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Investigations of Barley and Malt-Amylase

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When fractionating protein components of malt, Osborne [1] found that the amylase activity follows the albumin fraction. He isolated the albumins by precipitation with ammonium sulphate followed by dialysis. Out of the albumin solutions obtained in this way the albumins were precipitated with alcohol and dried over H_2SO_4. By this means a product of powerful amylase activity was obtained.

For the experiments described in this paper, Osborne's method of preparation has been mainly followed. Modifications have been introduced based on the experiences which were met with during earlier experiments to isolate the globulins from barley and malt [2,3]. Albumin is obtained from both barley and malt in exactly the same way, according to the method stated below.

METHOD OF PREPARATION

500 g of barley (or malt) are thoroughly ground and extracted at + 4° C with 2000 ml 1 *M* NaCl, buffered to pH = 7 by primary and secondary sodium phosphate. After centrifuging and filtering until clear, part of the proteins are precipitated by 4/10 saturation with anhydrous ammonium sulphate [2]. The precipitate obtained is discarded because it contains no albumin. The rest of the proteins are precipitated by 9/10 saturation with ammonium sulphate and the precipitate so obtained is separated by centrifugation and dissolved in 0.2 *M* NaCl at pH 7.0. The solution is then dialysed in a cellophane sack against distilled water at the lowest possible temperature (2—4° C) for 48 hours. The precipitate obtained by the dialysis consists of globulins, and has no amylase activity, being therefore centrifuged down and discarded. The solution from the dialysis is 7/10 saturated with ammonium sulphate and the precipitate formed is dissolved in buffer at pH 7.0 and dialysed anew. This procedure is repeated until a precipitate is no longer obtained on dialysis. The solution is then quite free from globulins. The preparation proceeds according to the following scheme of preparation.

1. Initial materials
2. Extraction with 1 *M* NaCl, pH 7
3. Precipitation by $(NH_4)_2SO_4$; 4/10 saturation

Precipitate | Solution

4. Precipitation by $(NH_4)_2SO_4$; 9/10

Precipitate | Solution

5. Dissolving in buffer at pH 7.0
6. Dialysis against distilled water

Precipitate | Solution

7. Precipitation by $(NH_4)_2SO_4$; 7/10

Precipitate | Solution

8. Dissolving in buffer at pH 7.0
9. Dialysis against distilled water

Precipitate | 10. Solution containing albumin

The solution obtained at stage 10 is treated according to stages 7—9 several times, until a precipitate is no longer obtained on dialysis against water.

The albumin fractions from barley and malt, prepared according to the scheme stated above, were tested in the ultracentrifuge (*cf.* Svedberg and Pedersen [4]). Exactly the same sedimentation diagram was obtained from both albumins (see Fig. 1).

The sedimentation constants were also identical. In earlier investigations of the albumin of barley, Quensel [5] determined the sedimentation constant of the albumin to be 3.5 S, but pointed out that the albumin fractions were not homogeneous. We also obtain this value of the sedimentation constant for those fractions which have not been precipitated a sufficient number of times. By careful purification of the albumin the value 4.6 S is obtained. This value remains constant for different preparations, and is independent of divergences from the scheme of preparation stated above, (*e. g.* precipitation at another concentration and dialysis at another pH).

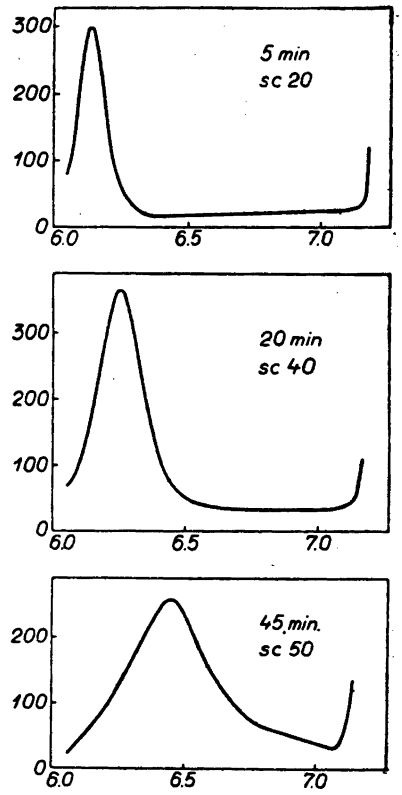

Figure 1. Sedimentation diagrams for barley and malt albumin.

DETERMINATION OF THE AMYLASE ACTIVITY

Despite the fact that the sedimentation constants of barley and malt albumin are identical, the amylase activities are quite different. The determination of the saccharification power has been carried out according to Windisch-Kolbach [6]. The value given by this test, which can be considered as a measure of the activity of β-amylase, is given as the number of grams of maltose formed from soluble starch by 100 g of enzyme preparation acting for 30 minutes at 20° C and pH $= 4.3 \pm 0.1$.

The determination of the dextrinizing power has been carried out according to a modified Wohlgemuth method [7], and is primarily a measure of the α-amylase activity. The result is given as the number of grams of starch which are broken down to dextrins by 100 g of enzyme preparation acting for 15 minutes at 30° C and pH $= 5.0$.

In those cases for which the enzyme activity was calculated from the dry

weight of the material the moisture determinations were carried out by means of drying in vacuum at 50° C.

Table 1. Effect of albumin purification on amylase activity.

	Dry substance %	Saccharification power (dry substance)	Dextrinizing power (dry substance)
Malt (from six-rowed barley)	93.0	680	770
Barley albumin solution no. 1	1.30	5400	0
» » » » 2	0.82	7800	0
Malt albumin » » 1	1.30	12600	12800
» » » » 2	1.27	13400	15400
» » » » 3	1.15	20500	25600
» » » » 4	0.85	32600	37500
» » » » 5	0.72	45600	51400
» » » » 6	0.55	59800	67100
» » » » 7	0.52	63700	60400

Through continued purification by precipitation and dialysis the enzyme activity reaches a maximum. Further manipulation tends to diminish the activity. The activity of α-amylase in malt albumin no. 7 is lower than in malt albumin no. 6 and it has in general been found that α-amylase in these preparations is more unstable than β-amylase, despite the fact that the pH is kept constant.

THE CHANGES OF THE ACTIVITY ON STORAGE

The aqueous solutions of malt albumin could be stored in a refrigerator (+ 1° C) for several weeks without any significant change of the activity being detectable. On longer storage ca. 3—4 weeks, a small part of the albumin precipitates in the form of extremely small amorphous particles. The precipitate easily redissolves on warming to room temperature, but appears again on recooling.

In two cases this precipitate was centrifuged down and redissolved, after which the activity was again determined.

Table 2. Amylase activity of albumin solutions and precipitates after storing at + 1° C for 4 weeks.

	Saccharification power (dry substance)	Dextrinizing power (dry substance)
Original solution no. 1	59800	67100
Precipitate by cooling	38400	112600
Original solution no. 2	63700	60400
Precipitate by cooling	96900	240800

By storage in the cold in this way the α-amylase is precipitated to a considerably higher degree than β-amylase. Consequently, it appears possible that by means of fractional precipitation in the cold, α-amylase may be separated from β-amylase. However, hitherto the precipitation has only been done by cooling to 1° C and at this temperature the separation is very slow and gives a low yield. Further attempts will therefore be made, by means of an improved method, to separate quantitatively both the amylases from each other and at the same time to obtain, if possible, a concentration.

CHANGES OF ACTIVITY ON PROLONGED STORAGE

The solutions of the enzymes have been kept in a refrigerator at + 4° C for a period of 3 months. At this temperature no precipitate was formed. Determinations of the activity were made at the beginning and end of this period. In the first column of Table 3 the original activity is given, in the second column the activity which was found after three months. In the third column appears the percentage of the original activity remaining after three months.

Table 3. Changes in amylase activity of albumin solutions after 3 months at + 4°C.

	Saccharification power			Dextrinizing power		
	Before	After	% Remaining	Before	After	% Remaining
Solution no. 1	418	324	78	522	429	82
» » 2	233	186	80	167	147	87
» » 3	—	—	—	2667	2224	83

The activity has consequently decreased by the same extent for all the solutions, or to about 80 % of the original value, in 3 months. The enzyme solutions are therefore relatively stable.

ULTRAFILTRATION OF ALBUMIN SOLUTIONS

In order to obtain concentrated solutions of malt albumin, experiments have been made with ultrafiltration [8] of the solutions. For this purpose a filter has been used which was obtained by dipping an alundum tube into a solution of collodion in glacial acetic acid. With a suitable thickness of the collodion membrane a filter is obtained which does not allow the albumin molecules to pass through. During the tests below the protein did not, on any occasion, pass through the filter. In Table 4 is presented the result of two tests with malt albumin.

Table 4. Effect of ultrafiltration on amylase activity.

	Saccharification power (dry substance)	Dextrinizing power (dry substance)
Untreated solution 1	29800	37300
Filtrate	0	0
Remainder	90000	55700
Untreated solution 2	29900	37200
Filtrate	0	0
Remainder	59100	75000

Ultrafiltration, therefore, offers two advantages: (1) concentration of the albumin and (2) increased enzyme activity.

DRYING OF ALBUMIN

The albumin solutions which had been concentrated by ultrafiltration can, after cooling down to $-16°$ C, be dried in vacuum over $CaSO_4$. By this means the albumin is obtained in the form of a brown, amorphous powder, which dissolves immediately and completely in distilled water. Chemical investigations of these dried albumin fractions show that the nitrogen content is about 11 %. Further investigations regarding the chemical composition will be made. In the dried condition the albumin is of unlimited stability. Both α- and β-amylase activities are largely retained. The drying entails some diminution of the amylase activities (see Table 5), but this is compensated by the fact that the enzyme becomes stable and easier to keep.

Table 5. Effect of drying on amylase activity.

	Saccharification power (dry substance)	Dextrinizing power (dry substance)
Albumin, no. 1		
before drying	59100	75000
after drying	45000	57400
Albumin, no. 2		
before drying	47500	62500
after drying	39200	55000

ELECTROPHORETIC EXPERIMENTS

The first problem with the electrophoretic experiments was to establish whether the α- and the β-amylase had the same mobility in an electric field. Since the enzymes could not be separated by centrifugation, it was hoped that this could be carried out with the help of electrophoresis.

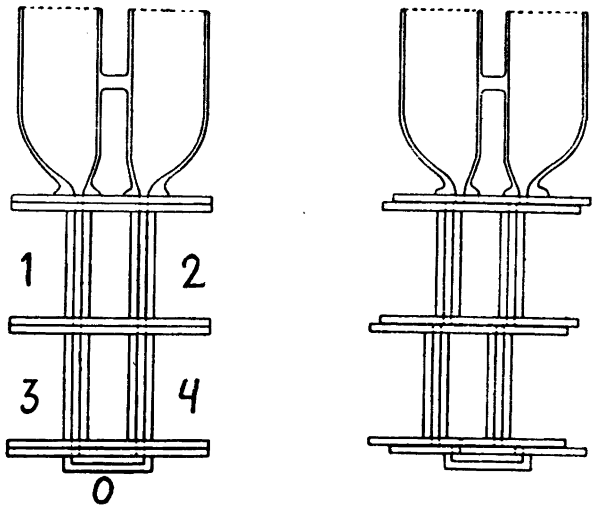

Figure 2. Electrophoretic cell (Tiselius).

The Tiselius apparatus [9] with the modifications indicated by Svensson [10] for the collection and chemical analysis of samples was used.

The method is as follows. At the beginning of the experiment the solution is placed in the cells 0, 3 and 4 (see Fig. 2). The cells 1 and 2 hold the solvent. The boundary between the solution and solvent is produced by the displacement of cells 3 and 4. When a direct current is passed through the solution, the active substance migrates under the influence of the current.

The experiment continues for a definite period, during which the current and voltage are determined. Conductivity and pH are also measured. At the end of the period, the cells are displaced in relation to each other, so that the solution in each one can be taken out with a syringe, after thorough mixing to equalize all the differences in concentration. The determination of the enzyme activity is then done for the 5 fractions, and in such a way that one obtains a relative measure of the enzyme concentration. In all the tests, all the samples have as far as possible, been treated in exactly the same way.

At the beginning of the experiment the enzyme activity is the same in cells 0, 3 and 4. In the cells 1 and 2 the concentration (a) is zero, since these cells contain pure solvent. After the time t the enzyme has migrated by the extent l into cell 1. If the volumes of the cells 1 and 3 are v_1 and v_3, cross sections $q_1 = q_3 = q$, and concentrations a_1 and a_3, one can write:

$$a_1 = \varrho_1 \cdot a_3 \qquad 0 < \varrho_1 < 1: \tag{1}$$
$$\varrho_1 = \frac{a_1}{a_3}$$

but

$$l_1 = \frac{\varrho_1 \; v_1}{q} = \frac{a_1 \; v_1}{q a_3} \tag{2}$$

now,

$$l_1 = \frac{u_1 \cdot i \cdot t}{q \cdot \varkappa} \tag{3}$$

where

u_1 = mobility measured in cell no. 1
i = current strength
t = time
$\varkappa$ = conductivity

one gets

$$u_1 = \frac{l_1 \cdot q \cdot \varkappa}{i \cdot t}$$

Substitution of l_1 from (2) gives

$$u_1 = \frac{a_1 \; v_1 \; \varkappa}{a_3 \cdot i \cdot t} \tag{4}$$

In this equation one can put $a_3 = a_0$, since the experiment is stopped before any change of concentration in cell 0 has taken place.

By observing the change in cell 4 a control value for the velocity is obtained. In an analogous way to the above is obtained:

$$a_4 = \varrho_2 \cdot a_0 \qquad 0 < \varrho_2 < 1 \tag{1'}$$
$$\varrho_2 = \frac{a_4}{a_0}$$

$$l_4 = \frac{(1 - \varrho_2) \; v_4}{q} \tag{2'}$$

From (1') and (2')

$$l_4 = \frac{v_4}{q} \cdot \frac{a_0 - a_4}{a_0} \tag{3'}$$

but

$$l_4 = u_4 \cdot \frac{i \; t}{q \; \varkappa}$$

and

$$u_4 = \frac{v_4 \; \varkappa}{i \cdot t} \cdot \frac{(a_0 - a_4)}{a_0} \tag{4'}$$

where u_4 is the mobility of the active substance, measured in cell 4.

With the help of these equations one can determine the mobility for both the α- and the β-amylase activities. The following example may be given:

Electrophoresis no. 17

Cell no. 1 positive pH 6.80

$i = 23.1$ mA

$\varkappa = 3.809 \cdot 10^{-3}$ ohm$^{-1} \cdot$ cm^{-1}

$t = 14400$ s

$v_1 = v_4 = 3.60$ ml

Concentrations were determined to be

$a_1^{\alpha} = 187$	$a_1^{\beta} = 292$
$a_3^{\alpha} = 414$	$a_3^{\beta} = 720$
$a_0^{\alpha} = 414$	$a_2^{\beta} = 729$
$a_4^{\alpha} = 213$	$a_4^{\beta} = 397$
$a_2^{\alpha} = 0$	$a_2^{\beta} = 0$

Formulae (4) and (4′) give with these values,

$u_1^{\alpha} = 1.67 \cdot 10^{-5}$ cm$^2 \cdot$ volt^{-1} s^{-1}

$u_4^{\alpha} = 1.87 \cdot 10^{-5}$ »

$u_1^{\beta} = 1.86 \cdot 10^{-5}$ »

$u_4^{\beta} = 2.00 \cdot 10^{-5}$ »

If the averages are taken,

$u^{\alpha} = 1.77 \cdot 10^{-5}$ cm$^2 \cdot$ volt^{-1} s^{-1}

$u^{\beta} = 1.93 \cdot 10^{-5}$ »

In this case the enzymes migrated towards the positive electrode, and were consequently negatively charged. The above-mentioned mobilities should therefore have negative signs.

By an exactly analogous way the values stated in Table 6 have been obtained.

Table 6. Electrophoretic mobility of amylase activity.

Buffer	pH	$u^{\alpha} \cdot 10^5$	$u^{\beta} \cdot 10^5$
0.175 *M* HAc 0.100 *M* NaAc	4.38	—	+ 1.81
0.002 *M* Na_2HPO 0.094 *M* NaH_2PO_4 0.05 *M* NaCl	5.26	+ 0.78	+ 0.78
0.00125 *M* Na_2HPO_4 0.0112 *M* NaH_2PO_4 0.05 *M* NaCl	5.98	— 0.44	— 0.51
0.00125 *M* Na_2HPO_4 0.0112 *M* Na_2HPO_4 0.05 *M* NaCl	6.15	— 0.68	— 0.64
0.0075 *M* Na_2HPO_4 0.005 *M* NaH_2PO_4 0.05 *M* NaCl	6.80	— 1.77	— 1.93
0.0075 *M* Na_2HPO_4 0.005 *M* NaH_2PO_4 0.05 *M* NaCl	6.85	— 1.25	— 1.19
0.004 *M* H_3PO_3 0.00125 *M* $Na_2B_4O_7$ 0.05 *M* NaCl	7.90	— 4.73	— 3.90
0.004 *M* H_3BO_3 0.00125 *M* $Na_2B_4O_7$	8.22	— 5.09	— 4.61

The results of Table 6 are shown graphically in Fig. 3.

Consequently α-amylase has exactly the same mobility in an electric field as β-amylase, over most of the pH range examined. A certain tendency to a different mobility appears at pH 8, but the differences are very small. It has not as yet been possible to separate the amylases from each other by the electrophoretic method. It appears from the diagram that the iso-electric point for both enzymes lies at pH 5.75. It is interesting to compare these determinations of the mobility of the two amylases with the values which have been obtained from electrophoretic experiments on the α-amylase from pancreas[11,12]. By means of optical determinations of the mobility these authors found $u = 3.09 \cdot 10^{-5}$ for the crystallized enzyme at pH 7.9. At pH 6.5 they

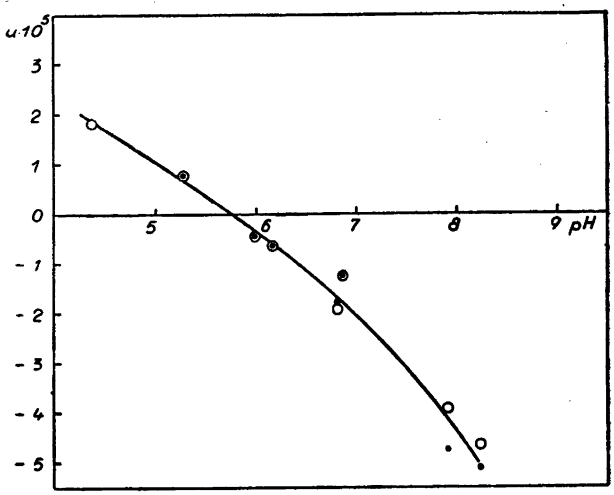

Figure 3. Electrophoretic mobility of amylase activity.

○ *Saccharification activity.*
● *Dextrinizing activity.*

found $u = 1.8 \cdot 10^{-5}$. From Fig. 3 the value $u = 4.3 \cdot 10^{-5}$ is given for malt amylase at pH 7.9, and at pH 6.5 the value is $u = 1.5 \cdot 10^{-5}$.

SUMMARY

1. The amylase activities of barley and malt have been localized in the albumin fractions. The amylase activity of the preparation increases with increasing purity of the albumin. Barley and malt albumins behave exactly alike in ultracentrifugation and both have a sedimentation constant of $s_{20} = 4.6$ S.

2. If aqueous solutions of pure malt albumin are kept in the cold (+ 1° C), α-amylase is precipitated after a time to a considerably higher degree than β-amylase. The aqueous solutions of albumin kept at + 4° C lose only 20 % of their activity after three months.

3. The albumin solutions can be concentrated and purified by means of ultrafiltration and by cooling and drying in vacuum over $CaSO_4$. From albumin solutions purified in this way a stable amylase preparation is obtained with high enzyme activity.

4. Electrophoretic experiments with malt albumin prove that the mobility for the α- and the β-amylase activity is practically the same in an electric field throughout the range pH = 4.4 to pH = 8.2. Only a very small difference is seen to be present at pH 8 and above. It is therefore probable that the α- and the β-amylase cannot be separated by means of electrophoresis.

5. The isoelectric point for the α- and the β-amylase has been determined to be pH 5.75.

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