and soluble in alcohol. It was purified by crystallization from 95% alcohol and separated in distorted prisms, which melted at 122–124°, to an oil. It did not give Millon's test and dissolved in concentrated sulfuric acid, forming a bright red solution. It was dried for analysis at 105°.

Calculated for $C_{14}H_{16}O_4N_2$: N, 10.14; found: 10.13.

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF COLUMBIA UNIVERSITY, NO. 224.]

STUDIES ON AMYLASES, V. EXPERIMENTS UPON THE PURIFICATION OF THE AMYLASE OF MALT.

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Many of the investigators of malt diastase have interested themselves only in its action, but a number have given more or less attention to the problem of the chemical nature of the enzyme substance. Among these may be mentioned in chronological order, Dubrunfaut,[1] Zulkowski,[2] Loew,[3] Lintner,[4] Hirschfeld,[5] Szilagyi,[6] Egoroff,[7] Osborne,[8] Wroblewski,[9] Seyffert,[10] Sykes and Hussey,[11] Friedenthal,[12] Frankel and Hamburg,[13] Hata,[14] Chrzaszez,[15] Wohl and Glimm,[16] Buroczewski, Krause and Krzemecki,[17] Lyalin,[18] Pribram,[19] Panzer,[20] Van Laer.[21]

Of these investigations that of Osborne is probably the most significant

[1] *Compt. rend.*, **66**, 274–5 (1868), and *Dingler's Polytech. J.*, **187**, 491–501 (1868).

[2] *Wien. Akad.*, **77**, II, 647–55; *Jahr. Thierchem.*, **8**, 356 (1878).

[3] *Pflüger's Arch. ges. Physiol.*, **27**, 203–14 (1882); also **36**, 170 (1885); *J. prakt. Chem.*, [2] **37**, 101–4 (1888).

[4] *J. prakt. Chem.*, [2] **34**, 378–94 (1886); **36**, 481–98 (1887); also *Z. ges. Brauw.*, **1886**, 479, 481; **1888**, 80; and *Woch. Brau.*, **16**, 166 (1899).

[5] *Arch. ges. Physiol.*, **39**, 499–574 (1886); see also reply by Lintner, *Ibid.*, **40**, 311–14 (1887).

[6] *Chem. Ztg.*, **15**, 349–51 (1891).

[7] *J. Rousskago Phys. Chem. Obchtchestva*, **25**, No. 2; *Mon. Sci.*, [4] **8**, II, 741–2 (1894).

[8] THIS JOURNAL, **17**, 587–603 (1895); **18**, 536–42 (1896); also *Ber.*, **31**, 254–9 (1898).

[9] *Ber.*, **30**, 2289–95 (1897); **31**, 1127–30, 1130–36 (1898); *Z. physiol. Chem.*, **24**, 173–223 (1897).

[10] *Z. ges. Brauw.*, **21**, 195–7, 221–3 (1898).

[11] *J. Fed. Inst. Brew.*, **4**, 527 (1898).

[12] His-Engelmann's *Arch. Anat. Physiol., Physiol. Abth.*, **1900**, 181–94.

[13] *Hofmeister's Beitr.*, **8**, 389 (1906).

[14] *Biochem. Z.*, **17**, 156–87 (1909).

[15] *Wochschr. Brau.*, **27** and **29**; also *Z. Spiritusind.*, **34**, 545; *Chem. Abs.*, **6**, 1050 (1912).

[16] *Biochem. Z.*, **27**, 349–75 (1910).

[17] *Bull. intern. acad. sci. Cracovie*, (*A*) **1911**, 369–70; *Chem. Abs.*, **6**, 1757 (1912).

[18] *J. Russ. Phys. Chem. Soc.*, **42**, 624–33; *Chem. Abs.*, **5**, 3833 (1911).

[19] *Biochem. Z.*, **44**, 293–302 (1912).

[20] *Z. physiol. Chem.*, **82**, 276–325, 377–90; **84**, 161–88 (1912–13).

[21] *Bull. Acad. roy. Belg.* (*Classe des sciences*), **1913**, No. 4 (April), 396–451.

because of the completeness and definitness of his records of methods and results and the fact that he has recorded systematic quantitative determinations of the diastatic powers of his preparations. Many of the records of other investigators can not be interpreted with any degree of certainty and are therefore entirely inconclusive (even when highly suggestive) because of the absence of accurate determinations of diastatic power. Without a quantitative measurement of activity, we cannot be sure that "purification" has really concentrated the enzyme, even though it may have removed certain impurities. The removal of a small amount of impurity may be accomplished at the cost of a change in the enzyme substance itself. Some investigators attempt to excuse their failure to record quantitative statements of diastatic power by advancing the argument that activity is an uncertain criterion in the case of purified preparations, since the purification may have removed some electrolyte whose presence is necessary to the normal activity of the enzyme. This argument is valid only on the assumption that the investigator is not sufficiently acquainted with the proper conditions for testing the diastatic power. Any adequate method for measuring enzyme action must provide an environment in which the enzyme can function normally. The necessity of adding an electrolyte in testing his purified preparations was noted by Osborne and has been more fully discussed by several writers since. We cannot assume that the investigators of the past eighteen years have been ignorant of Osborne's work or have failed to take advantage of it in testing their own preparations, and the evidence is therefore lacking that any of them has succeeded in obtaining material representing a higher concentration of actual (active) enzyme than did Osborne's preparation.

With full acknowledgment of our indebtedness to previous investigators, we shall not take space to review their work at this point. Many of the facts which they have recorded will be discussed in connection with our own findings later.

Experimental.

In general there was so little agreement among the previous investigators that it seemed best in beginning our attempts to purify this enzyme to base our procedures upon the experience which we had gained in our study of the pancreatic amylase,[1] rather than upon the conflicting statements to be found in the literature of malt amylase.

The purification experiments which have been carried out thus far have, for the most part, been based upon extraction of malt, dialysis of the extract, and final precipitation by alcohol or acetone. We are of course aware that contact with strong alcohol is apt to have a deleterious effect upon the enzyme; but by a sufficiently careful control of concentrations, times, and temperature we were able, by the method of alcohol

[1] THIS JOURNAL, 33, 1195; 34, 1104.

precipitation, to obtain from pancreatin uniform preparations of amylase much stronger than had previously been described from any source.[1] For this reason, and because we desire to make as systematic a comparison of the two amylases as is practicable, we have devoted some months to the preparation of malt amylase in solid form by the method of alcohol precipitation. Not only is such material essential to our comparative studies but from nearly every standpoint there are great advantages in working with a dry solid which can be kept for reasonable lengths of time and weighed out as desired for quantitative experiment, rather than with extracts or solutions which change rapidly and usually contain unknown amounts of accessory substances.

The method which we have used chiefly in the purification experiments thus far completed is essentially as follows: Ground malt is extracted with $2^1/_2$ times its weight of cold water, dilute alcohol, or very dilute acid phosphate solution, for $1^1/_2$ to 2 hours, the operations being carried out as far as possible at ice-box temperature (about 10°) and never allowed to rise to 20°. The extract is decanted or filtered and dialyzed in collodion bags against 10 times its volume of cold water (7° to 15°) for 24 to 42 hours with two or three changes of dialysate, then filtered and precipitated with alcohol or acetone, discarding the precipitate formed by the addition of an equal volume of alcohol or acetone and collecting the fraction which precipitates when a further quantity of alcohol or acetone, sufficient to bring the final concentration up to 65 or 70%, is added. This precipitate is dried in partial vacuum over sulfuric acid at ice-box temperature. So far as practicable a temperature of about 10° is maintained throughout the process; in those operations which cannot feasibly be carried out in the ice box we endeavor, by placing the containers in dishes of ice water, to keep the temperature below 20°.

In many cases we have been unable, with the facilities at hand, to maintain the desired conditions throughout, which doubtless accounts for much of the variation in power shown by successive preparations. It will also be apparent from the brief descriptions of individual preparations below that many of the details of the above outline have been modified from time to time for purposes of experiment.

By procedures essentially as outlined above we have made 38 experiments in purification, of which 34 have yielded products having powers above 400 on the scale used in this laboratory[2] (equivalent to over 600 on Lintner's scale) which, so far as we are aware, is the highest activity previously recorded for malt amylase and has apparently been reached in only one previous preparation.[3]

[1] THIS JOURNAL, **33**, 1195; **34**, 1104.

[2] Sherman, Kendall and Clark, THIS JOURNAL, **32**, 1082–84.

[3] Osborne, THIS JOURNAL, **17**, 598.

Of these preparations 13 had powers between 800 and 1200 on our scale, corresponding to 1200–1800 on Lintner's scale, and 7 had powers from 1200 to 1540 on our scale, corresponding to 1800 to 2300 on the scale proposed by Lintner. It may be recalled that a value of 100 on Lintner's scale represents the diastatic power of the most active preparation obtained by Lintner and which was apparently regarded at the time as pure diastase of maximum activity.

Twenty-nine other preparations made by essentially different methods have, in general, yielded much less satisfactory results.

The following brief statements regarding the individual preparations, including those made by the unsuccessful methods, are introduced here partly for the sake of completeness of record and convenience of reference in subsequent papers, and partly because a comparison of these with the corresponding data for pancreatic amylase[1] brings out some interesting differences.

Preparations 1–9 were devoted to preliminary experiments upon the influence of fineness of grinding of malt, separation of husks, extracting with 50% alcohol, precipitating and redissolving the enzyme before dialysis in 50% alcohol, as compared with dialyzing the original extract, and use of different concentrations of alcohol for final precipitation. It appeared that neither extreme fineness of grinding nor removal of husks was of any appreciable advantage in preparing the malt for extraction, that standing in solution in 50% alcohol involves a marked loss of diastatic power, and that in fractional precipitation by successive additions of strong alcohol most of the enzyme was obtained in the fraction between 50 and 65%.

Preparation 10.—Experiments having shown that the deterioration was less rapid in very dilute acid phosphate solution than in water or alcohol, malt was extracted with $2^1/_2$ times its weight of M/250 monosodium phosphate solution, the extract was filtered, 2% of maltose added[2] and the whole dialyzed against 10 volumes of the same phosphate solution at about 10° for 42 hours with 2 changes of dialysate. It was then filtered and precipitated fractionally by alcohol. The precipitate forming at 50% alcohol was discarded; that between 50 and 65% had, when air dry, a diastatic power of 1090; that between 65 and 71% had a power of 850. These diastatic powers are in terms of the "new scale" used in this laboratory; the same power expressed on Lintner's scale for diastase preparations would give figures approximately 50% higher. In subsequent preparations the final precipitation was in general as follows:

To the solution was added an equal volume of absolute alcohol or acetone, the precipitate thus obtained was discarded, and alcohol or acetone was added to the filtrate in sufficient quantity to bring the concentration up to 65 or 70% and the precipitate obtained at this point was collected and dried. The "amylase preparations" of this series consist therefore of material soluble in 50% but precipitated by 65 or 70% of alcohol or acetone.

Preparation 11.—Made as above, power 840.

Preparation 12.—Time and temperature not sufficiently controlled, power 360.

Preparation 13.—Made like Nos. 10 and 11, power 1052.

[1] THIS JOURNAL, 33, 1198–1201.

[2] This was a precaution taken as the result of our previous experience with pancreatic amylase but which later experiments indicated to be unnecessary for the amylase of malt.

Preparation 14.—Made as above, power 855; a portion dialyzed 67 hours instead of 42 gave a product whose power was only 390.

Preparations 15–20.—Here the method used in making Preparation 10 was modified in different ways, *e. g.*, by use of different solvents for extraction and dialysis, or use of trypsin in an attempt to facilitate removal of extraneous protein. These preparations showed no advance in diastatic power.

Preparation 21.—Made like No. 10, except that dialysis, during last 15 hours, was against pure water, and final precipitation was by means of Merck's (highest purity) acetone, the fraction between 55% and 63% being collected. Power, in air-dry condition, 1540.

Preparation 22 yielded a sticky precipitate which could not be handled; reason not ascertained.

Preparation 23.—Trypsin was added in neutral solution to the extract before dialysis. Power, 560.

Preparation 24.—Unsuccessful attempt to make use of kaolin in purification.

Preparations 25 and 26.—Made like No. 21; powers 690 and 575.

Preparation 27 yielded a sticky precipitate which could not be handled.

Preparation 28.—Temperature not sufficiently controlled, power 345.

Preparation 29.—Made like No. 21; precipitated finally in two fractions (1) by alcohol 50–65%, power 1230; (2) by alcohol 65–74%, power 1450.

Preparation 30.—Lost.

Preparations 31, 32, 33 were devoted to a comparison of alcohol and acetone as final precipitants. Powers of alcohol precipitate 417, 290, 585; of acetone precipitates, 450, 245, 393, respectively.

Preparations 34 and 35 were salted out by ammonium sulfate before dialysis and finally precipitated by alcohol or acetone as usual; powers 640 and 690.

Further experiments upon the use of ammonium sulfate are to be made.

Preparations 36, 37, 38, 40.—Unsuccessful attempts to purify by precipitation before dialysis.

Preparations 39, 41–44.—Made like No. 21; powers 205, 260, 725, 660, 755, respectively.

Preparation 45.—Lost.

Preparation 46.—Delayed in extraction and in filtering after precipitation; power 690.

Preparations 47 and 48.—Delayed and also exposed to room temperatures during purification; powers 220 and 240.

Preparations 49–52.—Made like No. 10 except that each was dialyzed 27 hours against M/250 monosodium phosphate with 2 changes and then 15 hours against pure water; finally precipitated by alcohol (between 50 and 66%); powers 960, 1230, 940, 1470, respectively.

Preparation 53.—Malt was extracted with 50% alcohol (without phosphate), otherwise conditions same as in preceding; power 1220.

Preparation 54.—Same, except that alcohol was used to extract the malt; contained acid phosphate (M/250); power 740.

Preparations 55, 56,[1] *58.*—Made in the same manner as preparation 52; powers 1230, 940, 830, respectively.

Preparations 59–61.—Extracts made with M/250 acid phosphate were inoculated with yeast before dialyzing. In preparation 59 the extract, after addition of yeast, was allowed to stand at 40° for 1/2 hour and then at room temperature for 1/2 hour before placing in the cold dialyzer; power 550. Preparations 60 and 61 were kept cold throughout; powers 1215 and 1090, respectively.

[1] Preparation 57 was lost.

Preparation 62.—Lost.

Preparation 63.—Time and temperature insufficiently controlled; power 580.

Preparation 64 was made by the same method as No. 52 but on a larger scale, the extract being clarified by means of a DeLaval separator; power 940.

Preparation 65.—A much larger quantity (3200 grams) of malt was treated by the same method as No. 64 but on account of mechanical difficulties the processes of extraction, clarification, and separation of the final precipitate from the strong alcohol were much delayed and the temperature could not be kept as low as usual. The diastatic power of this product was only 400.

Preparations 66–69.[1] *71–73.*—Preliminary experiments designed to diminish the amount of dextrin in the malt extract by fermentation with *Schizosaccharomyces pombe* before dialyzing. The powers of these preparations (air dry) were 400, 810, 1160, 450, 210, 575, 660, respectively. The conditions here have not yet been satisfactorily worked out; we intend to experiment further with this method.

Properties.

As our experiments upon purification of malt amylase are still in progress only a brief preliminary statement regarding the properties of the products will be given here.

The conditions used in determining the activity of pancreatic amylase were found unsuited to the testing of malt preparations, the latter requiring a lower concentration of hydroxyl ion. The powers given above were obtained by allowing the enzyme to act upon the soluble starch in a solution to which had been added 4 cc. of M/10 monosodium phosphate per 100 cc. of the total volume. In other respects the determinations were made in the same manner as with the pancreatic preparations.[2]

The appearance of these preparations was much like that of pancreatic amylase. They appeared somewhat less readily soluble in water. A 1% aqueous solution showed on heating a distinct opalescence at 60° which increased at 65°–75° but showed no distinct flocculation nor other change when held at this temperature for one hour but on being further heated it coagulated at about 80° into large flocks, which settled, leaving a clear supernatant liquid. The coagulum when dissolved in sodium hydroxide gave a distinct blue-violet biuret reaction and also showed the xanthoproteic test and Millon's reaction. The filtrate showed a rose-red biuret reaction.

These preparations also give the tryptophane reaction.

In so far as the amounts of material have permitted nitrogen determinations in individual preparations we have in general found the stronger preparations to have the higher nitrogen content. The most active preparations which were analyzed for nitrogen showed about 14%. This is lower than was found in our amylase preparations from the pancreas and also lower than Osborne's figures for nitrogen in malt diastase, though higher than the figures reported by many other observers. Since the

[1] Preparation 70 was lost.

[2] Sherman, Kendall and Clark, THIS JOURNAL, 32, 1082–85.

chemical nature of our substance is so much like that of Osborne's in other respects, we suspect that our material is still somewhat contaminated with carbohydrate. This point will be dealt with more fully in a later paper.

The diastatic powers of our preparations have been given in terms of the scale which has been in use in this laboratory during the past three years. Otherwise stated, a typical preparation (No. 29) showed a power of 2200 on Lintner's scale, or in terms of actual observations, when acting on "soluble" starch at 40°, it formed 3680 times its weight of maltose in 30 minutes.

Through the coöperation of Professor H. T. Beans we have made a few preliminary observations with the ultramicroscope. The enzyme solution showed the appearance of a fairly typical colloid. When brought in contact with "soluble" starch under the ultramicroscope a slight appearance of granulation of the starch solution was seen almost instantly and this increased until finally (at a point where the digestion mixture gave no color reaction with iodine) the appearance was that of a number of separate reef-like clusters showing practically no movement and widely separated in an otherwise clear field. We hope to extend these observations in the near future.

Summary.

The method which had served best for the purification of pancreatic amylase was found not to be applicable to malt but a method which yields products of much higher activity than have previously been recorded for malt preparations has been developed.

This product in its general properties corresponds with Osborne's description of malt diastase published in 1895–96. It is a yellowish white solid approaching the typical proteins in nitrogen content and showing typical protein reactions and the appearance of a colloid under the ultramicroscope. Heated in water solution it coagulates; the coagulum gives a violet-blue, and the filtrate a rose-red, biuret reaction.

Tested under proper conditions the best preparations showed diastatic powers of 1200 to 1500 "new scale" corresponding to Lintner figures of 1800 to 2200, an activity three to four times that of any malt diastase previously described and one-third to one-half that of the purified pancreatic amylase. The preparations from malt are much less uniform in diastatic power than those from the pancreas; the study of methods of purification is therefore being continued.

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