I.—AMYLASE FROM *ZEA MAIS*.

By Vinayak Narayan Patwardhan.

This investigation is the second of a series of studies on amylases of vegetable origin, the first one being the amylase from *Sorghum vulgare*. The results obtained from the latter have already been published (Patwardhan and Norris, *J. Indian Inst. Sci.*, 1928, 11A, 121). The results obtained with amylase from *Zea mais* as well as *Sorghum vulgare* fully warrant the view that it is hazardous to form any definite opinion on the distinct individuality of amylase till amylases from various origins are thoroughly examined.

The proportion of the liquefying and saccharifying components in an enzyme is not constant in the amylases so far examined. The ratio of saccharification to liquefaction in maize amylase exceeds that in barley amylase but is less than that in cholam amylase. The idea of influencing either the saccharification or liquefaction unequally by changing the conditions of reaction has again been followed in the present case. The effect of such changes, however, appears to be practically the same with this enzyme as in the previous cases investigated. The enzyme exerts its optimum saccharifying activity between 55° and 60°. The same can be said about the liquefaction. One fact which is noteworthy, however, is the very slow fall of activity as the temperature rises to 70°. In the case of barley and cholam amylases the activity at 70° is considerably smaller than that at the optimum temperature; but in this case the fall is relatively small. In other words the maize enzyme seems to be less liable to destruction by heat than that derived from cholam.

A study of the effect of change in the hydrogen-ion concentration shows that the enzyme behaves perfectly normally. The saccharifying activity increases slowly and constantly till the P_H 5·0 is reached, a gradual fall following. The region of the maximum liquefying activity is pushed further to the acid side, this being possibly due to the liquefying action of the acid itself which might to a certain extent compensate for the decreasing activity of the enzyme.

The enzyme has been found to pass through parchment membranes, but not through collodion membranes; it shows quite abnormal behaviour after dialysis, the enzyme in collodion bags having lost activity. The period of complete disappearance of activity is variable. In the case of maize-malt extract the activity is almost completely lost in three days, while the precipitated enzyme took longer. The inacti-

vation is such that the activity cannot be restored by any means tried, addition of salts, asparagine and buffer solution having been found unsuccessful. The idea that the enzyme protein might be broken down by proteolytic enzyme possibly present in the maize amylase was also investigated, but the precipitated enzyme was found to be without proteolytic action. The possibility that the dialysate might remove an amylase-component was also tested by adding the concentrated dialysate to the inactivated enzyme and testing the activity, but there was no activation. At present this inactivation remains unexplained, undialysed samples of the enzyme kept under similar conditions remaining active over the same period of time.

EXPERIMENTAL.

Malting and preparation of enzyme.—The grain was steeped in a slow stream of water for 60 hours at room temperature, spread on sand and allowed to germinate in the shade. After the plumules had grown to approximately half-an-inch the seeds were collected and dried in the sun for 6 hours. The sun-dried seeds were further dried in an oven, first at 50° for 24 hours, then at 70° for 24 hours and finally at 100° for 24 hours. After cooling, the malt was separated from dried plumules and radicles, and powdered. The powder could be stored without deterioration.

Dry malt powder (200 gms.) was extracted in cold water with 600 c.c. of 20 per cent. alcohol for 24 hours with frequent shaking. The extract was filtered and the enzyme precipitated from the filtrate by the slow addition of 1200–1300 c.c. of 95 per cent. alcohol with constant stirring. After 12 hours the supernatant liquid was siphoned from the precipitate which was washed successively with 98 per cent. and 99·5 per cent. alcohol and finally with ether, being then dried in vacuum over calcium chloride. The dry precipitate can be conveniently preserved for a long time without losing activity.

Experiment to follow the liquefaction and saccharification of the enzyme.—The liquid contained 50·0 c.c. starch paste (2 per cent.),

No.	Time in mins.	Maltose, mgms.	Viscosity in terms of t (secs.)	Colour with iodine
1	0	0	171·2	Deep blue
2	15	10·0	117·0	Violet
3	30	16·5	111·0	Very faint violet
4	60	24.0	107·2	Brown
5	100	29·0	106·0	Faint brown
6	260	42·5	...	...
7	1440	69·3	...	No change
8	4660	84·1	...	

12·5 c.c. enzyme (0·5 per cent.) and 37·5 c.c. water, 1 c.c. toluene being added for every 100 c.c.

Figures for comparison with the experiments on barley and cholam enzymes are given below :—

Time in hrs.	Saccharification Maltose (mgms.)		Time in hrs.	Liquefaction t in secs.	
	Barley	Cholam.		Barley	Cholam
0	0	0	0	165·5	177·7
15	39·4	3·3	15	109 2	136·0
30	58·0	5·5	30	107·5	128·8
60	69·5	11·4	60	106·2	117·8
100	76·8	18·4	100	106·0	112·0

Experiments to ascertain the temperature effect.—Eight flasks each contained 15 c.c. starch paste (2 per cent.) and 5 c.c. water. At the selected temperature the enzyme (10 c.c., 0·3 per cent. solution) was added and the flask maintained at that temperature. After half-an-hour 10 c.c. portions were removed for determination of sugar and 10 c.c. for precipitation by alcohol. A typical experiment is recorded below.

Temperature, degrees	Maltose, mgms.	Dry precipitate, mgms.
30	17	62·8
40	21·4	70·8
45	21·9	...
50	25·4	49·6
55	26·8	48·5
60	26·8	40·2
65	24·8	43·4
70	22·6	69·3

Effect of change in P_H.—Sixteen flasks each contained 15 c.c. starch paste 2 per cent. and 10 c.c. buffer solution (McIlvaine's); the enzyme (5 c.c., 0·6 per cent. solution) was added at 30° and the

reaction allowed to proceed for exactly fifteen minutes, when sugar and precipitable matter were estimated as usual.

No.	P_H	Maltose in 10 c.c. mgms.	Precipitate in 10 c.c. mgms.
1	6·90	9·3	176·1
2	6·50	18·2	173·3
3	6·05	21·7	161·4
4	5·85	22·8	160·6
5	5·70	22·6	169·9
6	5·50	23·2	156·0
7	5·20	24·1	146·1
8	5·00	24·8	139·4
9	4·75	24·1	135·0
10	4·55	23·6	122·9
11	4·40	23·2	115·2
12	4·15	22·8	120·3
13	4·00	20·2	130·0
14	3·80	18·0	122·4
15	3·65	15·3	123·3
16	3·55	12·0	...

Inactivation during dialysis. (*a*) *Malt extract.*—Malt (5 gms.) was shaken with water for an hour, filtered, and the filtrate dialysed in closed collodion bags with running water in constant presence of toluene. After 48 hours the dialysed residue was made up to 100 c.c. and activity tested. Reaction mixtures were composed of 15 c.c. soluble starch solution (2 per cent.), 10 c.c. dialysed enzyme and 5 c.c. water plus salt (0·4408 per cent.). The temperature was 30° and time of reaction 45 minutes and 18 hours; in neither case was any reduction observed.

(*b*) *Precipitated enzyme.*—Malted maize powder (25 gms.) was shaken with water for half an hour and filtered; to the stirred filtrate, which amounted to about 50 c.c., was added 120 c.c. of 95 per cent. alcohol which precipitated the enzyme. This was allowed to settle for three hours and filtered, the residue being shaken with water for half-an-hour and filtered. The filtrate was made up to 50 c.c., 40 c.c. being dialysed in collodion bags in presence of toluene while 10 c. c.

were kept in a tube in the dialyser for the same time. The dialysis was continued in running distilled water when loss of activity was found to be nearly complete on the third day, the product being without effect on starch paste, both alone and in presence of asparagine or sodium chloride.

Search for existence of a dialysable component.—Precipitated enzyme (0·3 gm.) was shaken with water for half-an-hour and filtered, the filtrate being enclosed in a collodion bag and toluene added. Dialysis was carried on in water saturated and covered with toluene, sometimes at room temperature but more frequently in an ice-chest. The liquid was collected at intervals and distilled under reduced pressure at 40–45°, but as this process was too laborious with the volume of liquid involved, concentration by freezing was subsequently adopted and the volume reduced to 100 c. c. After the lapse of five days the enzyme was found to be almost inactive towards starch paste in presence of McIlvaine's buffer solution.

Effect of Salt on the regeneration of inactivated enzyme at different P_H *values.*—The enzyme used in the previous experiment was employed in this series also. Reaction mixtures were made up in six flasks each containing 12·5 c.c. 2 per cent. starch paste to which 10 c.c. of inactivated enzyme was added. The reaction was allowed to continue at 30° for one hour and the sugar then estimated by Amos Peter's method.

P_H	Salt : c.c. of 0·4408 per cent.			Buffer : c. c.	Water : c. c.	Sugar : c. c. of thio-sulphate
6·9	I.	1.	7·5	10	0·0	0·10
		2.	0·0	10	7·5	0·35
5·7	II.	3.	7·5	10	0·0	0·20
		4.	0·0	10	7·5	0·20
4·75	III.	5.	7·5	10	0·0	0·15
		6.	0·0	10	7·5	0·20

The experiment clearly shows that there is no activation by the salt at the different hydrogen-ion concentrations used.

Identification of the sugar produced by hydrolysis.—Starch (20 gms.) was made into a paste with 1000 c.c. of water, cooled and mixed with 0·2 gm. of dry enzyme shaken with 50 c.c. of water. The reaction was conducted at 37° for 42 hours in presence of toluene and then stopped by boiling. After concentration on the water bath to 200 c.c. the

dextrins were precipitated by $2\frac{1}{2}$ vols. of 95 per cent. alcohol, allowed to settle and the supernatant liquid containing the sugar collected and distilled. The precipitate was again dissolved in water, concentrated and precipitated, the supernatant liquid being distilled with the liquid from the first precipitation. The sugar residue was dissolved in methyl alcohol, boiled for half-an-hour with norit and filtered through kieselguhr. Methyl alcohol was removed under reduced pressure and the last traces of moisture absorbed by heating the residue in vacuum over phosphorus pentoxide. The sugar was a white, hygroscopic powder giving $[\alpha]_D$ + 134°, and fermentable by *Saccharomyces cereviseae* to the extent of 85 per cent. estimated by decrease in the reducing power. Neither this residue nor the original sugar was hydrolysed by emulsin. These properties indicate maltose.

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II.—ENZYMES FROM THE SEEDS OF *CAESALPINIA BONDUCELLA.*

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The seeds of *Cæsalpinia bonducella* commonly known in the vernacular as *Gajga* have a very thick and hard seed-coat which is not easily softened. The decorticated resting seed, containing 15 to 20 per cent. of oil and 2 to 3 per cent. sugar, has been found to contain the following enzymes :—protease, urease, amylase, peroxidase, catalase and oxidases in very small quantity ; it does not contain invertase.

An unexpected result in this examination was that lipase could not be detected. Experiments with seeds containing fat and also with fat-free samples all failed to reveal this enzyme. After germination, which took nearly five weeks, the seed was again tested for lipolytic activity with the same result ; this is difficult to explain in view of the high percentage of oil stored in the cotyledons.

When searching for enzymes in resting seeds the seed-powder was previously extracted with ether for 40 hours, but as lipase sometimes becomes inactivated during extraction the untreated material was also used. In many experiments extracts were made with 20 per cent. glycerol, but this also may act as an inhibiting factor. In special experiments for lipase, therefore, water alone was used and the experiments conducted with shaking, without shaking,with finely emulsified oil and oil temporarily emulsified by shaking. Acetic acid having an accelerating influence on lipase was tried, but without success, and similarly esterase could not be detected, as was shown in experiments with ethyl butyrate. Conclusive proofs of the absence of lipase were obtained by examining the extract, the residue after centrifuging and the top layer of the centrifugate, which according to Hoyer contains most active lipase. Experiments to obtain lipase by extracting the centrifugate with petrol failed to give any results.

The activity of the other enzymes increases during germination.

EXPERIMENTAL.

Extraction of the Enzyme.—Dry fatty or fat-free seed-powder, as the case required, was ground in a mortar with sufficient 20 per cent. glycerol or water to make a loose paste. The mixture after half an hour was centrifuged at 4000–5000 r. p. m. for 10–15 minutes. The supernatant liquid was made up to a known volume, and with addition of a little toluene could be preserved at zero.

Methods used.—The substrates used for protease, urease, amylase and invertase were casein, urea, soluble starch and cane-sugar respectively, the first three giving positive results while inversion was not found in the case of sucrose. All the experiments were conducted at ordinary room temperature except in one or two tests for lipase when the reaction mixtures were incubated for some time at 37°. Tests for peroxidase were made with solutions of α-napthol, *p*-phenylenediamine hydrochloride and hydrogen peroxide solutions (indophenol reaction). Catalase was traced by its action on neutral hydrogen peroxide.

A typical example of experiments for investigating the lipase-content is as follows:—The fat-free seed-powder (10 gms.) was extracted with 20 per cent. glycerol, centrifuged and the centrifugate made up to 100 c.c. Reaction mixtures prepared from 5 gms. of oil, 10 c.c. of N/10 acetic acid, 25 c.c. of water and 10 c.c. of (*a*) extract and (*b*) boiled extract, each with a few drops of toluene, were shaken continually in a machine. Portions of 10 c.c. were withdrawn at intervals, 5 c.c. of alcoholic potash added and the mixture titrated with standard sulphuric acid, which showed no change at reaction periods of 19 hrs. 45 mins. and 43 hrs. 15 mins.

The residue was tested for lipase by extracting 10 gms. fat-free seed-powder with water and preparing reaction mixtures from 33 c.c. of oil, 10 c.c. of N/10 acetic acid with 20 c.c. of (*a*) residue, (*b*) the top-layer of centrifugate and (*c*) the remainder of the centrifugate. A few drops of toluene were added to each and the bottles shaken by hand at frequent intervals; 5 c.c. portions were titrated as above and showed no change after 24 hours.

Experiments with fatty seeds extracted with water or with water containing 10–15 c.c. of N/10 acetic acid and conducted on lines similar to above, failed to prove the presence of lipase. In some of the latter experiments the oil was emulsified with gum arabic.

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