Investigations on Barley Malt Amylases and Related Proteins

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Much work has been done on the well known barley and malt amylases, one of the earliest known enzyme systems. Ever since Wijsman¹ showed that there were two different components and demonstrated their different solubilities in ethyl alcohol, there have been many attempts to isolate them: by ammonium sulphate fractionation²⁻⁷, by ethyl alcohol^{8,9,3}, by tannine precipitation^{10,11} and by different adsorption methods^{5,11-15}. Important steps also used for the purification are the heating of the solutions to +70° C, which inactivates β -amylase and precipitates a lot of impurities¹⁶, and the dialysis of solutions which removes the globulins¹⁷⁻¹⁹.

Quite recently some investigators have reported crystalline barley malt amylase preparations: Schwimmer and Balls^{5,15} have prepared crystalline α -amylase by a combined heating and chromatographic fractionation procedure and Fischer, Meyer, Noelting and Piguet²⁰ claim to have crystallized β -amylase by combined fractionation with ammonium sulphate and acetone. But these methods involve steps (heating, acetone precipitation) which in general are considered to cause modifications in the physico-chemical state of enzymes.

The aim of this paper is to elucidate the properties of native enzymes and related proteins. For this purpose we have avoided precipitations with organic solvents and heating of solutions. But on the other hand even salting-out may alter the physico-chemical properties of enzymes: consequently the enzymes obtained by these methods are strictly speaking not native²¹. Thus the term native is used only in a relative sense.

The terms, albumin, globulin and glutelin, are defined as follows: albumins are soluble in salt-free water, globulins in approximately neutral salt solutions (pH 5—8) and glutelins in alkaline solutions.

EXPERIMENTAL METHODS

1. *Enzyme assay*: a. *Dextrinizing activity* was determined by a modified Wohlgemuth procedure according to Sandstedt, Kneen and Blish²². Spectrophotometric control of colour change according to Hultin²³ was sometimes used. The substrate, limit dextrin, was spectrophotometrically controlled before use. The temperature was +30° C and the pH 5.0.

Specific activity unit: The number of grams of starch hydrolyzed to dextrins, in one second under conditions specified above, to give the standard colour according to Sand-

stedt *et al.*²² on adding an amount of enzyme preparation containing one gram of nitrogen. The dextrinizing power was used as a measure of α -amylase activity.

Conversion factors: One of our units corresponds to about

14 400 units according to Danielsson and Sandegren¹⁹

575 » » » Kneen and Sandstedt²⁴

6.5 » » » Hultin²³

1.5 α -amylase units according to Schwimmer and Balls⁵

b. *Saccharifying activity* was determined by a modified Windisch-Kohlbach procedure²⁵ by titrating against Fehling's solution²⁶. Assays were performed at +20° C and at pH 4.5 in 2 % Lintner starch solution.

Specific activity unit: The number of grams of maltose produced in one second under conditions specified above on adding an amount of enzyme preparation containing one gram of nitrogen.

When the saccharifying activity of α -amylase, found to be approximately 0.0190 (± 0.0007) times the dextrinizing activity, was subtracted from the total saccharifying activity, the value found could be used as a measure of the β -amylase activity.

Conversion factors: One of our units corresponds to about

29 000 degrees Lintner (new units: g maltose in 30 min. per 100 g preparation)

575 units according to Kneen and Sandstedt²⁴.

The conversion factors are only approximate owing to the fact that the units above are defined on the dry weight basis or under other experimental conditions. In the latter case the conversion factors are determined experimentally whilst in the former it is assumed that the nitrogen content is 16 % (really 12–18 %) of the salt-free dry weight.

2. *Determination of nitrogen content.* The nitrogen content of the preparations was estimated by a semimicro Kjeldahl procedure according to Blom and Schwarz²⁷. The protein content was estimated by multiplying the nitrogen value by the usual factor 6.25, which however gives only rather approximate values. It can be calculated from the data in Table 3, that this factor really varies from 5.4 to 8.0.

Preparation of the amylase containing fraction of malt proteins

After several experiments it was found to be best to extract the finely crushed malt with distilled water containing 1 g calcium chloride and 0.5 g sodium bromate/l. The yield of α -amylase is said to be higher when the extraction is performed with 1 M sodium chloride²⁸. The content of globulins in such extracts is however higher. As the purity was more important for our purpose than the quantity, we did not use extraction with sodium chloride. The following preparation procedure was found to be the best one in order to obtain a purified fraction containing maximum amounts of both α - and β -amylase.

1. Extraction: 1 kg malt is mixed with 3 l dist. water containing 0.1 % CaCl_2 and 0.05 % NaBrO_3 and stirred for 1 hour at 20° C. Centrifugation.

2. Supernatant liquid (yield: 2 l): 410 g ammonium sulphate/l is added at 0° C. (63 % sat.) Centrifugation after two hours. Insoluble residue; discarded

3. Precipitate: suspended in 250 ml dist. water and dialyzed at 0° C for two days against dist. water containing 0.05 % CaCl_2 and NaBrO_3 . Solution is changed at least three times. The suspension is then centrifugated. Supernatant liquid: albumins, low molecular compounds, carbohydrates; discarded.

4. Supernatant liquid: the amylase-containing fraction. Precipitate: globulins, 10–20 % of α -amylase; discarded.

Table 1. Activity yield in the first purification steps.

Preparation phase (see scheme at p. 89)	Volume ml	Total N g/l	α -Activity		β -Activity	
			spec.	yield %	spec.	yield %
Calcium chloride extracted, kilned malt (phase 1) precipitated and dialyzed (phase 4)	2 100	1.70	4.4	100	0.72	100
	680	1.30	14	79	2.3	81
Calcium chloride extracted, freeze dried green malt (phase 1) precipitated and dialyzed (phase 4)	3 100	1.57	1.8	100	0.35	100
	270	3.2	7.2	72	1.56	82

The preparations obtained by this method contained principally albumins but also considerable amounts of globulins soluble in low salt concentrations. They could be precipitated by prolonged dialysis but we did not use this method because α -amylase was also precipitated but not inactivated as stated by several authors ^{29,30,11}.

Two examples of the effect of this procedure are shown in Table 1. The purification is three or four fold and the yield normally about 80 %. The carbohydrate content was about 1–3 % calculated on dry matter.

Precipitation of amylases with salt solutions

As it is known that the solubility of amylases in ammonium sulphate solutions varies with different preparations ^{31,6}, we have made several fractionation experiments with the following types of amylase preparations: I) crude extracts of malt which had been cautiously dried and kilned at +55° C, II) freshly milled green malt, III) green malt dried from the frozen state and IV) commercial barley flour. Some purified amylase-containing fractions corresponding to phase 4 in the scheme on p. 89 have also been fractionated. The experiments were carried out at varying pH values, from 4.9 to 7.3. In some cases tertiary potassium citrate was used instead of ammonium sulphate ³².

The precipitation procedure was generally as follows: In a sufficient number of 25 ml volumetric flasks equal amounts of a known buffered amylase solution and increasing amounts of dry ammonium sulphate (or potassium citrate) were added at +2° C. The flasks were made up to volume with dist. water, the salt cautiously dissolved (foaming must be avoided) and kept in the cold room at +2° C for ca 16 hours. The precipitate was then centrifuged off and the enzyme activity in the supernatant was determined.

As the supernatant in some solutions contained so much salt that it interfered with the enzyme assays, a correction factor was calculated by measuring the inhibitory power of different salt concentrations on known enzyme samples of about equal activity and purity. The correction factor was in no cases greater than 20 % of the value.

It can be pointed out that citrate ion, which forms soluble complexes with calcium, did not show any inhibitory power towards the α -amylase. The calcium ion concentration during the fractionation was calculated according to Hastings *et al.* ³³ to be not lower than 2×10^{-6} moles in any case. According to Hultin ³⁴ this calcium ion concentration is enough to stabilize the α -amylase, at least at pH 7.3.

The saturated ammonium sulphate solution was considered to be 4.14 molar at +20° C and the saturated potassium citrate 2.92 molar at +20° C.

The results of the experiments are shown graphically in Fig. 1. Most remarkable are the properties of freshly milled green malt. We did not obtain reproducible results with this type of extract. Figures C and D correspond to extracts prepared in exactly the same manner but the precipitation curves are quite different. The curves are also irregular owing that the amylases in these solutions do not follow, not even approximately,

Fig. 1. Salting-out amylases from different preparations. Abscissa: % saturation of the precipitating salt. Ordinate: % of amylase activity remaining in solution. Circles indicate α -amylase, triangles indicate β -amylase.

- A. Extract from malt kilned at $+55^{\circ}\text{C}$, am. sulphate fractionation at pH 5.9–6.1.
 B. Extract from freeze dried green malt, am. sulphate fractionation at pH 4.9.
 C. Extract from freshly milled green malt, am. sulphate fractionation at pH 4.9.
 D. Extract from freshly milled green malt, am. sulphate fractionation at pH 4.9.
 E. Malt protein fraction enriched on amylases, am. sulphate fractionation at pH 5.6.
 F. Malt protein fraction enriched on amylases, potass. citrate fractionation at pH 7.2–7.4.
 G. Extract from commercial barley flour, am. sulphate fractionation at pH 5.6–6.1.

Cohn's solubility law²². The solubility also seems to be very high. Some tentative experiments showed, however, that, when the amylases have once been precipitated from these solutions, the solubility is lowered to the normal level.

Our hypothesis concerning these irregularities is as follows: the amylases in the living cells of green malt are in another state than in dried malt (possibly bound to other proteins because a considerable quantity of total protein seems to precipitate together with the amylases). This state is evidently not broken up by milling or crushing but only by drying, salt precipitation or any other kind of dehydration.

The precipitation curves of the other preparations — those of freeze-dried malt as well as those of kilned malt — have all the normal S-shape showing that the amylases in these preparations follow approximately Cohn's solubility law²²: $\log S = \beta - K_s \mu$ (where S is the solubility, μ the ionic strength and β and K_s characteristic constants). To lower the amylase content from 90 % to 5 % an increase of the ionic strength by 2.2–2.3 is needed, i.e. K_s is really approximately constant. Although in more concentrated solutions the amylases are precipitated by lower salt concentrations, the term β is however not a constant — quite naturally because it is dependent on the variable solid phase. Therefore it seems practically very difficult to obtain constants appropriate for further fractionation experiments.

The results show also that α - and β -amylase are separated most effectively at pH-values 5.6–6.1, i.e. near their isoelectric points¹⁹. The results are well in accordance with those of Kneen, Sandstedt and Hollenbeck³.

Electrophoretic investigation of the malt protein fraction enriched on amylases

An electrophoretic investigation of the malt protein fraction enriched on amylases was performed in order to obtain knowledge of the character of impurities and of the absolute quantity of amylases in such preparations.

Malt from six-row barley, dried cautiously and then kilned at 55° C (K series) and green malt from two-row barley, dried from the frozen state and milled after drying (F series), were investigated. Fractions of both types of malt are prepared following the scheme on p. 89. The green malt preparation was, however, precipitated once more with 60 % ammonium sulphate in order to obtain a product more free from carbohydrates.

The amylase fraction of kiln-dried malt was further fractionated with potassium citrate. Potassium citrate was used in order to facilitate the determination of nitrogen content in the supernatant liquid. Since the green malt preparation could not be fractionated with potassium citrate because the colloidal precipitate was almost impossible to separate from the supernatant liquid, we used ammonium sulphate.

The fractionation was performed in the following way: the dialyzed amylase-containing solution was diluted to a protein content of 0.5 %. The amylase concentration was thus brought to about the same level as in the 1:3 extracts. Only water distilled in a glass apparatus was used. The sodium bromate concentration was held at 0.5 g/l.

Table 2. Specific and total activities of kiln-dried malt fractions investigated by electrophoresis.

No.	Saturation of potassium citrate	Fraction contains % of total-N	Specific activities		Total activities %		Molisch reaction
			α -Amylase	β -Amylase	α -Amylase	β -Amylase	
K I	21 %	6	66	0.06	39	0.1	weak
K II	28 »	9	50	3.7	42	11	»
K III	32 »	14.5	11	6.2	15	30	»
K IV	36 »	10.5	3.6	12.5	3.5	44.5	»
K V	40 »	13	—	2.4	—	11	»
K VI	58 »	32	—	0.3	—	3.4	strong
Rest	—	15	—	—	—	—	—

Table 3. *Specific and total activities of freeze-dried green malt fractions investigated by electrophoresis.*

No.	Saturation of ammonium sulphate	Fraction contains % of non-dialyzable dry weight	total N	Specific activities		Total activities %		Molisch reaction
				α -Amylase	β -Amylase	α -Amylase	β -Amylase	
F I	30 %	17.5	20.5	26	0.54	62.5	8.2	weak
F II	37.5 %	11.0	9.2	26	1.7	28.0	11.5	»
F III	42.5 »	13.4	13.0	5.8	2.6	8.6	25.1	very weak
F IV	47.5 »	19.1	18.2	0.42	2.8	0.8	36.2	» »
F V	52.5 »	13.8	13.4	0.09	1.7	0.1	17.5	weak
F VI	62.5 »	14.5	13.7	—	0.12	—	1.4	very weak
F VII	82.5 »	10.1	11.2	—	—	—	—	» »
Rest	—	0.6?	0.6	—	—	—	—	no reaction

Potassium citrate or ammonium sulphate was then successively added to the solutions. The ammonium sulphate fractionation was carried out at pH 4.9, the citrate fractionation at pH 7.2–7.4. The values of the concentration and the activities of fractions are shown in Tables 2 and 3. After each addition of salt the solution was left at +2° C for two days (potassium citrate) or for one day (ammonium sulphate), and then centrifuged. The precipitate was then dissolved in a convenient buffer solution, dialyzed against it and examined by electrophoresis.

It was observed, that the solutions remaining (58 % saturated potassium citrate and 82.5 % saturated ammonium sulphate) contained a lot of nitrogenous degradation products. Jensen³⁶ made similar observations when fractionating barley proteins. Only a small part of these products could be precipitated with trichloroacetic acid. This is explained by considering the action of the proteolytic enzymes during the slow fractionation procedure and assuming that sodium bromate is incapable of completely preventing their action. It seems however that this action, which during the fractionation in F series degraded nearly 30 % of proteins, does not considerably alter the relative amounts of the different components. The electrophoretic analysis of the initial fraction gave approximately the same result as could be calculated from analyses of all the fractions prepared from it.

The fractions of kiln-dried malt (K series) were examined in phosphate buffer at pH 7.6, with an ionic strength of 0.10 and a sodium chloride concentration of 0.08 M. The protein concentration varied from 0.6 to 1.2 %.

The fractions of freeze-dried green malt (F series) were examined in triethanolamine buffer³⁷ at pH 7.7, with ionic strength of 0.107 and sodium chloride concentration of 0.08 M. The protein concentration varied from 1.4 to 2.6 %.

The electrophoretic patterns are shown in Fig. 2 and 3. The composition of the fractions was calculated from the patterns of the negative limb. The ionic mobilities were calculated for cationic components from the positive limb and for anionic components from the negative limb, *i. e.* from ascending boundaries in both cases. This was done as mobility calculations from descending boundaries could not be done exactly due to anomalies caused by high total protein concentrations.

As shown in Fig. 3, there was a very marked anomalous boundary in the triethanolamine buffer in the positive limb (ϵ -boundary, Svensson³⁷), except for the fraction F VII, where the corresponding anomaly was observed in the negative limb.

The results of electrophoretic analyses are shown in Tables 4 and 5.

RESULTS OF ELECTROPHORETIC INVESTIGATIONS

The ionic mobilities of the different components do not vary in a random manner but fall with small variations around certain mean values. The proteins investigated can thus be divided into five groups and each of these into

Fig. 2. Electrophoretic patterns of kilned malt fractions in phosphate buffer at pH 7.6. Figures correspond to exposures after 4 hours with a potential gradient of about 5–6 volt cm^{-1} . The abscissa values represent ionic mobilities ($\times 10^6$).

Fig. 3. Electrophoretic patterns of freeze-dried green malt fractions in triethanolamine buffer at pH 7.7. Exposures and ionic mobilities as in Fig. 2.

Table 4. Electrophoretic analyses of kilned malt fractions corresponding to Fig. 2. Ionic mobilities ($\times 10^6$) in the column headings. Amounts of components in each fraction expressed as percentages of total N.

No. of fraction	% N in fraction	Group A		Group B		Group C		Group D	Group E
		a -(4.7 -3.8)	b -(3.6 -3.5)	a -(2.5 -2.2)	b -(2.0 -1.8)	a -(1.1 -0.6)	b -0.2 -+0.3	-(0.6 -2.0)	+(2.3 -3.0)
K I	6	4.1		1.3		0.2	0.4		
K II	8.7	3.8		2.5			1.3	1.1	
K III	14.3	1.3	3.4		5.2	0.4	2.1	1.9	
K IV	10.4		3.1		2.2	1.5	1.4	1.3	1.9
K V	13.1	3.9				3.9		4.5	0.8
K VI	32.2	23.2			1.5	1.9	4.7		1.0
Rest (not precip.)	15.3								
% of total N in each mobility group		36.3	6.5	3.8	8.9	7.9	9.9	8.8	2.7
% of precipitated N in each mobility group		43	7.5	4.5	10.5	9.5	11.5	10.5	3.0

two subgroups, as shown in Tables 4 and 5. These groups are also well distinguished in the pattern of the initial fraction in F series. The analysis of this fraction is also well in agreement with the sum of the analyses of all the fractions prepared from it as seen in Table 5.

None of these groups correspond, however, to any defined protein component. If the precipitation curves for all the groups are drawn there cannot

Table 5. Electrophoretic analyses of freeze-dried green malt fractions corresponding to Fig. 3. Ionic mobilities ($\times 10^6$) in the column headings. Amounts of components in each fraction expressed as percentages of non-dialyzable dry matter.

No. of fraction	% Dry weight in fraction	Group A		Group B		Group C		Group D		Group E	
		a -(4.7 -4.0)	b -(3.7 -3.4)	a -(2.8 -2.2)	b -(1.8 -1.5)	a -(0.8 -0.4)	b 0.0 -+0.2	a +(0.9 -1.3)	b +(1.6 -2.0)	a +(2.5 -2.7)	b +(3.0 -3.5)
Initial fraction	100	41	4	17	10	8	7	4.5	2	6	0.5
F I	17.4	12.4		2.4	0.1	0.8	0.8		0.2	0.3	
F II	11.0	6.5		2.4		0.9	0.5	0.1	0.4	0.2	
F III	13.4	6.4		2.0	1.7	0.7	1.1	0.2	0.5	0.7	
F IV	19.1	4.5	1.4	3.8	3.8	1.0	1.7	0.6	0.5	1.5	0.3
F V	13.8	3.4	1.0	2.3	1.9	1.5	1.4	0.5	0.5	1.3	0.1
F VI	14.5	5.7		1.2	1.9	1.9	1.8	0.6	0.3	1.2	
F VII	10.1	5.7		1.9		0.9		0.8		0.2	0.5
Rest (not precip.)	(0.6?)										
% of dry weight in each mobility group		44.6	2.4	16.0	9.4	7.7	7.3	2.8	2.4	5.4	0.8

be found any tendency towards regular S-shaped curves, as found for amylases (Fig. 1, p. 91) in similar conditions. Thus it can be concluded that the initial fraction comprised at least several tens of components of albumins and easily soluble globulins.

Group A comprises components with ionic mobilities from -4.7×10^{-5} to -3.4×10^{-5} . It is the largest group: 50 % in kilned malt, 45 % in freeze-dried malt. It contains both globulins precipitated at rather low salt concentration as well as albumins precipitated first at rather high concentrations.

The globulins of this group are carriers of the brownish colour which is intensified by oxidation. A part of them has a solubility very similar to that of α -amylase (cf. p. 98), and an ionic mobility and a sedimentation constant (6.1 S) resembling those of β -globulin as determined by Quensel³⁸. They can be divided into several components by electrophoresis at a pH of 5.9.

The albumins of group A are rather ill-defined components. Some of them at least have apparently rather high diffusion constants, as evidenced by the relatively rapid spreading of the boundary (observed in electrophoresis apparatus before the connection of the current). Consequently it can be supposed that they represent (at least partially) relatively low molecular components. They are present also in unmalted barley.

The B group includes components within the mobility range, -2.8×10^{-5} to -1.5×10^{-5} . It contains mostly albumins but also globulins. Both of the amylases belong to this group. This group is present principally in fractions precipitated between 40 and 60 % saturated ammonium sulphate (30 and 40 % saturated potassium citrate). They seem to be better defined components than those of the A group. Their amount is about 15 % in kilned malt, and 20 % in freeze-dried green malt fractions.

The proteins of the C group with mobilities ranging from -1.1×10^{-5} to $+0.3 \times 10^{-5}$ are present in all the fractions. The true percentage of proteins of this group is probably much lower (about 10 %) as shown in Tables 4 and 5, owing to the fact that the false δ and carbohydrate boundaries (especially in fraction K VI) are immobile. At least in the Ca group there are however some true albumin components, one of them following the β -amylase in further fractionation.

D and E groups include proteins of basic character. Some of them with relatively high mobilities at pH 7.6 (as for example a very well defined component in group Ea) must have their isoelectric points rather far on the basic side. Their amount is 12–13 % in fractions from both types of malt and is

Table 6. Ionic mobilities of α - and β -amylases in some different fractions, expressed in $\text{cm volt}^{-1} \text{sec}^{-1}$.

Buffer and malt type	Mobility of α -amylase	Mobility of β -amylase
Phosphate buffer, pH 7.6, kilned malt fractions (K series)	2.5×10^{-5}	2.0×10^{-5}
Triethanolamine buffer, pH 7.7, freeze dried green malt fractions (F series)	2.2×10^{-5}	1.9×10^{-5}

not diminished by prolonged dialysis: consequently there are no globulins. They are completely and reversibly precipitated by salts, which excludes the possibility that they are an easily soluble glutelin fraction. They are also present in barley.

The ionic mobilities of α - and β -amylase in some fractions were determined according to the method of Danielsson and Sandegren¹⁹. The results are shown in Table 6.

DISCUSSION

The results show clearly the complex nature of malt albumin. The conclusion is valid even for barley albumins because it has been found that barley and malt albumin contain the same principal components (*cf.* p. 102 and Fig. 6). Barley albumin or leucosin, prepared according to Osborne³⁹ cannot be regarded as a more or less homogeneous fraction with an isoelectric point at pH 4.5, as has hitherto sometimes been supposed^{40,41}.

The results also show that the globulin fraction precipitated between 15 and 40 % saturated ammonium sulphate contains several globulins, some of which have a certain resemblance to the β -globulin of Quensel³⁸.

When comparing the electrophoretic patterns of kilned malt fractions with those from freeze-dried green malt it was observed that the peaks were much more distinct and characteristic in the latter, *i. e.* the properties of malt proteins are altered during heating to 55° C.

An analogous observation has been made by Briggs and Mann^{42,43}, who have found that the originally distinct peaks in patterns of soybean extract partially combine when the extract is heated. They suppose that this is due to interaction between components with different mobilities. This explanation seems not to be valid in our case. The peaks of kilned malt fractions are larger than those of green malt indicating greater inhomogeneity. But if we suppose that the rather high kilning temperatures give rise to denatured components which then are partially regenerated, there may be components with slightly higher and lower mobilities than the original component^{44,21}. In this way the peaks become larger and lower.

The oxidation may also be a cause of the alteration of the electrophoretic mobilities. We have found that the mobility of Aa component in fraction F I is raised from -4.3×10^{-5} to -5.5×10^{-5} when the solution is exposed to air in an open beaker at +2° C for three weeks. Simultaneously the brownish colour is intensified.

We should not exclude, however, the possibility that there could be molecular associations. We have found that a fraction similar to K I when submitted to electrophoresis at pH 5.9 gives a more complicated pattern with several components. But the nature of the whole salt soluble barley protein fraction is evidently so complicated that more penetrating investigations are needed before more exact theories can be built up. The complexity of the salt soluble barley protein fraction has been recently emphasized by some authors⁴⁵⁻⁴⁸.

ALPHA-AMYLASE

A. Some purification experiments

As already pointed out, crystalline α -amylase has been prepared by Schwimmer and Balls⁵. However this preparation as also most other purified preparations has been obtained by heating the solution to $+70^{\circ}\text{C}$ whilst there are in literature very few recent investigations of the properties of unheated, "native" α -amylase.

a) *Salting out*: Repeated fractionation with ammonium sulphate of fractions similar to K I and F I has not led to essentially purer preparations. A globulin of Aa-group seems to have a solubility very similar to that of the α -amylase, and even three times reprecipitated fractions contained 60 % of this protein.

b) *Isoelectric precipitation*: As the globulins of Aa-group have some properties similar to those of the β -globulin of Quensel²⁸, we tried dialysis against very dilute calcium acetate buffer ($\mu = 0.002$) at pH 4.9 (the isoelectric point of β -globulin). The fraction precipitated was however small and contained some α -amylase.

c) *Adsorption*: We could not use the adsorption in ethanol solutions^{13,5} because the native α -amylase is precipitated even by 15–20 % ethanol. Some preliminary experiments (made when investigating the adsorption properties of β -amylase) showed that α -amylase is adsorbed almost completely by diatomaceous earth (Standard Super-Cel) in 5 % ammonium sulphate solution at pH 4.3. The yield by elution was however never higher than 60 %, which indicates that partial inactivation takes place. Thus the adsorption experiments were abandoned.

d) *Electrophoretic separation*: It seems probable that, using a suitable apparatus, the electrophoretic purification of fractions similar to K I might give highly active α -amylase preparations. We made an attempt at pH 5.9, but at this pH the proteins of the Aa-group migrated in both directions. The purification was only twofold. At pH 7–8 better results might however be obtainable.

B. Inactivation of α -amylase by dialysis

Several investigators^{29,30,11} have found, that α -amylase is inactivated by dialysis. According to our experience, however, the dialysis is not inactivating if calcium ions are not removed completely and if the water used is of the highest purity. The inactivation observed by the cited authors may be due to the following factors:

1) The native, unheated α -amylase is precipitated by dialysis in the same manner as many other easily soluble globulins. The precipitate is partially soluble in 1 *M* sodium chloride solutions. But even the finely divided insoluble suspension particles retain their activity. They can be centrifuged and resuspended without any loss of the total activity.

2) α -Amylase is inactivated irreversibly when it is dialyzed in such a way that the calcium ions are completely removed^{11,24}. The inactivation is observed when dialyzing against oxalate buffer, against distilled water which is often renewed or provided with a mixture of anionic and cationic exchange resins, or when electro dialyzing the solutions²⁹.

3) When purified α -amylase preparations are dialyzed against water distilled in a metallic apparatus, a loss of activity is often observed. This may be due to the action of heavy metal cations. The losses are avoided by using water distilled in glass.

We have never observed that α -amylase could pass through cellophane membranes during the dialysis as suggested by Hultin¹¹.

Fig. 4. Electrophoretic and ultracentrifugal patterns of the fraction K I. Electrophoresis was performed in phosphate buffer at pH 7.6. Ionic mobilities ($\times 10^6$) and corrected sedimentation constants (S_{20}) are marked in the figure.

C. On the chemical nature and absolute purity of α -amylase

Since the investigations of Osborne^{17,18}, α -amylase has been considered an albumin, although Osborne pointed out that the dextrinizing activity did not follow the albumin fraction as closely as did the saccharifying activity. But the experimental facts mentioned above indicate that the native α -amylase is more like a globulin: it is precipitated by dialysis (even in the presence of small amounts of calcium ions) as well as by ammonium sulphate and ethanol in low concentrations. Also the stability of α -amylase at relatively high temperatures is a property characteristic of globulins.

The electrophoretic pattern of fraction K I seemed rather simple: it showed a major fast-moving component and a minor component with the same mobility as α -amylase. When examined in the ultracentrifuge*, this fraction showed again two main components which were however not well defined, as is seen in Fig. 4. The major component had a sedimentation constant of 6.1 S and the minor, 2.65 S. We believe, however, that the similarity of electro-

* The ultracentrifugal runs were performed at the Institute of Physical Chemistry of Upsala University. For this help the authors wish to express their sincere thanks.

phoretic and ultracentrifugal patterns is accidental because it is hardly probable that the slower sedimentating minor component could be native α -amylase. Estimating from the sedimentation constant, the slower component must have a molecular weight much lower than 60 000, which value was found by Schwimmer and Balls¹⁵ for heat-treated, crystallized α -amylase.

Hultin⁴¹ has estimated that the pure α -amylase must have an activity greater than that of the crystallized α -amylase prepared by Schwimmer and Balls¹⁵. By calculating in a similar manner from the specific activity and the electrophoretic pattern of fraction K I, we have found a value which is also slightly higher than that of Schwimmer and Balls. But this value is based on the assumption that the slow moving component in this fraction is entirely α -amylase. Electrophoretic and ultracentrifugal experiments, however, have shown that this is probably not the case (see p. 98 and Fig. 4). Thus it should be possible to isolate a more active α -amylase than that prepared by Schwimmer and Balls. Using the experimentally determined conversion factors (p. 89) the specific activity of crystallized α -amylase was calculated to be 250 g sec⁻¹/g N, the corresponding value of pure α -amylase predicted by Hultin, should be 520 g sec⁻¹/g N, and the value based on our experiments, 310 g sec⁻¹/g N.

BETA-AMYLASE

A. Purification experiments

a) *Salting out.* The electrophoretic examination of malt albumin fractions has shown that there are several protein components with solubility and electrophoretic properties similar to those of β -amylase. Repeated salting out and subsequent electrophoretic control has shown that there is little chance of obtaining in this way essentially better β -amylase preparations than *e. g.* fractions K IV and F IV (*cf.* p. 94). These observations are in accordance with the findings of Meyer, Fischer and Piguet⁷.

b) *Dialysis.* As the β -amylase is a typical albumin, the globulins can be eliminated by dialysis. This is effectively performed with the aid of ion exchange resins. After the main part of the salts has been removed by usual dialysis, a suitable mixture of cation and anion exchange resins is put into the dialysis vessel (one volume of IR-120 and three volumes of IRA-400, supplied by Rohm and Haas Co). The dialysis is then continued for 3–5 days and the precipitated globulins centrifuged off and discarded.

c) *Alcohol precipitation.* In order to eliminate some impurities, especially globulins of group A, we have found it advantageous to use ethanol in low concentrations, *i. e.* in 15–20 % solutions (these operations are carried out in a cold room, as also the other operations). Under these conditions the β -amylase is not precipitated and its properties are not altered.

d) *Adsorption.* A lot of work was made in order to find out a suitable selective adsorbent for β -amylase. The following substances were tried: wheat starch, paper pulp, silica gel, silicious earth, aluminium oxide (γ -modification), aluminium hydroxide, and freshly precipitated zinc sulphide. The most effective adsorbent was zinc sulphide, but it had also a strongly inactivating effect. Silicious earth (Standard Super-Cel) showed in the first experiments very promising results: the adsorption rate was sufficient and the inactivation of adsorbed preparations was almost negligible at first. However, when applying the chromatographic techniques the inactivation rate was increased, probably due to successive adsorptions and elutions. As the purifying effect of these methods was also found to be rather slight, they were abandoned. The salting-out adsorption technique of Shepard and Tiselius⁴² showed a pronounced inactivation effect. Meyer, Fischer and Piguet⁷ had similar experiences when trying to purify β -amylase by adsorption.

One of the β -amylase preparations, made by the chromatographic column technique using zinc sulphide precipitated on diatomaceous earth as adsorbent, was investigated by

Fig. 5. Electrophoretic and ultracentrifugal patterns of a β -amylase-containing fraction prepared by adsorption technique (see text). Electrophoresis was performed in phosphate buffer at pH 7.6; exposure after 6 hours. Ionic mobilities ($\times 10^5$) are marked in the figure. Sedimentation constant $s_{20} = 4.5$ S.

electrophoresis (at pH 7.6) and in the ultracentrifuge. Both patterns are shown in Fig. 5. The preparation had a specific activity of $12.5 \text{ g sec}^{-1}/\text{g N}$ and was probably partially inactivated. The electrophoresis diagram shows several components but the ultracentrifuge pattern only one very sharp peak. The sedimentation constant 4.5 S is in accordance with the value of Danielsson⁵² for a common malt albumin preparation. The highest peak in the electrophoretic pattern has the same mobility as that of β -amylase.

B. A stable β -amylase preparation from barley and malt

A useful β -amylase preparation (for the purpose of α -amylase determination) can be made from the water extract of barley flour by taking the fraction between 30 and 57 % saturated ammonium sulphate and dialyzing this product successively against dilute (0.01 M) oxalate buffer at pH 3.6 (2 hours), 0.01 M sodium acetate (2 hours) and then against distilled water which is changed 3—4 times and finally provided with a suitable mixture of cationic and anionic exchange resins (as mentioned above).

Small amounts of impurities (of group A) can be removed by precipitating with 20 vol % ethanol at 0° C before dialysis. However, the ammonium sul-

Fig. 6. Electrophoretic patterns of barley and malt albumin fractions (see text). Phosphate buffer, pH 7.6, potential gradient 5.5 volt cm^{-1} . Exposures after 4 hours.

phate must be removed by dialysis before the addition of ethanol in order to avoid denaturation.

The electrophoretic pattern of such a barley β -amylase preparation is shown in Fig. 6 and compared with that of a similar malt β -amylase preparation. (Ethanol precipitation was used in both cases). Fig. 6 clearly shows the similar character of barley and malt albumins (*cf.* p. 97). The differences observed may be due to slight differences in the preparation method.

The specific activities of the final barley and malt products shown in Fig. 6 were 5.0 and 12.5 $\text{g sec}^{-1}/\text{g N}$ respectively. The malt β -amylase still contained slight traces of α -amylase but the barley preparation was completely free from it. We have found that the barley β -amylase is stable for at least a year (even in aqueous solution).

C. On the absolute purity of β -amylase

Fischer, Meyer, Noelting and Piguet ^{7,20} claim to have prepared crystalline, pure malt β -amylase. The purification was 15-fold, calculated on the basis of the starting material, a commercial malt extract which probably had an activity of the same order of magnitude as the usual extracts (estimated by a rough calculation from the data of Fischer, Meyer *et al.* ⁷).

As we obtained by simple ammonium sulphate fractionation and subsequent dialysis about 20-fold purification (fraction K IV), their results seem somewhat doubtful. Further, because the component with the same mobility as β -amylase amounts to only 21 % of the total protein in this fraction, the specific activity of pure β -amylase must be at least 100 times higher than that

in crude extracts. We have calculated the minimum value to be $60 \text{ g sec}^{-1}/\text{g N}$ but the true activity is probably still higher (*cf.* p. 96).

By fractionation with ammonium sulphate and ethanol, Caldwell and Doebbeling^{9,50} have prepared a β -amylase with an activity 130 times higher than that of crude malt extracts. This cereal β -amylase seems to be the most pure hitherto prepared. A "malt amylase" of Holmbergh⁵¹, containing also α -amylase, had also a remarkably higher specific activity than that of the β -amylase of Meyer, Fischer *et al.* and those of our preparations.

On the basis of our experiments we consider that the crystallized β -amylase of Meyer, Fischer *et al.* is a mixture of β -amylase and very similar albumins. As mentioned before (p. 100), the other albumins of Bb-group (and also some of Ca-group) are very difficult to separate from β -amylase. Further, we know, according to Danielsson⁵² and our own experiments (Fig. 5), that a great part of these proteins have the same sedimentation constant ($4.5 S$) and probably also the same molecular weight as β -amylase. Thus, the formation of mixed crystals would readily occur. It is possible that there is some intimate correlation between β -amylase and these albumins (which form the main part of barley and malt albumin) because it has been found by Sandegren and Klang⁵³ that the contents of β -amylase and of barley albumin are closely related.

SUMMARY

In trying to elucidate the properties of native barley malt amylases we have investigated the protein fraction of malt having similar solubility properties, *i. e.* precipitated by 60 % saturation with ammonium sulphate and remaining in solution when dialyzed against water containing 0.1 % calcium chloride and 0.05 % sodium bromate ($\mu \sim 0.03$).

- 1) A partial separation of α - and β -amylases can be obtained by salting-out from different extracts and from partially purified fractions. The precipitation curves have in general a regular shape. The separation is most effective in pH region, 5.6—6.1.

2. The precipitation of freshly milled green malt extracts by ammonium sulphate seems to be rather variable and irregular. This is supposed to be due to the combination of amylases with other proteins in the living cells.

3. The amylase-containing fraction is very complex when examined electrophoretically. Several tens of components, albumins as well as globulins, were found. 12—13 % of basic albumin-like proteins are also present.

4. The proteins of kilned malt are partially denaturated judging by the electrophoretic patterns.

5. The solubility properties of the native α -amylase are similar to those of a globulin. It is precipitated by dialysis but not inactivated provided that calcium ions are not completely removed. A protein fraction somewhat resembling β -globulin is coprecipitated with the native α -amylase.

6. The native β -amylase is a typical albumin. The fraction enriched on β -amylase contains several proteins with similar solubility properties to β -amylase and with almost identical sedimentation constants. The specific activity of pure β -amylase is estimated to be at least 100 times that of protein in water extracts from malt with high amylolytic activity.

7. The corresponding fraction of unmalted barley seems to have a very similar composition to that of malt.

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