

STUDIES IN ENZYMES. PART II. PURIFICATION OF AMYLASE FROM KASERU (*SCIRPUS GROSSUS*, LINN.).

By. J. P. SHUKLA.

Precipitation with ammonium sulphate, dialysis and subsequent precipitation with alcohol gave an active amylase from the herb *Kaseru* (*Scirpus grossus*, Linn.) having a high saccharogenic activity. The purified enzyme isolated has an optimum pH of 4.8 using acetate buffers.

In a previous paper the author (*J. Indian Chem. Soc.*, 1941, 18, 407) related to the presence of amylase in the tuberous root of the herb *Kaseru* (*Scirpus grossus*, Linn.) and has studied its optimum temperature and pH . In the present paper an attempt has been made to purify the enzyme and to investigate its nature together with its optimal conditions in the highly purified state.

EXPERIMENTAL.

A fresh lot of *kaseru* was obtained from the market and its skin removed. The white tubers were dried in the sun for 4 days and then ground to powder, sieved through a fine mesh screen and stored for use.

200 G. of starch, thus obtained, were mixed with dry neutral sand and 400 c.c. of 20% alcohol. The mass was kept at 37° for 40 hours and triturated from time to time to extract the enzyme. The filtrate was tested for activity per mg. of the substance. As it is likely that the extract may contain some sugar, the enzyme activity was determined by taking a difference in the weights of Cu_2O precipitated from 5 c.c. of the extract allowed to react without any lapse of time from the Cu_2O precipitated when the extract was allowed to react for exactly one hour at 37° on the same starch solution. The starch solution used was buffered to correspond to the optimum pH 5.6 as determined in the previous paper.

Diastatic value (Sherman, Kendall and Cierk. *J. Amer. Chem. Soc.*, 1910, 32, 1073) of the extract at 37° for 1 hour is $D^{37}_{1\text{hour}} = 12.2$.

Dialysis of the Extract.—One of the first steps recommended (*J. Amer. Chem. Soc.*, 1926, 48, 2947) for the purification of the enzyme is to dialyse the extract against distilled water in cold using collodion membrane. The specification and use of the membrane has been standardised by various authors (Pierce, *J. Biol. Chem.*, 1927, 76, 795). In the present investigation collodion membrane was prepared using a 6% solution of collodion in ether

and pouring the solution into a conical flask of the required size. The sack obtained was filled with distilled water and immersed for 24 hours in water before use. Several sacks were made under identical conditions and stored for use.

The enzyme extract was dialysed for 24 hours against distilled water at 14°. During dialysis the volume of the liquid in the sack increased from 100 c.c. to 172 c.c. Samples of the dialysate were taken out every hour up to 8 hours and the diastatic activity of the extract measured by Cu_2O precipitation. The dialysate was tested for proteins which are always associated with the amylase to ensure that the enzyme itself has not diffused out completely.

TABLE I.

Treatment.	Molisch test.	Binret test.	Cu_2O ppt.	Cu_2O due to extract.	Dry matter.	K per g. of solid at 37° for 1 hour.
Blank with starch	—	—	0.1283 g.	—	—	—
Original extract	+	+	0.3948	0.2665 g.	0.2606 g.	312
1 hour dialysis	+	+	0.2422	0.1139	0.232	163.6
2 " "	+	+	0.1950	0.0667	0.206	101.6
3 " "	+	+	0.1664	0.0381	0.178	67.8
4 " "	+	+	0.1512	0.0229	0.152	44.2
5 " "	+	+	—	—	—	—
6 " "	+	+	0.1455	0.0172	0.099	54.1
8 " "	+	+	—	—	—	21.6
24 " "	—	—	0.1293	0.0010	—	—

It may be seen from the above table that the value of K decreases from 312 to 44.2 and then rises to 54.1. On further dialysis it again begins to decrease and comes down to 21.6 after 8 hours' dialysis. The high initial value of K is due to the presence of reducing sugars originally present in the extract. The value of K falls for four hours during the progressive diffusion of the reducing sugars. The increase in the activity of the enzyme, which occurs subsequently, can be traced to the rise in purity. The fall in the value of K after further dialysis shows that during this stage considerable loss of enzyme takes place. A period of 6 hours was, therefore, fixed for dialysis.

Precipitation with Ammonium Sulphate.—At this stage there are two courses that are possible for further purification of the amylase *viz.*, (a) absorption of the amylase on alumina followed by subsequent elution as adopted by Wilstätter (Wilstätter, *et al.*, *Z. physiol. Chem.*, 1923, 121, 143; 1921 422, 72.), (b) precipitation of the amylase by $(\text{NH}_4)_2\text{SO}_4$ of a concentration of 25–35 g. of the salt per 100 c.c. of the extract as recommended by Sherman (Sherman, Caldwell and Doebbeling, *J. Biol. Chem.*, 1934, 104, 501), followed by dialysis to remove the salt. Since the work of Willstätter in this connection has been criticised by the later workers, the precipitation of the enzyme by $(\text{NH}_4)_2\text{SO}_4$ was adopted.

The dialysate obtained after 8 hours' dialysis was divided into 4 portions of 50 c.c. each and poured over weighed ammonium sulphate in stoppered bottles, well shaken and left for 23 hours in a frigidaire. The supernatant liquid was filtered off by upward microfiltration and the solids washed with alcohol and ether and weighed to get an approximate idea of the yields per 100 c.c. The weighed fractions, obtained at different concentration of the $(\text{NH}_4)_2\text{SO}_4$, were then dissolved in double-distilled water buffered at p_H 5.6 using acetate buffer and made up to 25 c.c. and the activity measured taking 5 c.c. of these solutions.

TABLE II.

Treatment.	Total moist solids.	Molisch test.	Cu_2O by 5 c.c. extract.	Total solids in 5 c.c. extract.	K per g. solids at 37°.
Extract.	—	+	0.0104 g.	0.2606 g.	12.2
Dialysed 8 hours	—	+	0.0072	0.0994	22.2
20 g. $(\text{NH}_4)_2\text{SO}_4$ per 100 c.c.	0.5750 g.	Doubtful	0.0057	—	—
30 g. „ „	0.8298	+	0.0162	0.0532	100.0
35 g. „ „	0.9394	+	0.0261	0.0405	219.2
40 g. „ „	0.9262	+	0.0100	0.0367	89

It would be seen from the data that 35 g. of $(\text{NH}_4)_2\text{SO}_4$ per 100 c.c. throw out the maximum quantity of enzyme of the highest activity and this concentration of ammonium sulphate, therefore, appears to be the best that could be utilised. This is also in accordance with the findings of Sherman *et al* (*loc. cit.*).

Precipitation with Alcohol.—The enzyme fraction precipitated out by 35 g. of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in a known quantity of double-distilled

water and dialysed till free from SO_4 . Total volume of the solution of enzyme, thus obtained, was kept as low as possible (85 c.c.) and enough absolute alcohol was mixed to obtain the resultant liquid of 50 % alcoholic strength (by vol.). On allowing to stand overnight at 4° in a fridaire, a precipitate was thrown out which settled at the bottom and was separated by upward filtration. The strength of the solution was then further raised to 60–75% alcoholic strength and the precipitate appearing was separated by upward filtration. It was dissolved in double-distilled water and its activity and optimum p_H determined using acetate buffers (*cf.* Clark, "The Determination of Hydrogen Ions," p. 219).

TABLE III.

Starch concentration = 1.5%. Time of reaction = 1 hour. Temperature = 37° .

p_H .	K per g. solids.	p_H .	K per g. solids.	p_H .	K per g. solids.
3.6	290.5	4.8	340.3	5.8	302.6
4.2	306.8	5.1	336.8	6.2	296.3
4.5	320.7	5.4	318.5	6.7	290.5
				7.0	286.8

The maximum value of the activity of the enzyme after the precipitation from $(\text{NH}_4)_2\text{SO}_4$ was 219.2. As would be seen from the above table the activity increases further to a value of 340.3 after alcoholic precipitation.

The optimum p_H of the amylase in the purified state was found to be 4.8. This value agrees with the findings of Sherman *et al.* (*loc. cit.*). The amylase obtained from *Kasaru*, was similar to the amylase from malt and had strong saccharogenic activity indicating the presence of β -amylase in larger proportions. Further investigation is in progress to separate the fractions during early stages of ammonium sulphate fractionation to recover the α -amylase possibly having a high amylolytic activity as against the amylase isolated in a purified state in the present paper.

The author acknowledges his thanks to R. C. Srivastava, O.B.E., Director and Dr. H. D. Sen, Biochemist, Imperial Institute of Sugar Technology, Cawnpore for their keen interest and encouragement in the work.