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BY

**Vinayak Narayan Patwardhan and Dattatreya Vishnu Karmarkar.**



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## CONTENTS.





BY

Vinayak Narayan Patwardhan and Dattatreya Vishnu Karmarkar.

# I.—AMYLASE FROM RICE.

*By Dattatreya Vishnu Karmarkar and Vinayak Narayan Patwardhan.*

The present communication forms a part of a series on amylases from cereals, of which some have already been published (*Journal Indian Inst. Sci.*, 1928, **11A**, 121; 1929, **12A**, 185; 1930, **13A**, 31, 159). The rice-malt amylase possesses properties similar to those of other cereal amylases examined in this laboratory. Ungerminated paddy does not contain any active enzyme.

## EXPERIMENTAL.

*Preparation of the enzyme.*—Paddy requires steeping for four days at room temperature (24-26°) before it can be spread out to germinate. After four days, when the plumules are about half an inch high, the seedlings are collected, sun-dried and treated as usual for preparation of dry malt.

Since rice contains only a small quantity of water-soluble proteins, it was very difficult to obtain an active precipitate of the enzyme by the method of Ling and Baker (*J. C. S.*, 1895, **67**, 702). An active preparation was, however, obtained on extracting the malt powder with a 0·6 per cent. solution of sodium bicarbonate and precipitating with 95 per cent. alcohol. It was also observed that if malted rice is allowed to stand overnight in contact with water and 95 per cent. alcohol then added to the extract, the precipitate is as active as that obtained from the alkaline extract of the malt. The reaction of the water extract after twenty-four hours was $P_H$ 4·8, that of the fresh extract being $P_H$ 5.9. Active enzyme was also obtained adjusting the reaction of the fresh extract to $P_H$ 4·8 by adding dilute acid followed by precipitation with alcohol. The enzymes obtained by the three methods mentioned above were found to be equally active.

### HYDROLYSIS OF STARCH BY RICE AMYLASE.

(*a*) *Saccharification.*—To 100 c.c. of 2 per cent. starch solution, 20 c.c. enzyme solution (0·02 per cent.) was added with 1 c.c. of toluene. The mixture was maintained at 38° and sugar estimated at

intervals. The results are compared with the corresponding figures for wheat-malt in Table I.

TABLE I.

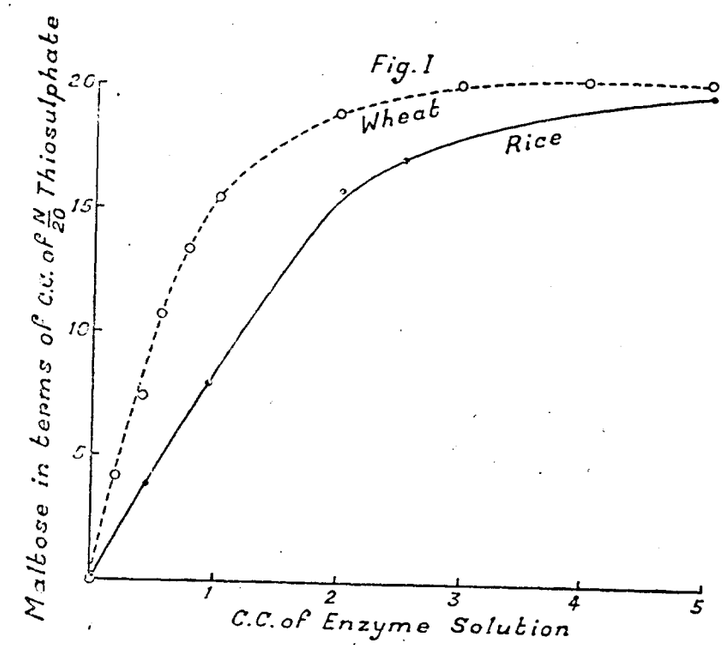
| Time in hours | Maltose in terms of c.c. N/20 thiosulphate | |
|---|---|---|
| | Rice | Wheat |
| ½ | 1·8 | 8·2 |
| 1 | 3·8 | 17·1 |
| 2 | 10·1 | 23·2 |
| 4 | 15·0 | 24·6 |
| 8 | 21·8 | 24·8 |

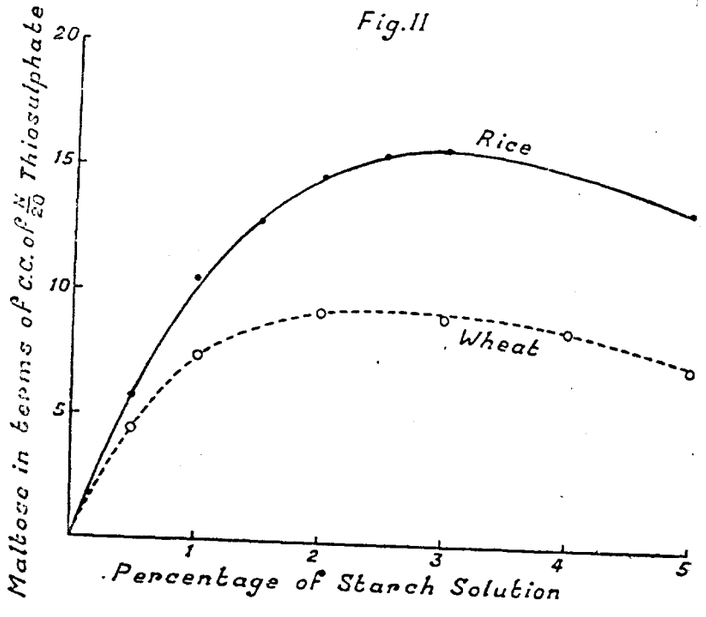
It may be noted that in the earlier stages the wheat-malt amylase is about five times as active as the one from rice-malt; in estimating liquefaction of potato-starch the enzymes were therefore taken in the ratio 1 : 5.

(*b*) *Liquefaction.*—Potato-starch paste (25 c.c. of 2 per cent.) was mixed with 25 c.c. of rice amylase (0·1 per cent.) at 30° and 10 c.c. of the mixture transferred to a viscometer maintained at 30°. The readings taken at intervals were compared with those in a similar experiment with a 0·02 per cent. solution of wheat-malt amylase (Table II).

TABLE II.

| Time in minutes | *t* in seconds | |
|---|---|---|
| | Rice | Wheat |
| 0 | 201 | 176 |
| 15 | 117 | 112 |
| 30 | 107 | 109 |
| 60 | 107 | 109 |
| 90 | 106 | 108 |
| 120 | 105 | 107 |






The observations show that rice amylase liquefies starch to the same extent as wheat amylase if the rates of saccharification are adjusted so as to be equal in both the cases.

*Effect of temperature on rice amylase.*—To each of a series of flasks containing 45 c.c. of starch solution (2 per cent.) 5 c.c. of malt extract (8 per cent.) was added after bringing the former to the desired temperature. The reaction was allowed to proceed for 30 minutes and then arrested by 5 drops of 30 per cent. sodium hydroxide. The mixtures were cooled and their respective sugar contents determined with the necessary correction for sugar content of malt.

TABLE III.

| Temperature ... | 25 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 75 |
|---|---|---|---|---|---|---|---|---|---|
| Maltose (c.c. of N/20 thiosulphate) ... | 9·1 | 10·6 | 11·6 | 14·5 | 15·1 | 15·5 | 15·8 | 14·1 | 3·7 |

*Effect of reaction.*—To each of a series of flasks containing 40 c.c. of starch solution (2 per cent.) and 10 c.c. McIlvaine's buffer solution adjusted to a particular reaction, 10 c.c. of enzyme solution (0·10 per cent.) were added and the mixtures incubated at 38° for 1 hour.

TABLE IV.

| PH ... | 3·8 | 4·0 | 4·2 | 4·4 | 4·6 | 4·8 | 5·0 | 5·2 | 5· | 5·6 | 5·8 | 6·0 | 6·4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maltose (c.c. of N/20 thiosulphate) ... | 8·8 | 9·6 | 10·2 | 11·61 | 1·7 | 11·4 | 11·2 | 11·0 | · · | 9·5 | 8·6 | 8·0 | 7·2 |

*Effect of concentration*:—(*a*) *Substrate.*—To seven flasks each containing 50 c.c. of starch solution of a particular strength 10 c.c. of rice amylase solution (0·08 per cent.) was added, and sugar estimated after incubation for one hour at 38°. The results are presented in Fig. I.

(*b*) *Enzyme.*—A series of nine flasks each containing 50 c.c. of starch solution (2 per cent.) and a particular quantity of water were arranged. The total volume was made up to 60 c.c. in each case by adding the required volume of enzyme solution (0·40 per cent.). The flasks were incubated for 1 hour at 38° and sugar determined as usual; the results are given in Fig. II.

*Effect of electrolytes and amino-acids on the activity of rice amylase.*—The experiments were conducted with electrolyte-free enzymes under controlled conditions of temperature and reaction, with and without addition of buffer solutions. The details of the experiments were the same as those described in the previous communications by Patwardhan and his co-workers (*loc. cit.*).

The electrolytes tested were the chlorides of potassium, sodium and calcium, and the sulphate, oxalate, malate and tartrate of potassium, in 0·0025, 0·005 and 0·010 molar concentrations. In no case could any effect on the activity of amylase be observed as the result of the adding of any of the electrolytes. The amino-acids, glycine, leucine, *l*-alanine, aspartic acid and hippuric acid in concentrations similar to those described above, also led to negative results.

*Effect of prolonged dialysis.*—The enzyme loses most of its activity on dialysis in collodion bags (6 per cent., 2 layers) prolonged for about fifteen days. The results of the above and other similar experiments are discussed in the following paper.

## SUMMARY.

1. An active preparation of rice amylase can be obtained by extracting the malt (*a*) with dilute (0·6 per cent.) alkali or (*b*) after adjusting the reaction to $P_H$ 4·8 followed by precipitation with 95 per cent. alcohol and subsequent dehydration with absolute alcohol and ether.

2. The activity of rice amylase is less than one quarter that of the corresponding wheat enzyme.

3. The optimum temperature of the rice-malt enzyme is 60° and optimum reaction, $P_H$ 4·6. The enzyme does not respond to addition of electrolytes or amino-acids, and on continued dialysis it loses its activity in fifteen days.

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[Accepted, 18-5-31.]



# II.—DIALYSIS OF SOME CEREAL AMYLASES.

*By Vinayak Narayan Patwardhan and Dattatreya Vishnu Karmarkar.*

Investigations on the properties of cereal amylases carried out in this laboratory have already shown that these enzymes lose activity when subjected to continued dialysis. Attempts to ascertain the cause of this phenomenon have led to the conclusion that it is due to the progressive destruction of the enzymes themselves owing to certain hitherto obscure causes. The present investigation has shown that the deterioration is not due to the relatively high room temperature, since it has been found that aqueous solutions of similar enzyme preparations retained more than 50–75 per cent. of their original activity even after more than two months in glass containers, while on prolonged dialysis nearly all their activity was lost within about three weeks.

Sherman and his co-workers (*J. Amer. Chem. Soc.*, 1919, **41**, 1867; 1921, **43**, 2461) suggested that amylase loses its activity owing to the hydrolysis of enzyme protein by proteases accompanying the enzyme. The present investigation has however shown that such a hypothesis is untenable. Supposing that the enzyme proteins were hydrolysed during dialysis, they would pass out of the collodion bag and thus bring about a steady decrease in the total nitrogen of the enzyme solution. Figures given in the experimental portion of the text show that with the progress of dialysis the nitrogen content reached a minimum and remained subsequently constant, while the activity continued to diminish to the very end. A direct proof of the untenability of Sherman's hypothesis was also obtained by testing the action of proteolytic enzymes on amylase solutions. The experimental details of such an experiment have been given in a previous paper (*J. Indian Inst. Sci.*, 1930, **13A**, 159), where it was shown that pepsin inactivated wheat amylase in aqueous solution, the rate depending upon the concentration of pepsin; papain and trypsin were without effect. All three proteolytic enzymes were allowed to act on amylase solutions at neutral reaction, since it was observed that loss of activity during dialysis took place in the neighbourhood of $P_H$ 6·7. Similar experiments carried out with amylases from rice, barley, cholam (*Sorghum vulgare*), maize, ragi (*Eleusine coracana*) and bajri (*Pennisetum typhoideum*) failed to show any inactivation due to the presence of proteolytic enzymes. These results do not support Sherman's hypothesis regarding the destruction of the enzyme nor do they supply evidence for the proteid nature of amylase as advocated by him.

Possibility of enzyme adsorption by collodion membranes was also tested by trying to elute the enzyme from the membrane, but no trace of adsorbed enzyme could be obtained by elution.

The dialyses were conducted carefully and sterile, toluenated water was used throughout. The bacterial count actually decreased with the progress of dialysis, and the possibility of bacterial decomposition of the dialysing enzyme was thus eliminated.

As a preliminary to experiments on dialysis, it was found that membranes made with two coats of 6 per cent. collodion did not allow the enzyme to pass through them. The same type of membranes were also used for electro-dialysis. Membranes prepared according to Frick and Kaja (*Ber.*, 1924, **57**, 310) were found to be unsatisfactory.

Some of the previous workers found electro-dialysis to be a rapid means of purifying amylases. Thus Narayanamurti and Norris (*J. Indian Inst. Sci.*, 1928, **12A**, 134) purified cholam malt amylase by electro-dialysing it through parchment membrane. Similar attempts to purify other amylases by electro-dialysis led, however, to almost complete destruction of the enzymes concerned. The details of our experiments were the same as those described by Narayanamurti and Norris except that rigorously tested collodion membranes were used in place of parchment ones. Of the seven amylases subjected to electro-dialysis, only wheat amylase retained its activity, all others being rendered inactive.

Rice amylase was first dialysed in the electro-dialyser at room temperature without passing the current. After three days, the terminals were connected to the main (110 D.C.) with a resistance of 2800 ohms in series. It was observed that the inactivation began at once, and at the end of four hours the activity was reduced by fifty per cent. It is possible that the destruction of the enzyme was due to the high potential between the terminals, because the enzyme could not have diffused out, or adsorbed on the membrane, or been precipitated with protein: it is however difficult to reconcile the above observation with those of Fricke and Kaja on commercial amylase (*loc. cit.*) Narayanamurti and Norris on cholam amylase (*loc. cit.*) and our own on wheat amylase.

In the present investigation, the activities of the enzyme solutions were compared on the volume basis rather than that of solid content as is usually done. Thus if a given solution of enzyme shows an activity $x$ remaining unchanged when a large amount of inert solids have passed out of the dialysing membrane, the activity can be said to have remained unaffected, even though according to the solid content estimations it must have increased. During dialysis a stage is reached when solid content remains constant while the activity continues to



diminish till it can be said to have been practically lost. This would mean that the solids undergo changes leading to the destruction of the hydrolytic properties of the enzymes and thereby converting them into inert substances. The mechanism of that process can be understood only by a physico-chemical study of the enzyme in solution. Investigation of such a nature has been undertaken in this laboratory and the results will be published shortly.

## EXPERIMENTAL.

The methods of preparing malts and enzymes are the same as those described in the previous communications (*loc. cit.*).

### I.—*Estimation of total and soluble nitrogen content of cereal malts and their enzymes.*

Since it is believed that proteins act as carriers of the enzyme and since there is considerable fall in the nitrogen content during dialysis, it was considered that a study of total and soluble nitrogen of these enzymes might be useful.

A weighed quantity of malt (3–4 g.) was digested with 100 c.c. of cold water during 1 hour, filtered and made up to 150 c.c. Concentrated sulphuric acid (5 c.c.) was added and the contents evaporated nearly to dryness on the water bath. Nitrogen was then determined by the kjeldahl method.

TABLE I.

| | Malts | | | Precipitated enzymes | |
|---|---|---|---|---|---|
| | Percentage | | Days required for inactivation | Percentage | |
| Malt | Total N | Soluble N | | Total N | Soluble N |
| Wheat ... | 2·43 | 0·58 | 15 | ... | 5·35 |
| Barley ... | 1·76 | 0·28 | 35(*cc.*) | ... | 5·85 |
| Rice ... | 1·32 | 0·17 | 15 | 0·85 | 2·30 |
| Maize ... | 1·77 | 0·26 | 3–4 | ... | 4·34 |
| Ragi ... | 1·33 | 0·27 | 9 | ... | 2·78 |
| Bajri ... | 1·74 | 0·43 | 13 | ... | 5·08 |
| Jawari ... | 1·53 | 0·21 | ... | ... | 1·56 |

II.—*Prolonged dialysis of cereal amylases.*

Portions of 20 c.c. of the malt extract (30 per cent.) were dialysed in a collodion bag, several being in one dialyser. At known intervals bags were removed, the contents filtered and made up to 100 c.c. Nitrogen, solid content and activity were determined. The results are given in tables II, III and IV.

TABLE II.

*Rice Amylase.*

| Days | $P_H$ | Solids per cent. | Nitrogen in c.c. of 0·1071 N alkali | Maltose in c.c. of N/20 thiosulphate |
|---|---|---|---|---|
| 0 | 5·9 | 2·13 | 6·75 | 15·00 |
| 1 | 6·4 | 0·62 | 1·50 | 14·00 |
| 3 | 6·7 | 0·04 | 1·00 | 13·90 |
| 6 | 6·5 | 0·05 | 0·50 | 6·80 |
| 8 | 6·7 | 0·05 | 0·50 | 6·10 |
| 15 | 6·7 | 0·05 | 0·50 | 1·70 |
| Control after 15 days ... | 5·9 | 2·12 | 6·75 | 15·0 |

TABLE III.

*Bajri Amylase.*

| Days | $P_H$ | Solids per cent. | Nitrogen in c.c. of 0·1071 N alkali. | Maltose in c.c. of N/20 thiosulphate |
|---|---|---|---|---|
| 0 | 5·7 | 4·20 | 15·50 | 14·70 |
| 2 | 6·2 | 0·49 | 3·50 | 11·70 |
| 5 | 6·5 | 0·10 | 1·50 | 4·20 |
| 7 | 6·7 | 0·08 | 1·00 | 3·30 |
| 10 | 6·9 | 0·08 | 1·00 | 1·80 |
| 13 | 6·9 | 0·08 | 1·00 | 0·70 |
| Control after 13 days ... | 5·7 | 4·20 | 15·00 | 13·50 |



TABLE IV.

*Barley Amylase.*

| Days | $P_H$ | Solids per cent. | Nitrogen in c.c. of 0·1071 N alkali | Maltose in c.c. of N/20 thiosulphate |
|---|---|---|---|---|
| 0 | 5·6 | 5·12 | 16·75 | 16·70 |
| 3 | 6·3 | 0·20 | 3·50 | 15·00 |
| 6 | 6·7 | 0·13 | 1·75 | 15·60 |
| 8 | 6·9 | 0·12 | 1·0 | 14·80 |
| 11 | 6·9 | 0·08 | 1·0 | 14·50 |
| 14 | 6·9 | 0·08 | 1·0 | 14·20 |
| 35 | ... | ... | ... | 0·30 |
| Control after 35 days ... | ... | 5·11 | 16·50 | 17·30 |

III.—*Effect of ageing on dialysed amylase.*

Portions of 20 c.c. of the malt extract (30 per cent.) of wheat and rice were dialysed during three days when the enzymes were practically free from electrolytes and reducing sugars. The solutions were preserved for over two months with added toluene, and their activities determined at intervals.

TABLE V.

| Date | Maltose in c.c. of N/20 thiosulphate | |
|---|---|---|
| | Wheat | Rice |
| 14—11—1930 | 15·4 | 12·1 |
| 3—12—1930 | 15·3 | 12·2 |
| 10—12—1930 | 15·5 | 11·9 |
| 18—12—1930 | 13·4 | 9·5 |
| 23— 1—1931 | 11·9 | 6·3 |

IV. *Testing for possible adsorption of enzyme by the collodion membrane.*

Four collodion bags, each containing 20 c.c. of the amylase from wheat enzyme solution (0·1 per cent.) were preserved for dialysis as

usual. After 15 days when the amylase was almost inactivated, the bags were removed, contents separated and the bags washed with small quantities of distilled water. Two bags were then cut to pieces and eluted with 20 c.c. of the elutive agent (100 c.c. consisting of 57 c.c. of 1 per cent. $(NH_4)_2HPO_4$, 3 c.c. of N ammonia and 40 c.c. of 87 per cent. glycerol) at $P_H$ 7·8. Two other bags were similarly treated with another elutive agent at $P_H$ 4·6 (100 c.c. consisting of 40 c.c. or McIlvaine's buffer of $P_H$ 4·6 and 60 c.c. of 40 per cent. glycerol). After four hours, 10 c.c. were taken from each flask and dialysed to remove glycerol. The activity was then tested against starch, and in no case could any activity be detected showing that the enzyme was not adsorbed on the membrane used for dialysis.

V. *Electro-dialysis of amylases.*

(*a*) An electro-dialyser very similar to that of Fricke and Kaja (*loc. cit.*) was used. Laboratory supply (110 volts, D.C.) with a resistance of 2,000 ohms in series was used as a source of current.

A stock solution of 500 c.c. of malt extract (15 per cent.) was prepared, 20 c.c. being made up to 50 c.c. and kept as control ; 400 c.c. was electro-dialysed till the conductivity approached that of distilled water. The solution was then removed, made up to 1000 c.c. and filtered, the solids being inert. The activity of the filtrate was then determined and the results are given in Table VI.

TABLE VI.

| Malt | Control | | Electro-dialysis | | |
|---|---|---|---|---|---|
| | $P_H$ | Maltose in c.c. of N/20 thiosulphate | Days | $P_H$ of middle cell | Maltose in c.c. of N/20 thiosulphate |
| Rice ... | 5·8 | 15·0 | 3 | 4·5 | 1·3 |
| Wheat ... | 5·9 | 14·8 | 2 | 4·6 | 14·7 |
| Barley ... | 5·6 | 16·1 | 3 | 4·4 | 0·9 |
| Maize ... | 5·6 | 8·4 | 3 | 4·6 | 1·2 |
| Ragi ... | 5·6 | 12·2 | 3 | 4·4 | 2·0 |
| Bajri ... | 5·7 | 12·9 | 3 | 4·5 | 0·2 |
| Cholam ... | 5·6 | 6·8 | 3 | 4·7 | 0·3 |

(*b*) Rice-malt extract was dialysed in the electro-dialyser without the passing current, which was then switched on and the enzyme electro-dialysed, in one case for four hours and in the other for six hours. Their respective activities are given in Table VII.



TABLE VII.

| Activity before electro-dialysis | Activity after electro-dialysis | | |
|---|---|---|---|
| Maltose in c.c. of N/20 thiosulphate | Electro-dialysis hours | $P_H$ | Maltose in c.c. of N/20 thiosulphate |
| 14·2 | 6 | 4·5 | 6·4 |
| 12·9 | 4 | 4·5 | 6·5 |

## SUMMARY.

1. All the cereal amylases hitherto investigated have been found to lose their activity on prolonged dialysis.

2. Electrolyte-free amylase in aqueous solution retained more than 50 per cent. of its original activity even after two months.

3. On electro-dialysis through collodion membranes, all cereal amylases excepting that from wheat lost their activity.

4. Probable causes of destruction of the enzymes have been investigated.

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