PREPARATION OF HIGHLY ACTIVE YEAST INVERTASE*

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(Received for publication, July 26, 1934)

In some preliminary experiments it was found that certain yeast invertase preparations which had been variously purified by adsorption to kaolin and alumina in accordance with the Willstatter procedures (1) were soluble in saturated aqueous solutions of ammonium sulfate. This solubility of the enzyme is interesting for two reasons. In the first place, saturated aqueous solution of ammonium sulfate is regarded as almost a universal agent for precipitating enzymes from their aqueous solutions, and in the second place, because saturated ammonium sulfate solutions precipitate practically all proteins so far tried. In other words, this peculiar behavior of the invertase brings up the question whether it contains an unusual protein, soluble in saturated aqueous solutions of ammonium sulfate, or whether it contains any protein at all. It seemed even more remarkable, when it was noticed that several other yeast invertase preparations, made in a similar manner, were almost entirely insoluble in this same reagent. This inconsistency was disconcerting until it was noticed that after the removal of the ammonium sulfate by dialysis, the soluble preparations were more active than before the ammonium sulfate treatment (2).

Because the autolytic processes by which the invertase preparations are rendered soluble in water consist of a degradation of substances constituting the yeast cell, by other enzymes present in the yeast (3, 4), it was suspected that the variations in the solubilities in saturated solutions of ammonium sulfate manifested

* The work described in this paper is based upon part of a thesis submitted by J. George Lutz in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.
The invertase preparations soluble in saturated aqueous ammonium sulfate solutions were also unique in that the precipitation tests, such as heat coagulation, phosphotungstic acid, trichloroacetic acid, tannin, and mercuric chloride, which are generally accepted as characteristic of protein, failed entirely. In carrying out these tests the solutions containing these preparations were concentrated so that 100 cc. contained at least 0.5 gm. of the enzyme preparation. In one case the solution was concentrated to a 1 per cent solution. Even in this case all of the protein tests were negative except those which are likewise given by protein degradation products, viz. the tryptophane, xanthoproteic, and biuret tests. Applying these same protein tests to highly active yeast invertase preparations insoluble in saturated aqueous solutions of ammonium sulfate gave good positive reactions in solutions containing as little as 24 mg. in 50 cc. The test for the presence of yeast gum in the preparation by means of the Salkowski method (5), viz. precipitation by means of hot alkaline copper solution, was negative. Likewise when heated for 2 hours with 20 per cent hydrochloric acid and then neutralized with alkali, the resulting solution gave no indication of cuprous oxide formation when treated with Fehling's solution. On the other hand, the invertase preparations soluble in saturated aqueous ammonium sulfate solution gave strong Molisch tests for carbohydrate, indicating the presence of the latter, and also the Adamkiewicz (6) test for tryptophane. The presence of tryptophane in preparations which fail to respond to characteristic protein reactions may be of interest in connection with Willstätter's (7) claim that he and coworkers have succeeded in obtaining preparations free from tryptophane.
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Nium sulfate solution appear to be as active per unit weight of dry material as any so far reported in the literature, one preparation having a time value¹ of 0.102 minute.

EXPERIMENTAL

Brewery yeast served as the source for the invertase preparations. It was found that "discard" yeast from the brewery was not satisfactory for the preparation of the enzyme, since not only is the invertase content usually very low, but considerable difficulty was encountered in the purification of the preparations. Through the kindness of the Jacob Ruppert Brewery, New York, yeast which had only been used in one or two brews was used. The invertase content of the yeast was increased by the method described by Willstätter, Lowry, and Schneider (9). The yeast so treated was about 4 times more active than before enrichment and had a time value of about 56 minutes.

Autolysis—The washed yeast was usually subjected to dilute acid autolysis. 10 kilos of washed and filtered yeast were mixed with 1 liter of toluene and kneaded until the mixture became thiny plastic (30 minutes to 1 hour). 20 liters of water, at 30⁰, were added and the mixture stirred mechanically until 80 to 100 per cent of the enzyme had become soluble. This usually required 2 to 3 days at 30⁰ or 5 to 10 days at room temperature. The pH of the autolyzing solution was maintained at about 5 by means of dilute acetic acid or dilute ammonia. After autolysis the clear filtrate was aged by storing under toluene for 2 or more weeks.

Precipitation by Alcohol—If in the aging of the autolysates any precipitate separated, this was filtered off and the clear liquid cooled by placing it in liter bottles and allowing it to stand in contact with cracked ice. Each cooled liter portion was mixed with a liter of ice-cold 95 per cent alcohol, infusorial earth was stirred into it, and the mixture was then filtered by suction

¹ O'Sullivan and Tompson (8) proposed what is known as the time value as a standard for measuring the degree of activity of invertase preparations. The time value is defined as the time in minutes required by 50 mg. of an invertase preparation for hydrolyzing 4 gm. of sucrose in 25 cc. of solution containing 0.25 gm. of monosodium phosphate monohydrate, at 15.5⁰ to such an extent that, after mutarotation, the rotation (measured at 20⁰) referred to the D line of sodium light is 0⁰. This corresponds to the hydrolysis of 75.93 per cent of the sucrose.
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through a pad of infusorial earth on a Buchner funnel. The precipitate on the funnel was next washed with cold 95 per cent alcohol and sucked as dry as possible. The operation up to this point required from 10 to 15 minutes. The precipitates from each liter portion together with the admixed earth were mixed each with 1 liter of water, and the accumulated mass was extracted by stirring from 6 to 10 hours and filtering. In this way, a yield of 95 to 100 per cent of the original invertase was obtained in solution.

*Kaozin Adsorption*—The Willstätter (10, 11) procedures for the use of kaolin in the purification of yeast invertase preparations were not found satisfactory for the preparation of the larger quantities of enzyme used in this work, because of the difficulty in handling such dilute solutions and also because the amount of acid used frequently inactivates considerable amounts of the enzyme. Furthermore the aqueous extracts of the alcohol precipitates always contain considerable inorganic phosphates, which seem to make adsorption of the invertase to kaolin and alumina difficult. By first dialyzing the aqueous solutions, after the alcohol treatment, it was found that more concentrated enzyme solutions could be adsorbed in the presence of considerably less acetic acid (0.1 to 0.01 M) with equally satisfactory results. The following procedure was therefore adopted.

The aqueous extracts from the alcohol precipitation were dialyzed in Visking sausage casings2 (12) for 24 to 36 hours against running tap water. The dialyzed solutions were diluted so that 1 liter contained about 10 units of invertase and cooled to 0°. After dialysis the pH of liter portions was made 3.5 by means of dilute acetic acid and 200 to 400 cc. of a 5 per cent kaolin suspension were stirred in. The exact amount of kaolin was determined on a few small trial samples and so chosen that 90 to 95 per cent of the invertase was adsorbed. The suspension was next filtered off and washed thoroughly with water.

*Elution from Kaolin*—The active material resulting was released from the kaolin by suspending it in an equal volume of 0.5 per cent secondary ammonium phosphate, pH about 8, and stirring the mixture for about 15 to 30 minutes. The eluate (the term

2 Visking Corporation, Chicago.
eluate has been used to designate the solutions obtained by the process of elution) usually contained from 70 to 100 per cent of the adsorbed invertase units. In one or two instances, however, only 50 per cent of the invertase originally adsorbed to the kaolin was recovered in the elution.

The principal effect of the adsorption to kaolin is the removal of yeast gum. Generally, after a single adsorption, the eluates gave no precipitate with alkaline copper solutions (Fehling's solution) nor did they reduce Fehling's solution after hydrolysis with acid. In the event that the gum was not entirely removed, then the eluate was dialyzed to remove salts, especially inorganic phosphates, and subjected again to adsorption to kaolin or alumina. If a second kaolin adsorption is resorted to, care must be taken, because the enzyme seems to be easily inactivated, even when very small amounts of acetic acid are used. It is also recommended to perform the adsorption at 0° and to wash with ice water. If considerable loss of enzyme occurs in spite of these precautions, then it is best to resort to adsorption to alumina in place of the second kaolin adsorption. It was usually found advisable to test a small portion of the preparation by means of the second adsorption to kaolin before deciding which of the two procedures should be adopted, there being taken into account not only the yield of enzyme in terms of units, but also the time value of the final product.

Adsorption to Alumina—The eluate from the kaolin adsorption was dialyzed free of inorganic phosphate, and enough 1 per cent alumina suspension to adsorb 90 per cent of the enzyme was added, the mixture stirred for 5 to 10 minutes, filtered by suction through a pad of infusorial earth, and finally washed thoroughly with cold water. The amount of alumina necessary to adsorb 90 per cent of the invertase present in the solution was determined by several preliminary adsorptions, small samples of the solution being used.

The invertase was eluted from the alumina by stirring with a volume of 0.5 per cent secondary sodium phosphate equal to the original amount of the invertase solution used in the adsorption to alumina. 10 minutes stirring usually were found to be sufficient for setting free 80 to 90 per cent of the enzyme. The suspension was clarified by filtering through a pad of infusorial earth and the
pH of the clear filtrate was adjusted to between 4 and 5. By dialyzing a portion of the filtrate against distilled water until free of phosphate, preparations were obtained having time values ranging from 0.3 to 0.2 minute.

Ammonium Sulfate Treatment—The main portion of the solution containing the invertase preparation eluted from the alumina was then concentrated by evaporation through Visking sausage casing until the concentration of the enzyme had reached the neighborhood of 50 units per 100 cc. and then filtered clear through infusorial earth, if necessary. Finely powdered ammonium sulfate was then stirred in until an excess had been added. The stirring was continued usually for 3 to 4 hours in order to make certain that the solution was completely saturated with ammonium sulfate. A cloudy precipitate always resulted from this treatment due to the separation of that part of the enzyme preparation insoluble in the saturated ammonium sulfate solution. By filtering through infusorial earth the ammonium sulfate extracts were obtained as a clear, colorless solution, containing from 70 to 90 per cent of the enzyme originally present in the solution obtained in the elution of the material from the alumina.

To remove the ammonium sulfate, the solutions were dialyzed in sausage casings, either against running tap water or better by placing the sausage casings in a rocking dialyzer (13) for a day, followed by 3 to 6 days against distilled water, pH 6.5, in the electric refrigerator, until a sample no longer gave a test for sulfate ion. After dialysis, the solution was passed through glass funnels, provided with fritted glass bottoms. In this way invertase preparations were obtained having time values of 0.102 to 0.11 minute.

Determination of Time Value—25 cc. of an invertase solution, containing a known weight of the enzyme preparation, were added to 100 cc. of a 20 per cent sucrose solution, buffered at pH 4.4 with 0.25 gm. of acid sodium phosphate monohydrate, and the solution was thoroughly shaken. Both solutions had been allowed to attain the temperature of the thermostat, 25°, ± 0.01° before mixing. The delivery time of the pipette used was 10 seconds. The delivery from the pipette was started 5 seconds before the time recorded as the starting time of the hydrolysis. Four 25 cc. samples were withdrawn at suitable time intervals and the inversion stopped by the addition of a drop of saturated sodium hy-
droxide solution. After 10 minutes (to allow for mutarotation) the rotations of the samples were determined in 2 dm. tubes at 25° with the mercury arc (λ = 546.1 μ) as the light source. The per cent sucrose inverted was calculated by means of: per cent inverted = \( \frac{(R - r)}{(R - L)} \times 100 \). \( R \) = initial dextro-rotation (25.04° for 16 per cent sucrose solution at 25°) and \( L \) = rotation after complete inversion (−7.32°). 0° rotation = 25.04° / 32.36° = 77.38 per cent inversion at 25°. The time required for 0° rotation was determined graphically by plotting per cent inverted against time and noting the time corresponding to 77.38 per cent inversion.

**Example**—1 cc. of a highly purified invertase preparation was diluted to 100 cc. and 25 cc. of the diluted solution were used in a hydrolysis as described above.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Rotation (degrees)</th>
<th>Inversion (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.04</td>
<td>72.0</td>
</tr>
<tr>
<td>65</td>
<td>1.45</td>
<td>76.3</td>
</tr>
<tr>
<td>70</td>
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<td>76.3</td>
</tr>
<tr>
<td>72</td>
<td>0.01</td>
<td>77.4</td>
</tr>
<tr>
<td>74</td>
<td>−0.30</td>
<td>78.5</td>
</tr>
</tbody>
</table>

Zero time at 25° equaled 72.0 minutes.

Since it is customary to report time values as based on hydrolyses conducted at 15.5° instead of 25°, the above value, 72.0 minutes, had to be multiplied by the factor 1.685, the ratio of the times required for the hydrolyses at the two respective temperatures, to reach 0° rotation. This ratio, 1.685, was determined by hydrolyzing several sucrose solutions, similar in all respects, at the two temperatures, 25° and 15.5°, and then plotting rotation against time and noting the times, for the two temperatures, corresponding to the respective 0° rotations. In this way it was found that the hydrolyses at 25° reached 0° rotation 1.685 times faster than the one run at 15.5°. Hence, in case of the example given above, the time required for 0° rotation at 15.5° would be equal to 72 × 1.685 or 121 minutes.

**Determination of Dry Weight**—Since the time value is based on the activity of 50 mg. of solid material, it was necessary to deter-
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mine the weight of the solid matter contained in a given volume of the enzyme solution. A method devised by Dr. E. L. Saul of this laboratory was used. Two thin walled glass bulbs of about 25 cc. capacity, provided with a drawn out narrow open tip on the top, were balanced one against the other. A given volume of the enzyme solution was introduced into one of the bulbs, and this and its empty tare were both placed in a drying oven heated electrically to 110°. Clean dry air was blown through capillary tubes into both bulbs until the enzyme solution was evaporated to dryness (10 hours). Both bulbs were then sealed while still in the oven, after which they were cooled in a desiccator and again counterbalanced. The increase in weight was the weight of the dry invertase preparation. This method was used because the thin film of material obtained when invertase preparations are dried is extremely hydroscopic. By sealing the dry residue in a bulb, the material was prevented from taking up moisture during the weighing.

Calculation of Time Value—With the invertase preparation used in the example of hydrolysis mentioned above, it was found that 16.3 cc. of the undiluted invertase solution contained 13.9 mg. of solids. Since 1 cc. of this solution was diluted 100 times and 25 cc. of the diluted enzyme solution were added to 100 cc. of the sugar solution, it follows that 25 cc. of the hydrolyzing sucrose solution contained 0.05 cc. of the undiluted enzyme solution, or 0.04264 mg. of solid enzyme preparation. From the definition for time value it will be seen that since 0.04264 mg. of enzyme preparation caused hydrolysis to 0° in 121 minutes at 15.5°, 50 mg. of the preparation will only require \((0.04264 \times 121)/50 = 0.103\) minute, which according to definition is the time value of the preparation.

BIBLIOGRAPHY

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