THE ENZYMES OF SOME ELASMOBRANCHS FROM BOMBAY

III. AMYLASES of *Scoliodon sorrakowah* and *Sphyrna blochii*.

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A vast amount of information on the amylases of tissues of higher animals and plants and those of yeasts molds and bacteria is available. The amylases of fish have not been studied much until recent times, except for the work of Yung (1898), Sullivan (1907), Kenyon (1925) and Yonge (1931), showing the presence of amylopsin in fish pancreas. The present investigation deals with the amylases of *S. sorrakowah* and *S. blochii*, the two varieties of Sharks from Bombay Waters.

Methods and Material

(i) 6% Aqueous extracts of various tissues were prepared by grinding the tissue in a mortar with glass powder. The extracts autolysed at 15° C. for 18 hours with toluene as the preservative were squeezed through muslin, and the filtrate in each case used as the source of enzyme.

pH 1 to 3·0 and 9 to 10·0 was maintained by M/10 glycine buffer, that from 4 to 5·0 by M/5 acetate buffer and from 6 to 8·0 by M/10 phosphate buffer.

The reaction mixture " in the proportion $2n$ c.c. Sub-strate, $2n$ c.c. buffer and n c.c. enzyme preparation (boiled in the case of control) " was set up at 37° C.

(ii) *Reagents.*—(*a*) Shaffer Somogyi copper reagent: 25 gm. anhydrous Na_2CO_3 and 25 gm. Rochelle salt were dissolved in 500 c.c. of water without the application of heat. To this 100 c.c. of 12% $CuSO_4$ $5H_2O$ (A.R.) solution were added by a pipette, its tip being kept under the solution of carbonate and tartrate to avoid losing of CO_2·20 gm. $NaHCO_3$, 160 gm. Na_2SO_4, 12 gm. KI and KIO_3 solution in volume according to the quantity of sugar to be analysed (here 120 c.c. of 0·4 N·KIO_3 were used) were added. Reagent was diluted to a litre, mixed and filtered, a few drops of toluene added as the preservative and stored in a brown bottle away from dust.

(*b*) Standard maltose solution was prepared by dissolving anhydrous maltose (Merck) in 0·25% benzoic acid solution.

(*c*) Stock iodine solution was prepared by dissolving 5·5 gm. of I_2 and 11·0 gm. of KI in 250 c.c. of water.

(*d*) *Dilute iodine solution.*—15 c.c. of stock iodine solution and 8 gm. of KI were diluted to 200 c.c. with water so that 1 c.c. sol. = 1·65 mg. I_2.

(*e*) *Standard dextrin solution.*—0·6 gm. Merck's reagent dextrin was suspended in small amount of cold water and then transferred to 400 c.c. of boiling water, after cooling the solution was made to a litre. The solution preserved under toluene in refrigerator keeps for several weeks. Red brown colour produced when a c.c. of dextrin solution is mixed with 5 c.c. of dilute iodine solution serves for colour comparisons.

(*f*) One gm. of corn amylum (Maize starch) made into a thin paste with little cold water was added slowly with constant stirring to hot water and the solution was boiled for fifteen minutes and cooled. 1% starch sol. adjusted to requisite pHs. served as the substrate. It was prepared fresh when required.

(iii) *Determination of enzyme activity.*—The saccharogenic activity of enzyme was determined by the estimation of maltose produced according to Shaffer and Somogyi (1933 and 1937). To draw a standard reference curve for maltose 2 c.c. sample containing 0·25 to 9·0 mgm. maltose were pipetted in a pyrex test-tube containing 5 c.c. of copper reagent. The contents were mixed and kept for 15 minutes in a boiling water-bath, then the tube was immersed in cold water and finally in freezing mixture to bring the contents to 1 to 4° C. One c.c. of $5N \cdot H_2SO_4$ was then introduced in test-tube, mixed and iodine liberated was titrated against N/100 $Na_2S_2O_3 \cdot 5H_2O$ with starch as an indicator. 4 c.c. reaction mixture were pipetted at zero time (*i.e.*, at the moment the enzyme was introduced) and after 20 minutes and were added to 5 c.c. of copper reagent in separate test-tubes, and other treatments followed subsequently as described. Activity of enzyme is expressed as the mgm. maltose produced by the enzyme present in 100 mgm. dry tissue, under the specified conditions.

The liquefying activity of amylases was measured viscometrically, the change in the initial rate of flow (in seconds) of 100 mgm. starch caused by the enzyme present in 30 mgm. dry tissue in 20 minutes at 37° C. being an index of enzyme activity.

The dextrinogenic activity of enzyme was followed by Sandstedt Kneen and Blish's (1939) modified Wohlgemuth procedure. One c.c. of reaction mixture removed at definite time interval was mixed with 5 c.c. of dilute iodine solution and colour comparisons were made with red brown colour

of standard dextrin solution, for the time interval "t" necessary for the end of dextrinization caused by the amount of enzyme "d.wt." represented by the dry weight of the enzyme preparation, amylase units were calculated as

$$\text{Amylase units} = \frac{\text{Wt. of starch} \times 60}{t \times \text{d.wt.}}$$

RESULTS

General survey of amylolytic activity of various tissues of the two fishes was made by the study of the dextrinogenic activity. Reactions were run at pH 7·0 and the readings were taken after 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 125, 140, 160, 180, 240, 280, 360 and 1,440 minutes to note the end of dextrinization. The reaction time when the red brown and achromic stage was reached for different amylase preparations is given in Table I.

TABLE I

General Survey of Amylolytic Activity by Modified Wohlgemuth's Procedure

Reaction mixture "containing 10 c.c. substrate + 10 c.c. Buffer (pH 7 0)+ 5 c.c. enzyme preparation" was set up at 37° C.

Fish	Organ	(Figures in bracket indicate amylase units) Time in minutes for	
		Red brown stage	Achromic stage
Scoliodon sorrakowah ..	Viscera	15 (13·33)	30
	Liver	40 (5·00)	60
	Spleen	55 (3·64)	240
	Kidney	55 (3·64)	240
	Heart	160 (1·25)	280
	Brain	160 (1·25)	280
	Muscle	..	1440
	Ampullæ of lorenzini	..	..
Sphyrna blochii ..	Viscera	15 (13·33)	25
	Liver	55 (3·64)	100
	Spleen	90 (2·22)	180
	Kidney	90 (2·22)	180
	Heart	140 (1 43)	240
	Brain	140 (1·43)	280
	Muscle	..	1440
	Ampullæ of lorenzini	..	..

TABLE II A

Saccharogenic Activity of Amylases at Various pHs.

Reaction mixture "containing 6 c.c. substrate + 6 c.c. buffer of required pH+3 c.c. enzyme preparation" was kept at 37° C. for 20 minutes.

Animal	pH	Enzyme activity/100 mgm. dry tissue, in terms of mgm. maltose							
		Organ							
		Pancreas	Liver	Spleen	Kidney	Brain	Muscle	Stomach	Intestine
Fish	2·0	..	1·16	..	0·0	..	..	0·0	..
Scoliodon	3·0	3·52	5·79	2·32	4·63	0·0	0·0	0·0	0·0
sorrakowah	4·0	8·15	15·05	5·29	8·15	0·0	0·0	0·0	4·63
	5·0	79·64	27·77	8·15	18·52	6·94	0·0	0·0	12·74
	6·0	243·20	16·20	17·36	9·26	0·0	0·0	0·0	0·0
	7·0	185·20	48·61	28·94	2·32	5·79	5·79	0·0	4·63
	8·0	11·10	33·57	4·63	2·32	0·0	0·0	0·0	0·0
Fish	2·0	..	0·0	..	..	0·0	..	0·0	..
Sphyrna	3·0	6·94	4·63	3·52	4·63	0·0	0·0	0·0	0·0
blochii	4·0	25·46	11·57	5·79	6·94	0·0	0·0	0·0	0·0
	5·0	127·40	34·71	8·15	22·01	6·94	0·0	0·0	0·0
	6·0	87·96	15·05	18·52	8·15	0·0	0·0	0·0	0·0
	7·0	182·80	37·03	32·41	2·32	2·32	4·63	0·0	6·94
	8·0	11·10	23·15	4·63	0·0	0·0	1·16	0·0	1·16
	9·0	74·08	15·05	0·0	0·0	0·0	0·0	0·0	0·0
Pig	2·0	..							
	3·0	5·79							
	4·0	50·93							
	5·0	189·80							
	6·0	402·80							
	7·0	240·70							
	8·0	125·00							
	9·0	74·08							

To know the distribution of amylases in different tissues of the two fishes and pig pancreas activity pH curve for each was studied. The saccharogenic and liquefying activities were determined and the results are represented by Tables II A and II B.

TABLE II B

Liquefying Activity of Amylases, at Various pHs.

Reaction mixture "containing 10 c.c. substrate + 10 c.c. buffer of required pH + 5 c.c. enzyme preparation" was set up at 37° C. for 20 minutes.

Animal	pH	Enzyme activity/30 mgm. dry tissue in terms of seconds			
		Organ			
		Pancreas	Liver	Spleen	Kidney
Fish *Scoliodon sorrakowah*	2·0	..	0·8	..	0·0
	3·0	4·0	7·0	0·0	1·0
	4·0	15·0	10·0	0·0	7·0
	5·0	26·0	23·0	2·0	12·0
	6·0	81·0	9·0	5·0	4·0
	7·0	64·0	12·5	9·0	0·5
	8·0	42·2	3·0	1·0	0·0
	9·0	30·3	1·0	0·0	..
Fish *Sphyrna blochii*	2·0	..	0·5	..	0·0
	3·0	5·0	7·5	0·0	0·0
	4·0	15·0	9·5	0·0	7·5
	5·0	68·5	26·0	1·0	14·0
	6·0	39·0	10·0	4·0	6·0
	7·0	52·0	12·5	8·0	0·0
	8·0	30·0	5·5	1·3	0·0
	9·0	19·0	2·0	0·0	..
Pig	3·0	11·0			
	4·0	21·0			
	5·0	53·0			
	6·0	94·0			
	7·0	64·0			
	8·0	40·0			
	9·0	29·0			

The distribution of α- and β-amylases in pancreas, liver, spleen and kidney of the two fishes and pig pancreas has been worked out by the application of Ohlsson's (1922, 1926 and 1930) technique. According to this technique original extract represented a mixture of α- and β-amylases, the original extract adjusted to pH 6·5 to 6·6 and then heated to 70° C. for 15 minutes is the source of α-amylase and the original extract brought to 1 to 2° C. and then adjusted to pH 3·4 represented β-amylase. Amount of maltose produced being the measure of enzyme activity, the results are embodied in Table III.

Effect of the presence of Ca^{++} in varying concentration in enzyme extract, on the application of Ohlsson's technique to pancreatic amylases of pig and *S. sorrakowah* has been studied. Enzyme activity was followed reductometrically by carrying out the reactions at pH 6·0, at 37° C. for 20 minutes. The results are shown by Figs. 1 A and and 1 B.

To study the solubility of amylases of pancreas, liver, spleen and kidney of *S. sorrakowah* and pancreas of *S. blochii*, homogenised tissue in each case was extracted separately with water and 10% (V/V) aqueous glycerol to prepare 6% extracts. All extracts were autolysed overnight at 15° C., squeezed through muslin and then filtered under suction. Residue in each case was suspended in the amount of extractant equal to that of the extract from which residue was obtained. Cloth filtrates, buchner filtrates and residue suspensions were tested for enzyme activity reductometrically, in a usual way by carrying out the reactions at the respective activity pH optimum or optima as exhibited by amylases of different organs. The results are given in Table IV.

Experiment was carried out to study the effects of varying H^+ concentration of pancreatic desmo- and lyo-amylases of *S. sorrakowah* and *S. blochii*. The solution of amylase under investigation was brought to 1 to 2° C. and was maintained at different pHs. for 15 minutes. The various test solutions thus prepared were used as the source of enzyme. Amylase activity was estimated reductometrically. The reactions in each case were run at the respective activi y pH optimum exhibited by the enzyme. The results are given in Table V. Percentage destruction of amylase by varying pH of enzyme solution is represented by Fig. 2.

TABLE III

Activity of α- and β-amylases, α-amylase and β-amylase at Various pHs.

Reaction mixture "containing 10 c.c. substrate + 10 c.c. buffer of required pH + 5 c.c. enzyme preparation" was set up at 37° C. for 20 minutes.

Animal	Organ	Amylase type	Enzyme activity/100 mgm. dry tissue in terms of mgm. maltose					
			pH					
			3·0	4·0	5·0	6·0	7·0	8·0
Fish : *Scoliodon sorrakowah*	Pancreas	α- & β-	2·29	5·83	57·90	180·60	113·30	46·25
		α-	0·0	0·00	23·12	64·79	30·00	11·66
		β-	0·0	0·0	43·95	120·40	60·00	20·83
	Liver	α- & β-	0·0	13·96	25·44	13·96	39·38	20·83
		α-	0·0	3·54	17·92	6·54	2·29	0·0
		β-	0·0	0·0	0·0	4·58	25·44	5·83
	Spleen	α- & β-	0·0	6·87	9·17	16·25	25·42	4·58
		α-	0·0	0·0	0·0	0·0	0·0	0·0
		β-	0·0	0·0	2·29	4·58	11·66	3·54
	Kidney	α- & β-	0·0	6·87	18·54	6·87	2·29	0·0
		α-	0·0	0·0	6·87	2·29	2·29	0·0
		β-	0·0	0·0	0·0	0·0	0·0	0·0

Fish: *Sphyrna blochii*							
Pancreas	α- & β-	11·66	43·95	127·3	71·66	159·8	92·28
	α-	0·0	4·58	78·24	27·71	0·0	0·0
	β-	0·0	0·0	23·12	55·62	141·2	74·15
Liver	α- & β-	0·0	13·96	27·71	20·83	43·95	20·83
	α-	0·0	4·58	16·25	2·29	0·0	0·0
	β-	0·0	0·0	0·0	11·66	39·59	9·17
Spleen	α- & β-	0·0	2·29	9·17	16·25	27·71	11·66
	α-	0·0	0·0	0·0	0·0	0·0	0·0
	β-	0·0	0·0	0·0	9·17	18·54	4·78
Kidney	α- & β-	0·0	6·87	18·54	9·17	0·0	0·0
	α-	0·0	4·58	18·54	6·87	0·0	0·0
	β-	0·0	0·0	0·0	0·0	0·0	0·0
Pig							
Pancreas	α- & β-	34·78	60·20	94·99	259·10	210·60	145·80
	α-	0·0	13·96	27·71	78·74	32·44	18·54
	β-	0·0	20·83	43·95	166·60	69·36	23·12

TABLE IV

Water and 10% Aqueous Glycerol as Extractants of Amylases

Reaction mixture "containing 6 c.c. substrate + 6 c.c. buffer of required pH + 3 c.c. enzyme preparation" was kept at 37° C. for 20 minutes.

Fish	Organ	pH	Enzyme activity/100 mgm. dry tissue in terms of mgm. maltose					
			Water extract			Glycerol extract		
			Cloth filtrate	Buchner filtrate	Residue sus	Cloth filtrate	Buchner filtrate	Residue sus
S. blochii	Pancreas	5·0	180·6	53·32	127·3	180·6	57·9	129·6
		7·0	208·3	208·3	25·42	208·3	201·3	23·12
S. sorrakowah	Pancreas	6·0	194·4	152·7	48·54	199·2	157·5	48·54
	Liver	5·0	27·71	6·87	25·42	30·0	6·87	27·71
		7·0	41·66	39·38	4·58	46·25	43·95	4·58
	Kidney	5·0	20·83	2·29	20·83			
	Spleen	7·0	27·71	30·0	0·0			

TABLE V

Effects of Varying pH of lyo- and desmo-form Pancreatic Enzyme Solutions on lyo- and desmo-amylases

Reaction mixture "containing 6 c.c. substrate +6 c.c. buffer of required pH + 3 c.c. enzyme solution" was set up at 37° C. for 20 minutes.

Enzyme activity/100 mgm. dry tissue in terms of mgm. maltose*

pH of amylase solution for 15 minutes	*Scolidon sorrakowah*		*Sphyrna blochii*	
	Lyo-amylase	Desmo-amylase	Lyo-amylase	Desmo-amylase
	Reaction pH			
	6·0	6·0	7·0	5·0
2·5	22·29 (13·9)	..	45·8 (25·0)	..
3·1	48·54 (29·5)	..	69·36 (37·9)	..
3·4	196·6 (119·6)	9·17 (18·8)	199·20 (108·9)	11·66 (9·0)
4·0	155·00 (94·3)	23·12 (47·6)	175·80 (96·1)	27·71 (21·4)
4·5	120·40 (72·2)	25·42 (52·4)	150·40 (82·2)	46·25 (35·7)
5·0	157·50 (95·8)	41·66 (85·8)	175·80 (96·1)	113·30 (87·5)
6·0	164·40 (100·0)	48·54 (100·0)	182·90 (100·0)	129·10 (100·0)
7·0	139·00 (84·5)	41·66 (85·8)	143·50 (78·5)	111·00 (85·7)
8·0	120·40 (73·4)	39·4 (81·1)	118·10 (64·6)	94·99 (73·3)
9·0	118·10 (71·9)	..	78·74 (43·0)	87·90 (67·8)

* ( ) % amylase in lyo- or desmo-form enzyme solution.

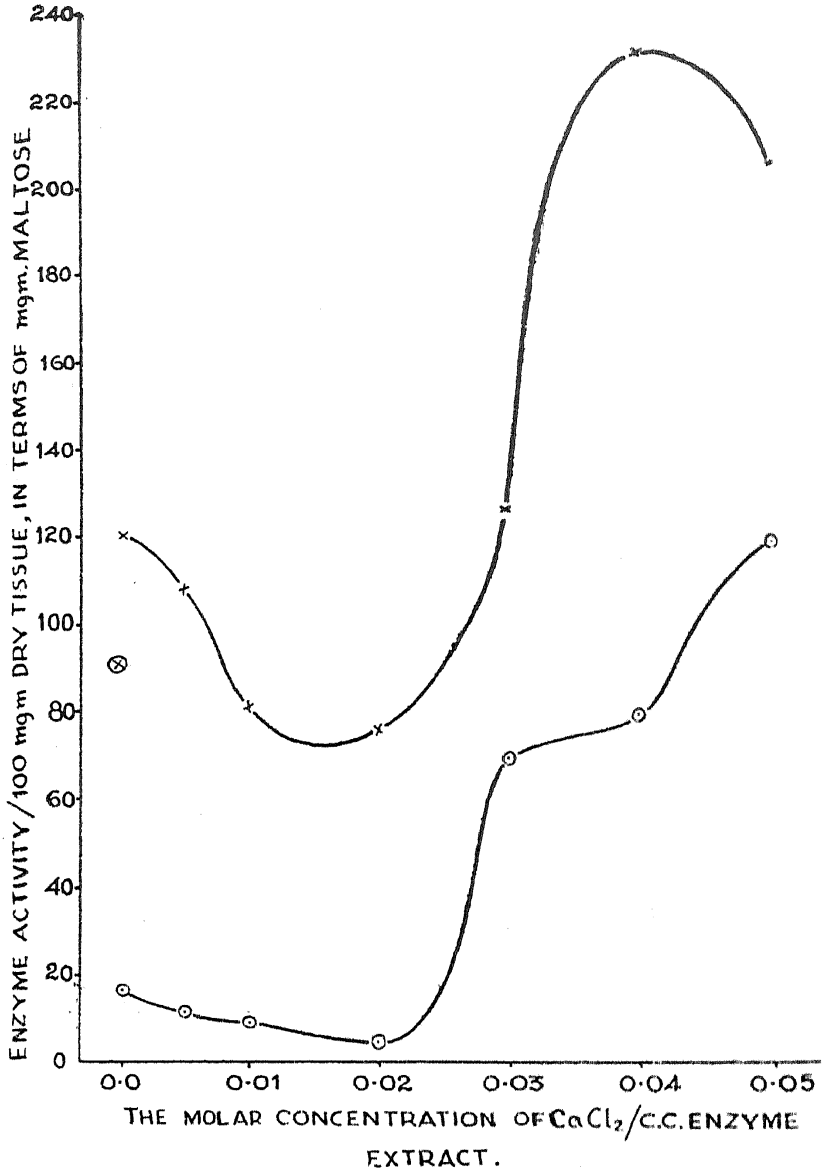

FIG. 1 A. Effect of Ca^{++} in varying concentration on the application of Ohlsson's technique to pancreatic amylases of pig.

⊗ α- and β-amylases
⊙–⊙–⊙ α-amylase
x-x-x- β-amylase

DISCUSSION

A general survey (Table I) of amylolytic activity of the various tissues, of the two fishes shows that the activity is maximum in pancreas, minimum in intestine, muscle, heart and brain, intermediate in liver, spleen and kidney and absent in stomach and ampullæ of lorenzini.

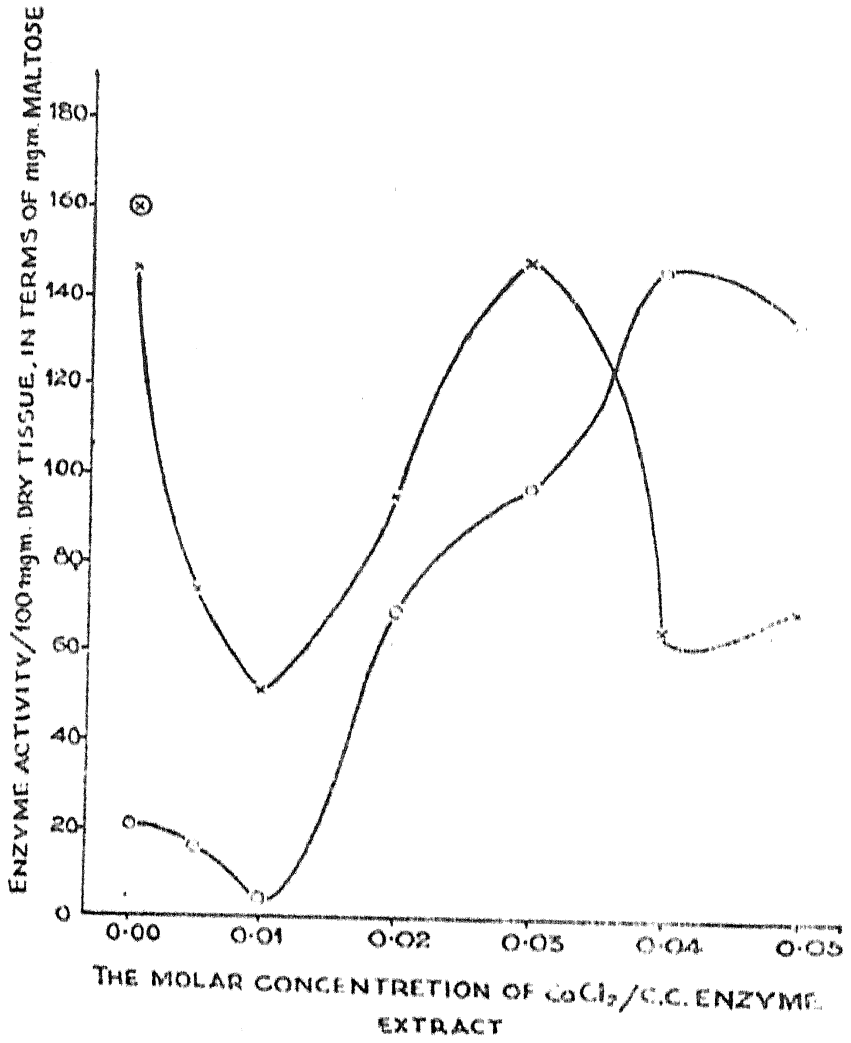

FIG. 1 B. Effect of Ca^{++} in varying concentration on the application of Ohlsson's technique to pancreatic amylases of *S. sorrakowah*.

⊗ α- and β-amylases
O-O-O α-amylase
x-x-x β-amylase.

It will be seen from the results in Table II A and II B that saccharogenic and liquefying activities of amylases of *S. blochii* pancreas and liver of both the fishes exhibit maxima at pH 5·0 and 7·0, of *S. sorrakowah* and pig pancreas exert optimum at pH 6·0 and in both the fishes those of kidney and spleen act best at pH 5·0 and 7·0 respectively. Optimal saccharogenic activity of intestinal and brain enzymes is at pH 5·0 and 7·0 and of muscle is at pH 7·0.

The two activity-pH-optima suggest the probable presence of two types of amylases. The higher saccharification and lower liquefaction at pH 7·0

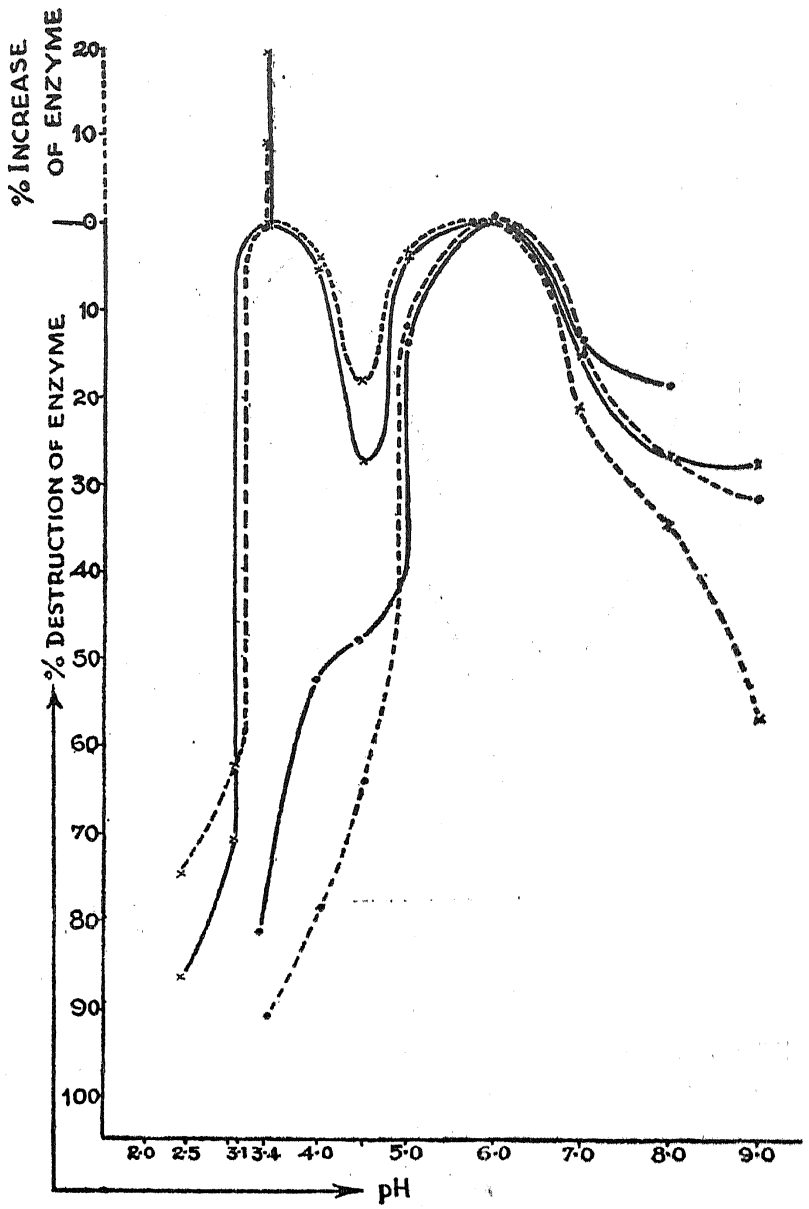

FIG. 2. % destruction of lyo- and desmo-form pencreatic amylases of fish, caused by varying H^+ concentration of enzyme solutions at 1 to 2° C.

S. sorrakowah	x—x—x	Lyo-amylase
	-●-●-●-	Desmo-amylase
S. blochii	-x-x-x-	Lyo-amylase
	-●-●-●-	Desmo-amylase

than at pH 5·0 give an indication of β-amylase to be acting best at pH 7·0 and α- at 5·0 or there may be a mixture of α- and β-amylases represented by two isodynamic systems at pH 5·0 and 7·0 respectively. The only one activity pH optimum tends to show the presence of either α- or β-amylase or there may be a mixture of the two acting best at the same pH.

Table III shows that *S. blochii* pancreatic and *S. blochii* and *S. sorrakowah* liver α- and β-amylases show optimum activity at pH 5·0 and 7·0 respectively, whereas those of *S. sorrakowah* and pig pancreas show maximum activity at pH 6·0. Kidney amylase is an α-amylase acting best at pH 5·0 and that of spleen is a β-amylase with optimal action at pH 7·0.

As per application of Ohlsson's technique (Fig. 1 A), pig pancreatic extract retains much of α-amylase, and β-amylase gets destroyed, when it is adjusted to pH 6·5 to 6·6 and heated to 70° C. for 15 minutes, while the extract at 1° to 2° C., if adjusted to pH 3·4, β-amylase is unaffected α-being destroyed. But when Ca^{++} is introduced in terms of increasing doses of $CaCl_2$ in enzyme extract, thermostability of α-amylase and acid stability of β-amylase starts decreasing, maximum decrease in each case is effected when Ca^{++}/c.c. extract is 0·02 M. Further addition of Ca^{++}, now causes increase in enzyme activity. Maximum increase in case of α-occurs when Ca^{++}/c.c. extract is 0·05 M. and that in the case of β-occurs when it is 0·04 M/c.c. extract.

A similar pattern of results (Fig. 1 B) has been observed with *S. sorrakowah* pancreatic amylases. Here the thermostability of α- and acid stability of β- fall to the maxima when Ca^{++}/c.c. extract is 0·01 M. Further addition of Ca^{++} turns out in the rise of enzyme activity. The optimum increase of α-amylase is indicated at 0·4 M. Ca^{++}/c.c. extract, and that of β-amylase at 0·03 M. Ca^{++}/c.c. extract.

The above results suggest the exchange of thermo-stability and acid-lability of α-amylase with the thermolability and acid stability of β-amylase, *i.e.*, the probable interconversion α-amylase $\rightleftharpoons$ β-amylase under the influence of Ca^{++} as indicated in the case of wheat amylases (1943).

The study of the solubility (Table IV) of amylases of pancreas liver spleen and kidney of *S. sorrakowah* and pancreas of *S. blochii* shows that both water and 10% aqueous glycerol are equivalent extractants of enzymes. α-type is a desmo-amylase and β-type a lyo-amylase.

The results in Table V and Fig. 2, show that in both the fishes destruction of desmo- and lyo-amylases starts when H^+ concentration of enzyme preparations is taken below or raised above pH 6·0 (original pH of enzyme extract). The destruction of desmo-amylase at pH 3·4 is 90·90%. The loss of lyo-amylase however, proceeds upto pH 4·5, at pH 4·0 it is less than what it was at pH 4·5, at pH 3·4 there is no fail in enzyme activity but the amount of active amylase content in solution is increased by 19·6% in *S. sorrakowah* and by 8·9% in *S. blochii.* The slight shift from pH 3·4 towards acidity is now followed by great losses in enzyme activity.

The acid lability of desmo-amylase and acid stability of lyo-amylase show that the former is predominantly of α-type and the latter of β-type.

The increase in active amylase content of pancreatic lyo-amylase solutions, when they are brought to 1 to 2° C. and adjusted to pH 3·4 may be due to the probable presence of Zymogen precursor of enzyme.

SUMMARY

1. In *S. sorrakowah* and *S. blochii* amylolytic activity is maximum in pancreas, minimum in intestine, muscle, heart and brain, intermediate in liver, spleen and kidney and absent in stomach and ampullæ of lorenzini.

2. Pancreas and liver of both the fishes and pig pancreas contain α- and β-amylases. Kidney amylase is an α-amylase and spleen amylase is a β-amylase.

3. In *S. sorrakowah* and pig there seems to be probable interconversion of pancreatic α-amylase $\rightleftharpoons$ β-amylase under the influence of Ca^{++}.

4. Both water and aqueous glycerol are equivalent extractants of enzyme. Desmo-amylase seems to be predominantly of α-type and lyo-amylase of β-type.

5. Pancreatic lyo-amylase of both the fishes shows the presence of pro-amylase.

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