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EFFECT OF AMINO ACIDS IN RETARDING THE HYDROLYTIC DECOMPOSITION OF AN ENZYME (PANCREATIC AMYLASE)

BY H. C. SHERMAN AND FLORENCE WALKER

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In recent work dealing with the effect of amino acids upon the action of amylases we have found that glycine, alanine, tyrosine, phenylalanine, aspartic acid and asparagine exert a favorable influence upon the enzymic hydrolysis of starch.

As more fully described in previous papers,[1,2] the activity of the amylase was measured by the amount of reducing sugar formed when the quantity of enzyme necessary to saccharify about $^1/_5$ of the substrate acted for $^1/_2$ hour at 40° on 100 cc. of a 1% dispersion of Lintner "soluble" starch containing optimum amounts of sodium chloride and disodium phosphate. The reducing sugar thus formed, chiefly maltose, was estimated by finding the weight of cuprous oxide precipitated as the result of the action of the digestion mixture on 50 cc. of mixed Fehling solution for 15 minutes in a boiling water-bath.

The carefully neutralized amino acids added to the starch pastes did not alter the hydrogen-ion concentration of the digestion mixtures. The favorable effect upon the activity of the enzyme was most marked in the case of purified pancreatic amylase, the amount of reducing sugar produced by this enzyme being about 15% greater in the presence of any one of the above-mentioned amino acids in a concentration of 0.05%. This enzyme was, therefore, used in the present study, in which a further explanation of the favorable effect of the amino acids was sought.

Experiments have already been reported[2] showing that: (1) the favorable influence of the amino acids cannot be attributed to their combination with some product or products of digestion which, if left free in solution might bring the starch-enzyme reaction to equilibrium, or else react with the enzyme itself, thereby rendering it inactive; (2) the effect is not due to a more favorable hydrogen-ion concentration induced by the presence of the amino acids, since in our experiments an optimum hydrogen-ion concentration was maintained throughout; (3) glycine is markedly effective in counteracting the deleterious influence of cupric sulfate on pancreatic amylase, thus suggesting that the amino acid may act by protecting the enzyme from some unknown injurious substance which may be present accidentally or as a constituent of the substrate or enzyme material; (4) positive evidence has been found that the favorable influence of amino

[1] Sherman and Walker, THIS JOURNAL, **41**, 1867 (1919).

[2] Sherman and Walker, *ibid.*, **43**, 2461 (1921).

acids is due at least in part to the fact that they prevent or retard the deterioration of the amylase in its aqueous solution.

In studying this last point, three methods of investigation have been used: (1) the resulting losses in activity of equal portions of the same enzyme solution were compared after these portions had stood in absence of substrate for a definite length of time at known temperatures, alanine having been added to some portions while others contained no amino acid; (2) the effect of temperature upon "activation" due to amino acids was determined; (3) a similar series of experiments was carried out in which the enzyme was allowed to act at these different temperatures for a longer time.

Protection of Pancreatic Amylase from Hydrolytic Destruction in Absence of Substrate

The enzyme was dissolved in (1) water alone, (2) water and alanine, 0.1%, (3) water with optimum concentrations of sodium chloride and disodium phosphate, (4) water with the same salts plus alanine. Such solutions were allowed to stand for 1 hour, in one series at 22° and in another at 40°, and their enzymic activities then determined, the results being expressed in terms of the weight of cuprous oxide obtained. In the determinations of enzymic activity, alanine was added to those solutions which had not previously contained it so as to have the same concentration of alanine present in all cases during the action of the enzyme upon the starch and the treatment of the resulting solution with the Fehling reagent.

The results are shown in Table I.

TABLE I

EFFECT OF ALANINE IN RETARDING DETERIORATION OF PANCREATIC AMYLASE IN SOLUTION AT 22° AND 40°

Alanine 0.1%	NaCl Optimum conc.	Na_2HPO_4 Optimum conc.	Cuprous oxide At 22° Mg.	Cuprous oxide At 40° Mg.
absent	present	present	274	144
present	present	present	275	198
present	absent	absent	133	...
absent	absent	absent	98	...

These experiments give direct evidence that the amino acid does retard the hydrolytic destruction of the amylase. Solutions of pancreatic amylase (containing optimum concentrations of sodium chloride and phosphate) which have stood 1 hour at 40° show about $^1/_3$ greater amylolytic activity when alanine has been added to the solution in advance. Under similar conditions except that the solution is kept at 22° instead of 40° there is no measurable difference, probably because the deterioration at this temperature and for this short time in the presence of optimum concentrations of salts is so small in either case that the effect of the amino acid is not demonstrable. Even at this lower temperature, however,

the deterioration of the enzyme in the absence of the salts was very marked and this deterioration was much retarded by the presence of the amino acid.

Effect of Variation of Temperature of Digestion

If protection against hydrolytic destruction explains the increased activity of enzymes in the presence of amino acids, it is logical to expect that conditions favoring the hydrolysis of the enzyme molecule, such as a higher temperature or subjection to a given temperature for a longer period, would increase the difference resulting from the presence of the amino acid. To test this point, a series of experiments was planned in which 30-minute and 60-minute digestions by pancreatic amylase with and without amino acid were carried out at temperatures ranging from 30° to 75°.

TABLE II

EFFECT OF TEMPERATURE ON APPARENT ACTIVATION OF PURIFIED PANCREATIC AMYLASE BY GLYCINE

Results expressed in terms of cuprous oxide

Temperature °C.	30-minute digestions: Glycine added 50 mg. Mg.	30-minute digestions: None Mg.	30-minute digestions: Increase of activity due to 50 mg. of glycine Mg.	30-minute digestions: Increase of activity due to 50 mg. of glycine %	60-minute digestions: Glycine added 50 mg. Mg.	60-minute digestions: None Mg.	60-minute digestions: Increase of activity due to 50 mg. of glycine Mg.	60-minute digestions: Increase of activity due to 50 mg. of glycine %
30	155	136	19	14	142	114	28	24
40	252	220	32	14	287	248	39	15
50	366	311	55	17	343	259	84	32
55	393	291	102	35	369	214	155	72
57	363	251	112	45	367	167	200	120
60	380	183	197	107	320	84	236	281
65	176	58	118	203	99	24	75	312
66.5	110	30	80	266	52	19	33	173
70	55	24	31	129	18	8	10	125
75	0	0	0	0	0	0	0	0

TABLE III

EFFECT OF TEMPERATURE UPON APPARENT ACTIVATION OF PURIFIED PANCREATIC AMYLASE BY PHENYLALANINE

Results expressed in terms of cuprous oxide

Temperature °C.	30-minute digestions: Phenylalanine added 50 mg. Mg.	30-minute digestions: None Mg.	30-minute digestions: Increase of activity due to phenylalanine Mg.	30-minute digestions: Increase of activity due to phenylalanine %	60-minute digestions: Phenylalanine added 50 mg. Mg.	60-minute digestions: None Mg.	60-minute digestions: Increase of activity due to phenylalanine Mg.	60-minute digestions: Increase of activity due to phenylalanine %
30	183	164	19	12	173	143	30	21
40	291	258	33	12	290	247	43	17
50	340	255	85	33	300	194	106	55
55	291	181	110	60	226	94	132	140
60	134	66	68	103	105	37	68	184
65	42	25	17	68	22	12	10	83
70	10	7	3	43	6	4	2	50

Table II shows the results obtained with glycine at different temperatures when 0.6 cc. of 0.01% enzyme solution acted upon 100 cc. of substrate

for 30 minutes and when $^1/_2$ the amount acted upon the same amount of substrate for 60 minutes. The experiments were repeated with phenylalanine replacing glycine, the results of which are given in Table III. The purified pancreatic amylase preparation employed in the phenylalanine experiments was about a year older than that used with glycine and only $^2/_3$ as active; consequently, $^1/_3$ more of the solution was added to each digestion mixture.

The results of the above experiments afford striking evidence that deterioration of the enzyme with increase in temperature is retarded by amino acids. For the 30-minute digestions, beginning with an increase at 30° of 19 mg. of cuprous oxide or 14%, the favorable influence of glycine reaches a maximum of 197 mg. of cuprous oxide or over 100% at 60°. Above this temperature the effect as represented by increase in milligrams of cuprous oxide declines sharply, although the percentage of apparent activation continues to increase up to 66.5°. The rapid falling off in activity above 60° is doubtless due to coagulation of the amylase which is not prevented by the amino acid.

The experiments with phenylalanine show the same general effect, though not in quite so marked degree as in the case of glycine, perhaps because of the lower molar concentration in which it was used. It will be observed from the tables that the 2 amylase preparations in absence of either amino acid behave somewhat differently, the less active one, employed in connection with phenylalanine, being destroyed more rapidly with increasing temperature. Glycine added to a substrate hydrolyzed by this enzyme at 60° gave practically the same result as obtained with phenylalanine. Hence the lack of perfect agreement in the temperature at which the optimum effect was found is probably due not to dissimilarity in the action of the two amino acids but rather to some difference in the amylase solutions connected with the deterioration which had already occurred in the less active preparation.

The most evident explanation of the marked temperature effect above shown is that the amino acids preserve the enzyme in solution from the destructive influence of hot water. Thus increase in temperature exerts two opposite influences upon amylolytic action. It accelerates the velocity of hydrolysis of starch into sugar by the amylase and at the same time increases the rate of deterioration (presumably hydrolytic destruction) of the enzyme. The second reaction being retarded by the presence of one of its products (amino acids), the first effect, that is increase in the rate of hydrolysis of the starch, becomes more noticeable.

When hydrolysis was continued for 60 minutes the amino acids produced a greater apparent activation at all temperatures (up to that at which coagulation of the enzyme occurred) than was observed in similar experiments of 30 minutes' duration. Digestions carried out at 40° for periods

of time from 20 minutes to 3 hours with and without glycine and tyrosine show the same increase in apparent activation with length of time of action of the amylase. This is what would be expected if the amino acid exerts its favorable influence by protecting against deterioration, since the longer the enzyme is subjected to an injurious temperature, the greater the deterioration and consequently the more apparent becomes the favorable effect of the conserving influence of the amino acid.

Summary and Conclusions

1. Highly purified preparations of pancreatic amylase, which deteriorate more rapidly in aqueous solution than the other amylases studied in this Laboratory, are also more affected by the presence of amino acids than are the other enzymes.

2. Solutions of pancreatic amylase (containing optimum concentrations of chloride and phosphate) which have stood 1 hour at 40° show considerably greater activity when alanine has been added to the solution in advance. Amylase solutions to which the chloride and phosphate have not been added deteriorate more rapidly; and with these the protective effect of the amino acid can be demonstrated at lower temperatures.

3. There is a striking increase in apparent activation by glycine and phenylalanine with increased temperature of digestion until coagulation of the enzyme occurs.

4. At the same temperatures there is greater apparent activation when hydrolysis is allowed to proceed for 1 hour than when the action is stopped at the end of 30 minutes.

5. All these facts point to the conclusion that the favorable influence of amino acids on the enzymic hydrolysis of starch is due, in large part at least and in all probability mainly, to a protection of the enzyme from deterioration in the aqueous dispersion in which it acts. This deterioration is probably due to gradual hydrolytic destruction.

6. That the presence of amino acids retards the hydrolytic destruction of the enzyme constitutes, as we have previously pointed out, an interesting addition to the evidence supporting the view that the enzyme itself is a substance of protein nature or one which contains protein as an essential constituent.

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