STUDY OF BARLEY AND MALT AMYLASES BY IMMUNOCHEMICAL METHODS[1]

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ABSTRACT

Proteins in saline extracts of barley and malt can be detected by the immunochemical methods of double diffusion and immunoelectrophoretic analysis. A technique of identifying alpha- and beta-amylase among the proteins thus detected has been perfected. Some results of the study of these amylases by immunochemical methods are described.

Although immunochemically identical, the beta-amylases of barley and malt are shown to present differences in at least two physicochemical properties. The beta-amylase of barley is less soluble in water and migrates more rapidly at pH 8.2 than beta-amylase of malt. It appears that alpha-amylase does not exist in barley, even in trace amounts or in an inactive form. Calculations of alpha- and beta-amylase mobilities and an evaluation of the diffusion coefficient of alpha-amylase are reported.

It is known that barley contains a beta-amylase and that malt contains both a beta- and an alpha-amylase, but the existence of an alpha-amylase in ripe seeds of barley is still in question (1,2).

The specificity of immunochemical methods makes it possible to enumerate and define the antigenic constituents of a complex mixture. Moreover, the reactions of specific precipitation are very sensitive, and even very small quantities of a substance can be detected. In some cases it is also possible to define constituents having an enzymatic activity by using proper substrates following the reaction of specific precipitation in immunoelectrophoretic analysis (IEA) or in double diffusion. In our previous studies we have enumerated as many as 20 constituents in the saline-soluble fraction of barley, and have defined them by their electrophoretic mobilities (3).

In the following, we present the results obtained by these methods in a study of the amylases of barley and malt. These results have allowed us to describe some of the properties of these amylases and to discuss the question of the existence of an alpha-amylase in barley.

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Materials and Methods

Preparation of Barley and Malt Extracts in Saline Solution. The Aurora variety of barley and the malt prepared from this same variety were used. Extractions were performed in the following manner. After careful washing and drying, the seeds were ground up fine in small amounts in a Turmix blender cooled by dry ice. The flour was suspended in phosphate buffer ($\mu = 0.1$) containing NaCl ($\mu = 0.4$) at pH 6.6 in the proportion of 2 ml. of buffer solution per 1 g. flour.

This suspension was agitated for 2 hr. at 4°C., then centrifuged and the supernatant was dialyzed for at least 48 hr. against the extraction buffer diluted three times. The pH remained nearly equal to 6.6. The solution was then centrifuged and dialyzed again.

Preparation of Albumin and Globulin. According to the method of Quensel (4), the barley and malt extracts were dialyzed against double-distilled water changed once a day for a period of at least 1 week. The precipitate was washed several times with double-distilled water and redissolved in the extraction buffer. This solution contained the "globulins" of barley (OG) and of malt (MG). The supernatant contained the water-soluble proteins, called "albumins" (OA for barley and MA for malt).

Electrophoresis. According to the technique of Grabar and Williams (5), electrophoresis in agar gel (EA) was performed under the following conditions: agar 1.25%; veronal buffer pH 8.2; 6–7 V/cm., duration 1 hr. 45 min. The electrophoresis was followed by fixation and coloration of the proteins (6).

The electrophoretic mobilities were calculated with the use of human serum albumin (HSA); its mobility in solution was measured in liquid media phase under the same conditions as those of our experiments.

The ratio of the mobilities of a protein, x, and of a protein used as reference (HSA, for example), must remain constant whatever the method of electrophoresis used. We can write:

$$\frac{U_x\,(\text{gel})}{U_{HSA}\,(\text{gel})} = \frac{U_x\,(\text{solution})}{U_{HSA}\,(\text{solution})}$$

Thus, U_x (gel)/U_{HSA} (gel) can be determined by simultaneous migration in gel of these proteins. Since the electrophoretic mobility of HSA in solution is known,[3] the mobility of constituent x in solution can be calculated.

The mobility U_x or U_{HSA} in gel is measured by taking account of

[3]The mobility of HSA at pH 8.2 in veronal buffer ($\mu = 0.05$) was calculated to be equal to -7.5×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ by M. de Mende, Laboratoire de Bellevue (S&O), France.

the electroendosmotic phenomenon. The proteins and a neutral substance, levane for example, are submitted to electrophoresis in the same conditions. The levane being transported during electrophoresis only by electroendosmosis, the distance separating the positions of the protein and the levane is proportional to the mobility in agar gel of this protein under the experimental conditions.

The position of a protein after electrophoresis can be determined by the spot it makes after staining with amido black or, more precisely, after IEA, by its arcs of specific precipitation. In the latter case, the point on the arc most remote from the axis of migration designates the average position of the protein.

Methods of Immunochemical Analysis. The antisera used in these analyses were obtained by immunizing rabbits with extracts of barley or malt in saline solution. The standard technique of double diffusion according to Ouchterlony (7) and the immunoelectrophoretic analysis according to Grabar and Williams (5,6) were used.

For the Ouchterlony technique, a glass plate is covered with a layer of buffered agar 3 mm. deep, and 6-mm. cylindrical holes are punched in the gel. Certain of these reservoirs are filled with the solutions of antigen and the others are filled with the antisera; the number, position, and contents of these reservoirs are decided according to the experimental design. The antigens and antibodies diffuse toward one another in the gel and, where they meet in the correct proportions, a band of precipitation is formed.

Where two bands join to form a single line, there is "identity" between the two antigens forming the precipitates. When the lines cross, however, the antigens in question must differ in their antigenic structures.

For the IEA technique, the proteins are first separated by electrophoresis in veronal buffer 0.05*M*, pH 8.2, and an immune serum containing antibodies specific for the antigenic mixture studied is diffused perpendicularly to the electrophoretic migration axis. These antibodies form arcs of precipitation with their homologous antigens. In this method, proteins are defined by their immunochemical specificity and by their mobility; this allows one to distinguish between proteins of identical or close mobilities, and to detect small quantities of a substance (a few micrograms).

Evaluation of the diffusion coefficient of the alpha-amylase was performed in agar gel according to Allison and Humphrey (8). When the antigen and the antibody are placed in troughs at a 90° angle in such proportions that the specific precipitate forms along a plane which, seen from above, appears as a straight line, the ratio of the

diffusion coefficient of antigen D_g and that of the antibody D_b is given by the relation:

$$\left(\frac{D_g}{D_b}\right)^{1/2} = t_g\,\Theta$$

where Θ is the angle formed by the precipitation line and the well of the antigen. Since D_b is known for rabbit antibodies (3.8×10^{-7} cm.^{2} sec.$^{-1}$), it is possible to calculate D_g.

Characterization of Amylases. The alpha- and beta-amylases are known to possess the following properties:

1. Both amylases can degrade amylose, which is colored blue by iodine, into sugars and dextrins which are no longer colored by iodine (9).

2. The enzymatic activity of the beta-amylase can be inhibited much more easily than that of the alpha-, by salts of heavy metals such as Hg^{++}, Ag^{+}, and Cu^{++} (9,10). Using these properties of the two amylases, we have developed a characterization of the amylases in barley and malt extracts. After electrophoresis in agar gel or after IEA, and, in the latter case, after washing of the gel, this gel is immersed in a 0.25% amylose bath with a pH of about 4.5 for 2 hr. The slides carrying the gel are brought to 37°C. for about 1 hr. and dipped in a 0.25% iodine bath. The amylase activities appear as transparent zones contrasting with the blue background. This method detects both alpha- and beta-amylases. When the same method is used but $5 \times 10^{-6}M$ $HgCl_2$ (or $5 \times 10^{-4}M$ $AgNO_3$ or $10^{-3}M$ $CuSO_4$) is added to the gel before EA, or the gels are soaked after IEA or double diffusion in a $HgCl_2$ solution for 1 hr., only the alpha-amylase activity can appear. This technique has enabled us to localize and identify the alpha- and beta-amylase among the precipitation lines.

Results

The technique of characterization of the alpha- and beta-amylases described above was used to define the constituents of barley and malt extracts possessing these activities. It was then possible, using the immunochemical methods, to describe some of the physicochemical properties of these constituents.

Characterization of the Alpha- and Beta-Amylases. After the EA of a saline extract of malt, a zone of alpha-amylase activity appears which cannot be seen with barley extracts. A zone of beta-amylase activity appears with barley extracts, but it has not been possible to discover this activity in malt extracts; it is known, however, that this activity is present in malt, but its presence is masked in our characterization method by the activity of alpha-amylase.

IEA enables us to further identify the amylases in malt and barley extracts. With malt extract, alpha-amylase activity is detected along one arc of precipitation and beta-amylase activity along another. With barley extract, beta-amylase is also detected along an arc of specific precipitation (Fig. 1). It is apparent from IEA that the mobilities of

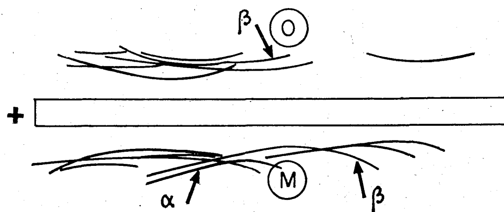

Fig. 1. Schema of IEA of saline extract of barley (O) and malt (M) developed with an antimalt serum. The arrows indicate the precipitin lines corresponding to alpha- and beta-amylases.

the alpha- and beta-amylases of malt are close together, a fact which was predictable from the results of the EA, and that the beta-amylase extracted from malt migrates more slowly than the beta-amylase extracted from barley.

Study of Some Physicochemical Properties of the Alpha- and Beta-Amylases by Immunochemical Methods. (a) Alpha-amylase. The mobility of the alpha-amylase in veronal buffer 0.05M, pH 8.2, has been calculated to be equal to -3×10^{-5} cm.^{2}volt^{-1} sec.$^{-1}$.

The approximate value of the diffusion coefficient has been found to lie between 5.7 and 6.4×10^{-7} cm.^{2} sec.$^{-1}$ at 18°C., by the Allison and Humphrey method (8), with malt extracts and an antimalt serum in which the antibodies other than those against the alpha-amylase have been eliminated by absorption with a barley extract.

Separation of the proteins of malt extract into albumins and globulins was performed as described in the section on techniques. The double diffusion of these fractions with an antimalt serum, first absorbed by a barley extract and containing only the antialpha-amylase antibody, shows that the alpha-amylase is in the globulin fraction (Fig. 2).

(b) Beta-amylase. As already mentioned, beta-amylases of barley and malt have different mobilities in veronal buffer pH 8.2. These have been calculated to be -2.7×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ for barley and -2×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ for malt.

These two proteins, though they possess different mobilities, show an immunochemical identity (Fig. 3). Separation of the saline-soluble

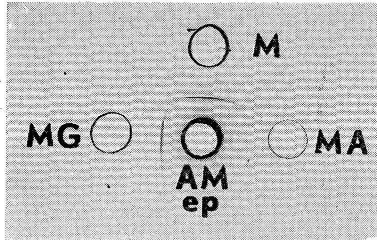

Fig. 2. Double diffusion of malt extract (M) of the albumin fraction of malt (MA) and of the globulin fraction of malt (MG) with an antimalt serum absorbed with barley flour (AMep).

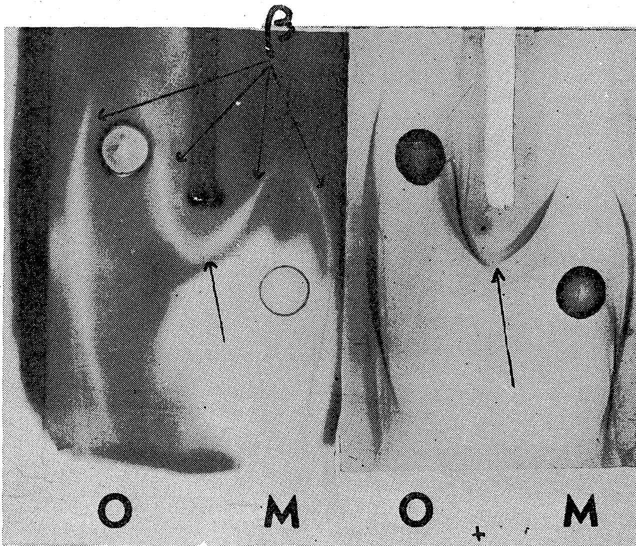

Fig. 3. IEA of saline extract of barley (O) and malt (M) with an antimalt serum. Left: characterization of amylases. Right: protein staining of precipitin lines. The mobilities of barley and malt beta-amylases are quite different (see also Fig. 1). The continuity of the lines of precipitation (arrow) proves the immunochemical identity of the beta-amylase in barley and malt.

proteins into globulins and albumins points out another difference between the beta-amylase extracted from barley and the beta-amylase extracted from malt. In double diffusion experiments the beta-amylase is detected by the characterization reaction with amylose. This precipitin line (Fig. 4) appears intensely with the OA and MA fractions, less intensely with OG fraction, and very weakly with the MG fraction. Thus, the beta-amylase of barley seems to be only partially soluble in water, whereas that of malt is almost completely soluble in water.

Attempts to Detect Alpha-Amylase in Barley by Immunochemical Methods. (a) Double diffusion. In a first series of experiments, we tried

to see if, among the proteins of malt extract, the one which possesses alpha-amylase activity corresponds immunochemically to one or another of the barley extract proteins. We observed in double-diffusion reactions, using barley and malt extracts and antimalt sera, that several

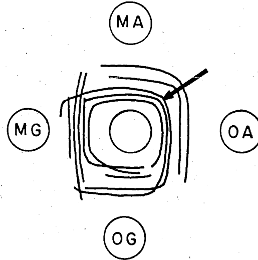

Fig. 4. Schema of double diffusion of albumin fractions of barley (OA) and malt (MA) and of globulin fractions of barley (OG) and malt (MG) with an antimalt serum. The arrow indicates the continuous line which has also been characterized by its activity proving the presence of beta-amylase in OA, MA, and OG fractions.

lines of precipitation are common to the two extracts. But at least one line of precipitation is specific to the malt extract. Alpha-amylase characterization shows that it is precisely this precipitin line along which is found the alpha-amylase activity. As shown in Fig. 5 the

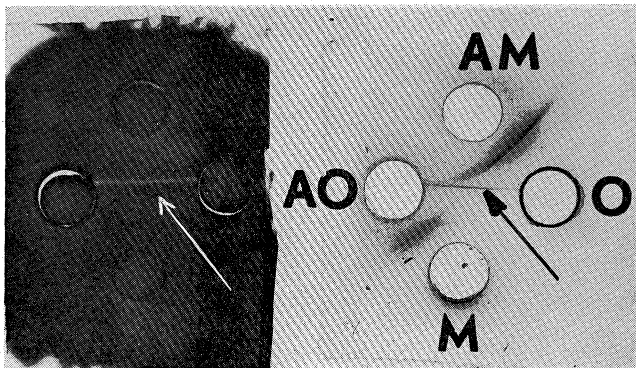

Fig. 5. Double diffusion of barley (O) and malt (M) extracts with antibarley (AO) and antimalt (AM) sera. Left: characterization of alpha-amylase. Right: protein staining of precipitin lines. The identical configuration is used for both parts of the figure.

precipitation line indicated by the arrow corresponds to the alpha-amylase of malt as indicated by the characterization reaction. But this specific precipitate formed by an antimalt serum does not correspond to any protein of barley extract, because, if it did, this line of precipita-

tion should deviate near the well containing the barley extract. Moreover, since this line of precipitation does not deviate near the well containing the antibarley serum, this serum must not contain antialpha-amylase antibodies. And yet, since antibodies can be formed against very small quantities of antigens present in the immunizing doses, it seems justified to state that the alpha-amylase does not exist, even in trace amounts, in ripe barley extracts.

(b) Absorption of immune sera. Using barley flour, we absorbed from antimalt serum all antibodies which would react with any barley constituents. Barley flour was suspended in antimalt serum and, after agitation for 2 hr. at 37°C., the suspension was centrifuged. This operation was repeated twice. Using double diffusion, we made certain that this absorbed serum did not contain any more antibodies reacting with the proteins of the saline extract of barley. But it still reacted with malt extract, giving a specific line of precipitation. And, again, it was just this line which showed alpha-amylase activity (Fig. 6). According

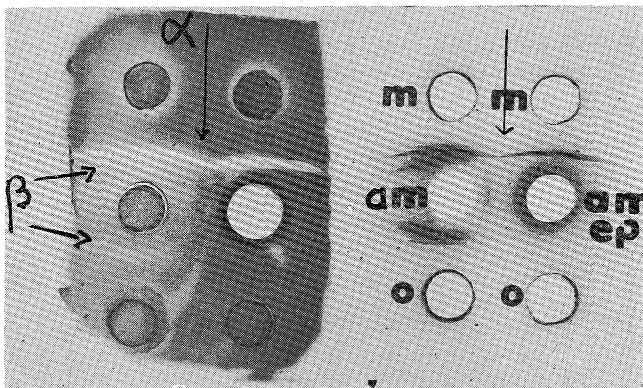

Fig. 6. Double diffusion of barley (O) and malt (M) extracts with an antimalt serum (AM) and with the same antimalt serum absorbed with barley flour (AMep). Left: characterization of alpha-amylase. Right: protein staining of precipitin lines. The arrow indicates the precipitin lines corresponding to the alpha- and beta-amylases.

to some authors, hydrogen sulfide is capable of enhancing the beta-amylase activity at the time of extraction (11). We also have tried to use this technique for the alpha-amylase by passing a current of H_2S through the flour-serum suspension during absorption of the serum. Experiments performed with an antiserum thus absorbed did not change the above results. It appears, therefore, that barley flour does not contain an antigen able to react with the antibody corresponding to the alpha-amylase of malt, and that this alpha-amylase does not exist in barley.

Discussion

By using a substrate that can be sufficiently degraded by the alpha- and beta-amylase and by using inhibitors more specific for the beta- than for the alpha-amylase, we were able to identify the two enzymes along specific arcs of precipitation after IEA or double diffusion.

The separation of the proteins of malt extracts into albumins and globulins has helped to confirm by immunochemical methods the insolubility in distilled water of the alpha-amylase and the solubility in water of the beta-amylase. The differences noted between the beta-amylases of barley and malt show that operations of malting alter the beta-amylase of barley. But the immunochemical identity of this enzyme in the two extracts proves that at least the parts of these proteins which make up the antigenic sites are identical. Thus, the beta-amylase of malt most probably derives from the beta-amylase of barley. Moreover, the fact that a total identity is detected between beta-amylase of barley and malt indicates that the beta-amylase of barley keeps its antigenic structure during malting.

Attempts to detect in the saline extract of barley a protein with the same immunochemical properties as that of the alpha-amylase of malt indicate complete absence in barley extract of any substance possessing identical or analogous immunochemical character. Moreover, absorption of antibodies from antimalt serum with whole barley flour, even when performed in H_2S atmosphere (a condition described as allowing maximum extraction of beta-amylase, by liberation of the latent enzyme), never absorbed the antibodies corresponding to the alpha-amylase in a malt antiserum. It appears, therefore, that the alpha-amylase is quite specific to malt. If it pre-exists in barley its structure must be quite different, because we know that the immunochemical properties of an antigen molecule relate to its stereochemical structure and that it is more than probable that a molecule of proenzyme should have at least in part the same structure as the active enzyme. It is possible, however, that the weak alpha-amylase activity detected in saline extracts of barley (1) might be due to factors other than the presence of the alpha-amylase of malt, as has already been proposed (1). Therefore, we believe we can conclude that this amylase is synthesized during germination, as has already been suggested (12).

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