Studies on Sulphatases

26. ARYLSULPHATASE ACTIVITY IN THE DIGESTIVE JUICE AND DIGESTIVE GLAND OF *HELIX POMATIA**

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During the course of studies on arylsulphatases in these Laboratories evidence has accumulated which suggests that these enzymes can be classified into two types. The type I enzymes are particularly active towards simple arylsulphates such as potassium *p*-nitrophenyl sulphate and potassium *p*-acetylphenyl sulphate. They are inhibited by cyanide but not by phosphate and fluoride. In contrast the type II enzymes show relatively greater activity towards more complex arylsulphates such as dipotassium 2-hydroxy-5-nitrophenyl sulphate, and their behaviour towards cyanide, phosphate and fluoride is the reverse of that shown by the type I enzymes.

Recently arylsulphatases from a variety of organisms have been investigated in attempts to provide additional evidence for the existence of two distinct types of enzyme. One of the enzymes which was of particular interest was that present in the edible snail, *Helix pomatia*. This arylsulphatase has previously been studied in some detail by Jarrige & Henry (1952) and by Dastugue, Bastide & Tronche (1957), but from their results it is not possible to deduce with certainty whether the enzyme can be classified as type I or II. In particular these two groups of workers differed in their findings about the effect of phosphate on enzyme activity. Jarrige & Henry (1952), using potassium *p*-nitrophenyl sulphate as the substrate, reported only 48% inhibition of enzyme activity by 0·1M-phosphate, whereas Dastugue *et al.* (1957), using sodium 2-carboxyphenyl sulphate as substrate, obtained 98% inhibition with 0·01M-phosphate.

In an attempt to resolve these and other discrepancies *Helix* arylsulphatase has been re-examined in these Laboratories. Initially, digestive juice obtained from the crop of the organism was used as the enzyme source but it was subsequently necessary (for reasons outlined in the text) to abandon this in favour of extracts of the digestive gland (hepatopancreas). The results described in this and the next paper show that, in certain respects, the *Helix* enzyme appears to be unlike any arylsulphatase previously described.

* Part 25: Spencer (1959).

MATERIALS AND METHODS

Snails. These were obtained from L. Haig, Beambrook, Newdigate, Surrey and were starved for 4 days before use. Occasionally during the winter months it was necessary to use hibernating snails. In such cases the organisms were kept in a humid atmosphere at a temperature of about 25° for a week, when most of them became active and began to feed. In general, the arylsulphatase activity of hibernating snails was appreciably lower than that of organisms collected in spring and summer.

Substrates. Potassium *p*-nitrophenyl sulphate (NPS) was prepared by the method of Burkhardt & Lapworth (1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) by the method of Roy (1953) modified as described by Dodgson & Spencer (1956).

Determination of enzyme activity. Arylsulphatase activity towards NPS and NCS was measured by spectrophotometric estimation of the anionic form of the liberated phenolic moiety, at the appropriate wavelength of maximum absorption, after the addition of alkali to the incubation mixture (see Dodgson & Spencer, 1957). With crude digestive-gland extracts when NCS was the substrate the colour of the anionic form of the liberated 4-nitrocatechol tended to fade rather rapidly. This could be eliminated by developing the colour with the alkaline quinol–sodium sulphite reagent described by Roy (1953). Except where otherwise stated, incubation of enzyme (0·2 ml.) and substrate (0·2 ml.) was for 30 min. (for the digestive-juice enzyme) or 15 min. (digestive-gland extract) at 38° in the presence of 0·25M-acetic acid–sodium acetate buffer at the appropriate pH or 0·25M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) which had been adjusted to the required pH with acetic acid. On some occasions acetic acid–sodium acetate buffer was used at pH values where its buffering power was low. However, separate experiments, in which the pH of the incubation mixture was checked at the beginning and the end of the incubation period, showed that no significant change in pH had occurred. Preliminary experiments were made in order to establish the concentration of enzyme appropriate to the range of accuracy of the assay method.

EXPERIMENTAL AND RESULTS

Experiments with digestive juice

Preliminary experiments were made on a sample of the digestive juice which had been stored at 0° for several months. In later experiments digestive

juice was expressed from the crop of freshly dissected snails. Results obtained with both fresh and stored juice were substantially the same. The arylsulphatase activity of the juice is extremely high (cf. Jarrige & Henry, 1952) and the preparations had to be diluted approximately 10 000-fold to bring the activity within the range of the assay methods.

Effect of pH *and substrate concentration.* Jarrige & Henry (1952) have reported that *Helix* arylsulphatase shows maximum activity at pH 5·2 and at a substrate (NPS) concentration of 0·5 mM. Attempts were made to confirm these findings with both dialysed and non-dialysed samples of the juice. During the course of these experiments it became apparent that the optimum pH of the enzyme towards NPS varied appreciably with the concentration of substrate used and, similarly, the optimum substrate concentration varied with pH. Fig. 1 shows the effect of increasing substrate concentration on the optimum pH of a fixed amount of enzyme. There is a gradual shift in the optimum pH from 6·0 in the presence of 0·5 mM-NPS to 7·3 in the presence of 15 mM-NPS. Further increase in substrate concentration to 30 mM did not result in a further shift in pH optimum. No attempt was made to determine pH optima at NPS concentrations below 0·5 mM because a high proportion (> 40 %) of the substrate present in the incubation mixture would have been hydrolysed before suitable spectrophotometric readings could be made. Separate experiments showed that at pH 6·0 maximum enzyme activity was obtained with 6 mM-NPS, whereas at pH 7·0 and 7·3 the corresponding values were 12 and 15 mM. Similar results were obtained with dialysed and non-dialysed digestive juice.

Fig. 2 shows that similar shifts in the pH optimum were obtained with NCS, the range of the shift being from pH 6·5 at 0·5 mM-NCS to pH 7·5 at 15 mM. There was a further slight shift to pH 7·6 when the NCS concentration was increased to 30 mM. The increase in substrate concentration

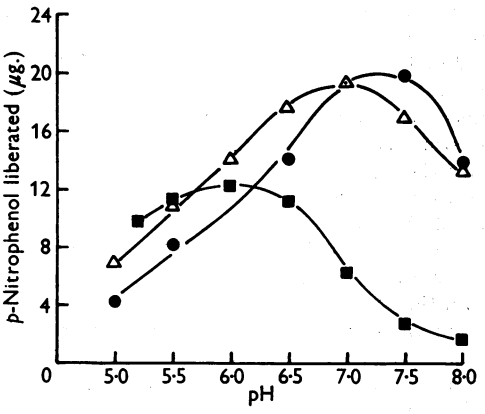

Fig. 1. Effect of potassium *p*-nitrophenyl sulphate concentration on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting in the presence of 0·25 M-acetic acid–sodium acetate buffer. ■, 0·5 mM; △, 6 mM; ●, 15 mM. The curves are quantitatively related to each other.

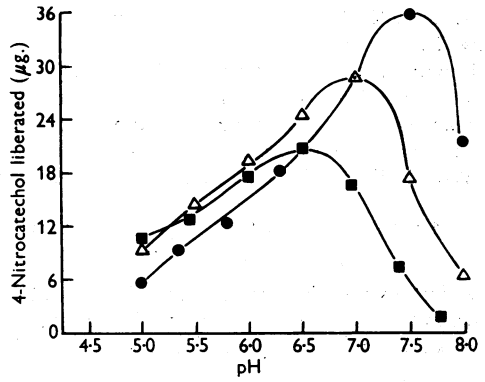

Fig. 2. Effect of dipotassium 2-hydroxy-5-nitrophenyl sulphate concentration on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting in the presence of 0·25 M-acetic acid–sodium acetate buffer. ■, 0·5 mM, △, 1·5 mM; ●, 15 mM. The curves are quantitatively related to each other.

Table 1. *Enzyme activities and Michaelis constants of the arylsulphatase of the digestive juice of* Helix pomatia *acting on potassium* p*-nitrophenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate at the lower and upper limits of the* pH *shift*

Arylsulphatase activity is expressed in terms of μg. of *p*-nitrophenol or 4-nitrocatechol liberated/ml. of digestive juice in 30 min. at 38° in the presence of 0·25 M-acetic acid–sodium acetate buffer.

Substrate	Substrate concn. (mM)	pH	Arylsulphatase activity	$10^4 K_m$
NPS	0·5	6·0	420 000	1·9
	15·0	7·3	700 000	17·9
NCS	0·5	6·5	620 000	0·3
	15·0	7·5	1060 000	12·5

which occurred with increasing pH was similar to that obtained with NPS.

Table 1 shows the K_m values (calculated from the plot of v against $v/[S]$) for NPS and NCS at the upper and lower limits of the pH shift. The table also records the activities of the juice at pH 6·0 and 0·5 mM-NPS, pH 7·3 and 15 mM-NPS, pH 6·5 and 0·5 mM-NCS and pH 7·5 and 15 mM-NCS. The ratio of the activities towards NPS under the two experimental conditions was of the order of 1:1·7; the corresponding ratio for NCS was also 1:1·7. This ratio of activities will be referred to hereafter as the ratio of activities at low and high pH.

NCS was used as substrate in subsequent experiments, because of its appreciable buffering power at high pH (cf. Roy, 1953).

Effect of time of incubation and enzyme concentration. The possibility existed that the shift in pH with substrate concentration might arise from an anomalous behaviour of the enzyme with the time of incubation similar to that observed for arylsulphatase A of human liver (Baum, Dodgson & Spencer, 1958; Baum & Dodgson, 1958). However, the time–activity curves at both low and high pH showed no anomalies although there was a steady decline in enzyme activity with time. Enzyme concentration–activity curves at low and high pH values were virtually linear and gave no indication of the presence of an enzyme with anomalous kinetics similar to those of human arylsulphatase A.

Attempted demonstration of the presence of two arylsulphatases

Inhibition experiments. The shift in pH optimum described earlier would be expected if the digestive juice contained two arylsulphatases, one having a high affinity for NPS and NCS and an optimum pH in the region of 6·0–6·5 and the other having a lower affinity for these substrates and an optimum pH in the region of 7·3–7·5. A similar multiplicity of arylsulphatases is known to occur in other organisms; for example, three distinct arylsulphatases occur in human and ox liver (Dodgson, Spencer & Thomas, 1955; Roy, 1956). An attempt was made to test this possibility by examining the effect of pH on *Helix* arylsulphatase in the presence of 25 mM-CN^- ions (an inhibitor of the type I enzymes) and 25 mM-PO_4^{3-} ions (inhibitory towards type II enzymes). An experiment of this type was used by Roy (1956) to distinguish between the types I and II arylsulphatases of ox liver. In the present work a substrate (NCS) concentration of 1·5 mM was selected, this being intermediate between the optimum concentrations obtained at pH 6·5 and 7·5 respectively. Fig. 3 shows that the pH optimum was not affected by cyanide or phosphate although the latter caused appreciable inhibition of enzyme activity. The curves provide no evidence for the co-existence of types I and II arylsulphatases in the digestive juice.

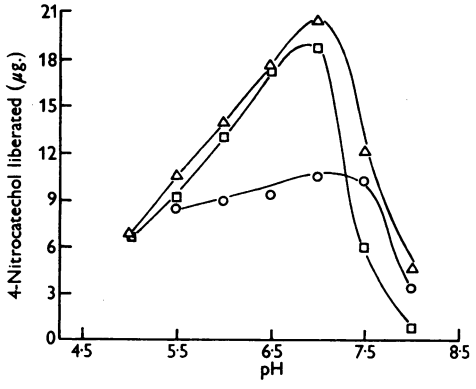

Fig. 3. Effect of phosphate and cyanide on the pH optimum of the arylsulphatase of the digestive juice of *Helix pomatia* acting on 1·5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer. △, Control curve; □, 25 mM-$NaCN$; ○, 25 mM-Na_2HPO_4–NaH_2PO_4.

Enzyme-fractionation experiments. The experiment described in the previous section did not eliminate the possibility that two arylsulphatases of the same type were present in the digestive juice (cf. the type II arylsulphatases A and B of human liver; Dodgson, Spencer & Wynn, 1956). Attempts were therefore made to separate two such enzymes by fractionation of the digestive juice with acetone and $(NH_4)_2SO_4$.

Two series of acetone fractionations were carried out, one at pH 7·0 when the juice (diluted 30-fold with water) was fractionated at 0° between the limits 0–45 and 45–70% (v/v) of acetone, and the other at pH 5·0 when seven successive fractions covering the range 0·80% (v/v) of acetone were obtained. The ratio of activities at low and high pH of each of the fractions was not significantly different from that of the unfractionated juice (Table 2). No fraction showed a reversal of the ratio such as might have been expected had separation of two arylsulphatases been achieved. pH–Activity curves of the 0–35 and 50–60% acetone fractions at NCS concentrations of 0·5 mM and 15 mM were virtually identical with those obtained with the unfractionated juice.

Similar fractionation experiments were carried out at 0° with $(NH_4)_2SO_4$. In one experiment a tenfold dilution of the juice was fractionated between the limits 0–45 and 45–70% saturation at pH 6·5, and in another a similar dilution of juice in 0·5 M-acetic acid–sodium acetate buffer was fractionated between the limits 0–40, 40–60 and 60–75% saturation at pH 5·5. Each fraction was dialysed for 18 hr. before assay at low and high pH

Table 2. *Distribution of arylsulphtase activity towards dipotassium 2-hydroxy-5-nitrophenyl sulphate after fractionation of the digestive juice of* Helix pomatia *with acetone or ammonium sulphate*

Experimental conditions for determination of the ratios of enzyme activity at low and high pH are given in the text.

Method of fractionation	pH of fractionation	Concn. of acetone (%, v/v) or $(NH_4)_2SO_4$ (% saturation)	Activity of fractions (% of total initial activity)	Ratio of enzyme activity at low and high pH
Acetone	7·0	0–45	6·5	1·0:1·7
		45–70	72·0	1·0:1·5
	5·0	0–35	1·3	1·0:1·8
		35–40	0·8	1·0:1·7
		40–45	1·3	1·0:1·8
		45–50	1·6	1·0:1·9
		50–60	45·0	1·0:1·7
		60–70	22·6	1·0:1·7
		70–80	0·4	1·0:1·4
$(NH_4)_2SO_4$	6·5	0–45	—	1·0:1·4
		45–70	—	1·0:1·5
	5·5	0–40	4·0	1·0:1·5
		40–60	42·0	1·0:1·7
		60–75	21·0	1·0:1·8

as before. The ratios of activities for these fractions (Table 2) were again very similar.

Electrophoresis experiments. Attempts were made to separate two enzymes by electrophoresis (horizontal) on Whatman no. 100 or 3MM paper in the presence of 0·1M-acetic acid–sodium acetate buffer at pH varying from 4·1 to 7·5. Electrophoresis was for 18 hr. at an ambient temperature of 4° and a potential gradient of 7v/cm. Strips (1 cm.) of the paper were subsequently incubated with NCS at low and high pH and enzyme activity was located by visual assessment of the 4-nitrocatechol colour after the addition of N-NaOH. Although appreciable movement of the enzyme (towards the cathode) occurred at below pH 6·0 separation of two enzymes was not achieved. At pH greater than 7·0 considerable loss of arylsulphatase activity was experienced. This appeared to be due to destruction of enzyme rather than removal of some cofactor or activator since the loss in activity was not restored when eluates of the 1 cm. strips were combined. Addition of boiled digestive juice or Mg^{2+} and Mn^{2+} ions to the pooled eluates did not restore enzyme activity.

Attempted removal of carbohydrate material from the juice. It will be observed from Table 2 that some enzyme activity appeared in all the fractions during the fractionation experiments. It seemed possible that the high concentration of polysaccharide material in the digestive juice was interfering with the fractionation procedures. Attempts were made to remove this polysaccharide material by the use at 0° of the $CHCl_3$ treatment of Sevag (1934). Although much polysaccharide was eliminated in this way, appreciable amounts still remained and the procedure was discontinued in view of the high losses of enzyme activity which were experienced. However, during the course of these experiments no significant change in the ratio of activities at low and high pH was observed.

In view of the problems posed by the presence of such large amounts of polysaccharide material it was decided to abandon the digestive juice as a source of arylsulphatase and investigate the possibility of using extracts of the digestive gland for further experiments.

Experiments with digestive-gland extracts

Preliminary work showed that the digestive gland was, as expected, a rich source of arylsulphatase, the enzyme being readily extracted from the gland with comparatively little associated carbohydrate material.

Enzyme extracts. The digestive glands (a total of 45 g. from 24 snails) were washed with ice-cold water and stored in the frozen state overnight. After thawing, the glands were suspended in 150 ml. of ice-cold water with the aid of a glass homogenizer (Potter & Elvehjem, 1936). The suspension was then incubated at 38° for 20 min. before centrifuging for 30 min. at 0° and 78 000 *g* av. (head no. 30) in the Spinco preparative ultracentrifuge. Aggregated fatty material was removed from the surface of the supernatant and the latter was then re-centrifuged at 0° and 105 000 *g* av. for 60 min. The clear supernatant was divided into two equal portions, one of which was dialysed at 2° against several changes of water for 40 hr. whereas the other was dialysed at 2° for 16 hr. against 60 vol. of 0·1M-disodium ethylenediaminetetra-acetate (EDTA) which had been adjusted to

pH 8·0 with ethanolamine. This portion was subsequently dialysed for 40 hr. at 2° against several changes of water. Each portion was adjusted to the same volume with water and stored in the frozen state. The preparations had to be diluted approximately 100 times for the enzyme experiments.

Effect of pH *and substrate concentration.* The behaviour of the arylsulphatase of the digestive gland towards variation of pH and substrate concentration in the presence of acetic acid–sodium acetate buffer was virtually identical with that shown by the digestive-juice enzyme. In the presence of 0·25 M-acetic acid–tris buffer there was greater enzyme activity (approx. 100 % increase) and the pH shift varied from pH 6·6 at 0·5 mM-NCS to pH 7·4 at 15 mM-NCS. These experimental conditions were subsequently used to measure the ratio of activities at low and high pH respectively (cf. experiments on the digestive juice), the ratio for the extract being 1:1·5. K_m values obtained in the presence of 0·25 M-acetic acid–tris buffer at pH 6·6 and 7·4 (0·14 mM and 0·17 mM respectively) were appreciably different from those obtained at pH 6·5 and 7·5 respectively in 0·25 M-acetic acid–sodium acetate buffer (cf. Table 1). The enzyme activity of a typical preparation was such that in 15 min. at 38° approximately 3 mg. of 4-nitrocatechol was liberated/mg. of protein at low pH whereas the corresponding figure for high pH was 4·5 mg. No significant differences were observed between the EDTA-treated and the untreated preparation.

Effect of buffer concentration. Enzyme activity increased appreciably with increasing concentration of either acetic acid–sodium acetate or acetic acid–tris buffer (Fig. 4). In each case similar increases were obtained at both low and high pH. Subsequently acetic acid–tris buffer was used in most enzyme experiments because of its superior buffering power at high pH.

Effect of time of incubation and enzyme concentration. The effect of the time of incubation on enzyme activity towards NCS is shown in Fig. 5. There was a considerable decline in activity as incubation proceeded, particularly at the high pH. The enzyme could be pre-incubated for 60 min. before the addition of substrate, with only slight loss in activity. Linear enzyme concentration–activity curves were obtained at both low and high pH (Fig. 5).

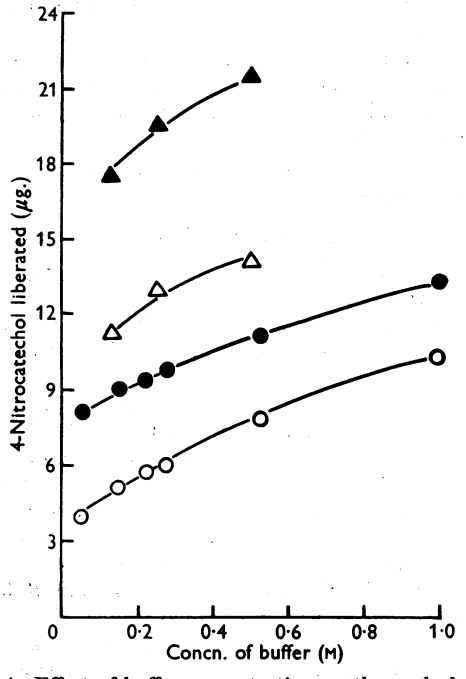

Fig. 4. Effect of buffer concentration on the arylsulphatase activity of a fixed amount of an extract of *Helix pomatia* digestive gland acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS). ○, Acetic acid–sodium acetate buffer at 0·5 mM-NCS, pH 6·6; ●, acetic acid–sodium acetate buffer at 15 mM-NCS, pH 7·4; △, acetic acid–tris buffer at 0·5 mM-NCS, pH 6·6; ▲, acetic acid–tris buffer at 15 mM-NCS, pH 7·4.

DISCUSSION

Although many molluscs are known to be particularly rich in arylsulphatase (Dodgson & Spencer, 1953; Soda, 1936), *Helix pomatia* is undoubtedly the most potent source of the enzyme which has yet been described. The activity of the digestive juice which was observed during the present investigation is considerably higher than that quoted by Jarrige & Henry (1952). However, direct comparison of activities is not possible since the experimental conditions used by these workers for

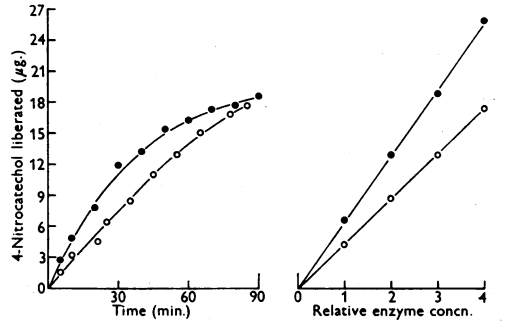

Fig. 5. Effect of time of incubation and enzyme concentration on the arylsulphatase of an extract of *Helix pomatia* digestive gland acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer. ○, 0·5 mM-NCS, pH 6·6; ●, 15 mM-NCS, pH 7·4.

measuring activity (pH 5·2 and 0·5 mM-NPS in 0·1 M-acetic acid–sodium acetate buffer) were different from those used in the present work.

The significance of the high arylsulphatase activity of *Helix* is quite obscure, although it is possible that the enzyme might participate in the transfer of sulphate from arylsulphates to polysaccharides in a way similar to that recently suggested (Suzuki, Takahashi & Egami, 1959) for the arylsulphatase of the marine mollusc, *Charonia lampas*. Certainly *Helix* digestive juice contains appreciable amounts of sulphated polysaccharide material (Levene, 1925).

The shift in the optimum pH of *Helix* arylsulphatase which occurs with increasing substrate concentration is of interest. Similar shifts have been observed with other hydrolytic enzymes, for example, alkaline phosphatase (Morton, 1957) and β-glycosidase (Hofstee, 1955). No shifts of this type have previously been observed with other arylsulphatases although it must be stressed that in two cases only, namely the enzymes of *Patella vulgata* (Dodgson & Powell, unpublished results) and *Proteus vulgaris* (Dodgson, 1959), has any direct search for such shifts been made. It has already been mentioned that the co-existence of two distinct arylsulphatases in *Helix* could be responsible for the observed effects. The results described in this paper suggest that a single enzyme is responsible for the shifts; nevertheless direct proof of this was not obtained. If it is assumed that the effects can be attributed to a single enzyme then several possible explanations of the phenomenon can be advanced. The studies described in the next paper provide further evidence that a single enzyme is involved and eliminate a number of these possible explanations.

SUMMARY

1. A study has been made of the properties of a highly active arylsulphatase which is present in the digestive juice and in extracts of the digestive gland of the edible snail, *Helix pomatia*.

2. Within limits, the pH optimum of the enzyme shifts in the direction of higher pH as the concentration of substrate (either potassium *p*-nitrophenyl sulphate or dipotassium 2-hydroxy-5-nitrophenyl sulphate) is increased.

3. Fractionation of the digestive juice under varying conditions with acetone or ammonium sulphate together with paper-electrophoresis experiments suggest that the pH shift cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation.

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