (Table 2). Compared with the amino acid composition of several yeasts (Kim *et al.*, 1992; DeMot and Verachtert, 1987; Forgaty and Kelly, 1980) and of mold glucoamylases (Forgaty and Kelly, 1980; Saha and Zeikus, 1989), the *L. knonenkoae* CBS 5608 mutant enzymes contain an unusually high content of proline. In this respect these enzymes were very similar to the composition of glucoamylase P from *Hormocois resinae* (Fagerstrom *et al.*, 1990) However, a common feature is the presence of a relatively large number of acidic and hydroxy amino acids (threonine and serine). They are major potential glycosylated sites (DeMot and Verachtert, 1987). GI exhibits a pI of 5.6 and GII of 5.4, indicating that the *L. kononenkoae* glucoamylases are acidic protein like other yeast or fungal enzymes (Kim *et al.*, 1992; Forgarty and Kelly, 1980; Saha and Zeikus, 1989).

**Table 2.** Amino acid composition of glucoamylase I and II of *Lipomyces kononenkoae* CBS mutant

| Amino acid | Molar ratio (%) | |
|---|---|---|
| | G I | G II |
| Cya | 0.72 | 0.84 |
| Asx | 8.90 | 8.65 |
| Glx | 6.12 | 5.83 |
| Ser | 6.90 | 7.68 |
| Gly | 7.46 | 7.71 |
| His | 2.44 | 2.46 |
| Arg | 2.24 | 2.28 |
| Thr | 7.90 | 8.65 |
| Ala | 6.40 | 6.88 |
| Pro | 12.25 | 10.95 |
| Tyr | 9.68 | 7.75 |
| Val | 6.43 | 7.12 |
| Met | 0.69 | 0.91 |
| Ile | 4.96 | 5.18 |
| Leu | 6.98 | 7.39 |
| Phe | 4.40 | 4.57 |
| Trp | 3.18 | 2.65 |
| Lys | 2.34 | 2.50 |

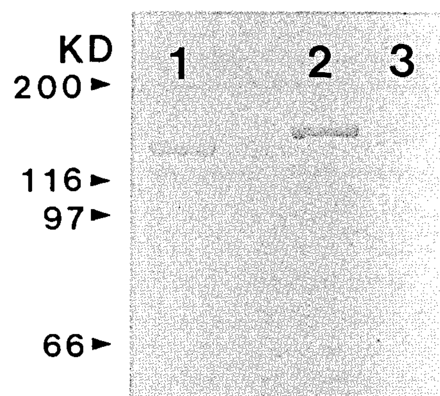


**Fig. 6.** Immunoblots of purified glucoamylase I, II and crude enzyme from *L. kononenkoae* CBS 5608 mutant. Purified and crude enzymes were separated by SDS-PAGE and probed with antibody raised against 150 kDa of G I. Lanes : 1, purified G II; 2, purified G I; 3, crude enzymes as control.

## Enzyme formation

The monoclonal antibody raised against purified 150 kDa of G I exhibited strong cross-reactivity to G II

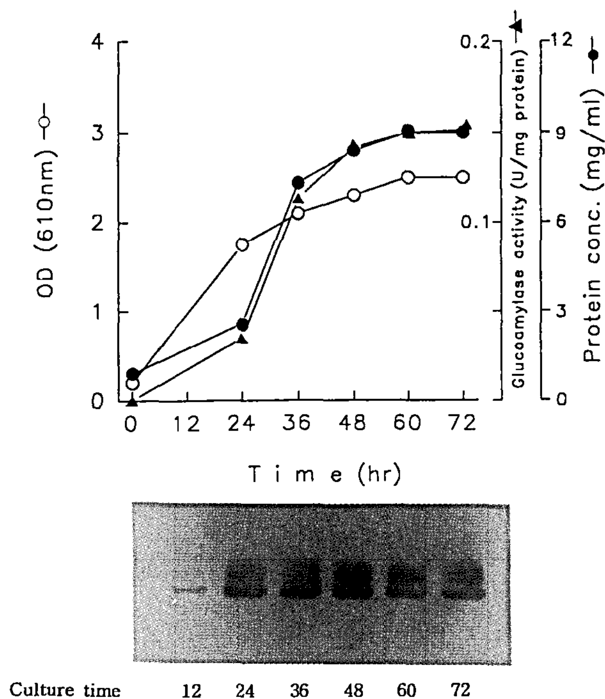


**Fig. 7.** *Time course pattern of growth, protein content and extracellular glucoamylase synthesis by L. kononenkoae* CBS 5608 *mutant grown on starch medium. Glucoamylase in the culture fluid collected at each reaction time point were separated by* SDS-PAGE *and probed with antiserum against* 150 kDa *of* G I. *Each lane correspond to the culture times. Enzyme activity and protein content were estimated as described in Materials and Method*

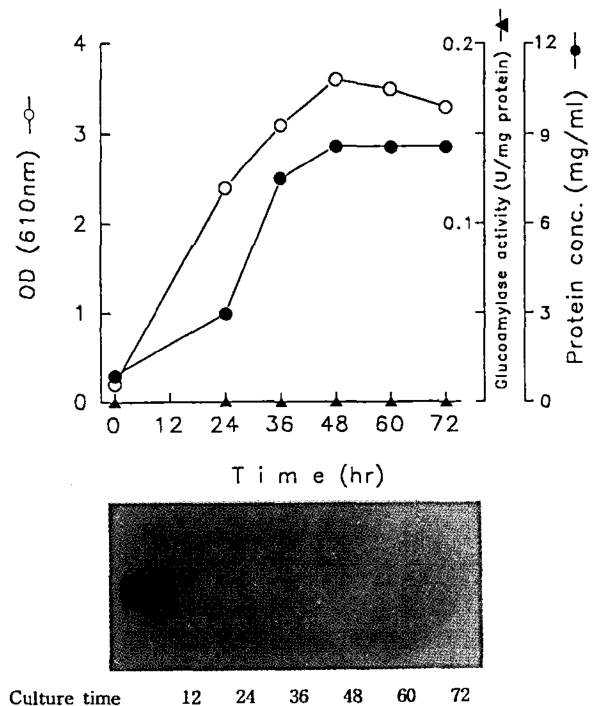


**Fig. 8.** *Time course study of growth, protein content and extracellular glucoamylase synthesis by L. kononenkoae* CBS 5608 *mutant grown on starch medium. Experimental procedures were the same as described in Fig. 7. Lane S; cluture fluid from cells grown on starch for* 24 h *as control.*

(Fig. 6) but no reactivity against glucoamylase from *Schwanniomyces allvius* or *Candida tsukubaensis* (data not shown). This indicates that both enzymes may include some similar antigenic determinants in either carbohydrate or protein portions. Using this antibody as a probe, we examined the time course production of the two glucoamylases on starch or glucose. Western blot analysis showed that for both enzymes synthesis in the presence of starch reached a maximal level in 48 h and maintained a similar level till 72 h, and that band intensities of immunoblot were parallel with enzymes activities (Fig. 7). This result suggests that synthesis of both enzymes might be regulated at the transcriptional level rather than at the translational or posttranslational level. However, the enzyme synthesis is not induced in media containing glucose as the sole carbon source (Fig. 8), indicating that both enzymes are subject to glucose repression.

The immunoloigcal result together with the similarity in both enzyme's properties or amino acid composition suggests that G I and G II could be the product of a single gene. However, the possibility of products of being the two distinct genes could not be excluded. Fagerstrom *et al.* (1990) have reported that two glucoamylases from *Hormoconis resinae* had very similar amino acid composition and cross-reactivity with polyclonal antiserum against one of the two enzymes, but differed in peptide mapping and N-terminal amino acid sequence. It has been well documented that glucoamylases produced by fungi and yeast, especially an *Aspergillus* sp, occur in multiple forms (Lineback and Aria, 1972; Yamashita *et al.*, 1989; Saha and Zeikus, 1989). Alternative splicing of mRNA transcribed from a single gene (Boel *et al.*, 1984), proteolysis of the larger form (Dowanick *et al.*, 1990), difference in carbohydrate content (Hayashida and Yashino, 1978), or the difference in purification procedure (Lineback *et al.*, 1978) are responsible for multiple forms of glucoamylases. Therefore, further studies are needed to determine if GI and GII are products of single or of two separate genes. Molecular cloning of genes encoding *L. kononenkoae* glucoamylase are under investigation to obtain a better understanding.

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