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SULPHOGALACTOLIPID SULPHOHYDROLASE ACTIVITY OF ARYLSULPHATASE PURIFIED FROM A MARINE GASTROPOD *CHARONIA LAMPAS*

HIROSHI HATANAKA [a], FUJIO EGAMI [a], INEO ISHIZUKA [b] and YOSHITAKA NAGAI [b]

[a] *Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya Machida-shi, Tokyo 194 and* [b] *Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173 (Japan)*

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Summary

Sulphatide, cerebroside 3-sulphate, was hydrolyzed at a considerable rate by arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) purified from a marine gastropod, *Charonia lampas*. However, it was scarcely hydrolyzed by glycosulphatase (sugar-sulphate sulphohydrolase, EC 3.1.6.3) from the same origin. The same was observed with seminolipid, a sulphoglycerogalactolipid.

The enzymatic characteristics of both sulphogalactolipid and sulphohydrolase activities of the arylsulphatase were determined as follows. The enzyme activities are stimulated by the addition of sodium taurodeoxycholate and $MnCl_2$. The pH optimum of sulphatide sulphohydrolase activity was pH 5.0, while seminolipid sulphohydrolase activity had maximum activity at pH 5.5. Both of these pH versus activity curves were broad. The K_m value was $6.22 \cdot 10^{-5}$ M for both substrates. However, the V values with sulphatide were lower by a factor of one-third than those with seminolipid. These enzyme activities were inhibited by substrates of the arylsulphatase, i.e., *p*-nitrophenyl sulphate, *p*-nitrocatechol sulphate, ascorbate 2-sulphate and each other sulphogalactolipid, but not by glucose 6-sulphate. Sulphate and phosphate anions inhibited both of the enzyme activities.

Introduction

It has been established that the brain sulphatide (cerebroside 3-sulphate) was one of the physiological substrates of mammalian arylsulphatase A (arylsulphate sulphohydrolase, EC 3.1.6.1) [1,2]. Rcemtly, the testicular seminolipid (sulphoglycerogalactolipid) was reported to be hydrolyzed by the

same enzyme [3,4]. These two sulphogalactolipids have very close similarity in structure, but have a slight difference in lipid moieties. The sulphate group of these sulphogalactolipids is attached at position 3 of the galactose moiety [5,6]. On the other hand, glycosulphatase (sugar-sulphate sulphohydrolase, EC 3.1.6.3], which is known to hydrolyze the sulphate ester linkage at position 6 of glucose, has been found in microorganisms and in molluscs [7]. So far it has not been investigated whether these sulphogalactolipids can be hydrolyzed by glycosulphatase. This is probably because the occurrence of glycosulphatase in higher animals seems to be doubtful [7]. The presence investigation was undertaken with enzyme preparations from a marine gastropod, *Charonia lampas*, the liver of which is well-known to have higher activities of both glycosulphatase and arylsulphatase [8,9].

In the present paper, we report that sulphogalactolipids, sulphatide and seminolipid, were easily hydrolyzed by arylsulphatase but scarcely by glycosulphatase purified from the liver of *C. lampas*. A part of the present study has been published as a preliminary note [10].

Materials and Methods

Materials

$H_2{}^{35}SO_4$ (carrier-free) was purchased from Daiichi Pure Chemicals. Sodium taurodeoxycholate, sodium cholate and potassium *p*-nitrocatechol sulphate were obtained from Sigma Chemicals, sodium deoxycholate from Merck and potassium *p*-nitrophenyl sulphate from Boehringer Corporation. Synthetic lecithin, DL-α-dipalmitoylphosphatidylcholine, and egg yolk lysolecithin were purchased from Sigma Chemicals, and crude soy bean lecithin from Iwai Chemicals. Potassium L-ascorbate 2-sulphate was synthesized by the method of Hatanaka et al. [11]. Potassium D-glucose 6-sulphate was purchased from Seikagaku Kogyo. Other chemicals were of reagent grade.

Preparation of [^{35}S] sulphatide and [^{35}S] seminolipid from rat tissues

Five male Wistar rats, 3-week-old, were each injected intraperitoneally with 100 μCi of carrier-free $H_2{}^{35}SO_4$ dissolved in 0.1 M sodium phosphate buffer, pH 6.8, to give a concentration of 1 mCi/ml. After 24 h the animals were decapitated and the organs removed. The brains (7.0 g) and testes (2.0 g), which were carefully freed from spinal cords or tunicae, were homogenized in a Waring blender with 19 vol. of a 2 : 1 (by vol.) mixture of chloroform and methanol for a few minutes and filtered through filter paper. The lipid extracts were evaporated to dryness, redissolved in 50 ml of chloroform/methanol (2 : 1) and submitted to Folch's partition procedure [12] to remove nonlipid contaminants. The partitioning was carried out once against 0.88% KCl solution and twice against Folch's theoretical upper phase [12]. The lower chloroform phase was evaporated to dryness and the residual lipids taken up in a small amount of chloroform were applied to a small column of Florisol (Floridin) containing 7% water. The column was eluted successively with 2 vol. each of chloroform, 5%, 30%, 50% and 60% methanol in chloroform. All radioactivities were recovered in 30% methanol fraction in the case of testes, but in the case of brain were eluted in both 30% and 50% methanol fractions.

The radioactive lipid fractions from testes and brain were evaporated to dryness, redissolved in 5 ml (testes) and 50 ml (brain) of chloroform/methanol (1 : 1), and applied to 1-ml and 5-ml columns of DEAE-Sephadex A-25 (acetate form, Pharmacia), respectively. After removal of neutral lipids with 10 column vol. of the same solvent, acidic lipids were eluted with 15 column vol. of 0.8 M ammonium acetate in chloroform/methanol (1 : 1). The radioactive eluates were dialyzed against water and concentrated.

The incorporation of the radioactive sulphate into the testes lipid fraction was similar to that reported previously in the case of mice [13]. The uptake of sulphate into brain lipids of rats was comparable to that reported by Green [14].

To examine the purity of labeled compounds, approx. 10 000 dpm of each sulpholipid were located on a 0.25 mm Silicagel thin-layer plate (Merck) using the solvent system of chloroform/methanol/acetone/acetic acid/water, 10 : 2 : 4 : 2 : 1 (by vol.) [4]. Radioscanning of the chromatogram was performed using a Nuclear Chicago, Actigraph III apparatus. Radioautography was performed by exposing the plate against Kodak RP Royal X-Omat medical X-ray film for 4 days. The glycolipid spots were also visualized by spraying with anthrone/H_2SO_4 and heating at 120°C [5]. By all the above criteria radioactive seminolipid isolated from testes was found to be homogeneous, while the brain sulphatide preparation contained a very small amount of seminolipid (less than one tenth of sulphatide, judged from the radioactivity). The galactose contents were measured by the anthrone/H_2SO_4 method [15]. Non-labeled seminolipid and sulphatides were prepared from boar tested [6] and bovine spinal cord, respectively, and used for diluting radioactive substrates to the suitable specific activities.

Preparation of glycosulphatase and arylsulphatase from the liver of C. lampas

The purified enzyme preparations of three species of sulphatase, namely glycosulphatases I and II, and arylsulphatase were the same as described in the previous paper [16].

Arylsulphatase was purified essentially as described in a previous paper [11], and was further separated from glycosulphatases by electrofocussing. Its specific activity was 7.38 μmol of *p*-nitrophenyl sulphate hydrolyzed/mg protein (123 nkat/mg). The extent of purification was 77.7-fold. Glycosulphatase I and II were separated from each other using a column of Concanavalin A-Sepharose gel, on which only the latter enzyme was retained. These enzymes were purified 31.3 and 33.9 fold, respectively, from a crude extract of *C. lampus* liver These specific activities were 2.22 and 2.41 μmol of glucose 6-sulphate hydrolyzed/mg protein (37.0 and 40.2 nkat/mg, respectively). By means of the above purification procedures, these three enzyme activities were almost completely separated from each other.

These activities were measured according to the method as previously described [11], and then the determination of protein was done by the method of Lowry et al. [17] with bovine serum albumin as a standard.

Determination of sulphatide and seminolipid sulphohydrolase activities

These enzyme activities were determined essentially according to the methods of Porter et al. [18] and Yamato et al. [3]. The reaction mixture, in a final

volume of 200 μl, contained 18 nmol of rat brain [^{35}S]sulphatide (379 dpm/nmol) or 19 nmol of rat testicular [^{35}S]seminolipid (341 dpm/nmol), 4 μmol of $MnCl_2$ and 250 μg of sodium taurodeoxycholate. 24 μmol of sodium acetate/acetic acid buffer, pH 5.0 (with sulphatide) or pH 5.5 (with seminolipid) and 3 μg protein of arylsulphatase (with sulphatide) or 1.8 μg protein of arylsulphatase (with seminolipid). At first, the required amounts of substrate and sodium taurodeoxycholate dissolved in chloroform/methanol solution were put in glass-stoppered test tubes (1.5 × 15 cm), and then evaporated to dryness at about 50° C with a stream of nitrogen gas. After adding the other constituents, except for the enzyme, the suspension was preincubated at 37° C for 10 min with constant "8-shaped" shaking (63 times/min), and then stirred vigorously with a Thermo Mixer. The enzyme was added and the complete reaction mixture was incubated for 60 min in the same conditions as for the preliminary incubation. The reaction was terminated by the addition of 2.4 ml of chloroform/methanol (2 : 1). After partitioning with 0.4 ml of 0.12 M NaCl containing 0.4 mM Na_2SO_4, the aliquot (usually 1 ml) of the upper phase was washed with 1 ml of Folch's theoretical lower phase [12]. To complete the separation this suspension was centrifuged at 3000 rev./min for 10 min with a Kubota centrifuge apparatus, model KC-70. The aliquot (usually 0.5 ml) of the resulting clear upper phase was transfered to a Watmann GF/D glass-fiber paper disc, 24 mm diameter. After drying, the disc was counted in 5 ml of a toluene scintillator fluid containing 4 g/l of 2,5-diphenyl-oxazole and 0.1 g/l of 1,4-bis-2-(5-phenyloxazolyl)benzene by a Backman model LS-250 liquid scintillation spectrometer. Its counting efficiency was 95.0%.

Results

Enzymatic hydrolysis of sulphatide and seminolipid by three species of sulphatase purified from C. lampas liver

The hydrolytic activities of 3 species of sulphatases, i.e., glycosulphatase I and II and arylsulphatase, toward sulphatide and seminolipid were quantitatively measured and are shown by means of enzyme concentration versus sulphate liberation curves in Fig. 1, a, b and c. Based on the results of Fig. 1, the specific activities toward sulphatide on glycosulphatase I and II, and arylsulphatase were calculated as 1.92 pkat, 4.35 pkat and 2.27 nkat/mg protein, respectively, and then these toward seminolipid were similarly calculated as 2.68 pkat, 2.82 pkat and 4.97 nkat/mg protein, respectively. These results show that sulphogalactolipids of sulphatide and seminolipid were hydrolyzed by arylsulphatase purified from *C. lampas* liver which contained a large amount of glycosulphatase. We have found also that ascorbate 2-sulphate was hydrolyzed by *C. lampas* arylsulphatase [11]. In a separate experiment, it was also confirmed by using the present enzyme preparation. Its specific activity toward ascorbate 2-sulphate with arylsulphatase was observed to be 5.85 nkat/mg protein. The activities by glycosulphatase I and II were less than 8 pkat/mg protein.

Requirements for sulphatide and seminolipid sulphohydrolase activities

The extent of hydrolysis of two sulpholipids under the omission of one of the components in the complete assay mixture is shown in Table I. The small

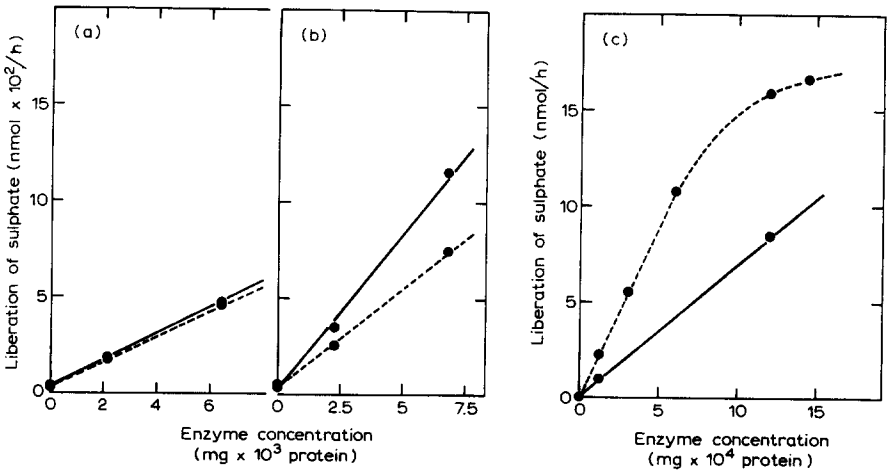

Fig. 1. Effects of protein concentration of *C. lampas* glycosulphatase I (a) and II (b), and arylsulphatase (c) on the hydrolytic activities with sulphatide (———) and with seminolipid (- - - - -). Enzyme assays were as described in the text. Glycosulphatase I and II was incubated overnight (16 h).

activity in each case was observed even in the absence of the detergent, sodium taurodeoxycholate. These enzyme activities in the absence of detergent were not enhanced by the preliminary treatment in a sonicated bath (Bransonic. 50 kHz) for 60 min, for the purpose of better substrate micelle formation. A number of detergents other than sodium taurodeoxycholate were examined for their effects on sulphatide sulphohydrolase activity as shown in Table II. Sodium teurodeoxycholate and sodium cholate were effective in this respect, whereas non-ionic detergents such as Triron X-100 and Tween 80 had little or no effect. The strong cationic and anionic detergents such as cetyltrimethylammonium bromide and sodium dodecyl sulphate, respectively, were inhibitory. As shown in Fig. 2, the optimal concentration of sodium taurodeoxycholate on the sulphatide sulphohydrolase activity was 1 mg/ml. Its value was lower than that of sodium cholate (shown in Table II).

TABLE I

REQUIREMENTS FOR THE SULPHATIDE AND SEMINOLIPID SULPHOHYDROLASE ACTIVITIES ON *C. LAMPAS* ARYLSULPHATASE

The complete reaction mixture contained 0.09 mM [^{35}S]sulphatide or 0.095 mM [^{35}S]seminolipid, 20 mM $MnCl_2$, 1.25 mg/ml sodium taurodeoxycholate, 120 mM sodium acetate/acetic acid buffer, pH 5.0 (with sulphatide) or pH 5.5 (with seminolipid), and 15 μg/ml (with sulphatide) or 9 μg/ml (with seminolipid) or arylsulphatase. Other assay conditions were the same as described in the text. Enzyme activities represent the amount of sulphate liberated from radioactive substrates per hour.

Reaction mixtures	Enzyme activities (nmol/h) with	
	sulphatide	seminolipid
Complete	2.58	5.75
minus sodium taurodeoxycholate	0.46	0.58
minus $MnCl_2$	1.07	3.69
minus $MnCl_2$ plus 1 mM EDTA	0.94	3.73
minus arylsulphatase	0.00	0.00

TABLE II

EFFECTS OF VARIOUS DETERGENTS ON THE SULPHATIDE SULPHOHYDROLASE ACTIVITY

Enzymatic assays are the same as described in the text.

Deptergents	Concentration (mg/ml)	Enzyme activity (nmol/h)
None	0	0.56
Sodium taurodeoxycholate	1.25	2.07
Sodium deoxycholate	1.25	0.41
Sodium cholate	0.25	2.13
Sodium cholate	0.63	4.22
Sodium cholate	1.25	4.67
Sodium cholate	2.50	3.50
Sodium dodecyl sulphate	0.25	0.12
Cetyl trimethylammonium bromide	0.25	0.08
Tween 80	1.25	0.15
Triton X-100	1.25	0.92

The requirement for metal ions was also investigated. As can be seen in Table I, omission of $MnCl_2$ from the complete assay mixture caused a half reduction of both sulphatide and seminolipid sulphohydrolase activities. Addition of 1 mM EDTA caused no more reduction of either activity. A number of metal ions were tested for their effects on sulphatide sulphohydrolase activity in a separate experiment. The activity was increased two fold by the addition of 20 mM $MgCl_2$, $CaCl_2$ and $CoCl_2$, each in a similar manner as $MnCl_2$, but to a lesser extent by the addition of 20 mM $ZnCl_2$ and $CuCl_2$. $HgCl_2$ was inhibitory. The extent of stimulation of sulphatide sulphohydrolase activity by $MnCl_2$ was the same over a concentration range of 10—50 mM.

The pH optima at pH 5.0 and 5.5 of sulphatide and seminolipid sulphohydrolase activities, respectively, were observed as shown in Fig. 3. These pH versus activity curves were broad.

Both the sulphatide and seminolipid sulphohydrolase reactions seemed to follow Michaelis-Menten kinetics (shown in Fig. 4). The apparent K_m value for both sulphogalactolipids was calculated as $6.22 \cdot 10^{-5}$ M. As shown in Fig. 4, however, the maximal velocities were different, i.e., 5.12 nkat/mg protein for sulphatide and 14.4 nkat/mg protein for seminolipid sulphohydrolase activities.

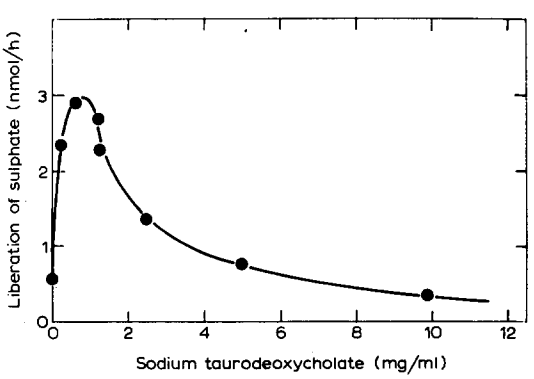

Fig. 2. Effect of sodium taurodeoxycholate on the sulphatide sylphohydrolase activity. Enzyme assay was as described in the text.

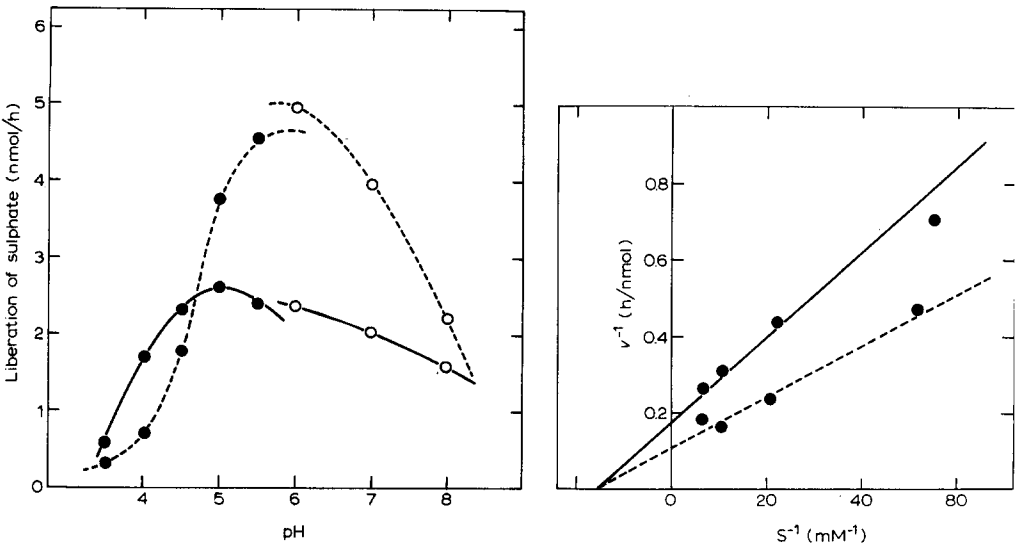

Fig. 3. Effect of the pH on the sulphatide (———) and seminolipid (- - - - - -) sulphohydrolase activities. Enzyme assay was as described in the text. These activities were measured in sodium acetate/acetic acid buffer (●, pH 3.5—5.5) and Tris/acetic acid buffer (○, pH 6.0—8.0).

Fig. 4. Effect of substrate concentration on the sulphatide (———) and seminolipid (- - - - - -) sulphohydrolase activities. Enzyme assay was as described in the text.

Effects of the various additions on sulpholipid sulphohydrolase activities

The sulphatide sulphohydrolase activity was considerably inhibited by a number of phospholipids as shown in Table III. The addition of these phospholipids in the absence and presence of sodium taurodeoxycholate was inhibitory.

Table IV shows the effects of several sulphate esters and the other ions on sulphatide and seminolipid sulphohydrolase activities. The sulphate and phosphate anions considerably inhibited both sulpholipid sulphohydrolase activities. The phosphate inhibition was stronger. The substrates of arylsulphatase such as

TABLE III

EFFECTS OF PHOSPHOLIPIDS ON THE SULPHATIDE SULPHOHYDROLASE ACTIVITY

Reaction mixture was described in the legend of Table I. The preincubation before the addition of enzyme was carried out in a sonicated bath (Bransonic, 50 KHz) at 50°C for 60 min. Other assay conditions were the same as described in the text.

Phospholipids	Concentration (mg/ml)	Enzyme activities (nmol/h)	
		without sodium taurodeoxycholate	with sodium taurodeoxycholate
None		0.56	2.44
D,L-α-Dipalmitoyllecithin	0.25	0.09	1.75
D,L-α-Dipalmitoyllecithin	1.25	0.03	0.57
D,L-α-Dipalmitoyllecithin	5.00	0.03	0.08
Soy bean lecithin (crude)	1.25	0.02	0.20
Egg yoke lysolecithin	1.25	0.04	0.87

TABLE IV

EFFECTS OF VARIOUS ESTERS AND THE OTHER IONS ON THE SULPHATIDE AND SEMINOLIPID SULPHOHYDROLASE ACTIVITIES

Enzyme assay was the same as described in the text.

Additions	Concentration (mM)	Percent enzyme activity with	
		sulphatide	seminolipid
None	0	100	100
Sodium sulphate	1.0	91,5	— *
Sodium sulphate	5.0	66.2	75.8
Potassium phosphate	0.5	32.8	37.5
Potassium phosphate	5.0	5.0	—
Sodium chloride	500	74.3	—
Sodium chloride	2000	47.4	—
Ascorbate 2-sulphate	7.5	98.4	—
Ascorbate 2-sulphate	75	70.1	81.8
Glucose 6-sulphate	7.5	97.8	—
Glucose 6-sulphate	75	87.9	93.7
p-Nitrophenyl sulphate	0.75	39.5	—
p-Nitrophenyl sulphate	7.5	5.9	—
p-Nitrocatechol sulphate	7.5	0.9	—
Seminolipid	0.5	20.5	—
Sulphatide	0.5	—	55.2

* Not determined.

p-nitrophenyl sulphate and *p*-nitrocatechol sulphate were potent inhibitors. Ascorbate 2-sulphate also inhibited to a small extent, while glucose 6-sulphate scarcely inhibited both the sulpholipid sukphohydrolase activities. These two enzyme activities were strongly inhibited by each other substrate.

Discussion

We found in the present investigation that all of the natural sugar sulphates, i.e., sulphatide, seminolipid and ascorbate 2-sulphate, were easily hydrolyzed by the arylsulphatase purified from the liver of the marine gastropod, *C. lampas*. The fact that these sugar sulphates were scarcely hydrolyzed by glycosulphatase from the same organism seems to be remarkable. Glycosulphatase activity is usually measured using glucose 6-sulphate as the substrate [7]. However, the substrates most frequently used for arylsulphatase are artificial ones such as *p*-nitrophenyl sulphate and *p*-nitrocatechol sulphate [19]. Now the nomenclature of sulphatases must be revised, because in spite of their names, glycosulphatase is inactive on such natural sugar sulphates and certain arylsulphatases are active. Further studies on the substrate specificity using various sugar sulphates of both arylsulphatase and glycosulphatase will be required.

The sulphatide sulphohydrolase activity in invertebrates, including molluscs but not including *C. lampas*, was first investigated by Mraz and Jatzkewitz [20]. They have shown that the acidic forms of the arylsulphatase, separated by electrofocussing, possess sulphatide sulphohydrolase activities. In the present study, we examined further the enzymatic characteristics using the partially purified arylsulphatase preparation from *C. lampas* liver, and found the same

hydrolyzing activity for seminolipid, a sulphoglycerogalactolipid.

The enzymatic properties of *C. lampas* sulphogalactolipid sulphohydrolase activities were somewhat different from those of the mammalian enzyme: (i) The activity ratio with sulpholipids and artificial arylsulphate as the substrate was almost the same [1—4,21]. The activity with seminolipid was higher than that with sulphatide in the case of *C. lampas* arylsulphatase, but the reverse has been observed in the mammalian arylsulphatase A [3,4]. (ii) The K_m value, $6.22 \cdot 10^{-5}$ M, of *C. lampas* arylsulphatase for sulphatide and seminolipid as the substrates was lower by a factor of one order of magnitude compared to the value of mammalian enzymes [1,2,4,17,21]. (iii) The broad pH optima, pH 5.0 with sulphatide and pH 5.5 with seminolipid, were rather different from the narrow pH optimum of pH 4.5 in mammalian case [1—4,18,21]. (iv) The requirement of detergent, sodium taurodeoxycholate or sodium cholate, was not obligatory. On the other hand, an absolute requirement for the detergent has been observed for mammalian sulphatide and seminolipid sulphohydrolase activities. The detergent is required for the micelle formation of substrates in which the enzyme is active [1—4,18,21]. The substrate micelle formed even in the absence of a detergent might be able to be recognized by *C. lampas* arylsulphatase, which had a higher affinity (low K_m value) for sulphogalactolipid.

So far, sulphogalactolipids have not been searched for in the marine molluscs such as *C. lampas*. However, these substrances or similar compounds might be the physiological substrate of the *C. lampas* arylsulphatase. Further studies on other organisms are required.

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References

1 Mehl, E. and Jatzkewitz, H. (1968) Biochim. Biophys. Acta 151, 619—627
2 Jerfy, A. and Roy, A.B. (1973) Biochim. Biophys. Acta 293, 178—190
3 Yamato, K., Handa, S. and Yamakawa, T. (1974) J. Biochem. Tokyo 75, 1241—1247
4 Fluharty, A.L., Stevens, R.L., Miller, R.T. and Kihara, H. (1974) Biochem. Biophys. Res. Commun. 61, 348—354
5 Yamakawa, T., Kiso, N., Handa, S., Makita, A. and Yokoyama, S. (1962) J. Biochem. Tokyo 52, 226—228
6 Ishizuka, I., Suzuki, M. and Yamakawa, T. (1973) J. Biochem. Tokyo 73, 77—87
7 Roy, A.B. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 5, 3rd edn., pp. 1—19, Academic Press, New York
8 Takahashi, N. (1960) J. Biochem. Tokyo 47, 230—237
9 Takahashi, N. and Egami, F. (1961) Biochem. J. 80, 384—386
10 Hatanaka, H., Ogawa, Y., Egami, F., Ishizuka, I. and Nagai, Y. (1975) J. Biochem. Tokyo 78, 427—429
11 Hatanaka, H., Ogawa, Y. and Egami, F. (1975) J. Biochem. Tokyo 77, 353—359
12 Folch, J., Lees, M.J. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
13 Handa, S., Yamato, K., Ishizuka, I., Suzuki, A. and Yamakawa, T. (1974) J. Biochem. Tokyo 75, 77—83

14 Green, J.P. and Robinson, Jr., J.D. (1960) J. Biol. Chem. 235, 1621—1625
15 Yamakawa, T., Irie, R. and Iwanaga, M. (1960) J. Biochem. Tokyo 48, 490—507
16 Hatanaka, H., Ogawa, Y. and Egami, F. (1976) J. Biochem. Tokyo 79, 27—34
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) J. Biol. Chem. 193, 265—275
18 Porter, M.T., Fluharty, A.L., De La Flor, S.D. and Kihara, H. (1972) Biochim. Biophys. Acta 258, 769—778
19 Nicholls, R.G. and Roy, A.B. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 5, 3rd edn., pp. 21—41, Academic Press, New York
20 Mraz, W. and Jatzkewitz, H. (1974) Hoppe-Seyler's Zo. Physiol. Chem. 355, 33—44
21 Stevens, R.L., Fluharty, A.L., Skokut, M.H. and Kihara, H. (1975) J. Biol. Chem. 250, 2495—2501