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Isolation and Characterization of *Schwanniomyces alluvius* Amylolytic Enzymes

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The extracellular amylolytic enzymes of *Schwanniomyces alluvius* were studied to determine future optimization of this yeast for the production of industrial ethanol from starch. Both α-amylase and glucoamylase were isolated and purified. α-Amylase had an optimum pH of 6.3 and was stable from pH 4.5 to 7.5. The optimum temperature for the enzyme was 40°C, but it was quickly inactivated at temperatures above 40°C. The K_m for soluble starch was 0.364 mg/ml. The molecular weight was calculated to be 61,900 ± 700. α-Amylase was capable of releasing glucose from starch, but not from pullulan. Glucoamylase had an optimum pH of 5.0 and was stable from pH 4.0 to >8.0. The optimum temperature for the enzyme was 50°C, and although less heat sensitive than α-amylase, it was quickly inactivated at 60°C. K_m values were 12.67 mg/ml for soluble starch and 0.72 mM for maltose. The molecular weight was calculated to be 155,000 ± 3,000. Glucoamylase released only glucose from both soluble starch and pullulan. *S. alluvius* is one of the very few yeasts to possess both α-amylase and glucoamylase as well as some fermentative capacity to produce ethanol.

The recent increase in the production of industrial ethanol by fermentation of grain starch has led to intensive research into improving the conventional fermentation procedures. Although many advances have been reported dealing with continuous fermentations, yeast recycling, immobilization, and ethanol recovery techniques (i.e., vacuum distillation and solvent extraction), little work has been reported on the starch conversion process. Currently, the use of nonrenewable, commercial bacterial and fungal amylase preparations is common for the liquefaction and saccharification of the starch substrate. We believe that a considerable cost saving could be realized by the use of a yeast which produces its own α-amylase and glucoamylase for starch conversion and which is also capable of fermentation.

Very few yeasts have been reported which possess both α-amylase and glucoamylase. These include *Saccharomycopsis fibuligera* (formerly *Endomycopsis fibuligera*) (3, 8), *Saccharomycopsis capsularis* (2), and more recently *Lipomyces kononenkoae* (7), *Lipomyces starkeyi* (6), and *Schwanniomyces castellii* (K. Oteng-Gyang, Ph.D. thesis, Université des Sciences et Techniques du Languedoc, Montpellier, France, 1979). The *Lipomyces* spp. are nonfermentative yeasts. *Saccharomycopsis fibuligera* has been used to ferment premalted 20% wheat starch, however, actual yields reached only 54.0% of the theoretical (9). *Schwanniomyces alluvius* was found to produce ethanol yields of 70.3% of the theoretical from 2% soluble starch and 67.4% of the theoretical yields from 2% wheat starch (J. J. Wilson, Ph.D. thesis, University of Saskatchewan, Saskatoon, Canada, 1981).

To optimize the starch conversion by the amylolytic system of *S. alluvius*, the component enzymes were isolated and characterized.

MATERIALS AND METHODS

Yeast strain. *S. alluvius* UCD 54-83 was obtained from the culture collection of the National Research Council of Canada Prairie Regional Laboratory, Saskatoon, Saskatchewan.

Media. Cultures were grown at 30°C on YPS slants (2% peptone, 2% soluble starch [Difco Laboratories], 1% yeast extract, 2% agar) and maintained at 4°C. Amylase production medium consisted of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer (pH 6.3) containing 1% soluble starch (Sigma Chemical Co., S-2630) which was autoclaved before the addition of 0.67% filter-sterilized yeast nitrogen base without amino acids (Difco).

Preparation of crude enzyme. Two 500-ml cultures of *S. alluvius* in amylase production medium were grown at 30°C and a 220-rpm agitation to the stationary phase. The cultures were then centrifuged at 6,000 × *g* for 10 min at 4°C, and the supernatant (950 ml) containing the extracellular enzymes was collected and used as the crude enzyme preparation.

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Ultrafiltration. The 950 ml of crude enzyme preparation was concentrated by ultrafiltration at 25°C under 60 lb/in^2 of nitrogen with a Millipore Hi-flux 90-mm cell fitted with a PSAC pellicon membrane (1,000-molecular-weight cutoff). The 21 ml retained after 4 h was adjusted to pH 5.6 with 10 ml of 0.04 M acetic acid before chromatography.

Ion-exchange chromatography. A column (2.6 by 32 cm) was packed at 4°C with 200 ml of DEAE-Sephacel (Pharmacia Fine Chemicals) ion-exchange resin equilibrated in 0.04 M sodium acetate buffer (pH 5.6). A 20-ml sample was applied and elution was carried out at a flow rate of 0.2 ml min^{-1} at 4°C, using an NaCl gradient from 0 to 0.5 M developed in 700 ml of elution buffer. Collected fraction volumes were 3.5 ml. Protein peaks were monitored by UV absorbance at 280 nm. Pooled peak fractions were desalted and reduced 10-fold by ultrafiltration as described above.

Gel filtration. A column (1.6 by 60 cm) was packed at 4°C, using 7 g of Sephadex G-150 (Pharmacia) swelled in 0.04 M sodium acetate buffer (pH 5.6). Samples of 1.5 to 2.5 ml of concentrated protein peaks from the ion-exchange step were eluted with the same buffer at a flow rate of 0.14 ml min^{-1}. The 3.5-ml fractions containing the amylolytic activity were pooled and used as the partially purified enzyme preparations for the characterization studies.

α-Amylase assay. The substrate consisted of 0.2% soluble starch (Sigma, S-2630) dissolved in boiling 0.05 M KH_2PO_4-NaOH buffer (pH 6.0) and cooled to 40°C. The iodine reagent was prepared fresh by diluting 1 ml of stock solution (0.5% I_2 in 5.0% KI) into 500 ml of deionized water containing 5 ml of 5 N HCl. For the assay, 1.0 ml of enzyme solution was placed in a test tube (16 by 150 mm) and warmed to 40°C in a water bath. At 10 min after the addition of 2.0 ml of starch substrate, the reaction was stopped by taking a 0.2-ml sample and adding it to 5.0 ml of iodine reagent. The absorbance at 620 nm was measured against a blank (0.2 ml of water in 5 ml of iodine reagent). The substrate control used 1.0 ml of buffer in place of the enzyme. Amylase activity was calculated from the absorbances by using the equation: α-amylase units per milliliter = [(control − test)/control] × 40D, where D is the enzyme dilution factor, and 40 represents the 4.0 mg of starch present in the reaction tube times 10. One unit of α-amylase is defined as the amount of enzyme that will hydrolyze 0.1 mg of starch in 10 min at 40°C when 4.0 mg of starch is present. Activities which resulted in absorbances of <0.125 after 10 min required dilution to give linear reactions over the 10-min period.

Glucoamylase assay. Glucose liberation from starch was measured by the peroxidase-glucose oxidase assay (Sigma technical bulletin no. 510). The substrate was 0.5% soluble starch dissolved in boiling 0.05 M KH_2PO_4-NaOH buffer (pH 5.0) and cooled to 40°C. For the assay, 5.0 ml of starch substrate was added to 1.0 ml of prewarmed enzyme dilution, and after 10 min at 40°C the reaction was stopped by taking a 0.5-ml sample which was placed in a test tube and boiled for 5 min. After cooling in an ice bath, 5 ml of peroxidase-glucose oxidase reagent was added and the tube was incubated at 40°C for 30 min. The absorbance was measured at 450 nm against a blank (0.5 ml buffer plus peroxidase-glucose oxidase reagent). The substrate control used 1.0 ml of buffer in place of enzyme. Glucoamylase activity was determined from the absorbances by using the equation: glucoamylase units per milliliter = [(test − control)/standard] × 1.667D, where D is the enzyme dilution factor, and 1.667 is the conversion factor to give units defined as the amount of enzyme that will liberate 1 μmol of glucose in 10 min at 40°C when 25 mg of starch is present. The standard is the same as that used in the Sigma peroxidase-glucose oxidase assay. Enzyme activities which resulted in absorbances of >0.73 were no longer linear over the 10-min reaction and required dilution before the assay.

SDS-gel electrophoresis. A 10% polyacrylamide slab gel was prepared by mixing 10 ml of 30% acrylamide containing 0.8% bisacrylamide, 7.5 ml of 1.5 M Tris-hydrochloride buffer, (pH 8.8), and 12.2 ml of deionized water. After degassing, 0.3 ml of 10% sodium dodecyl sulfate (SDS), 0.3 ml of ammonium persulfate (75 mg ml^{-1}), and 15 μl of tetraethylenediamine were added, and this mixture was used to prepare a 1-mm-thick slab gel. The 3.2% stacking gel was prepared as above except that 3.2 ml of acrylamide, 19.2 ml of water and 0.25 M Tris-hydrochloride buffer (pH 6.8) were used. The ratio of the stacking gel to the running gel was 1:6. The running buffer (pH 8.3) consisted of 14.4 g of glycine, 3.0 g of Tris, 10 ml of 10% SDS, and deionized water to make 1 liter.

The enzyme samples (1 ml) were precipitated with cold trichloroacetic acid and centrifuged at 10,000 × g in a Beckman Microfuge. The pellets were washed twice without resuspension with 95% ethanol at −20°C and then dissolved in 5 μl of 1% ammonium hydroxide. To each sample was added 20 μl of 10% SDS, 25 μl of 0.25M Tris-hydrochloride (pH 6.8), 10 μl of 75% sucrose, and 5 μl of 0.04% bromophenol blue. Samples were boiled for 2 min before 50 μl of each was applied to the gel.

High- and low-molecular-weight protein standards (Bio-Rad Laboratories) were similarly treated and applied to the gel. A voltage of 300 V at 8.5 mA was applied for 1 h and then reduced to 150 V at 8.25 mA for 4 h. The gel was stained for 2 h at 30°C with Coomassie blue (0.2% in 375 ml of methanol–250 ml of glacial acetic acid–1,373 ml of water). The gel was destained for 6 h with the same solution without the dye.

Chromatography of hydrolysis products. The enzymatic action on soluble starch and pullulan (Sigma) was determined after 6 h at 30°C by paper chromatography. Samples (15 μl) were spotted onto Whatman no. 1 paper, and triple-ascent chromatography was done at 25°C with a 2:2:3 water–pyridine–n-butanol solvent system. The chromatogram was developed using the silver nitrate dip described by Brewer et al. (1).

Total protein. Protein was determined by the method of Lowry et al. (4), using egg albumin to prepare the standard curve.

RESULTS

Enzyme purification. Two major proteins were isolated from the crude enzyme preparation after ion-exchange chromatography (Fig. 1). Both peaks possessed "glucoamylase" activity, but only the smaller peak had α-amylase activity. The small amounts of glucose released from

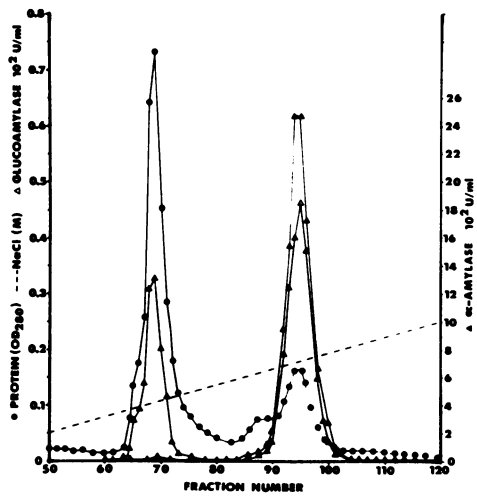

FIG. 1. Elution profile after ion-exchange chromatography.

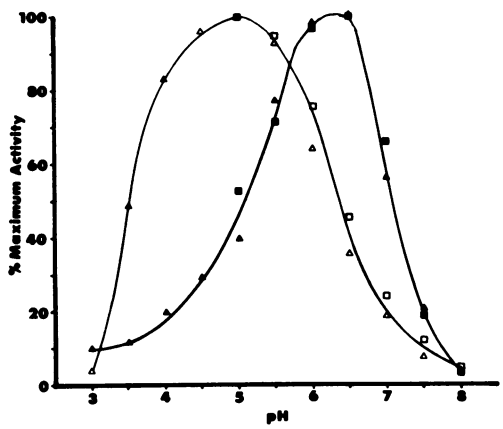

FIG. 2. Optimum pH for α-amylase and glucoamylase. Solid symbols, α-amylase; open symbols, glucoamylase; △ and ▲, McIlvaine citrate phosphate buffer; □ and ■, 0.1 M sodium phosphate buffer.

starch by high levels of α-amylase activity would result in the apparent glucoamylase activity as glucose liberation is the basis of the assay.

Each peak was further purified by gel filtration through Sephadex G-150. α-Amylase was separated from two contaminating proteins, neither of which had any amylolytic activity. Glucoamylase was separated from a single contaminating protein which had no amylolytic activity. Glucoamylase eluted soon after the void volume, indicating a greater molecular weight than that of α-amylase.

A summary of results from the purification of α-amylase is given in Table 1. A 10.8-fold purification of the α-amylase was achieved with a yield of 17.1% of the original activity. Because of the interference of the α-amylase in the glucoamylase assay, the purification and yield of glucoamylase from the original enzyme preparation could not be determined; however, values are given in parentheses based on the activity found after the initial isolation following ion-exchange chromatography. The extensive loss of protein during ultrafiltration is probably due

TABLE 1. Enzyme purification summary

Purification step	Enzyme	Total vol (ml)	Activity (U/ml)	Protein (mg/ml)	Sp act (U/mg of protein)	Total activity[a] (U)	Yield[a,b] (%)	Purification[b]
Crude extract	α-Amylase	950	187	0.039	4,795	177,650	100	1
	Glucoamylase	950	5.4	0.039	138	5,130	100	1
Ultrafiltration I	α-Amylase	31	5,598	0.67	8,355	173,538	97.6	1.74
	Glucoamylase	31	158.3	0.67	236.3	4,907	95.6	1.71
Ion-exchange pooled fractions	α-Amylase	31.5	1,327	0.093	14,269	64,790	36.5	2.98
	Glucoamylase	28.0	15.9	0.329	48.3	690	(100)	(1)
Ultrafiltration II	α-Amylase	2.0	19,309	1.04	18,566	59,858	33.7	3.87
	Glucoamylase	3.0	139.7	2.41	58.0	649	(94.1)	(1.2)
Gel filtration pooled fractions	α-Amylase	17.5	1,301	0.025	52,040	30,357	17.1	10.8
	Glucoamylase	10.5	21.9	0.071	308.4	276	(40.0)	(6.4)

[a] Extrapolated to activity present if total recovered volumes from each ultrafiltration step were used in subsequent purification steps (i.e., 20 of 31 ml from ultrafiltration I and 1.5 of 2.0 ml of α-amylase and 2.5 of 3.0 ml of glucoamylase from ultrafiltration II were used).

[b] Due to interference of α-amylase in the glucoamylase assay, purification and yield of separated glucoamylase cannot be compared with the mixed enzymes. Values in parentheses are therefore relative to ion-exchange-purified glucoamylase.

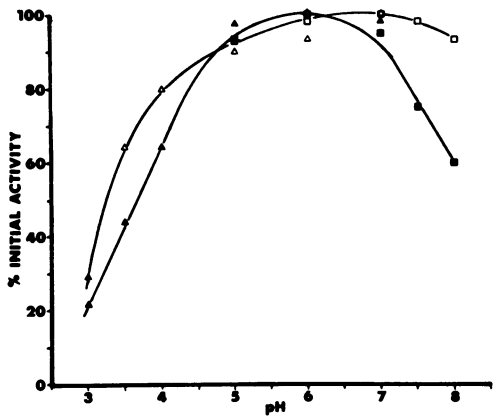

FIG. 3. pH stability of α-amylase and glucoamylase. Solid symbols, α-amylase; open symbols, glucoamylase; △ and ▲, McIlvaine citrate phosphate buffer; □ and ■, 0.1 M sodium phosphate buffer.

to denaturation of some protein by the agitation mechanism of the Millipore Hi-flux ultrafiltration system.

Enzyme characterization. The pH optima were 6.3 for α-amylase and 5.0 for glucoamylase (Fig. 2). α-Amylase was more sensitive to changes in pH as <80% activity remained at pH 5.5 and <70% activity remained at pH 7.0. Glucoamylase retained <80% activity at pH values of between 4.0 and 5.8, but was less active above pH 6.0.

The pH stability of the enzymes was tested after 24 h at 30°C at each pH. The pH was adjusted to 6.0 for α-amylase and 5.0 for glucoamylase with 1.0 N HCl or NaOH before the residual activity was assayed under standard conditions. Glucoamylase remained stable over a wide range, with >80% activity retained between pH 4.0 and 8.0 (Fig. 3). α-Amylase was stable over a narrower range from pH 4.4 to 7.5.

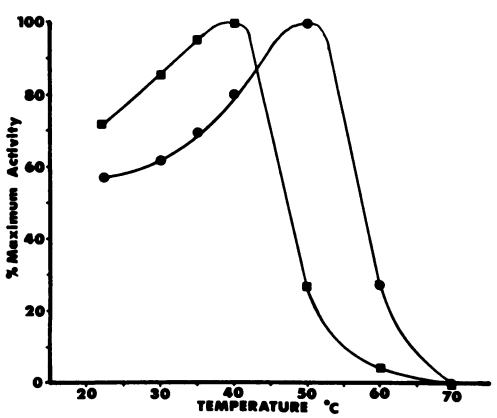

FIG. 4. Optimum temperature for α-amylase and glucoamylase. Symbols: ■, α-amylase; ●, glucoamylase.

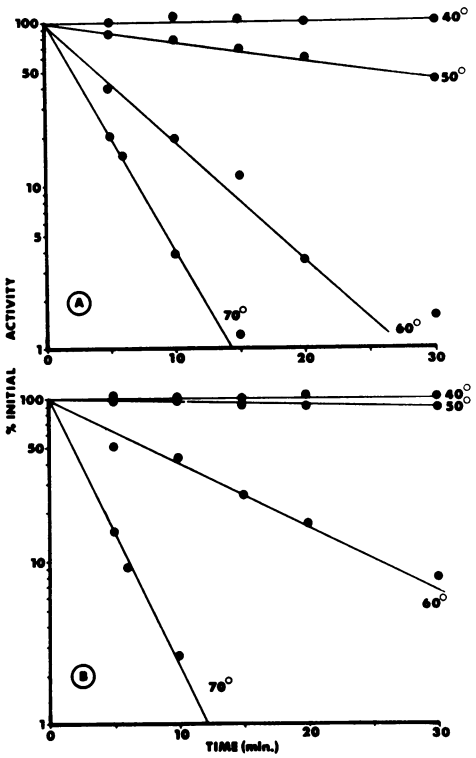

FIG. 5. Thermal inactivation of α-amylase (A) and glucoamylase (B).

The optimum temperatures were 40°C for α-amylase and 50°C for glucoamylase (Fig. 4). Thermal inactivation was determined at 40, 50, 60, and 70°C after exposure to each temperature for from 5 to 30 min. Residual activities were assayed at 40°C and were compared with the initial activity present before heat treatment (Fig. 5). The buffer used during heating was 0.05 M KH_2PO_4-NaOH at pH 6.0 (α-amylase) and pH 5.0 (glucoamylase). Protein contents of the enzyme preparations were 0.50 μg ml^{-1} (α-amylase) and 2.84 μg ml^{-1} (glucoamylase). Both enzymes were stable after 30 min at 40°C. Glucoamylase retained 88% of the initial activity after 30 min at 50°C, whereas α-amylase retained only 44%. Both enzymes were rapidly inactivated at 60°C.

The K_m values, determined from Lineweaver-Burk plots, for soluble starch were 0.364 mg ml^{-1} for α-amylase and 12.67 mg ml^{-1} for glucoamylase. The K_m for maltose was 0.72 mM for glucoamylase.

The results of SDS-gel electrophoresis are shown in Fig. 6 and Table 2. α-Amylase yielded one major protein band (molecular weight,

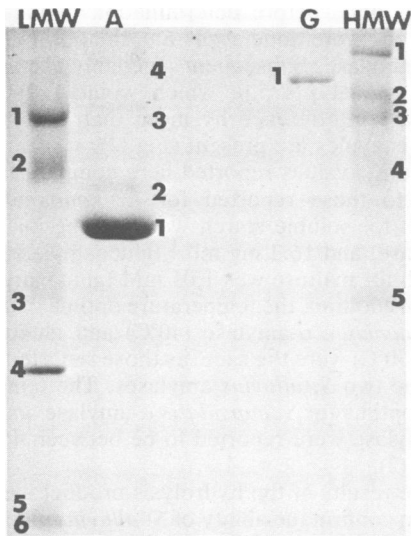

FIG. 6. SDS-polyacrylamide gel electrophoresis. Abbrevations: LMW, low-molecular-weight standard; HMW, high-molecular-weight standard; A, α-amylase; G, glucoamylase. Band numbers refer to Table 2.

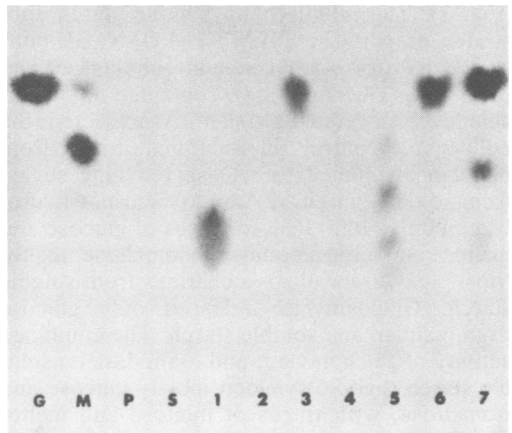

FIG. 7. Paper chromatography of hydrolysis products. Abbreviations: G, glucose; M, maltose; P, pullulan; S, soluble starch. 1, Pullulan plus pullulanase; 2, pullulan plus α-amylase; 3, pullulan plus glucoamylase; 4, soluble starch plus pullulanase; 5, soluble starch plus α-amylase; 6, soluble starch plus glucoamylase; 7, soluble starch plus α-amylase and glucoamylase.

61,900 ± 700) and three minor bands (molecular weights, 69,800, 119,000 and 171,300). Glucoamylase yielded a single protein band (molecular weight 155,000 ± 3,000). Molecular weights were calculated by using the standard curves for low and high molecular weights as determined by linear regression of the relative mobilities of the protein standards.

The hydrolysis products of pullulan and soluble starch were analyzed by paper chromatography after 6-h treatments with pullulanase (Sigma) and *S. alluvius* α-amylase and glucoamylase

TABLE 2. Relative mobilities in SDS-gel electrophoresis

Sample	Band[a] no.	Mol wt	Relative mobility (R_f)
High-molecular-weight standard	1	200,000	0.073 ± 0.002
	2	116,250	0.150 ± 0.004
	3	92,500	0.188 ± 0.004
	4	66,200	0.291 ± 0.008
	5	45,000	0.526 ± 0.002[b]
Glucoamylase	1	155,000 ± 3,000[c]	0.115 ± 0.004
Low-molecular-weight standard	1	92,500	0.201 ± 0.002
	2	66,200	0.291 ± 0.008
	3	45,000	0.521 ± 0.008
	4	31,000	0.662 ± 0.004
	5	21,500	0.906 ± 0.004
	6	14,400	0.949 ± 0.004
α-Amylase	1	61,900 ± 700[d]	0.406 ± 0.008
	2	69,800 ± 300[d]	0.320 ± 0.004
	3	119,000[c]	0.175
	4	171,300[c]	0.087

[a] Band numbers refer to Fig. 6.
[b] Omitted from linear regression analysis of high-molecular-weight standard.
[c] Calculated from high-molecular-weight standard curve.
[d] Calculated from low-molecular-weight standard curve.

(Fig. 7). The pullulan and soluble starch substrates, prepared in 0.05 M KH_2PO_4-NaOH buffer, pH 6.0 (for α-amylase and pullulanase) and pH 5.0 (for glucoamylase), contained no detectable levels of reducing sugars. As expected, the pullulanase control released maltotriose from pullulan and failed to release reducing sugars from soluble starch. α-Amylase did not hydrolyze pullulan but released traces of glucose and maltose and large amounts of isomaltose, maltotriose, and larger oligosaccharides from soluble starch. Glucoamylase released only glucose from pullulan and soluble starch. The combined activity of glucoamylase and α-amylase on soluble starch (pH 5.5) yielded mostly glucose and isomaltose, with traces of maltose and maltotriose.

DISCUSSION

S. alluvius produces both extracellular α-amylase and extracellular glucoamylase. *S. castellii* has recently been found to produce α-amylase and two forms of glucoamylase, with all three enzymes having pH and temperature optima of 6.0 and 60°C, respectively (Oteng-Gyang, Ph.D. thesis). Oteng-Gyang reported that glucoamylase eluted after α-amylase (the reverse order from that of *S. alluvius*). As with the *S. castellii* glucoamylase II, the glucoamylase of *S. alluvius* was the major protein peak after ion-exchange chromatography. The pH optimum of 6.3 found for the α-amylase from *S. alluvius* agrees with that reported for *S. castellii* α-amylase; however, the pH optimum of 5.0 for the glucoamylase of *S. alluvius* is 1 pH unit less than that for *S. castellii* glucoamylases. Both *S. alluvius* α-amylase and *S. alluvius* glucoamylase appear to be less heat resistant and to have lower temperature optima than the 60°C reported for the *S. castellii* α-amylase and glucoamylases.

While this work was being written up (Wilson, Ph.D. thesis), the isolation and partial characterization of α-amylase from *S. alluvius* UCD 54-83 was reported in a preliminary communication which listed the pH optimum at 4.0 to 6.0, the optimum temperature at approximately 45°C, and the molecular weight at 52,000 as determined by SDS-gel electrophoresis (Nasim et al., Proc. 3rd Bioenergy R and D Semin., Ottawa, Ontario, Canada, abstr. no. P3-3, p. 141, March, 1981). Although the optimum temperature agrees with our results, little experimental protocol was given to allow for an explanation of the lower pH optimum and much lower molecular weight than the 61,900 ± 700 reported here for *S. alluvius* α-amylase (see below).

Interference of α-amylase in the glucoamylase assay reported in column chromatography was probably intensified because the glucoamylase assays done before determination of the optimum pH were done at pH 6.0, the optimum pH of α-amylase. Subsequent glucoamylase assays were run at pH 5.0, which would reduce α-amylase interference by more than 50% when both enzymes are present (Fig. 2).

The K_m values reported here compare favorably to those reported for *L. kononenkoae*, which for soluble starch were 2.7 mg ml^{-1} (α-amylase) and 16.2 mg ml^{-1} (glucoamylase) and which for maltose was 1.05 mM (glucoamylase) (7). In addition, the temperature optima of the *L. kononenkoae* α-amylase (40°C) and glucoamylase (50°C) were the same as those reported here for the two *S. alluvius* amylases. The temperature optima for *S. capsularis* α-amylase and glucoamylase were reported to be between 40 and 50°C (2).

The results of the hydrolysis product identification confirm the ability of *S. alluvius* glucoamylase to hydrolyze both the α(1-4)- and the α(1-6)-glycosidic linkages in pullulan as revealed by the release of substantial glucose from pullulan (Fig. 7). Similarly, the endohydrolysis action of the α-amylase was confirmed by the release of maltose, maltotriose, and other oligosaccharides from soluble starch. The α-amylase was also able to release glucose, albeit slowly, from soluble starch.

The molecular weight of *S. alluvius* glucoamylase, estimated to be 155,000 ± 3,000, was considerably larger than the 81,500 reported for *L. kononenkoae* (7), but within the range of molecular weights of five forms of glucoamylase isolated from *Saccharomyces diastaticus* ranging from 98,500 to 305,000 after SDS-gel electrophoresis (J. A. Erratt, Ph.D. thesis, University of Western Ontario, London, Canada, 1980). No other values for the molecular weights of glucoamylases from the genus *Schwanniomyces* have been reported to date. The molecular weight of *S. alluvius* α-amylase was 61,900 ± 700, assuming that the major protein band in Fig. 6 possessed the α-amylase activity. None of the other three proteins isolated from the α-amylase preparation had the same molecular weight as the glucoamylase, which indicates that the apparent glucoamylase activity found with the α-amylase in Fig. 1 and 2 was, in fact, due to the ability of α-amylase to liberate glucose from soluble starch. *L. kononenkoae* α-amylase has a molecular weight of only 38,000 (7). An α-amylase of *Aspergillus niger* had a molecular weight of 61,000 (5).

The demonstration of both α-amylase and glucoamylase in *S. alluvius*, an alcohol-producing yeast, has stimulated genetic work on intraspecific protoplast fusion to create polypoloid industrial strains (J. J. Wilson, G. G. Khachatourians, and W. M. Ingledew, Mol. Gen. Gen-

et., in press). A derepressed hyperproducing mutant for α-amylase synthesis has also been isolated (J. Wilson, Ph.D. thesis; J. J. Wilson, G. G. Khachatourians, and W. M. Ingledew, Biotechnol. Lett., in press).

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LITERATURE CITED

1. **Brewer, J. M., A. J. Pesce, and R. B. Ashworth.** 1974. Experimental techniques in biochemistry, p. 340. Prentice-Hall, Inc., Englewood Cliffs, N.J.
2. **Ebertova, H.** 1966. Amylolytic enzymes of *Endomycopsis capsularis* II. A study of the properties of isolated α-amylase, amyloglucosidase, and maltose-transglucosidase. Folia Microbiol. (Prague) **11**:422–438.
3. **Fukumoto, J., Y. Tsujisaka, and M. Araki.** 1960. Studies of amylase of *Endomycopsis*. Kagaku To Kogyo (Osaka) **34**:423 (Chem. Abstr. **55**:24,917, 1961.)
4. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
5. **Minoda, Y., M. Arai, Y. Torigoe, and K. Yamada.** 1968. Acid-stable α-amylase of black aspergilli. III. Separation of acid-stable α-amylase from the same mold amylase preparation. Agric. Biol. Chem. **32**:110–116.
6. **Moulin, G., and P. Galzy.** 1977. Study of an amylase and its regulation in *Lipomyces starkeyi*. Agric. Biol. Chem. **43**:1165–1171.
7. **Spencer-Martins, I., and N. van Uden.** 1979. Extracellular amylolytic system of the yeast *Lipomyces kononenkoae*. Eur. J. Appl. Microbiol. Biotechnol. **6**:241–250.
8. **Stepanov, A. I., V. P. Afanas'eva, G. V. Zaitseva, A. P. Mednokova, and I. B. Lupandina.** 1975. Regulation of the biosynthesis of the enzymes of the amylolytic complex of *Endomycopsis fibuligera* strain in 20–9. Prikl. Biokhim. Mikrobiol. **11**:682–685.
9. **Wickerham, L. J., L. B. Lockwood, O. G. Pettijohn, and G. E. Ward.** 1944. Starch hydrolysis and fermentation by the yeast *Endomycopsis fibuliger*. J. Bacteriol. **48**:413–427.