Separation of α- and β-Amylase Enzymes from Barley Malt by Ion-Exchange Chromatography[1]

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ABSTRACT

Active forms of barley-malt α- and β-amylases were separated from one another by ion-exchange chromatography on carboxymethyl cellulose. Each enzyme contained two components. The enzymes were also separated on ion-exchange cellulose by a stepwise elution technique. The identity of each enzyme was confirmed by analyzing the hydrolysis products of amylose by α-amylases and the hydrolysis products of starch by β-amylases. A 70°C. heat treatment completely inactivated β-amylase in extracts of barley malt, but the chromatographic properties of the α-amylases were not altered.

α-Amylase is widely distributed in nature and has been isolated and purified by a variety of techniques from higher plants, mammals, bacteria, and fungi (1). β-Amylase, however, occurs only in higher plants, and cereal grains are an important source of the enzyme. The removal of one amylase from preparations containing

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both α- and β-amylases is a major problem when purifying these enzymes from cereal sources. In most methods used for the purification of α-amylase, the crude extract is heated to 70°C. to preferentially inactivate the heat-labile β-amylase enzymes (2). To remove α-amylase from preparations of β-amylase, the crude extract is maintained at pH 3.6 and low temperature for several hours; α-amylase is acid-labile and is preferentially inactivated (3).

These preferential inactivation techniques are not entirely satisfactory. There is evidence that the low-pH treatment is not efficient if large amounts of α-amylase are present (4). In addition, the effect of these rather severe treatments on the physical properties of the cereal amylases has not been investigated. By use of preferential inactivation techniques, only one type of amylase enzyme at a time is obtained from extracts containing both α- and β-amylase enzymes. A method for separating and recovering each enzyme in active form from the same extract would certainly be advantageous.

This report describes methods for separating active forms of the amylase components of barley malt by ion-exchange chromatography on carboxymethyl cellulose (CMC). Gradient elution (5) and stepwise elution procedures were used. α- and β-Amylases were completely separated from one another, and each enzyme was shown to contain two components. The identity of each α-amylase was confirmed by studying its action pattern on amylose, and the β-amylases were similarly identified by their action on starch.

MATERIALS AND METHODS

Sample of Barley Malt

Malt was prepared from Conquest barley using the steeping and germination units described previously (6). Green malt was removed from the germination unit after 144 hr., frozen immediately, and freeze-dried to a moisture content of 4%. The rootlets were removed, and the malt was ground in a Wiley mill through a 1-min. sieve.

Extraction of Barley Malt

Ground malt (75 g.) was extracted with 210 ml. of acetate buffer (0.02M, pH 4.75, containing 0.001M calcium chloride ($CaCl_2$) and 0.001M 1-thioglycerol) for 5 min. in a Waring Blendor at 5°C. The extract was centrifuged (7,000 X g, 15 min.), and the supernatant solution was filtered through glass wool. The residue was re-extracted with 135 ml. of the same buffer and centrifuged as before. The clarified supernatant solutions were combined and dialyzed against further portions of acetate buffer at 5°C. Precipitated material was removed from the dialyzed extract by centrifugation (7,000 X g, 15 min.), and 100-ml. portions of the supernatant solutions were partitioned on CMC columns.

Heat Treatment of Malt Extracts

Ground barley malt (75 g.) was extracted as already described. Half of the extract was heated at 70°C. for 15 min., cooled quickly to room temperature, and centrifuged (7,000 X g, 15 min.). Both the heated and unheated extracts were prepared for chromatography and partitioned on CMC by gradient elution, as described below. Amylase activities and protein nitrogen determinations were performed at all stages of the experiment.

Ion-Exchange Chromatography on CMC Columns

Gradient Elution. The fines from Whatman CM-32 cellulose were removed by decantation, and the residual cellulose was cycled through 0.5N NaOH and 0.5N HCl prior to equilibrating with 0.02M acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. A slurry of this cellulose was used to pack 2 × 90-cm. columns.

Extracts of barley malt were partitioned on CMC by means of a sodium-ion gradient produced by a four-chambered variable gradient device (7). Each gradient vessel contained 500 g. of degassed acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. The sodium-ion concentration of each gradient vessel was as follows:

Gradient vessel	4	3	2	1	
Sodium-ion concentration (M)	0.75 →	0.02 →	0.15 →	0.02 →	column

Chromatography was performed at 5°C. and at a flow rate of 1.25 ml. per min. Column fractions were collected at 15-min. intervals.

Stepwise Elution. To develop a method for large-scale separations (150 g. of malt) of barley-malt α- and β-amylases, samples of these enzymes which had been partially purified by gradient elution on CMC (Fig. 1) were used to determine effective fractionation conditions. The following portions of the column effluent were pooled: 600 to 675 ml. (α-amylase I); 950 to 1,050 ml. (β-amylase III); 1,200 to 1,350 ml. (β-amylase IV); and 1,600 to 1,900 ml. (α-amylase II). The enzyme solutions were dialyzed for 24 hr. at 5°C. against 0.01M acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. Each dialyzed enzyme solution was washed carefully into separate 6 × 1.5-cm. columns of Whatman CM-32 cellulose equilibrated with 0.01M acetate starting buffer. Portions (30 ml.) of acetate buffer containing increasing concentrations of Na^+ (from 0.01 to 0.17 moles per liter in 0.01-mole increments) were passed through the columns. The effluent obtained with each buffer was assayed for amylase activity and the Na^+ concentration required to elute each amylase was determined.

Determination of Amylase Activities

β-Amylase activity of the column fractions was determined by the dinitrosalicylic acid (DNS) assay procedure (8) and is given in terms of the absorbance produced by 0.05 ml. of enzyme during 5 min. of incubation with 1% starch at 20°C. and pH 4.5.

α-Amylase activities were determined as described previously, using the β-limit dextrin of waxy maize starch as substrate (9). The α-amylase activities of column fractions are given in terms of (change in absorbance of a standard digest per ml. of enzyme solution) × 2.

Action Patterns of α- and β-Amylases

Aliquot samples of α-amylase enzymes (500 units of activity), obtained by partitioning extracts of barley malt on CMC, and purified further by ion-exchange chromatography (method to be published elsewhere) and selective precipitation with glycogen (10), were added to solutions of amylose (2 mg. per ml.) in acetate buffer (0.01M, pH 5.5, containing 0.001M $CaCl_2$). The samples were incubated at

room temperature and, after intervals of 5, 11, 20, 29, 49, and 69 hr., 5-ml. aliquots were removed, boiled for 2 to 3 min., frozen, and freeze-dried. Each sample was dissolved in 0.1 ml. of water and spotted on Whatman 3MM chromatography paper together with standard solutions of glucose (G_1), maltose (G_2), maltotriose (G_3), and maltotetraose (G_4). The samples were partitioned by descending chromatography for 16 hr. using ethyl acetate:pyridine:water (10:4:3) as solvent. Spots were developed by the method of Trevelyan (11).

Similarly, the two β-amylase enzymes that were obtained were further purified (method to be published elsewhere) and incubated for 30 min. with a 2% starch solution. The remaining starch was precipitated with 80% ethanol and removed by filtration. The digests were concentrated by rotary evaporation and analyzed by paper chromatography, as described above, and by ion-exchange chromatography on polystyrene resin (12).

Determination of Protein Nitrogen

The method of Mitcheson and Stowell (13) was used for digesting samples, and nitrogen was determined with Nessler reagent (14).

Amylose

Amylose was isolated from potato starch by the aqueous dispersion method previously described (15).

Maltodextrins

Maltodextrins were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of an extract of barley malt partitioned on CMC by gradient elution. Two α-amylase enzymes, referred to as α-amylase I and α-amylase II, were detected. In addition, two β-amylase enzymes were also found. These are referred to as β-amylases III and IV (5) to distinguish them from β-amylases I and II, which are found in extracts of barley (16), and which can also be fractionated on CMC.

The first three amylase enzymes were eluted with a shallow Na^+ gradient, but a much steeper gradient was required to elute α-amylase II as a compact peak. α-Amylase II is strongly adsorbed to CMC, and hence is the most basic of the four enzymes at pH 4.75. It should be emphasized that pure samples of the individual enzymes are not obtained after one chromatographic separation on CMC, but the system described is a useful first step in the separation and purification of these enzymes.

It is well established that α-amylase requires Ca^{++} to maintain full activity (17), and this is the reason for including 10^{-3}M $CaCl_2$ in the extraction and chromatography buffers. Before determining α-amylase activities of column effluents, it was necessary to dilute column fractions as much as 400 times. If these dilutions were made with water, the diluted fractions quickly lost α-amylase activity. When dilutions were made with 10^{-3}M $CaCl_2$, loss of α-amylase activity was very small over a period of 2 to 3 hr.

Thioglycerol (0.001M) was included in the extraction and chromatography buffers because of the unusual elution behavior of β-amylase enzymes in the

absence of reducing agents (5). The concentration of thioglycerol was kept low in order to have a minimum adverse effect on the DNS assay procedure (18) and to minimize extraction of "bound" β-amylase that is readily extracted by use of greater concentrations of thiols (16).

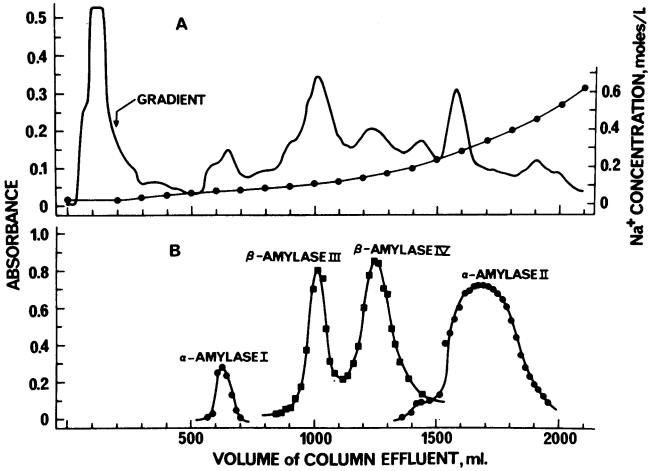

Fig. 1. Fractionation of barley-malt extracts by gradient elution chromatography on carboxymethyl cellulose. In A, solid line = absorbance of column effluent at 280 nm.; closed circles = Na^+ gradient. B = amylase activities.

The separation of α- and β-amylases into two components of each is real and is not caused by artifacts arising from experimental procedures or from proteolysis, as an extract that was divided into four equal portions and dialyzed for 1, 3, 6, and 10 days before gradient chromatography produced protein and amylase profiles similar to the chromatograms shown in Fig. 1. In addition, it was shown that separation of α- and β-amylases into two active components of each is not related to aggregation or dissociation of monomeric forms of the enzymes. When column fractions containing α-amylase I, α-amylase II, β-amylase III, and β-amylase IV were pooled separately and repartitioned on CMC, each enzyme was eluted in the same position as before (Fig. 1), and very little activity was lost during the process. This also eliminates the possibility that α-amylase I is a "break-through" portion of α-amylase II produced by overloading the CMC columns.

To confirm that the enzymes being studied were indeed α-amylases and not a form of glucoamylase or mixture of β-amylase and R-enzyme that would also hydrolyze the β-limit dextrin of waxy maize starch, the action pattern of purified samples of α-amylase on amylose was studied (Fig. 2). The chromatogram shows that, up to 11 hr. of incubation, small amounts of G_2, G_3, G_4, and G_5, and larger amounts of G_6 and higher maltodextins were produced. After 20 hr., G_7 was the largest dextrin found in the digests. The dextrin pattern remained stable for a period of 49 hr. Therefore, α-amylases I and II readily hydrolyze dextrins larger than G_7, but smaller dextrins are hydrolyzed much more slowly, if at all. These results are characteristic of cereal α-amylases.

The β-amylase enzymes were also further purified (method to be published

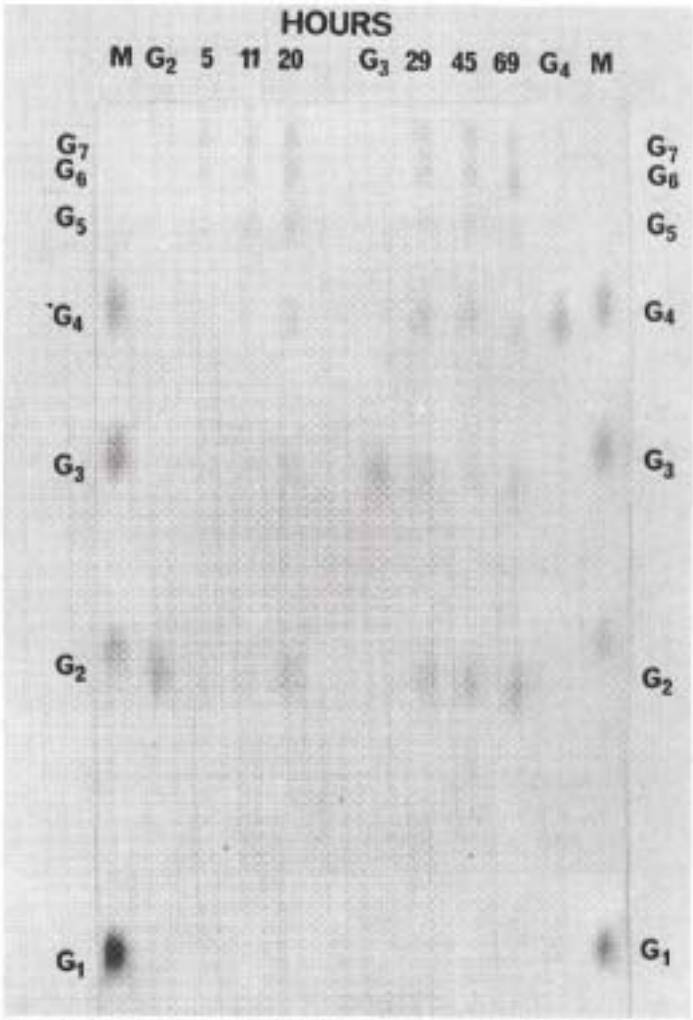

Fig. 2. Products of hydrolysis of amylose by barley-malt α-amylase I. Samples of the digest were removed at the times indicated, treated as described in the experimental section, and spotted on chromatography paper. G_1, G_2, G_3, etc., represent glucose, maltose, maltotriose, etc. G_2, G_3, and G_4 standards were also run on the chromatogram. M is a mixture of G_1, G_2, G_3, and G_4.

elsewhere) to eliminate traces of α-amylase and other carbohydrase enzymes. The end products of their hydrolysis of soluble starch were determined by paper chromatography. Only maltose was detected. This result was confirmed by ion-exchange chromatography as shown in Fig. 3 (12). Both enzymes hydrolyzed 58% of the starch to maltose. This is characteristic of the action of β-amylase on whole starch.

A large number of gradient-column chromatography runs on CMC are required to

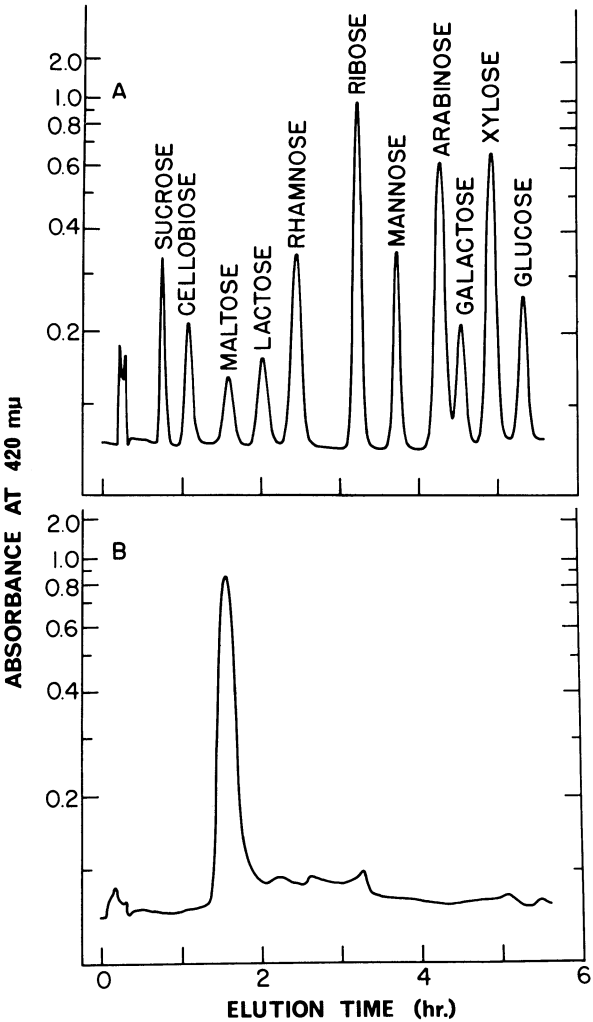

Fig. 3. Ion-exchange chromatography, on polystyrene resin, of the end products of starch hydrolysis by barley-malt β-amylases. A = Chromatogram of standard sugars; B = chromatogram of starch hydrolysis products.

obtain amylase enzymes in reasonable quantities. It is difficult to increase the scale of this technique and, at the same time, to maintain efficiency of separation. Therefore, conditions for the stepwise elution of these enzymes from CMC were determined. The results are shown in Fig. 4.

Samples of each malt enzyme, obtained by gradient elution, were applied to small columns of CMC and eluted with acetate buffer containing increasing concentrations of salt. The optimum salt concentrations for elution of the enzymes were found to be 0.04M for α-amylase I, 0.08M for β-amylase III, and 0.14M for β-amylase IV and α-amylase II.

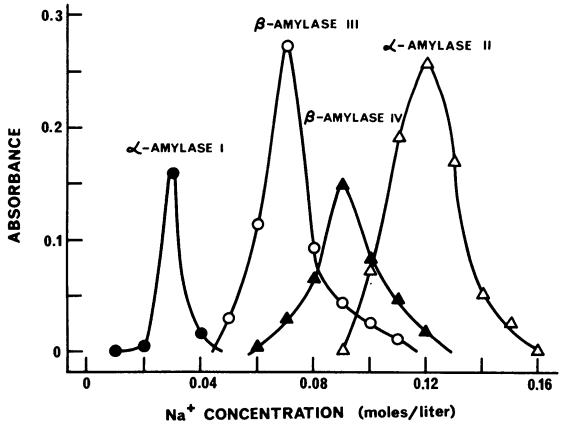

Fig. 4. Stepwise elution of barley-malt amylases on carboxymethyl cellulose. Each enzyme was eluted from a different column.

These salt concentrations were used in large-scale fractionations of barley-malt extracts to give samples of α-amylase I and β-amylase III. A mixture of β-amylase IV and α-amylase II was obtained. Complete separation of these two enzymes was then achieved by gradient elution on CMC using the normal Na^+ gradient.

The effect of a 70°C. heat treatment on the α-amylase enzymes of a malt extract is shown in Table I. The total α-amylase activity of the heated extract fell by 8%, but the specific activity increased slightly owing to preferential removal of other proteins. The specific activity of both extracts increased after dialysis, as a result of loss of amino acids and small peptides by dialysis and precipitation of inert proteins during dialysis. Total α-amylase activity decreased slightly during dialysis.

TABLE I. EFFECT OF 70°C. HEAT TREATMENT ON MALTED BARLEY α-AMYLASES

Treatment	Total α-Amylase Activity	Nitrogen mg.	α-Amylase Specific Activity
Extract before dialysis	3,480,000	130.3	26,600
Extract after dialysis	3,010,000	62.8	48,000
Heated extract before dialysis	3,190,000	104.2	30,600
Heated extract after dialysis	3,020,000	52.5	57,600
α-Amylase I from extract	124,584	12.8	9,770
α-Amylase II from extract	2,171,400	21.3	102,200
α-Amylase I from heated extract	105,028	11.1	9,430
α-Amylase II from heated extract	1,886,920	18.4	102,400

It is difficult to determine accurately the β-amylase activity of an extract in the presence of large amounts of α-amylase and so it was not possible to assess directly the efficiency of thy heat treatment for inactivating β-amylase in the original extract. Howev er, when the heated extract was fractionated on CMC, only an extremely small amount of β-amylase was detected in the eluate, indicating that the heat treatment was almost completely effective.

The 70°C. heat treatment did not alter the chromatographic properties or the

relative proportions of α-amylases I and II. Identical amylase profiles were obtained with the heated and unheated extracts. α-Amylase I accounted for 5.4% of the total α-amylase activity in both extracts.

The particular chromatographic conditions used in this study (pH and strength of buffer, and Na^+ gradient) were chosen to give maximum resolution of α- and β-amylases, both in active form, from barley malt. Although the techniques described should be applicable to separating amylases from sources other than barley malt, chromatographic conditions may have to be altered to suit the chemical and physical properties of the particular amylases.

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Literature Cited

1. GREENWOOD, C. T., and MILNE, E. A. Starch degrading and synthesizing enzymes: A discussion of their properties and action pattern. Advan. Carbohyd. Chem. 23: 281 (1968).
2. SCHWIMMER, S., and BALLS, A. K. Isolation and properties of crystalline α-amylase from germinated barley. J. Biol. Chem. 179: 1063 (1949).
3. MEYER, K. H., FISCHER, E. H., and PIGUET, A. Amylolytic enzymes. XVI. Purification and crystallization of malt beta-amylase. Helv. Chim. Acta 34: 316 (1951).
4. GREENWOOD, C. T., and MacGREGOR, A. W. The isolation of α-amylase from barley and malted barley, and a study of the properties and action-patterns of the enzymes. J. Inst. Brew., London 71: 405 (1965).
5. LaBERGE, D. E., and MEREDITH, W. O. S. The chromatographic properties of barley and malt β-amylases. J. Inst. Brew., London 75: 19 (1968).
6. BETTNER, R. E., MEREDITH, W. O. S., and ANDERSON, J. A. Laboratory drum-malting equipment. II. Multiple units. Amer. Soc. Brew. Chem., Proc. 1962, p. 5.
7. PETERSON, E. A., and SOBER, H. A. Variable gradient device for chromatography. Anal. Chem. 31: 857 (1959).
8. BENDELOW, V. M. Modified procedure for the determination of diastatic activity and α-amylase. J. Inst. Brew., London 69: 467 (1963).
9. MacGREGOR, A. W., LaBERGE, D. E., and MEREDITH, W. O. S. Changes in barley kernels during growth and maturation. Cereal Chem. 48: 255 (1971).
10. LOYTER, A., and SCHRAMM, M. The glycogen-amylase complex as a means of obtaining highly purified alpha-amylases. Biochim, Biophys. Acta 65: 200 (1962).
11. TREVELYAN, W. E., PROCTER, D. P., and HARRISON, J. S. Detection of sugars on paper chromatograms. Nature (London) 166: 444 (1950).
12. LaBERGE, D. E., and MEREDITH, W. O. S. Factors affecting losses of sugars during acid hydrolysis of polysaccharides. Lab. Pract. 19: 1121 (1970).
13. MITCHESON, R. C., and STOWELL, K. C. Application of new analytical techniques to routine malt analysis. I. Determination of barley and malt nitrogen content using an AutoAnalyzer technique. J. Inst. Brew., London 76: 335 (1970).
14. WILLIAMS, P. C. The colorimetric determination of total nitrogen in feeding stuffs. Analyst (London) 89: 276 (1964).
15. BANKS, W., GREENWOOD, C. T., and THOMSON, J. The properties of amylose as related to the fractionation and subfractionation of starch. Makromol. Chem. 31: 197 (1959).
16. LaBERGE, D. E., CLAYTON, J. W., and MEREDITH, W. O. S. The effect of thioglycerol on the chromatographic and electrophoretic behavior of barley beta-amylases. Amer. Soc. Brew. Chem., Proc. 1967, p. 18.
17. VALLÉE, B. L., STEIN, E. A., SUMERWELL, W. N., and FISCHER, E. H. Metal content of alpha-amylases of various origins. J. Biol. Chem. 234: 2901 (1959).
18. CLAYTON, J. W., and MEREDITH, W. O. S. The effect of thiols on the dinitrosalicylic acid test for reducing sugars. J. Inst. Brew., London 72: 537 (1966).
18a. WALDSCHMIDT-LEITZ, E., and SIGRIST, C. Separation of α- and β-amylases in active forms from malt. Hoppe-Seyler's Z. Physiol. Chem. 351: 1571 (1970).

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