Phytochemistry, 1972, Vol. 11, pp. 1673 to 1676. Pergamon Press. Printed in England.

SHORT COMMUNICATION

HOMOGENEOUS α-AMYLASE FROM MALTED BARLEY*

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(*Received* 20 *July* 1971, *in revised form* 6 *November* 1971)

Abstract—α-Amylase (α-1,4-gluconoglucohydrolase, E.C. 3.2.1.1.) from malted barley was purified by gel filtration. The homogeneity was determined by gel electrophoresis and sedimentation velocity measurements. This enzyme contains a carbohydrate moiety that is bound to the protein. The carbohydrate moiety contains glucosamine, galactose, xylose, fucose and mannose and appears to act as a protective agent against denaturation and proteolysis.

INTRODUCTION

THE α-AMYLASE (E.C. 3.2.1.1) of the mold *Aspergillus oryzae* has been particularly well studied,[1,2] as has that from saliva, blood, urine and serum of man and certain animals.[1–4] Purified preparations contain from one to five electrophoretically different components.[4–7] The presence of multiple forms of α-amylase has also been demonstrated in higher plants.[8,9] Greenwood and Milne[8] reported that α-amylases from ungerminated and germinated cereal grain had similar modes of action and molecular size. Olered and Jönsson[9] showed that there are two forms of α-amylase in wheat. Schwimmer and Balls[10] prepared α-amylase from barley malt and Waldschmidt-Leitz and Sigrist[11] from green malt, but the homogeneities of these preparations were not determined.

This paper reports the purification of α-amylase from malted barley, using a modification of the procedure of Schwimmer and Balls.[10] Some of the physical and chemical properties of barley α-amylase are described.

RESULTS

Chromatography on Bio-Gel P-100

The eluant obtained from the starch column[10] was treated with solid $(NH_4)_2SO_4$ (0·35 g/ml). The precipitated protein was centrifuged at 2000 *g* for 20 min at 0°, dialysed against 1 mM acetate pH 5·5 with 10 mM $CaCl_2$, and placed on a 60 × 2·5 cm Bio-Gel P-100

* Journal Article 2262 of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma. This research was supported by Grant Number AM 13489 from the National Institutes of Health, and was carried out with the technical assistance of Miss Su Wang.

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column equilibrated with the same buffer at 5°. Three-ml fractions were collected at a flow rate of 17 ml/hr cm^2. The most active fractions (15–25) were pooled and precipitated with $(NH_4)_2SO_4$ (0·35 g ml), resuspended and dialysed against 1 mM acetate buffer pH 5·5 containing 1 mM $CaCl_2$. Vacuum dialysis was used to concentrate the protein. The purification factor was usually in the range of 50–60 fold from our designated, crude extract.

Homogeneity

The purified sample from the Bio-Gel column was analyzed in polyacrylamide gel disc gel electrophoresis, where a single band was obtained, and by analytical ultracentrifugation (Fig. 1). The single symmetrical pattern sedimented at an apparent sedimentation coefficient value of 3·92 S and the mol. wt. was calculated to be 50 000. The protein had the same specific activity after centrifugation as before.

Carbohydrate Determination

The anthrone method according to Roe[12] was used for the determination of total carbohydrate. The total carbohydrate content was determined as glucose. Amino sugars were determined on the amino acid analyzer.[13] For identification of the neutral sugars and amino sugars, 7·5 mg of purified α amylase protein was hydrolyzed with 0·8 ml of 2 N H_2SO_4 for 4 hr in a sealed tube. The solution was evaporated under vacuum and the solid residue was dissolved in 2 ml water. This hydrolysate was passed through a Dowex 50 X 4 200–400 H^+ column that was placed on top of a Dowex 1 × 8 200–400 formate column and eluted with 80 ml of water. The effluent contained the neutral sugars and the Dowex 80 column was washed with 12 ml 2 N HCl to obtain the amino sugars. The neutral sugars were analyzed

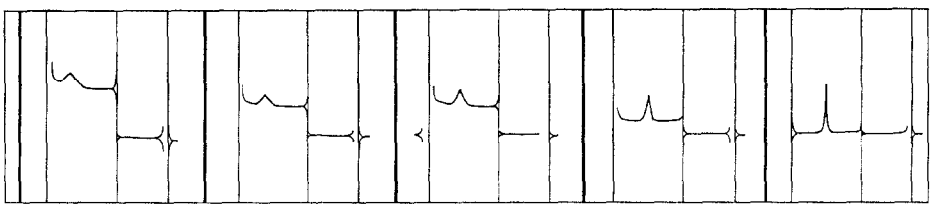

FIG. 1. THE SCHLIEREN PATTERNS OF PURIFIED α-AMYLASE SEDIMENTARY FROM RIGHT TO LEFT.
The details were taken 0°, 5, 7, 11 and 15 min after the rotor had attained maximum speed. The details of the run are in the text.

by paper chromatography using pyridine–EtOAc–HOAc–H_2O (20:30:21:7).[14] The chromatograms were developed with $AgNO_3$ and fixed with X-ray fixer according to Benson *et al.*[15] The neutral sugars were also analyzed by a Technicon Carbohydrate Analyzer as described by Kesler.[16] Glucose, galactose, mannose, fucose and xylose were detected.

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DISCUSSION

The α-amylase from barley was isolated by a comparatively simple scheme and an apparently homogenous protein was obtained (Fig. 1). The carbohydrate analysis shows 3·0% of the enzyme as carbohydrate (glucose units) and < 1% as glucosamine. The fuchsin stain shows good carbohydrate pattern in disc gels before Step 5 (Table 1) initially associated

TABLE 1. PURIFICATION OF α-AMYLASE FROM MALTED BARLEY

Step	Procedure	Volume (ml)	Protein (mg/ml)	Conc. units (ml)	Total units ($\times 10^{-3}$)	Sp Act (units/mg)	Purification
1	Crude extract	2000	12·3	63·4	126	5·16	1·0
2	45% $(NH_4)_2SO_4$ saturation	100	10·2	64·9	6·49	6·37	1·2
3	Dialysis, lyophilization	40	23·8	530·0	16·20	22·27	4·3
4	Starch column	200	0·55	89·6	17·92	163·0	31·5
5	Bio-Gel-P-100	3	0·66	198·0	0·49	300·0	58·0

with the purification process; however, the purified α-amylase gives very weak carbohydrate staining with the fuchsin. There is thus some dialyzable carbohydrate non-covalently bound to the enzyme, the presence of which in the interstices of the protein would suppress the accessibility of sites of hydration by water molecules which in turn would prevent swelling of the protein.

This carbohydrate moiety may also protect the protein from proteolysis. We have been able to detect inhibition of α-amylase activity by trypsin after treatment of the enzyme with periodate; but, with the carbohydrate unit intact trypsin does not destroy enzyme activity (Table 2). Periodate treatment alone causes a 65% inhibition in activity, which is not overcome by the presence of calcium. Thus periodate may also attack the peptide moiety. Further studies of this phenomenon are in progress.

TABLE 2. PERIODATE INHIBITION STUDIES ON α-AMYLASE

Treatment*	% Activity
None	100
Periodate + Ca^{2+}	35
Periodate + Trypsin + Ca^{2+}	0
Periodate + Trypsin + EDTA	0
Trypsin + Ca^{2+}	95
Trypsin + EDTA	45

* The treatments consisted of 20 μmol periodate, 10 μmol EDTA, 10 μM $CaCl_2$ 1·7 μg trypsin protein and 10 μg α-amylase protein in 1 ml of 10 mM acetate buffer pH 5·5. After 6 hr incubation 0·5 m of the medium was removed and assayed according to the procedure of Experimental.

EXPERIMENTAL

Protein concentration was measured by the method of Lowry *et al.*[17] Malted barley was obtained as a gift from Kurth Malting Company, Milwaukee, Wisconsin, Fleischmann Malting Company, Minneapolis and Minnesota Malting Company, Connon Falls, Minn.

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Enzyme preparation. A 400-g quantity of freshly ground malted barley was extracted with 2 l. of 0·1 M NaOAc buffer pH 6·0 with 0·01 M $CaCl_2$. The material was heated at 45° for 30 min with constant stirring followed by heating at 70° for 15 min. The suspension was rapidly cooled in an ice-bath and the precipitated solids were removed by centrifugation at 555 *g* followed by filtration. The purification steps are shown in Table 1. The filtrate was designated as the crude extract (Step 1). $(NH_4)_2SO_4$ (45% saturation, 277 g/l.) was added to the filtrate and the precipitate was collected by centrifugation at 2000 *g* for 20 min at 0° (Step 2). The precipitate was resuspended in 100 ml of 0·01 M acetate buffer pH 6·0 and 0·01 M $CaCl_2$, dialyzed and then lyophilized (Step 3). This material was then dissolved in 40 ml H_2O saturated with $CaSO_4$ and added to 40 ml of 80% EtOH. The 40% alcoholic solution of amylase was absorbed onto starch at room temp. and eluted according to the method of Schwimmer and Balls.[11] The active fractions were pooled and concentrated by vacuum dialysis (Step 4). This material was then chromatographed on a 60 × 2·5 cm Bio-Gel P-100 column.

α-Amylase activity. In this method, a modification of that used by Schuster and Gifford,[18] activity is expressed as mg of starch hydrolyzed/min as determined by the starch–iodine color. A stock solution of approximately 1·0 mg/ml of soluble starch in 1 mM acetate buffer pH 4·8 was used in which an absorbance (A_{620}) of 0·80 is attained with the starch–iodine color. One ml of starch solution in 10 mM acetate buffer pH 4·8 with 10 mM $CaCl_2$ was placed in a test tube at 30° and 1 ml of the properly diluted enzyme solution in the same buffer was added with vigorous mixing. After 1 min, the enzyme reaction was stopped by the addition of 1 ml of 5 mM KI_3 in 0·05 N HCl. This mixture was then diluted to 8 ml and A_{620} was determined. One unit of activity is defined as the mg of starch hydrolyzed per minute and specific activity is defined as units/mg of protein.

Purity of enzyme: Electrophoresis. Disc gel electrophoresis was performed using 0·1 M phosphate buffer pH 7·2 or 0·1 M phosphate buffer at pH 7·2 with SDS. Gels were prepared with polyacrylamide concn varying from 5–15%. Protein samples, 10–100 μg, were polymerized into sample gels and electrophoresis was performed for 90 min using 3 mA/tube. Carbohydrate components were stained with fuchsin-sulphite dye after removing the SDS according to Glossman.[19] This procedure involves removing SDS by washing the gels with 40% MeOH–7% HOAc for 24 hr. The gels were stained with aniline blue black or Coomassie Blue for one hr followed by diffusion destaining with 7% HOAc. α-Amylase activity in the gels was determined by slicing the gels into 1 mm sections and assaying for starch hydrolyzing activity.

Sedimentation velocity measurements. Purified enzyme solution was concentrated to 1 mg/ml of protein by vacuum dialysis against 0·01 M acetate buffer pH 4·8 containing 0·01 M $CaCl_2$ and 0·15 M KCl. Sedimentation velocity experiments were conducted using the synthetic boundary cell at a constant temp. of 20° with a rotor speed of 59 780 rpm using a Spinco Model E analytical Ultracentrifuge. Fifteen photographs were obtained at time intervals of 4 min.

The homogeneity of sedimentation of the purified enzyme solution was evaluated by a detailed boundary analysis of the sedimentation velocity experiments. The apparent diffusion coefficient was calculated for each picture taken during the experiment by the height area method and plotted against time to determine linearity.

Acknowledgement—The author thanks Dr. R. E. Koeppe for reading this manuscript.

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Key Word Index—Hordeum vulgare; Gramineae; malted barley; α-amylase activity; bound carbohydrate.