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THE PROTEOLYTIC ACTIVITY OF PANCREATIC AMYLASE PREPARATIONS.

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Previous papers from this laboratory[1] have described the purification and properties of pancreatic amylase preparations which, while not chemically pure, appear to constitute a definite product of protein nature, similar in most of its chemical characteristics to the malt amylase described by Osborne.[2] but showing much greater enzymic activity. Several years' study leads us to conclude in regard to our pancreatic amylase, as did Osborne in his study of the amylase of malt, that the protein of which the final preparation is composed is not merely a carrier, but probably more nearly represents an approximate isolation of the enzyme itself, though as we have pointed out elsewhere,[3] there is reason to believe that in each case a part of the enzyme may have undergone, during its purification, some hydrolysis or chemical rearrangement with a corresponding diminution of amylolytic power.

Last year we showed in some detail[4] that the purified pancreatic amylase preparations made in this laboratory exhibit also a proteolytic activity similar to that of trypsin, and fully equal in intensity to that of the best commercial trypsins which we have tested, though they are not so active proteolytically as another of our preparations from the pancreas.

The marked proteolytic activity of our purified pancreatic amylase preparation is characteristic of it, as contrasted with our purified amylase preparations from malt and from *Aspergillus oryzae*. It is well known that extracts of malt and of *Aspergillus oryzae* contain proteases, as do also commerical taka-diastase and crude precipitated malt diastase; but the purified malt amylase prepared as described in a previous paper[5] has now been tested throughout a wide range of hydrogen ion concentration without revealing any proteolytic action, and the amylase preparation from *Aspergillus oryzae*, in such degree of purity as has so far been obtained, shows only about one-fortieth of the proteolytic activity of the purified pancreatic amylase preparation.

The proteolytic activity thus shown by the pancreatic amylase preparation might be attributed to the presence in the pancreas of a protease independent of the amylase but having so nearly the same solubilities

[1] Sherman and Schlesinger, THIS JOURNAL, **33**, 1195 (1911); **34**, 1104 (1912); **37**, 1305 (1915).

[2] Osborne, *Ibid.*, **17**, 587 (1895); *Ibid.*, **18**, 536 (1896).

[3] Sherman and Schlesinger, *Ibid.*, **37**, 1305 (1915).

[4] Sherman and Neun, *Ibid.*, **40**, 1138 (1918).

[5] Sherman and Schlesinger, *Ibid.*, **37**, 643 (1915).

as to remain admixed with the amylase throughout the purification process to which the latter has been subjected. Possibly a part of the trypsin may thus accompany the amylase.

The facts observed and to be described below also suggest the possibility that the amylolytic and proteolytic activities may in this case be the characteristic properties of interrelated substances or may conceivably be two properties of the same substance, analogous to the finding of Osborne and Wells[1] that a single isolated protein (hordein or gliadin) may contain more than one antigenic radical. One is also reminded of the problem of the possible identity of pepsin and rennin.

The experiments described below were undertaken with a view to throwing some further light upon the nature of the relationship which exists between the amylolytic and proteolytic activities of our purified pancreatic amylase preparations.

Experimental.

In our usual method of purification,[2] high grade commercial pancreatin is extracted with 50% alcohol, this filtrate is precipitated with alcohol-ether mixture and the precipitate dissolved in water and precipitated with absolute alcohol, then dissolved and dialyzed in 50% alcohol containing maltose to retard deterioration, and finally precipitated with an equal volume of 1 : 1 alcohol-ether mixture. In this procedure there settles out in the dialyzing sacs some material which, originally soluble in 50% alcohol, has now become insoluble in the same solvent apparently as a result of the preceding precipitation with absolute alcohol or standing in 50% alcohol, or both. This product, "sac precipitate," has little, if any, diastatic power, but has higher proteolytic activity than has the final amylase preparation. In fact, it is much the most active protease of which we have knowledge.

In order to test whether the amylase and protease activities of the final product of our usual method of purification are due to admixed substances which can be separated by differing solubilities in alcohol, we substituted for the usual final precipitation with 1 : 1 alcohol-ether, a precipitation with an equal volume of a mixture of two parts alcohol to one of ether, the product thus obtained being called Precipitate A, after which a second precipitate, B, was obtained by adding more ether. Precipitate A was separated by centrifugal force, with the additional precaution of cooling the centrifuge with liquid air,[3] and decantation; Precipitate B, by filtration after the addition to the solution decanted from Precipitate A, of an amount of ether equal to half its volume.

[1] Osborne and Wells, *J. Infec. Diseases*, 12, 341 (1913).

[2] Sherman and Schlesinger, THIS JOURNAL, **37**, 1305 (1915).

[3] This precaution appeared important because otherwise there was a large loss of amylolytic power in the precipitation and handling of the final products.

Typically the amylolytic activity of Precipitate A was lower than that of Precipitate B; but the latter was not more active than our usual amylase preparations.

The proteolytic activity was higher in Precipitate A than in Precipitate B.

The full description of a single typical experiment follows:

Preparation 78 (N. 14):—20 g. of air-dry pancreatin was mixed with 200 cc. of 50% alcohol at 5° and allowed to stand 20 minutes surrounded by a freezing mixture, then filtered, and the filtrate received in cylinders surrounded by a freezing mixture. The residue was washed with absolute alcohol and ether, then dried in a vacuum desiccator. (The washings were kept separate from the filtrate and were rejected.) The filtrate or 50% alcohol extract, which amounted to 142 cc. was poured into one liter of alcohol-ether mixture (1 : 4) and after standing 20 minutes surrounded by a freezing mixture, the supernatant liquid was decanted and the residue was dissolved in 57 cc. of triply distilled water at 1° and this solution was poured into 430 cc. of absolute alcohol which had been chilled in a freezing mixture. The resulting flocculent precipitate was made to settle by centrifuging for 15 minutes, liquid air being continuously introduced into the centrifuge to keep it cold. The temperature of the liquid was thus kept below 14° even after continuous centrifuging for 15 minutes. The precipitate was dissolved in 250 cc. of 50% alcohol containing 5 g. of maltose and dialyzed in two collodion sacs against 2 liters of cold 50% alcohol for 40 hours, changing the dialysate morning and evening. The 3 dialysates were rejected. The precipitate which appeared in the dialyzing sac was filtered in an ice chest, the filtrate[1] being received in cylinders packed in a freezing mixture. This filtrate (330 cc.) was mixed with an equal volume of alcohol-ether mixture (2 : 1) and the resulting flocculent precipitate was collected by centrifuging for 15 minutes with a continuous stream of liquid air passing into the centrifuge as described above. (In this case a maximum temperature of 19° was reached.) The precipitate thus obtained was scraped out of the centrifuge cups, dried *in vacuo* and weighed as "Precipitate A." The filtrate was poured into half its volume of ether, the resulting precipitate separated by filtration at ice box temperature, dried *in vacuo*, and weighed as "Precipitate B."

The weights and activities of these products are shown in Table IV.

Amylolytic Activity.—To permit of comparison with other preparations, the amylolytic activities of the 4 fractions obtained in each of several experiments are given in Table I, both in terms of the "new scale" diastatic powers as generally used in this laboratory since 1910, and as "saccharogenic powers" defined as the number of mg. of maltose formed per hour at 40° by each mg. of enzyme acting upon a sufficient excess of substrate.

As was to be expected in view of earlier work, the extractions, dialyses and precipitations with alcohol necessarily involve more or less destruction or inactivation of the amylase (probably at least half of that present), which together with experimental errors, make the results somewhat irregular when the activities of the different fractions are stated in quantitative terms. The general trend of the results is, however, quite pro-

[1] The precipitate was subsequently washed 3 times with cold 50% alcohol by centrifuging and decantation, the washings rejected and the washed precipitate dried *in vacuo*.

nounced and uniform. The residue from extraction with 50% alcohol has much less amylolytic power than the original pancreatin, while that of the "sac precipitate" is much smaller still, negligible, in fact, as compared with the activity of the material which remains to be precipitated by alcohol. The amylase activities of the two alcohol precipitates (A and B) are of the same order of magnitude both with each other and with the final products of the method of purification which we have usually employed in the past. Typically, as in Preparation 78 (N. 14), the amylolytic power of Precipitate B is higher than that of Precipitate A. It is, however, not higher than has been obtained by the method of purification previously employed.

TABLE I.—AMYLOLYTIC ACTIVITIES OF PANCREAS PRODUCTS.

Preparation number.	Residue.	Sac precipitate.	Precipitate A.	Precipitate B.
	Diastatic powers "new scale of 1910."			
72 (N.8)	65	20	2394	3000
75 (N.11)	82	51	1955	2195
77 (N.13)	133	23	2677	2500
78 (N.14)	152	51	2624	3700
81 (N.17)	81	49	2922	2800
	Saccharogenic powers.			
72	330	110	11580	14280
75	410	256	9550	10500
77	680	110	12800	10150
78	756	256	12600	17370
81	410	250	13900	13400

Proteolytic Activity.—If we express proteolytic activity in terms of the number of parts of casein nitrogen converted, (*a*) to soluble products, or (*b*) to amino nitrogen, by one part of enzyme preparation per hour at 40°, then the data for the proteolytic activities of the fractions obtained in typical experiments are as shown in Table II.

TABLE II.—PROTEOLYTIC ACTIVITIES OF PANCREAS PRODUCTS.

Preparation number.	Residue.	Sac precipitate.	Precipitate A.	Precipitate B.
	Nitrogen digested to soluble forms (*a*).			
72	12	256	80	55
75	12	256	89	63
77	13	257	90	55
78	13	252	83	68
81	12	214	94	69
	Nitrogen digested to amino form (*b*).			
72	1.3	26.4	7.3	5.5
75	1.3	29.6	12.5	5.7
77	1.4	28.6	8.8	5.7
78	1.4	31.2	11.5	6.2
81	1.1	25.8	8.6	5.4

It will be seen that the sac precipitates have much higher proteolytic activities than either of the later alcohol precipitates, A or B. As between

the latter, Precipitate A always exceeds Precipitate B in proteolytic activity. These relations hold uniformly true whether the proteolytic activity be judged from the total "soluble" nitrogen or the amino nitrogen of the cleavage products of the casein. In order to make the proteolytic powers comparable with the amylolytic (saccharogenic) powers, they are best expressed in terms of protein digested per hour by unit quantity of enzyme preparation. The data (*a*) of Table II then become as shown in Table III.

TABLE III.—PROTEOLYTIC ACTIVITIES IN TERMS OF CASEIN DIGESTED TO SOLUBLE PRODUCTS.

Preparation number	Residue.	Sac precipitate.	Precipitate A.	Precipitate B.
72	75	1600	500	344
75	75	1600	556	394
77	81	1607	563	344
78	81	1575	519	425
81	75	1338	588	431

Of the total enzymic activity of the pancreatin powder employed in such experiments a larger proportion of the proteolytic than of the amylolytic power is recovered in the 4 products here described. This is shown in quantitative terms in Table IV.

Discussion.—The exceedingly unstable nature of the material under purification, the relatively large losses which appear to be unavoidably involved in precipitation and reprecipitation if the characteristic amylolytic activity is not to be destroyed in the process, the dependence of the yields of precipitate upon minute details of manipulation which probably cannot be completely formulated and standardized until our knowledge of the behavior of colloidal substances is further developed, are all conditions which tend against closely concordant results in successive experiments, even after long practice has acquainted one with the many sources of difficulty and with the necessity for the utmost care and caution at every step.

For the same reasons and particularly because of the great tendency to loss of amylolytic activity when pancreatic amylase in the absence of salts and carbohydrates is held in solution or subjected to precipitation, it was not feasible to push the fractionation further by repeatedly dissolving and reprecipitating as would have been done in a similar study of a simpler or more stable substance. This deterioration in activity of the enzyme in solution which has been emphasized and discussed in previous papers and is much more pronounced in the case of amylolytic than of proteolytic action might of itself explain the fact that of the total enzyme activity of the original pancreatin employed in the experiments referred to in Table IV, about 3 times as large a proportion of the proteolytic as of the amylolytic activity was recovered.

TABLE IV.—YIELDS OF AMYLASE AND PROTEASE IN FRACTIONATION.

		Amylase			Protease.		
Description.	Weight. G.	"Power" of preparation.	Units of activity.	Per cent of original total.	"Power" of preparation.	Units of activity.	Per cent of original total.
Pancreatin 7	20.000	1795	35900	100.00	82	1640	100.00
Preparation 72:							
Residue	10.515	329	3459	9.64	75	789	48.11
Sac ppt.	0.140	117	16	0.04	1600	224	13.66
Ppt. A.	0.042	11580	486	1.35	500	21	1.28
Ppt. B.	0.062	14275	885	2.47	344	21	1.28
				13.50			64.33
Preparation 75:							
Residue	10.175	410	4172	11.62	75	763	46.52
Sac ppt.	0.187	260	49	0.14	1600	299	18.23
Ppt. A.	0.126	9550	1203	3.35	556	69	4.20
Ppt. B.	0.159	10500	1670	4.65	394	61	3.72
				19.76			72.67
Preparation 77:							
Residue	8.906	686	6110	17.02	81	721	43.96
Sac ppt.	0.237	114	27	0.08	1607	381	23.23
Ppt. A.	0.076	12820	974	2.71	563	43	2.62
Ppt. B.	0.171	10130	1732	4.82	344	59	3.60
				24.63			73.41
Preparation 78:							
Residue	9.226	756	6972	19.42	81	748	45.60
Sac ppt.	0.151	256	39	0.11	1575	238	14.51
Ppt. A.	0.044	12600	555	1.55	519	23	1.40
Ppt. B.	0.139	17370	2414	6.73	425	59	3.60
				27.81			65.11
Preparation 81:							
Residue	9.864	409	4034	11.24	75	740	45.12
Sac ppt.	0.203	246	50	0.14	1338	272	16.59
Ppt. A.	0.077	13920	1072	2.98	588	45	2.74
Ppt. B.	0.284	13390	3803	10.59	431	122	7.44
				24.95			71.89

The following notes on data of individual experiments may also be considered at this point:

Preparation 78 (N. 14) has been described as typical since we regard it as the most representative in details of method and in results. In the tables, however, are also included the data of 4 other experiments, each of which shows some appreciable difference from No. 78 either in the yield or activity of one or more of the products obtained. The principal differences of each of these from No. 78 may be briefly noted.

In No. 72 the amylolytic activity of the residue was less well conserved and the yield of Precipitate B was much lower. Moreover, a higher temperature was reached

during centrifugation than in the case of No. 78, hence the percentage of original enzyme activity recovered in the 4 products was somewhat lower for proteolytic and much lower for amylolytic power.

In No. 75 the final alcoholic Precipitates A and B, especially the latter, suffered great loss of amylolytic power because of insufficient control of temperature, especially during centrifugation. The larger yields resulted in a relatively high total recovery of proteolytic activity.

In No. 77 the weight of the "sac precipitate" and Precipitate A are higher and their powers are normal, while the weight of Precipitate B is also higher (than in Preparation 78) but its enzymic activity, both amylolytic and proteolytic, is low. The sum of the 4 fractions shows lower amylolytic and higher proteolytic power than in Preparation 78.

In No. 81 there appears to have been a better extraction of amylase from the pancreatin at the beginning of the process and hence a larger yield of each of the 3 precipitates obtained later. It appears, however, from their amylolytic powers, that Precipitates A and B are not as well separated from each other as in Preparation 78 and also that in the present experiment Precipitate B has either suffered a deterioration of amylolytic power or is contaminated with inert material as in No. 77, or both.

Since the sum of either the weights or the units of amylase or of protease action of the 4 fractions here prepared from pancreatin is much below the total of the pancreatin powder which served as starting material, there is evident need of caution in quantitative interpretation of the results. It is, however, apparent from the data tabulated above that the purification process results in a marked concentration of amylolytic power in the final Precipitates A and B. Thus, in Preparation 78, Precipitate A has about seven times, and Precipitate B about 10 times the amylolytic activity of the original pancreatin. Proteolytic activity is concentrated to a much greater extent in the "sac precipitate," and to a lesser but considerable extent in the final Precipitates A and B.

Special interest attaches to the comparison of the amylase and protease activities of the Precipitates A and B with each other and with our usual amylase preparations. In the case of a typical fractionation experiment such as that described above as Preparation 78, the high proteolytic activity of Precipitate A obviously suggests that our usual amylase preparation may be a mixture of amylase and protease, of which the protease is here concentrated in the first precipitate. In this case Precipitate B should have shown a higher concentration of amylase than our usual amylase preparations, except that because of the added manipulation a greater deterioration of amylase was necessarily involved.

On the whole, it seems probable that a partial separation of a mixture of amylase and protease was accomplished but that amylolytic activity was partially lost because of the extra manipulation, since amylolytic activity deteriorates much more rapidly than proteolytic, at least under conditions such as obtained in these experiments.

The further possibility that there are enzyme particles, which have both amylolytic and proteolytic activities is not excluded. Both possibilities

should be taken into account in future consideration of the problem of specificity of enzyme action.

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THE ACTION OF BASIC REAGENTS ON SCHIFF'S BASES. I. THE CHLORALNITRANILINE GROUP.

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Theoretically a great many compounds are included under the designation of Schiff's Bases, since it comprehends all condensation products of primary aromatic amines with aldehydes and ketones of every description. Actually a considerable number of Schiff's Bases have been described, beginning with the first mention of such products by Laurent and Gerhardt,[1] who condensed aniline with benzaldehyde. But H. Schiff[2] made the first real study of the reaction, condensing a humber of amines and aldehydes. The reaction is indicated by the equation:

$$R.CHO + 2R.NH_2 = R.CH(NHR)_2 + H_2O.$$

These bases are stable, as a rule though a small number are sensitive to light and may be decomposed readily by hot water. Among these are compounds of the *meta* series and certain naphthylamine derivatives. The investigations of Schiff's Bases in the organic laboratory of the University of North Carolina have dealt only with three aldehydes, chloral, vanillin and piperonal, but with a wide variety of amines.

It has been noted generally by observers that Schiff's Bases are sensitive to acids, being resolved into their constituents by strong hydrochloric acid. Their behavior toward basic reagents however has been quite generally overlooked. Wallach[3] noted that the condensation product of aniline and chloral was slowly decomposed by aqueous potash but rapidly by alcoholic potash, giving aniline, chloroform and phenylisocyanide. The presence of the two latter substances indicate that the decomposition takes place in 3 stages. This uninteresting behavior with alcoholic potash undoubtedly caused later investigators of Schiff's Bases to pass by this reaction. One of us[4] observed that the condensation product of chloral and *p*-nitraniline was changed immediately by alcoholic potash from a yellow substance to a brilliant red one. Upon puri-

[1] *Compt. rend.*, **30**, 404 (1850).

[2] *Ann. Chem. Pharm.*, **131**, 118 (1864).

[3] *Ann.*, **173**, 278 (1874).

[4] Wheeler and Glenn, *J. Elisha Mitchell Sci. Soc.*, **19**, 63 (1903).