ISOLATION AND PROPERTIES OF CRYSTALLINE α-AMYLASE FROM GERMINATED BARLEY

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The isolation of crystalline α-amylase has been reported in a preliminary communication (1). Details of the method of isolation, behavior of the fractions leading to crystallization, and some properties of the crystalline material are reported here. This material constitutes the first instance of the crystallization of an α-amylase from a higher plant and is probably the only protein crystallized from barley malt. The only other amylase crystallized from a higher plant is the β-amylase protein obtained from the sweet potato by Balls, Walden, and Thompson (2).

Methods of Assay

Amylase and Other Enzymes—When α- and β-amylases were both present, each enzyme was determined by the methods of Olson, Evans, and Dickson (3). After the β enzyme had been destroyed (by heat) in the course of purifying α-amylase, the α-amylolytic activity was determined as described by Schwimmer (4), by measuring the rate of change in the intensity of the color formed when iodine is added to the digesting starch. Under controlled conditions, the time required to arrive at any specified intensity is inversely proportional to the amount of α-amylase. This proportionality holds over a wide range of enzyme concentrations and of color intensities, but measurements are best when the system transmits about half the incident light.

For reasons of convenience and somewhat increased accuracy, a new unit of α-amylase will be used here and in later communications. The new unit is defined as that amount of enzyme which (with starch as the initial substrate) gives 50 per cent transmission at 660 mμ in 10 minutes under the previously specified conditions.1 The units may be read off by interpolation on an ordinary curve of time versus per cent transmission, made with any preparation of malt α-amylase that has previously been heated to 70°

1 α-amylase unit as described here is equivalent to 0.16 of the unit of Sandstedt, Kneen, and Blish (5), and to 0.0026 mg. of protein nitrogen in the form of the twice crystallized protein described here.
Fig. 1. The change in color of the dextrin-iodine complex and the per cent of glucosidic bonds hydrolyzed as the hydrolysis by \(\alpha\)-amylase proceeds.

Fig. 2. Relation between optical density of malt protein solutions and protein nitrogen content as determined by micro-Kjeldahl. Curve A, optical density of turbid solutions determined at 400 m\(\mu\) in an Evelyn colorimeter. The solutions were prepared by mixing 1.0 ml. of a (suitably diluted) crude protein solution with 10.0 ml. of 2 per cent trichloroacetic acid. Concentration is expressed as mg. of nitrogen per ml. of the diluted protein solution before addition of the trichloroacetic acid. Curve B, optical density of 1 cm. of solutions of crystalline \(\alpha\)-amylase read at 281 m\(\mu\) in a Beckman spectrophotometer. Protein nitrogen concentration is expressed as mg. per 10 ml. of solution.
for 15 minutes to free it from the $\beta$ enzyme. Such a curve is shown in Fig. 1. For comparison the fraction of total glycosidic bonds hydrolyzed in the same time is shown.

Hydrolysis of glycosidic bonds was followed by a modification (6) of the Willstätter-Schudel reduction of hypoiodite (7). This method was also used to determine maltase activity. Phosphatase was assayed by the method of Axelrod (8), which is based on the rate of hydrolysis of $p$-nitrophenyl phosphate.

**Protein Nitrogen and Specific Activity**—The specific activity is expressed as $\alpha$-amylase units per mg. of protein nitrogen, [u./g]. Protein nitrogen was estimated by one of three methods. For routine analysis of crude preparations a turbidimetric measurement of the precipitate with trichloroacetic acid was made in an Evelyn colorimeter at 400 m$\mu$. Protein nitrogen in colorless, purified preparations was determined by the ultraviolet absorption of the protein solution at 281 m$\mu$ measured by a Beckman spectrophotometer. The relation between optical density and protein nitrogen in these two cases is given by Fig. 2. These methods were checked by usual micro-Kjeldahl determinations on washed trichloroacetic acid precipitates.

**Procedure for Isolation**

**Characteristics of Crude Concentrate**—Commercial malt syrup was used as source material. This extract was very high in sugar and contained salt and sulfate as preservatives. Its activity was about 1.5 times that of the same weight of a highly diastatic malt; however, the specific activity of the $\alpha$-amylase therein was 3 to 4 times as high. When heated to 70° for 16 minutes, over half of the protein, including all of the $\beta$-amylase, was removed, but there was no loss of $\alpha$-amylase activity (Table I). In contrast to dilute malt extracts (4), $\alpha$-amylase cannot be removed from this material by bentonite. No additional calcium salt was required to stabilize the enzyme during heating. As a routine procedure, 2 liters of this concentrate were heated to 70° for 15 minutes and then filtered on a Büchner funnel with 60 gm. of Celite. The residue was then washed with calcium sulfate solution until the final volume was again 2 liters. This filtrate is Line 2 in Table II, where the entire procedure is summarized and the specific activity and yield are shown at each step.

**Precipitation by Ammonium Sulfate**—Practically all the $\alpha$-amylase was precipitated from the filtrate just described by making it 0.43 to 0.50 saturated with ammonium sulfate, and adjusting the pH to 5.6 to 6.0 with ammonia.$^4$ After 2 hours at room temperature, the precipitate was filtered

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$^2$ Obtained through the courtesy of Dr. Alexander Frieden of the Pabst Brewing Company, to whom the authors express their best thanks.

$^4$ Solid ammonium sulfate was used in the amount required if the liquid was water (instead of being a strong solution of maltose).
onto fluted paper (Schleicher and Schüll, No. 588), and washed with 5 or 6 times its volume of 0.33 saturated ammonium sulfate solution (pH 5.6 to 6.0) containing 2 gm. of calcium sulfate per liter. The washed precipitate is Line 3 in Table II.

This precipitate may be considerably purified by repeated fractional precipitations with ammonium sulfate. A specific activity of 340 was once obtained in this way, to be compared with 390 found later for the crystalline protein. The yields were invariably low, and the material never got beyond the “globular stage,” but much information of subsequent value was obtained by studying these preparations. The enzyme exhibited a minimum solubility in dilute ammonium sulfate (0.28 saturated) in the neighborhood of pH 6.0. Furthermore, more protein impurities remained in solution when precipitation was made at pH 6.0 to 7.0 than otherwise.

### Table I

<table>
<thead>
<tr>
<th>Time of heating</th>
<th>Activity [α u./ml]</th>
<th>Protein nitrogen [mg. per ml]</th>
<th>Specific activity [α u./PN]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.0</td>
<td>2.35</td>
<td>6.7</td>
</tr>
<tr>
<td>4</td>
<td>16.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>15.9</td>
<td>1.41</td>
<td>11.3</td>
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<tr>
<td>10</td>
<td>15.8</td>
<td>1.25</td>
<td>12.8</td>
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<tr>
<td>16</td>
<td>16.0</td>
<td>1.10</td>
<td>14.6</td>
</tr>
<tr>
<td>24</td>
<td>14.0</td>
<td>1.07</td>
<td>13.1</td>
</tr>
<tr>
<td>35</td>
<td>13.0</td>
<td>0.95</td>
<td>13.7</td>
</tr>
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</table>

### Table II

<table>
<thead>
<tr>
<th>Summary of Isolation of Malt α-Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>1. Crude malt extract</td>
</tr>
<tr>
<td>2. Heated to 70° and filtered</td>
</tr>
<tr>
<td>3. Ppt. at 0.43 saturated ammonium sulfate</td>
</tr>
<tr>
<td>4. Alcoholic solution of partly purified enzyme</td>
</tr>
<tr>
<td>5. Combined eluates</td>
</tr>
<tr>
<td>6. Ppt. from eluate</td>
</tr>
<tr>
<td>7. Suspension of 1st crystals</td>
</tr>
<tr>
<td>8. &quot; of crystals, 4 times recrystallized</td>
</tr>
</tbody>
</table>

obtained in this way, to be compared with 390 found later for the crystalline protein. The yields were invariably low, and the material never got beyond the “globular stage,” but much information of subsequent value was obtained by studying these preparations. The enzyme exhibited a minimum solubility in dilute ammonium sulfate (0.28 saturated) in the neighborhood of pH 6.0. Furthermore, more protein impurities remained in solution when precipitation was made at pH 6.0 to 7.0 than otherwise.
Thus the specific activity of the precipitate obtained at 0.28 saturation of a partially purified enzyme solution that contained 81 units per ml. was 210 at pH 5.1, 260 at pH 6.0, 240 at pH 7.0, and 205 at pH 7.5.

An attempt was also made to predict the specific activity of the pure enzyme by the method of Falconer and Taylor (9). To this end, a solution of partly purified enzyme was precipitated at different concentrations of ammonium sulfate. At zero ammonium sulfate (i.e., when diluted with water instead) the solution contained 135 units of α-amylase per ml. with a specific activity of 197 units per mg. of protein nitrogen. Fig. 3, b is plotted from the solubility data of Fig. 3, a. According to Falconer and Taylor (9) the tangent DB represents the behavior of a system in which the enzyme is pure. Therefore, in the preparation used, 48 per cent of the total protein is inert, and the specific activity of the remaining 52 per cent is 197 × 1.93, or 380. This is in remarkably close agreement with the maximum activity, 390, found for the material crystallized later. The activity and total protein content of the soluble phase are shown in Fig. 3, a. The enzyme is seen to be less soluble than the remainder of the protein.

The stability of the partially purified protein toward methanol, ethanol, and acetone was tested. Of these reagents, ethanol seemed to be least destructive. In 40 volume per cent ethanol about half the enzymic activity was lost in 6 hours at 25°, while at 5° no loss whatever occurred in
24 hours. The enzyme was found to be quite soluble in ethanol solutions up to 55 volumes per cent, at temperatures around -20°.

Adsorption on Starch Granules—The adsorption of amylase on starch was reported by Starkenstein in 1910 (10). In 1933, Holmberg (11, 12) found that the \(\alpha\) component of malt amylase could be separated from the \(\beta\) component by adsorption of the former on starch, but apparently this method was not used extensively. Hockenhull and Herbert (13) found that the amylase of \textit{Clostridium acetobutylicum} is adsorbed onto potato starch in the presence of alcohol at low salt concentrations.

It was found, starting with material corresponding to Line 3, Table II, that the \(\alpha\)-amylase could be adsorbed to some extent on raw wheat starch from aqueous solutions, but much more completely from solutions containing ethanol. Unlike most adsorptions, that of the amylase required considerable time. In one experiment, 100 cc. of 40 per cent ethanol containing 10,500 units of enzyme was stirred at 0° with 20 gm. of raw starch. In 30 minutes, 50 per cent of the activity was adsorbed, 65 per cent in 80 minutes, and 70 per cent (the maximum) in 90 minutes. Some non-selective adsorption of protein also took place. Better results were obtained by the use of a Zechmeister column, usually 40 mm. in diameter, filled to a depth of 10 to 12 cm. with a mixture of equal weights of Celite and wheat starch. The column was used as follows:

The washed precipitate (Line 3, Table II) was made up to a volume of 50 ml. in water; then an equal volume of cold 80 per cent ethanol containing 5 gm. of calcium chloride per liter was added. The insoluble material was filtered out with a little Celite and washed with diluted alcoholic calcium solution (40 per cent alcohol) until the total volume of filtrate and washings was 200 ml. (Line 4, Table II). This was kept for 30 to 60 minutes at +5° and filtered again if a precipitate (CaSO\(_4\)) appeared. It was poured onto a dry well packed column of starch and Celite and sucked through. The column was usually kept at room temperature. It was next washed with diluted alcohol (40 per cent) alcohol until the outflow was colorless. Elution was made by pouring 50 ml. portions of water saturated with calcium sulfate through the column at room temperature. Each portion of eluate was kept separate until assayed and only those containing much enzyme were combined and used (Line 5, Table II).

An experiment was made to determine the distribution of enzyme in the column after the washing procedure. The data are given in Table III. Most of the enzyme remained near the top, but the enzyme toward the bottom was somewhat purer.

Final Purification—The enzyme was precipitated from the combined eluates (about 150 cc.) by 0.66 saturation with ammonium sulfate (pH 6.0). The precipitate (Line 6, Table II) was collected in a little Celite on a small
Biúchner funnel, and washed thereon with about 20 ml. of half saturated ammonium sulfate (pH 6.0). It was then dissolved while on the filter in as small a volume as convenient (15 to 20 ml.) of half saturated calcium sulfate. At this stage the enzyme solution was clear, colorless, and contained about 2 to 3 mg. per ml. of protein nitrogen. The pH of the solution was adjusted, when necessary, to 5.9 to 6.0 with 0.1N ammonia, and saturated ammonium sulfate solution at the same pH was added slowly up to 0.26 saturation. When placed at 30–33°, crystals of the enzyme appeared

| TABLE III |
| Distribution of α-Amylase Activity in Starch-Celite Column after Washing* |

<table>
<thead>
<tr>
<th>Portion No.</th>
<th>Length cm.</th>
<th>Per cent of total activity</th>
<th>Per cent of total protein nitrogen</th>
<th>Relative purity</th>
<th>Per cent activity per cm. of depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8</td>
<td>12</td>
<td>13</td>
<td>92</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>42</td>
<td>45</td>
<td>93</td>
<td>13</td>
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<tr>
<td>3</td>
<td>4.6</td>
<td>35</td>
<td>32</td>
<td>110</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>11</td>
<td>10</td>
<td>110</td>
<td>6</td>
</tr>
</tbody>
</table>

* The column was divided into four portions, No. 1 being the topmost. Portions 1 and 3 are slightly higher in Celite content, since a pad of Celite was placed at either end.

| TABLE IV |
| Yield and Specific Activity of Malt α-Amylase on Recrystallization |

<table>
<thead>
<tr>
<th>No. of crystallization</th>
<th>Suspension</th>
<th>Supernatant therefrom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity [α u.]</td>
<td>Specific activity [α u./F N]</td>
</tr>
<tr>
<td>1st</td>
<td>30,500</td>
<td>345</td>
</tr>
<tr>
<td>2nd</td>
<td>22,100</td>
<td>388</td>
</tr>
<tr>
<td>3rd</td>
<td>19,300</td>
<td>396</td>
</tr>
<tr>
<td>4th</td>
<td>15,800</td>
<td>389</td>
</tr>
</tbody>
</table>

in 1 to 3 hours. Later, ammonium sulfate was added to 0.33 saturation, and after standing overnight in the incubator the suspension was centrifuged (Line 7, Table II). For recrystallization, the sedimented crystals were taken up in 15 ml. of cold half saturated calcium sulfate solution and the crystallization carried out as before. The protein seems to be more soluble in the cold. Crystal formation was not observed below 25° or below pH 5.6. The specific activity and recovery of enzyme through four successive crystallizations are shown in Table IV. The purity of the mother liquors gradually approached that of the crystals which separated
from them. Analysis (Kjeldahl) of the twice crystallized material gave 13.4 per cent nitrogen after prolonged dialysis against distilled water.

The crystals (Fig. 4) are hexagonal prisms, about 13 μ in length, capped by pyramids. Larger crystals (about 30 μ in length) have been occasionally obtained when an amorphous precipitate appeared at first and did not crystallize for several hours. They were usually accompanied by large amounts of amorphous protein.

![Crystals of the α-amylase of barley malt (× 580)](image)

**Several Properties of Crystalline Protein**

**Solubility Curve**—The solubility of the crystalline protein in half saturated ammonium sulfate (adjusted to pH 5.95 with ammonia) was determined in the presence of increasing amounts of the crystals, according to the technique described by Herriott (14). The crystals were washed with the solvent and then suspended in it. The suspensions were diluted to constant volume with more solvent and the containers were slowly rotated on a horizontal axis for 16 hours at 25°. Thereafter the solid phase was removed by centrifugation, and the absorption of the supernatant liquids was measured at 281 mμ in a Beckman spectrophotometer as a measure of their protein content. Analysis of the curve shown in Fig. 5 indicates, according to the discussion of Herriott, the presence of 95 to 97 per cent of one protein component.

The ultraviolet absorption spectrum of a twice crystallized preparation
is shown in Fig. 6. It exhibits the usual maxima, owing to tyrosine, and tryptophan.

Osmotic Pressure, Molecular Weight, and Turnover Number—The molecular weight of four times crystallized protein was calculated from osmotic pressure measurements made by the procedure of Bull (15). The enzyme was dissolved in 0.2 M acetate buffer, pH 6.1, made up with water saturated with calcium sulfate. The protein was dialyzed against the solvent for 1 week at 5°. Equilibrium in the osmometers was reached from both sides in 5 days at 30°. The final values were 2.86 and 2.72 cm. of water for the osmotic pressure of solutions containing respectively 0.656 and 0.643 gm. of protein per 100 gm. of solvent. The molecular weight was therefore calculated as 59,500 ± 900.

Calculation of the turnover number from this value and the data of Fig. 7 indicates 19,000 bonds hydrolyzed per minute per molecule of enzyme (molecular weight taken as 59,500) when the total number of glycosidic bonds per molecule of enzyme in the digestion mixture was $4 \times 10^6$. The turnover number for crystalline pancreatic amylase, calculated from the data of Meyer, Fischer, and Bernfeld (16) and by Fischer and Bernfeld (17),
is 25,000, with 45,000 as the molecular weight when $4 \times 10^4$ glycosidic bonds were present per molecule of enzyme.

**Enzymic Action**—The elimination of $\beta$-amylase in the early stages may be justly assumed, since this enzyme in malt is known to be relatively sensitive to heat. Furthermore, the rate of production of reducing substances from starch (Fig. 7) is not in accord with the effect of even a small amount of the $\beta$ enzyme. Thus the addition of calcium sequestering agents

![Ultraviolet absorption of crystalline malt $\alpha$-amylase (0.0083 mg. of protein nitrogen per ml. in 0.1 M acetate, pH 6.0, containing CaSO$_4$).](http://www.jbc.org/)

(0.01 M "tetraphosphate" or 0.01 M hexametaphosphate, pH 5.0) was found to have no effect upon $\beta$-amylase, but destroyed all the amylolytic activity of the $\alpha$ preparations.

The extent of digestion of soluble starch solutions by two different concentrations of crystalline amylase is shown in Fig. 7. Formation of reducing substances appears to follow the same kinetics as observed by Bernfeld and Studer-Pécha (18) with partially purified malt $\alpha$-amylase acting on amylose. This behavior has been regarded as characteristic of
\(\alpha\)-amylase action. On the other hand, Fig. 1 indicates that the reducing value at the "achromic point" (no color of the digest with iodine) was 24 per cent compared to the value of 30 per cent reported by Hanes and Cattle (19). However, this difference may be due to the differences in the iodide and iodine concentrations used to obtain the color. Swanson (20) has shown that the absorption maxima of the dextrin-iodine complexes shift towards smaller wave-lengths during digestion of amylose by \(\alpha\)-amylase.

Whereas partially purified preparations of malt \(\alpha\)-amylase rapidly lost all activity when dialyzed against distilled water, it was difficult to destroy the activity of a solution of crystals completely by the most thorough dialysis (20 ml. was dialyzed for 1 week against a total of 30 liters of distilled water). Such dialyzed enzyme lost 84 per cent of the activity and 59 per cent of the protein became insoluble. This preparation still gave a positive flame test for calcium. Furthermore, crystals which were washed thoroughly against 0.4 saturated ammonium sulfate containing no calcium still contained 0.13 per cent of calcium. In this preparation the molar ratio of protein of molecular weight 60,000 to calcium is 2. It would seem that crystalline preparations still require calcium for activity, but that it is more difficult to remove it from the crystalline enzyme. Lyophilization of a solution of crystallized enzyme dissolved in saturated calcium sulfate water resulted in almost complete loss of activity.
According to Lane and Williams (21), inositol is an active constituent of pancreatic α-amylase. They found that the γ isomer of hexachlorocyclohexane acted as an inhibitor and that inositol reversed this inhibition. When crystalline malt α-amylase was dissolved in 50 per cent dioxane and 2 mg. per ml. of a mixture of isomers of hexachlorocyclohexane were added, no decrease in activity was observed after 24 hours at 5°. A control containing dioxane but no hexachlorocyclohexane also lost no activity under the same conditions. Qualitative tests for inositol as described by Salkowski (22) were negative.

**SUMMARY**

Isolation and crystallization of the α-amylase of germinated barley (malt) has been described in detail. The procedure consists essentially of heating concentrated malt extract, precipitating the remaining protein with ammonium sulfate, adsorption of the enzyme from an alcoholic solution on wheat starch granules, and crystallization of the eluted enzyme from ammonium sulfate. Behavior of the fractions leading to crystallization and some of the properties of the crystalline substance, including molecular weight, purity, and ultraviolet absorption spectrum, are reported. The kinetics of hydrolysis, the requirement of the enzyme for calcium, and the apparent absence of inositol in the enzyme are also discussed.

**BIBLIOGRAPHY**

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