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ENZYME PURIFICATION BY ADSORPTION: AN INVESTIGATION OF PANCREATIC AMYLASE[1]

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Willstätter and his co-workers have investigated the behavior of the pancreatic enzymes toward adsorbents and have found that pancreatic amylase can be adsorbed from an aqueous-alcohol–glycerol solution by alumina gel and can be recovered by extraction with a glycerol–ammonium-phosphate–ammonia solution. They have described[1a] a method of purifying pancreatic amylase based upon the use of kaolin and alumina gel as adsorbents by means of which they report that they attained an unprecedented degree of purification and state that their purified preparation gives none of the usual protein reactions.

The purpose of the present paper is to record briefly the results of our investigation of the adsorption method of purifying pancreatic amylase. In the course of our work we have repeated that of Willstätter and his co-workers and confirmed and extended their findings as to the behavior of this enzyme in relation to certain adsorbents, but have refuted the claim that their method results in a higher degree of purification of this enzyme than had previously been attained by the method developed in this Laboratory[2] and have shown that the negative evidence which they offer as bearing upon the chemical nature of the enzyme is not valid.

In addition to testing the conclusions of Willstätter, Waldschmidt-Leitz and Hesse, our experiments were also designed to extend our knowledge of the physicochemical behavior of pancreatic amylase, especially with reference to its amphoteric nature as indicated by a careful investigation of the conditions governing the adsorption and recovery of this typical enzyme.

For the sake of economy of space we omit both the full presentation of our numerical data and anything in the nature of a general review of earlier work upon the adsorption relations of enzymes.

Experimental Part

The procedure of Willstätter, Waldschmidt-Leitz and Hesse[1a] for the purification of pancreatic amylase, involves the following steps: (*a*) extraction of fat-free pancreas powder with 87% aqueous glycerol solution,

[1] We are greatly indebted to the Carnegie Institution of Washington for grants in aid of this investigation.

[1a] Willstätter, Waldschmidt-Leitz and Hesse, *Z. physiol. Chem.*, **126**, 143 (1923); **142**, 14 (1925).

[2] Sherman and Schlesinger, THIS JOURNAL, **33**, 1195 (1911); **34**, 1104 (1912). Sherman, *Proc. Nat. Acad. Sci.*, **9**, 81 (1923).

with removal of solids by centrifuging; (*b*) dilution of the glycerol extract with five times its volume of water, and clarification by centrifuge; (*c*) acidification of solution and treatment (twice) with alumina gel to remove lipase by adsorption; (*d*) treatment of the acid solution from *c* with kaolin (twice) for removal of protease; (*e*) solution from *d*, made slightly alkaline and mixed with alcohol to 50% by volume, is treated with alumina gel to adsorb the amylase; (*f*) extraction of amylase from alumina gel of *d* by means of a solution containing ammonium phosphate and ammonia; this solution is then tested for enzymic activity; (*g*) dialysis of solution from *f*, which contains active amylase, against running water for a week, to remove glycerol and salts; (*h*) evaporation of the dialyzed solution which no longer contains active amylase and use of the residue as a measure of the weight of enzyme material whose activity was measured in *f*, and for qualitative tests as to its protein nature.

Our first attempts to repeat the work of these investigators by simply following their own description of procedure showed that critical study of several steps was needed. In order to economize space we shall here combine our findings and our discussion on each successive step in the process.

Investigation of Successive Steps

The use of glycerol as a solvent to prevent or retard the deterioration of the enzyme in solution is an expedient which has frequently been employed for several decades past, but was set aside by Osborne, and later in the work of this Laboratory, in favor of procedures capable of yielding the purified active enzyme as a dry product.[2] This was not accomplished, nor even approximated, in the work of Willstätter, Waldschmidt-Leitz and Hesse,[1a] and the presence of glycerol as used by them introduces a serious, and apparently as yet insuperable, difficulty in the subsequent attempts to measure the activity of the purified enzyme or determine its chemical nature. In order satisfactorily to discuss enzymic activities, it is essential to have a basis for the expression of results. The weight of total solids of a preparation has been the usual basis of reference, but the small amount of enzyme solids usually present cannot be determined quantitatively in solutions containing 15 to 35% of glycerol such as those resulting from the Willstätter method. Attempts to remove the glycerol by superheated-steam distillations were found to be unsatisfactory with these solutions. The errors involved in attempts to determine the glycerol quantitatively in the organic material remaining after its partial removal were also too great. The use of dialysis to remove the glycerol as employed by Willstätter, Waldschmidt-Leitz and Hesse[1a] does not solve the problem, as the activity of the amylase is lost and the enzyme itself is more or less completely removed with the glycerol.

A possible basis of reference seemed to be offered by a systematic study of the nitrogen content of all solids and solutions obtained in the process. In addition to supplying a comparative basis for calculating activities, data thus gained promised to be of direct interest by indicating the progress of the purification. In order to be able to correlate enzymic activity with nitrogen content it was necessary to avoid the introduction of nitrogenous reagents such as the ammonium-phosphate—ammonia solution used by Willstätter for the extraction of the amylase from the alumina gel by which it had been adsorbed. A slightly alkaline solution of 0.05 *M* (mono- and di-) sodium phosphate was found to be as efficient as the ammonium-phosphate—ammonia solution for this purpose and had the added advantage of permitting accurate control of the reaction or Sørensen value, (P_H). A systematic study of the influence of the reaction of the solutions upon the adsorption and extraction of the enzyme appeared to be of primary importance in order to establish definite and reproducible conditions.

It was found practicable to apply to the dilute glycerol and alcohol solutions the e.m.f. measurements usually made in determinations of Sørensen values in aqueous solutions. Steady e.m.f. readings were obtained and solutions could readily be reproduced or titrated to the same values. These values were calculated to Sørensen values in the usual way. It was recognized that these Sørensen values may not bear the same relation to the hydrogen-ion concentrations or activities of the solutions as they would in aqueous solutions containing no glycerol or alcohol. This was not significant in our work, however, as aqueous solutions made up in the same way but containing no glycerol or alcohol gave e.m.f. values which agreed with those given by the solutions containing the glycerol.

Experiments were carried out in which the Sørensen values of the solutions at each stage in the process were varied between wide limits. In stating the reaction at which an adsorbent was used, the Sørensen value of the solution at the end of the adsorption is given.

After the optimum hydrogen-ion activity had been established for each step, the process of purification as a whole was carried through several times under the most favorable conditions. The nitrogen content of all solutions and solids obtained in the process was determined by the Kjeldahl method.

All measurements of amylase activity were made in buffered 2% starch solutions at 40° under the conditions for testing saccharogenic activity which have been described in previous papers from this Laboratory.[3] In addition to the careful regulation of the reaction of all solutions for adsorption experiments and for the tests of activity, other precautions which have been found advantageous, such as keeping the solutions and

[3] Sherman and Walker, THIS JOURNAL, **43**, 2461 (1921).

reagents cold and guarding them from the unfavorable influence of light were observed. The solids were tested for activity in suitable aliquot portions of their suspensions in buffered solutions. The amounts of alumina and kaolin thus introduced were found by suitable blank experiments not to influence the accuracy of the method for measuring the amylase activity under the conditions used.

The amylase activities of both solids and solutions at each step were tested so that the total percentage of amylase recovered after each treatment could be calculated, and also to obtain a measure of the adsorption taking place. By measuring the activities of both solids and solutions, the losses of activity due to inactivation of the enzyme were not added to the effects of adsorption, that is, the decreases in activity occurring in the solutions were not assumed to be due entirely to increases in the activity of the solids.

The dilution of the glycerol extract with five times its volume of water causes the precipitation of a relatively large amount of inert material, with the result that the amylase activity expressed as milligrams of maltose formed per milligram of nitrogen of the enzyme solution is increased. Thus in typical cases the activity so expressed was 5700 to 6500 in the extract, and 8000 to 8900 in the clarified solution. As mere dilution of the glycerol extract with water throws down inert material, it is plain that the initial extraction with glycerol takes into solution with the enzyme an unnecessary amount of other material. Thus, while the deterioration of the enzyme is retarded, its purification is also retarded by the use of glycerol.

The treatments of the glycerol-water solution with alumina gel introduced by Willstätter and his co-workers for the removal of lipase[4] were found to cause loss of amylase (15 to 25%) without increasing appreciably the purification as a whole. Changes in the Sørensen values of the solutions from P_H 6.1 to P_H 8.4 did not appreciably influence the activity of the amylase recovered after the treatments. No measurements of the lipase activities of the solutions were made in this work but when the solutions were adjusted to P_H values of 6.1 to 6.7, which would correspond to the conditions of "slightly acid" specified by Willstätter for the most complete removal of the lipase, the increase in amylase activity per milligram of nitrogen as compared with that of the solution before the treatments was negligible. In some typical experiments, the amylase activity per milligram of nitrogen in the solutions was 7000 to 10,000 mg. before, and 7000 to 11,000 mg. after the treatments.

The treatments of the aqueous solutions with kaolin were introduced by Willstätter and co-workers for the purpose of separating the protease from the amylase. We have repeated these steps using suspensions of commercial kaolin (Merck's) and also of electro-osmotically purified

[4] Willstätter and Waldschmidt-Leitz, *Z. physiol. Chem.*, 125, 132 (1923).

kaolin[5] as specified by Willstätter and co-workers. As the latter appeared more effective it was used in the work reported here. The solutions and residues obtained after the treatments with kaolin were tested quantitatively both for amylase and protease activities. It was found possible to remove various proportions of the proteolytic activity depending upon the reaction of the solution. At an effective hydrogen-ion concentration of about P_H 5.6 to 5.8, most of the proteolytic activity of these glycerol-water extracts is removed with relatively little loss (about 17 to 19%) of the amylase activity.

Willstätter, Waldschmidt-Leitz and Hesse report removal of all of the protease by these treatments of the solution with kaolin. This we have not been able to confirm but find in the case of their method, as in that of Sherman and Schlesinger, that the purified pancreatic amylase still shows some proteolytic activity. It seems probable that the negative results obtained by them in their tests for protease activity may have been due to the inadequacy of their method. It has been shown[6] that the method used by us, that of measuring the increase of soluble nitrogen in a protein solution, is both more delicate and more reliable as a test for protease activity than that of depending upon the increase in titratable acidity used by Willstätter and co-workers.

The adsorption of pancreatic amylase by alumina gel which is relatively slight from an aqueous solution, even when this is tested over a wide range of Sørensen values, becomes marked from a 50% alcohol solution. This was also noted by Willstätter and his co-workers. In addition, we have established a definite relationship between the adsorption of the amylase by the alumina gel and the Sørensen value of its alcoholic solution. There is a gradual increase in adsorption of the amylase with decrease in the acidity of the solutions from those of about P_H 5.0 to those of about P_H 7.3. There is a decided decrease in the adsorption of the amylase in more alkaline solutions of about P_H 8.0 to 8.5. The most effective adsorption with the least destruction of amylase appeared to occur from solutions of about P_H 7.3.

A more definite and very suggestive correlation was found to exist between the P_H of the aqueous sodium phosphate solution and the effectiveness with which it could extract the amylase from the alumina gel in which it had been adsorbed. Solutions of about P_H 7.1 to 7.5 were found to be most efficient. Solutions that were more acid than this did not extract the amylase from the alumina gel so well, and solutions more alkaline than this gave more variable results because of a greater tendency for in-

[5] The electro-osmotically purified kaolin was obtained from Karlsbader Kaolin Elektro-Osmose Aktiengesellschaft and is believed to have been prepared in precisely the same manner as that used in the Willstätter Laboratory.

[6] Sherman and Neun, THIS JOURNAL, **38**, 2199 (1916). Sherman, Garard and LaMer, *ibid.*, **42**, 1904 (1920).

activation of the enzyme to take place. The results with these more alkaline solutions did not indicate a higher retention of the amylase by the alumina.

The relationship between the Sørensen values of the sodium phosphate solution and its ability to extract pancreatic amylase from alumina gel may be taken as an indication of the amphoteric nature of the enzyme, and thus constitutes an added link in the chain of evidence pointing toward its protein nature. Aluminum hydroxide is an amphoteric electrolyte having an iso-electric point in the neighborhood of P_H 5.6. It would therefore exist as an anion in solutions more alkaline than this. The fact that the amylase is least readily extracted from alumina gel by solutions more acid than P_H 7.0 would indicate that the enzyme may be cationic at this value and is therefore more ready to react with and be bound by the anionic alumina gel under these conditions. The readiness with which solutions of about P_H 7.0 extract the amylase from the alumina gel would indicate that under these conditions the amylase may exist in its iso-electric condition and thus be least ionized and less firmly bound by the alumina gel. The results showing no increase in the tendency of the amylase to remain adsorbed by the alumina gel when washed by more alkaline solutions would also agree with this interpretation as both the aluminum hydroxide and the enzyme would presumably exist as anions and have little tendency to interact.

Our results indicate that the two types of alumina gel introduced by Willstätter and his co-workers for the adsorption of the lipase and amylase, respectively,[7] can be used interchangeably for the adsorption of the amylase from its alcoholic solution if the Sørensen value of the solutions for the adsorption is the same.

Whereas the treatments of the aqueous-glycerol solutions with alumina gel and kaolin appear to effect the separation of relatively little inert material as shown by the very small increases in the amylase activities per milligram of nitrogen in the solutions, the adsorption of the amylase from its alcohol-glycerol-water solution by alumina gel and its subsequent extraction from the alumina gel by the sodium phosphate solution (of about P_H 7) were found to be very effective in the purification of the amylase. The amylase activity per milligram of nitrogen in the solution was usually doubled or even more than doubled in this step. Thus the activity of the solution before the adsorption of the amylase was 8000 to 10,000 mg. of maltose per milligram of nitrogen while an activity of 20,000 to 26,000 mg. of maltose per milligram of nitrogen was found in the sodium phosphate solution containing the enzyme extracted from the alumina gel.

An experiment in which the amylase was adsorbed directly from the

[7] (a) Willstätter, *Ann.*, **422**, 72 (1921). (b) Ref. 4, p. 180. (c) Willstätter and Kraut, *Ber.*, **56**, 149 (1923).

clarified solution by treatment with alcohol and alumina gel with the omission of the intermediate treatments of the aqueous glycerol solution with alumina gel and kaolin showed a higher total percentage of amylase activity recovered in the final sodium phosphate solution with only a slightly lower activity per milligram of nitrogen than was obtained after the longer process. Thus the activity in the final enzyme solution in a typical experiment which included the treatments with alumina gel and kaolin was 67% of the activity in the clarified solution and the amylase activity per milligram of nitrogen was 23,000 mg. of maltose, while after the shorter process, the activity in the final solution was 80% of that in the clarified solution and the activity was 20,000 mg. of maltose per milligram of nitrogen. Apparently, the omission of the steps designed to remove lipase and protease did not appreciably decrease the degree of purification of the amylase.

The process of purification by adsorption, as advocated by Willstätter and co-workers and as carried out by us under the most favorable conditions, caused the amylase activity, expressed per milligram of nitrogen, in the solution to be increased between three- and four-fold, that is, the activity in the final enzyme solution was usually about three to four times as high per milligram of nitrogen as that of the original glycerol extract of pancreatin whereas, when the activity of the highly active solid preparations of pancreatic amylase which have been repeatedly obtained in this Laboratory was calculated on the same basis, it was found that they are about twice as active per milligram of nitrogen as the final solution obtained after this adsorption process. The pancreatic amylase preparations previously described by us in THIS JOURNAL[2] appear to represent the highest degree of purification and enzymic activity yet obtained.

As was pointed out above, Willstätter, Waldschmidt-Leitz and Hesse base their conclusions concerning the activity and chemical nature of the amylase upon the weights and qualitative protein reactions of the solids resulting from the evaporation of the enzymically inactive solutions which they obtained after prolonged dialysis against running water, introduced to remove glycerol and salts from their solutions of purified amylase. The errors involved in this procedure are such as vitiate their conclusions on these two points. The results of experiments in this Laboratory with solutions treated in the same way are instructive. Collodion membranes were used in our work and the dialyses were carried out against distilled water which was changed every 24 hours instead of against running distilled water. In this way tests could be made in the solutions from the outside as well as in those from inside of the dialyzers. As there was in this way some chance for the dialyzing solutions to come to equilibrium with the outside solutions, the dialysis was less drastic in our experiments than in those of Willstätter and co-workers.

It was found that even under our conditions there was a gradual loss of amylase activity in the dialyzing solutions. The solutions in the bags were found to lose their amylase activity entirely in from three to five days. Positive evidence of the presence of protein or of its decomposition products was obtained in the outer as well as in the inner solutions by means of ninhydrin, Millon, xanthoproteic and biuret tests. These tests were always positive in any solution which showed amylase activity. The intensity of the color tests was found to decrease as the dialysis continued and it became necessary to concentrate some solutions before positive tests were obtained.

In one case the final, active, purified enzyme solution obtained after carrying out the adsorption process under the most favorable conditions was subjected to fractional precipitation by alcohol and ether as in the method of Sherman and Schlesinger.[2] As the optimum conditions for the combined methods had not been established, the yield was not quantitative, but a solid product was obtained which was active and which gave a positive biuret reaction. This is additional evidence of the protein nature of the active material even as purified by the methods of Willstätter and his co-workers.

While this work was in progress a paper that is of interest in this connection was published by Luers and Sellner.[8] In it they describe the results obtained by them in some recent experiments dealing with the purification of malt amylase. By the use of a modification of the adsorption method proposed by Willstätter and co-workers for the purification of pancreatic amylase combined with a modification of the method used by Sherman and Schlesinger[9] for the purification of malt amylase, they obtain a highly active preparation which shows typical protein reactions.

Discussion and Summary

A critical study of the use of adsorption by alumina gel and kaolin as recommended by Willstätter, Waldschmidt-Leitz and Hesse for the purification of pancreatic amylase has been made. Special attention has been directed to establishing and controlling the most favorable conditions for each step.

The fact already reported by Willstätter and co-workers that pancreatic amylase is not appreciably adsorbed from its aqueous solutions by alumina gel has been verified and extended by the finding that changes in the reaction of the solution within wide limits do not influence the adsorption.

It was found feasible in testing the reaction of the aqueous glycerol and alcohol solutions obtained to make electrometric measurements and calculate them to Sørensen (P_H) values.

[8] Luers and Sellner, *Wochschr. Brau.*, **42**, 97, 103, 110 (1925).

[9] Sherman and Schlesinger, THIS JOURNAL, **35**, 1617 (1913); **37**, 643 (1915).

Pancreatic amylase is adsorbed to a slight extent from its aqueous solutions by kaolin. The Sørensen value of the solutions was found to influence this. The efficiency of the adsorption increased with increasing acidity but inactivation also increased.

It was not found possible to effect a complete separation of proteolytic from amylolytic activity by means of adsorption with kaolin.

Pancreatic amylase is readily adsorbed from 50% alcohol solutions by alumina gel. There is a definite relationship between the adsorption of the amylase and the hydrogen-ion activity of the solution. The most effective adsorption with the least destruction of amylase was found at about P_H 7.3.

A slightly alkaline solution of 0.05 *M* (mono- and di-) sodium phosphate (P_H 7.1 to 7.5) is as efficient as the ammonium-phosphate–ammonia-water solution recommended by previous investigators for the extraction of the amylase from the alumina gel in which it has been adsorbed and has the advantage of being more readily reproduced, and of not introducing nitrogenous reagents.

A suggestive correlation between the hydrogen-ion activity of the phosphate solution and the effectiveness of its extraction of active amylase from alumina gel has been established. These observations, especially when studied in connection with the results obtained in other phases of our investigation, indicate that pancreatic amylase is amphoteric and has an iso-electric zone in the neighborhood of P_H 7. This would mean that in the case of pancreatic amylase (as in the case of malt amylase, the investigation of which has already been reported)[10] the iso-electric zone of the enzyme coincides with its zone of optimum activity—a point of distinct theoretical interest.

The process of purification of pancreatic amylase proposed by Willstätter, Waldschmidt-Leitz and Hesse has been studied critically step by step and then repeated several times under the most favorable conditions. By it the amylase activity per milligram of nitrogen is increased three- to four-fold, that is, the activity per milligram of nitrogen is three to four times as high in the final purified enzyme solution as in the original glycerol extract of pancreatin. This activity is about half that of the purified preparations which have been repeatedly obtained by the method previously developed in this Laboratory.

Invariably, when sufficiently concentrated, the final active solution gave positive color reactions for proteins (or their derivatives).

The amylase activity was gradually lost during dialysis, protein or its decomposition products simultaneously dialyzing away as shown by positive color tests and by quantitative determinations of nitrogen in the inner and outer solutions. These tests were always positive in any solution which showed amylase activity.

[10] Sherman, Thomas and Caldwell, THIS JOURNAL, **46**, 1711 (1924).

The results of this study give added evidence, to that already obtained in other ways, that pancreatic amylase either is protein or contains protein as an essential constituent. This is shown to be true of the enzyme as purified by the adsorption method of Willstätter, Waldschmidt-Leitz and Hesse, as well as of that purified by the method of Sherman and Schlesinger.

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SOME REACTIONS OF THIOCARBANILIDE

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Although thiocarbanilide is a very commonly known industrial material its chemical properties have not been studied exhaustively. This paper contributes to the knowledge of its oxidations, nitrations and capacity to form additive compounds.

Various oxidations in acid[1] and neutral[2] solutions have been effected, but hitherto no studies have been attempted in alkaline solution, notwithstanding that thiocarbanilide is soluble in alkalies[3] and such solutions afford ready contact with alkaline oxidizing agents. The primary effect of oxidizing agents is to substitute the sulfur atom by oxygen, thus yielding diphenylurea, a compound lending itself to easy separation and identification. When acid solutions are employed, thiocarbanilide tends[4] to yield aniline, phenyl mustard oil and triphenylguanidine; alkaline oxidizing reagents avoid these by-products.

In the following experiments oxidations were effected by various reagents, most of which gave good yields of diphenylurea, but the sodium peroxide method excels in simplicity of separation and yield of pure product. Some of the reagents separated free sulfur, and others oxidized it to alkali sulfate.

Various other desulfurizing reagents have been employed in contact with thiocarbanilide as, for example, mercuric oxide, lead acetate, etc. The casual view is that double decomposition results in the process; however, closer study indicates that addition reactions are involved. That the addition is not on nitrogen is precluded by the inability of diphenylurea to form corresponding additive compounds. That the addition is on the sulfur atom seems the alternative conclusion; observations that are to be reported on later, however, seem not to substantiate even this con-

[1] Guareschi, *Gazz. chim. ital.*, **8**, 246 (1878). de Connick, *Compt. rend.*, **128**, 365 (1899).

[2] Hofmann, *Ann.*, **70**, 148 (1849). Herzog, *Z. angew. Chem.*, **33**, 140 (1920). Jacobson, *Ber.*, 19, 1077 (1885). Vanino and Schinner, *Ber.*, **47**, 699 (1913).

[3] Rathke, *Ber.*, 12, 772 (1882).

[4] Hofmann, *Jahresber.*, **1858**, 349.