Studies on the Phospholipid-Splitting Enzyme of *Vibrio* El Tor

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(Received 22 September 1961)

Vibrio cholera (including *Vibrio* El Tor) secretes a lecithin-splitting enzyme whose nature has not been ascertained (Ruata & Caneva, 1901; Kraij & Wolff, 1923; Felsenfeld, 1944). Mitra & Chatterjee (1961) reported that the lecithin-splitting enzyme is a phospholipase B (lysophospholipase) though it is not identical with the similar enzyme from other sources. The present paper gives a more detailed account about the characterization of the enzyme as isolated from the culture filtrates of *Vibrio* El Tor, which contain much more of the enzyme than do the filtrates of Ogawa and Inaba strains of *Vibrio cholera*.

METHODS AND MATERIALS

Organism. Strains of *Vibrio cholera* including *Vibrio* El Tor were kindly supplied by Dr D. K. Roy, Indian Institute for Biochemistry and Experimental Medicine, Calcutta, India.

Preparation of enzyme. The enzyme was precipitated from the culture filtrates of *Vibrio* El Tor grown for 48 hr. in a peptone medium (0.5% Bactopeptone, 1% sodium chloride, pH 7.6) by ammonium sulphate fractionation between 50 and 75% saturation and subsequent dialysis, with stirring, for 1 hr. against 0.01M-phosphate buffer (pH 7.4). The enzyme could be stored at -15° for up to 4 weeks without appreciable loss of activity. This enzyme preparation was used in all experiments.

Materials. Lecithin was prepared from egg yolk according to the method of Pangborn (1950, 1951). Since this lecithin preparation contained impurities, it was further purified by silicic acid column chromatography (Lea, Rhodes & Stoll, 1955). Lysolecithin was prepared from this purified lecithin with the help of cobra venom as described by Hanahan (1954). A sample of glycerophosphorylcholine was kindly supplied by Dr R. M. C. Dawson, Institute of Animal Physiology, Cambridge.

Incubation mixture. The incubation mixture usually consisted of enzyme (approx. 0.15 mg. of protein) and 0.75 μ -mole of phospholipid substrate, dried and emulsified in 0.5 ml. of 0.05M-barbital buffer (sodium salt), pH 8.0, in a total volume of 1.0 ml. The incubation period was 60 min. at 37° , unless otherwise stated.

Assay of enzyme activity. Enzyme activity was assayed according to the method of Dawson (1956) by measurement of the amount of glycerophosphorylcholine liberated from the substrate lysolecithin. The glycerophosphorylcholine was estimated by determining the choline content after acid hydrolysis. The reaction was stopped by the addition of

trichloroacetic acid solution (final concn. 5%, w/v). Then bovine serum albumin was added to co-precipitate the unchanged lipid. This precipitate was spun down and the supernatant was extracted once with 3 vol. of a chloroform-isobutanol (2:1, v/v) mixture. The aqueous layer was separated by centrifuging and samples were hydrolysed with 0.25 vol. of 5N-hydrochloric acid at 100° for 20 min. The precipitate formed during the hydrolysis was spun down and discarded, and the choline content of the supernatant determined.

Estimations. Choline was estimated according to the method of Appleton, La Du, Levy, Steele & Brodie (1953). Ester groups in the reaction mixture were estimated, after chloroform extraction, by the method of Snyder & Stephens (1959). Total phosphorus was determined by the method of Lepage (1957). Estimation of protein was done according to the biuret method (Gornall, Bardawill & David, 1949) or with the Lowry reagent (Lowry, Rosebrough, Farr & Randall, 1951).

Chromatographic identification of the hydrolysis products of lysolecithin. The hydrolysis products were identified by paper chromatography according to Dawson & Rowlands (1959). The chromatogram was developed with the Hanes & Isherwood (1949) reagent as described by Bandurski & Axelrod (1951) and irradiated with u.v. light for 15 min.

RESULTS

Characterization of the enzyme. The enzyme, isolated by ammonium sulphate fractionation, slowly attacks lecithin preparations obtained according to the method of Pangborn (1950, 1951), but it is completely inactive towards lecithin purified further by silicic acid column chromatography (Lea *et al.* 1955). However, it splits lysolecithin quite vigorously with the liberation of an acid-soluble phosphorus and choline compound without liberation of free choline (Table 1). There is also a simultaneous decrease in the fatty acyl

Table 1. Activity of *Vibrio* El Tor phospholipase towards various lecithins

The contents of the incubation mixture and the method of estimation were as described in the text. Concn. of each substrate was 0.75 μ mole/ml.

Substrate	Release of acid-labile choline compounds (μ mole/hr.)
Lysolecithin	0.23
Lecithin (Pangborn, 1951)	0.07
Chromatographically purified lecithin	0.03

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ester bond after enzyme action shown by estimating the ester groups in the reaction mixture after extraction with chloroform. The liberated choline compound is acid-labile and on paper chromatographic analysis this has been identified as glycerophosphorylcholine. The enzyme is therefore a phospholipase B.

Enzyme secretion during the growth of *Vibrio El Tor*. Enzyme secretion increases simultaneously with the growth of the organism (Fig. 1), attaining its maximum between the late log phase and the beginning of the stationary phase of growth. The amount of secreted enzyme thereafter remains constant for up to 72 hr. of incubation.

Properties of the enzyme. The maximal liberation of glycerophosphorylcholine from lysolecithin occurs at pH 8.0 (Fig. 2). Citrate-phosphate, acetate,

barbital and tris buffers of various pH at 0.05M-concentration were used. With phosphate, tris or barbital buffers of identical pH, the enzyme activity is identical. The composition of the buffer thus appears to have no effect on the enzyme activity.

The enzyme is fairly stable towards heat denaturation and, although activity is lost on heating at 100° for 15 min. at pH 7.4, heating for 5 min. at this temperature brings about a loss of only 50 % of activity.

The percentage of lysolecithin split first increases with increasing substrate concentration and then declines (Fig. 3). The net amount of conversion of lysolecithin (0.75 μ mole) into glycerophosphorylcholine during 60 min. of incubation increases with increasing amounts of enzyme (Fig. 4), and with

Fig. 1. Secretion of phospholipase B during the growth of *Vibrio El Tor*: ○, growth; ●, enzyme content. The enzyme was assayed in 0.2 ml. of culture filtrate with 0.75 μ mole of lysolecithin as substrate (total volume 1 ml.); incubation was for 1 hr. at 37°. Klett reading 190 was equivalent to 0.55 mg. of dry cells/ml. of suspension.

Fig. 2. Optimum pH of *Vibrio El Tor* phospholipase B. Enzyme (0.15 mg. of protein) was incubated with 0.75 μ mole of lysolecithin in barbital buffer (0.05M) or, for pH 6.6, citrate-phosphate buffer (0.05M). Incubation was for 1 hr. at 37°.

Fig. 3. Effect of substrate concentration on the activity of *Vibrio El Tor* phospholipase B. The enzyme sample used contained 0.15 mg. of protein.

Fig. 4. Effect of enzyme concentration on the hydrolysis of lysolecithin by *Vibrio El Tor* phospholipase B.

Table 2. *Effect of inhibitors and certain sulphhydryl-reacting compounds on the activity of Vibrio El Tor phospholipase B*

The reaction mixture consisted of enzyme (0.15 mg. of protein) and 0.75 μ mole of lysolecithin emulsified in 0.5 ml. of 0.05M-barbital buffer (pH 8.0) in a total volume of 1.0 ml. The enzyme was preincubated for 15 min. with the various compounds; after addition of substrate incubation was continued for a further 60 min.

Additions	Concn. (mm)	Activity (%)
None	—	100
KCN	1	100
Dinitrophenol	1	80-85
NaN ₃	1	80-85
NaF	10	50-60
<i>p</i> -Chloromercuribenzoate	1	50-55
EDTA	1	60-65
<i>p</i> -Chloromercuribenzoate + glutathione	10	95-105
EDTA + calcium chloride	10	95-100
Cysteine	10	105-110
Glutathione	10	115-120

approximately 0.4 mg. (protein equivalent) of enzyme this conversion reaches about 60%. On further increasing the enzyme no increase in the conversion above this value has been noted.

Copper, manganese, nickel, cobalt, zinc, calcium and magnesium salts were used at a final concentration of 1 mM to study their effects on the enzyme reaction at pH 8.0. The enzyme was preincubated with the respective metal ions for 15 min. before incubation with the substrate for another 60 min. The enzyme is fairly insensitive to the action of metal ions. Only Zn²⁺ ions inhibit the enzyme to the extent of 40%, whereas Ni²⁺ and Ca²⁺ ions cause approximately 10% inhibition.

Dinitrophenol, sodium fluoride and *p*-chloromercuribenzoate cause about 45% inhibition and EDTA about 40%, whereas potassium cyanide has no effect (Table 2). The inhibition by *p*-chloromercuribenzoate and by EDTA can be fully reversed by the addition of glutathione and Ca²⁺ ions respectively. In all these experiments the enzyme was preincubated with the inhibitors for 15 min. before addition of the substrate. Glutathione and cysteine alone stimulate the enzyme activity by 20 and 10% respectively.

DISCUSSION

The cholera-producing organism *Vibrio cholera* and also *Vibrio El Tor* were known to secrete a lecithin-splitting enzyme, and the report by Felsenfeld (1944) gave an indication of the complex nature of the enzyme. He concluded that a mixture of enzymes such as 'lecithinase B', 'glycerophosphatase' and 'choline phosphatase'

are secreted by the organism and are responsible for the degradation of the phospholipid molecule. The substrate used, however, was crude and did not allow adequate characterization of the enzyme. The present work was undertaken with purified substrate and partially purified enzyme, and shows that it is a phospholipase B (lysolecithinase). The enzyme after isolation by ammonium sulphate fractionation appears to be quite stable, unlike the similar enzymes obtained from pancreas or *Penicillium notatum* (Dawson, 1959a).

Unlike any other lysolecithinase from moulds (Dawson, 1959a; Francioli, 1935; Contardi & Ercoli, 1933; Fairbairn, 1948), bacteria (Hayaishi & Kornberg, 1954), or animal tissues (Shapiro, 1953; Dawson, 1956), this lysophospholipase has a pH optimum in the alkaline range, at 8.0. This optimum seems to be similar only to that of the lysolecithinase of rumen micro-organisms of the sheep (Dawson, 1959b). The enzyme is fairly stable towards heat inactivation, unlike the similar enzymes from moulds or from animal tissues which are fairly labile and are destroyed completely on heating at 60° for 15 min. (Shapiro, 1953; Fairbairn, 1948). It should be noted that Hayaishi & Kornberg (1954) found that bacterial lysolecithinase was stable to heat. The inhibition of lysolecithinase by high substrate concentrations previously noted for the liver (Dawson, 1956) and pancreas enzymes (Shapiro, 1953) has also been found with the *Vibrio El Tor* enzyme.

The observation by Fairbairn (1948) that *P. notatum* phospholipase B is almost insensitive to the action of metal ions has been confirmed with the *Vibrio El Tor* enzyme and only Zn²⁺ ions bring about substantial inhibition (40% at 1 mM). The breakdown of lysolecithin by phospholipase B of *Vibrio El Tor*, *P. notatum* (Dawson, 1959a) or pancreas (Shapiro, 1953) is not influenced by Ca²⁺ ions, though *Aspergillus oryzae* phospholipase B (Contardi & Ercoli, 1933) is much stimulated. Potassium cyanide has no effect on the *Vibrio El Tor* phospholipase B, as noted with the *P. notatum* enzyme (Dawson, 1959a). An esterase inhibitor, sodium fluoride, which is known to inhibit the phospholipase B of pancreas (Dawson, 1959a) and of *P. notatum* (Dawson, 1958), inhibits this enzyme to the extent of about 45% at 1 mM. Unlike liver phospholipase B (Dawson, 1956), the *Vibrio El Tor* enzyme is inhibited by EDTA and the inhibition can be reversed by the addition of Ca²⁺ ions. The inhibition by *p*-chloromercuribenzoate, which is quite appreciable, can be completely reversed by glutathione, indicating the importance of sulphhydryl groups for the enzyme activity. The activation of the enzyme by glutathione and also by cysteine to a lesser extent also suggests that sulphhydryl groups are necessary for enzyme activity.

SUMMARY

1. A phospholipid-splitting enzyme of *Vibrio* El Tor has been characterized as a phospholipase B (lysolecithinase).

2. The enzyme is secreted during the late log phase of growth and no further increase in the excretion has been observed during the stationary phase.

3. The enzyme has been isolated from the culture filtrates by ammonium sulphate fractionation between 50 and 75 % saturation and some of the properties of the enzyme preparation have been studied.

4. This *Vibrio* El Tor lysolecithinase has a pH optimum at 8.0, and is fairly stable towards inactivation by heat. The enzyme is inhibited by high concentrations of substrate. It is fairly insensitive to the action of metal ions but is inhibited by Zn^{2+} ions (mM) to the extent of about 40 %.

The authors are indebted to Professor B. C. Guha for his kind interest, to Dr R. M. C. Dawson, Cambridge, for his gift of a sample of glycerophosphorylcholine, and to Dr K. L. Roy, Department of Applied Chemistry, Calcutta University, for his help in the preparation of ferric perchlorate.

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Biochem. J. (1962) **83**, 387

Regulation of Glucose Uptake by Muscle

6. FRUCTOSE 1,6-DIPHOSPHATASE ACTIVITY OF RAT HEART AND RAT DIAPHRAGM*

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(Received 7 November 1961)

Newsholme & Randle (1961) presented evidence that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate in perfused rat heart is increased by anoxia and decreased by starvation in a specific way. Essentially similar findings with anoxia have been reported by Regen, Davis & Morgan (1961) who also observed that the overall rate of phosphorylation of fructose 6-phosphate is decreased in alloxan diabetes. The

conclusion was drawn that these agents must affect either the rate of conversion of fructose 6-phosphate into the diphosphate (catalysed by phosphofructokinase) or that of the reverse reaction [catalysed by fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11)]. The present paper is concerned with the fructose 1,6-diphosphatase activity of extracts of hearts from normal and alloxan-diabetic rats and from normal rats subjected to prolonged starvation.

* Part 5: Newsholme & Randle (1961).