Biochimica et Biophysica Acta, 525 (1978) 87—92

BBA 68470

CHARACTERIZATION OF STEROL-ESTER HYDROLASE IN *SACCHAROMYCES CEREVISIAE*

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(Received December 16th, 1977)

Summary

A homgenate of *Saccharomyces cerevisiae* grown under semi-anaerobic as well as aerobic conditions was found to catalyze the hydrolysis of fatty acid esters of sterols in the presence of Triton X-100. The enzyme levels in cells grown under various conditions were similar and the enzyme had a broad substrate specificity for sterol esters. The enzyme was localized in the mitochondrial fraction for the aerobically grown cells and in the mitochondrial and cytosolic fractions for the semi-anaerobically grown cells.

Introduction

Sterol-ester hydrolases (EC 3.1.1.13), which catalyze the hydrolysis of fatty acid esters of sterol, have been found in a variety of mammalian tissues [1—5]. Studies on kinetic and physicochemical properties and the physiological role of the mammalian cholesterol esterase were recently reviewed [6].

Only few studies have been made on the enzymes of micro-organisms. In 1974, Havie [7] reported that the α-toxin produced by *Staphylococcus aureus* showed a sterol-ester hydrolase activity. Uwajima and Terada [8] reported that *Pseudomonas fluorescens* grown on a medium containing sterol ester as a sole carbon source produced an extracellular sterol-ester hydrolase. However, physiological roles of the above enzymes are unclear, since these bacteria cannot synthesize sterols and its ester. With the fungal species *Fusarium*, Okawa and Yamaguchi [9] reported that sterol-ester hydrolase was produced extracellularly in large amounts.

Although yeasts form a large amount of sterol esters, the enzymes involved in the formation, hydrolysis and physiological role of the ester seem not to

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have been reported. Some recent papers dealing with sterol esters of yeast have now appeared [10—12], and we have reported that a large amount of sterol ester, synthesized by aerobically grown cells, were rapidly hydrolyzed when the cells were then grown under anaerobic conditions [13]. This observation led us to investigate the sterol-ester hydrolase of yeast.

Using Triton X-100, we could detect a sterol-ester hydrolase from yeast cells grown under anaerobic, as well as aerobic, conditions. Some properties and subcellular distribution of the enzyme were clarified.

Materials and Methods

Chemicals. L-[*Me*-^{14}C]Methionine (5.6 Ci/mol), DL-[2-^{14}C]mevalonolactone (5 Ci/mol) and [4-^{14}C]cholesterol (10 Ci/mol) were purchased from Daiichi Pure Chemicals Co., Tokyo. Zymolyase 5000 (5 units/mg) was obtained from Kirin Brewery Co., Ltd., Tokyo. Dry yeasts were from Oriental Yeast Co., Ltd., Tokyo. Wako Gel B-5 (silica gel G) was obtained from Wako Pure Chemicals Industries, Kyoto. Oleoyl chloride, palmitoyl chloride, and other organic reagents were from Nakarai Chemicals Co., Kyoto.

Substrate. [^{14}C]Cholesteryl oleate (0.17 Ci/mol) and [^{14}C]cholesteryl palmitate (0.17 Ci/mol) were prepared by reaction of [^{14}C]cholesterol with the corresponding fatty acyl chloride by the method of Deykin and Goodman [1]. [28-^{14}C]Ergosterol (0.48 Ci/mol) was prepared by the method of Fryberg et al. [14]. [^{14}C]Lanosterol was prepared as follows: Dry yeast (1 g dry weight) and 5 μCi DL-[2-^{14}C]mevalonate were incubated for 4 h at 30°C with shaking, as described previously [15]. The mixture was saponified and non-saponifiable lipids were extracted with light petroleum. The extract was concentrated and applied to a thin-layer chromatographic plate (Wako Gel B-5). After developing the plate with methanol/benzene (2 : 98, v/v), the fraction of lanosterol (R_F 0.35—0.40) was scraped from the plate and eluted with 5 ml methanol. Specific radioactivity of [^{14}C]lanosterol obtained was 0.98 Ci per mol. Each of [^{14}C]-ergosterol and [^{14}C]lanosterol obtained both gave a single peak in gas-liquid chromatography. [^{14}C]Ergosteryl oleate (0.48 Ci/mol) and [^{14}C]lanosteryl oleate (0.98 Ci/mol) were prepared as described above. [^{14}C]Cholesteryl acetate (0.17 Ci/mol) was prepared using [^{14}C]cholesterol and acetic anhydride by the method of Fryberg et al. [14]. Determination of sterols was carried out by gas-liquid chromatography, as described previously [16].

Growth of yeast. For aerobic cultivation, *Saccharomyces cerevisiae* (ATCC 12341) was grown in a cultivation medium at 30°C with continuous shaking [17].

For semi-anaerobic cultivation, the cells were grown in the same cultivation medium at 30°C [15]. The cells grown under both of these conditions were harvested at the logarithmic growth phase and washed twice with cold water.

For aerobic adaptation, the cells semi-anaerobically grown for 48 h were shaken aerobically in 0.1 M potassium phosphate buffer (pH 6.2)/2% glucose (10 mg wet cells/ml) for 2 h [12]. Labeling of C-28 sterols and their esters with L-[*Me*-^{14}C]methionine during aerobic adaptation was carried out as described previously [12].

Preparation of sterol-ester hydrolase. The cells were suspended in 0.1 M

Tris · HCl buffer (pH 7.4)/5 mM 2-mercaptoethanol (500 mg/ml cells, wet weight) and disrupted twice in an Ohtake French pressure cell at 600 kg/cm^2 pressure at 4°C. Unbroken cells and cell debris were removed by centrifugation at 3000 × *g* for 20 min. The resulting supernatant was centrifuged at 100 000 × *g* for 60 min. After the floating lipid layer on the top of the supernatant was removed, the pellet was remixed with the supernatant with a Teflon homogenizer. Protein concentration was determined by the method of Lowry et al. [18].

Assay of sterol-ester hydrolase. To 0.5 ml [^{14}C]cholesteryl oleate (10 000 cpm, 26 nmol) in diethyl ether were added 30 μl 10% Triton X-100 (0.3% final concentration) and 0.7 ml 0.1 M Tris · HCl (pH 7.4). The mixture was mixed thoroughly and the ether was evaporated with a N_2 stream. The enzyme was added to it (total volume 1 ml) and incubation was proceeded for 1 h at 30°C, with shaking. The reaction was stopped by the addition of 3 ml chloroform/methanol (2 : 1, v/v) and 1 ml 1% NaCl. The extraction of lipids was carried out three times with chloroform/methanol. An aliquot of the extract was concentrated with a N_2 stream and applied to a thin-layer chromatographic plate (Wako Gel B-5). After developing with methanol/benzene (2 : 98, v/v), the fraction of free sterols (R_F 0.1—0.2) was scraped from the plate into counting vial. Radioactivity of ^{14}C-labeled free sterols was measured in a Beckman LS-230 liquid scintillation spectrometer using toluene-Triton X-100 scintillator [19].

Isolation of intracellular organelles. In order to obtain spheroplasts of the aerobically and semi-anaerobically grown cells, the cells (3.4 g wet weight) were suspended in 6.8 ml 50 mM Tris · HCl buffer (pH 7.4)/1 mM EDTA/0.6 M mannitol/120 mM 2-mercaptoethanol, and the cell suspension was incubated for 40 min at 30°C. After the cells were harvested, they were washed twice with the above buffer and digested with Zymolyase, according to Kitamura and Yamamoto [20]. Disruption of the spheroplasts was carried out with a Teflon homogenizer, as described by Shimizu et al. [17]. The treatments of the semi-anaerobically grown cells were carried out under nitrogen and those of the aerobically grown cells were done in air. Intracellular organelles were fractionated by centrifugation as described by Shimizu et al. [17].

Results

Detection and kinetics of sterol-ester hydrolase

The assay of sterol ester hydrolase was carried out in the presence of Triton X-100 throughout the present study. The presence of Triton X-100 was not only useful for dispersion of the substrate, but also indispensable for the appearance of the activity. The use of 0.3% Tween 80 or 0.4% acetone was without effect. The enzyme activity increased with increasing concentration of Triton X-100, showing an optimum at 0.3%. The activity was inhibited at the higher concentrations.

As previously reported [13], net hydrolysis of sterol ester did not occur in the aerobically grown cells, whereas a marked hydrolysis of the ester did occur in the anaerobically grown cells. From the consideration that the variation of sterol ester may be due to variation of the enzyme level accompanying the

change of growth condition, the enzyme levels in the semi-anaerobically grown, aerobically grown, and aerobically adapted cells were determined. However, no significant difference in the level was observed among the cells under the three conditions: the levels under the above three conditions were 0.13—0.23, 0.10—0.20 and 0.12—0.17 nmol of cholesteryl oleate hydrolyzed per mg protein per h, respectively.

Using the homogenate or the 100 000 $\times g$ pellet prepared from the semi-anaerobically grown cells, some properties of the enzyme were investigated. The hydrolytic reaction of cholesteryl oleate by the enzyme proceeded linearly with time up to 1 h, but no more hydrolysis occurred by further incubation. It is worth noting that the hydrolysis occurred with no more than 14% of the substrate added. In order to determine if the above phenomenon was due to the product inhibition or due to the inactivation of the enzyme during incubation, the following experiments were carried out: When the reaction was carried out in the presence of 30 nmol cholesterol and 30 nmol oleate, no inhibition was observed; and when the enzyme was preincubated for 1 h in the absence of the substrate, no effect was observed in the enzyme activity (unpublished data). No further attempt was made to elucidate the reason why the reaction did not proceed after 1 h.

The enzyme had a relatively high activity in a range from pH 6 to 8, showing an optimum at pH 7.4 (unpublished data).

The enzyme activity increased linearly with increasing concentration of enzyme up to 20 mg of protein. No more hydrolysis than 12% of the substrate added was observed by increasing the amount of the enzyme (unpublished data).

The enzyme activity increased linearly by increasing the concentration of substrate up to 26 μM (unpublished data). The apparent K_m value for cholesteryl oleate in the standard assay condition was estimated to be about 22 μM.

Substrate specificity of sterol-ester hydrolase

Substrate specificity of the enzyme was examined through measurement of the activities towards some sterol esters (22 μM) under the standard assay condition. The relative activities were as follows: cholesteryl oleate; 100; ergosteryl oleate, 91; cholesteryl palmitate, 77; lanosteryl oleate, 10; and cholesteryl acetate, 8. These results indicate a rather broad substrate specificity of the enzyme.

Subcellular distribution of sterol-ester hydrolase

Subcellular distributions of the enzyme in the cells grown under aerobic and semi-anaerobic conditions were examined. Cell walls of the cells grown under both conditions were digested enzymatically and the resulting spheroplasts were lyzed. The homogenates were fractionated by centrifugation [17]. Table I shows the distribution in the aerobically and semi-anaerobically grown cells. In the aerobically grown cells, the mitochondrial fraction contained 45% of the total recovered activity, and the nuclear fraction 40%. A small amount of activity was found in the cytosol. These results indicate that large parts of the enzyme are localized in the mitochondrial fraction in aerobically grown cells. In the semi-anaerobically grown cells, however, a considerable amount of the

TABLE I

SUBCELLULAR DISTRIBUTION OF STEROL-ESTER HYDROLASE IN THE AEROBICALLY AND SEMI-ANAEROBICALLY GROWN CELLS

The cells were treated with Zymolyase and the resulting spheroplasts were disrupted. The subcellular fractionations were carried out by the method of Shimizu et al. [17], except that the mitochondrial fraction was precipitated by centrifugation at 25 000 × *g* for 5 min in the case of the semi-anaerobically grown cells. The enzyme activity of each particulate fraction was assayed after sonicated for 1 min at 4°C. The reaction mixture and the assay were as described in Materials and Methods.

Fraction	Aerobically grown cells		Semi-anaerobically grown cells	
	Specific * activity	Percent of total activity	Specific * activity	Percent of total activity
Nuclei	0.21	40	0.07	20
Mitochondria	0.37	45	0.23	13
Mitochondrial supernatant	0.04	1	0.45	19
Microsomes	0.11	10	0.25	13
Cytosol	0.06	4	0.20	33

* nmol of cholesteryl oleate hydrolyzed per mg protein per h.

activity was found in the cytosolic fraction and mitochondrial supernatant, besides the mitochondrial fraction. These results indicate that the subcellular distribution of the enzyme in the semi-anaerobically grown cells is different from that in aerobically grown cells. As an index of the fractionation, the subcellular distributions of succinate dehydrogenase (EC 1.3.99.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were also examined. The recoveries of the activity of succinate dehydrogenase which is a marker enzyme of mitochondria were 40—50% in the mitochondrial fraction, 30—40% in the nuclear fraction and 0—3% in the microsomal fraction. The recoveries of the activity of glucose-6-phosphate dehydrogenase which is a marker enzyme of cytosol were 55—60% in the cytosolic fraction and 5—6% in the mitochondrial fraction (unpublished data).

Does the endogenous sterol ester undergo a hydrolysis in cell-free reaction system?

As described above, hydrolysis of the added substrate occurred with no more than 12—14% in the incubation with the homogenate of the semi-anaerobically grown cells. From consideration of the possibility that the amount of endogenous sterol esters may affect the extent of hydrolysis of ^{14}C-labeled substrate added, effect of concentration of the enzyme on the activity was examined with the homogenate of the aerobically grown cells which contain more than 10-fold as much the ester as that of the semi-anaerobically grown cells [13]. The activity increased linearly up to 12% hydrolysis of the substrate added. However, no more hydrolysis occurred by increasing the concentration of the enzyme similarly to the above-described experiments (unpublished data). For further examination of utilizability of the endogenous ester, the homogenate containing endogenous ^{14}C-labeled sterol esters which were prepared as described in Materials and Methods, was incubated without the addition of exogenous substrate. After free and esterified sterols were extracted and separated, radioactivities in both sterol fractions were measured. Virtually no endogenous

^{14}C-labeled ester was found to be hydrolyzed during the incubation (unpublished data).

Discussion

In the previous paper, we reported that hydrolytic reaction of fatty acid esters of sterols occurred in the anaerobically grown cells but not in the aerobically grown cells [13]. The possibility that this phenomenon is either due to induction of sterol ester hydrolase in the former cells or due to repression of the enzyme in the latter cells was excluded by the present investigation. The enzyme level was shown to be rather constant, not depending on the growth condition. The present investigation also shows that the enzyme is present in the mitochondrial fraction in the aerobically grown cells but in the mitochondrial and cytosolic fractions in the anaerobically grown cells. It is well known that the development of mitochondria in yeast is influenced by the growth condition and that some enzymes are differently distributed in the cells depending on the condition in association with the mitochondriogenesis [21]. The phenomenon that the hydrolysis of sterol ester occurs in the anaerobically grown cells but not in the aerobically grown cells might be associated with this problem.

It was shown in the present investigation that the exogenously added sterol esters undergo the enzyme reaction but not the endogenous sterol esters under the experimental conditions. In this connection, we reported that the ^{14}C-labeled sterol esters were more rapidly hydrolyzed than non-labeled sterol esters in yeast cells [13]. Although some explanations are possible for these phenomena, it can be said that the sterol esters are present in heterogeneous states, at least in states susceptible and non-susceptible to hydrolytic reaction. Studies on the interaction of sterols and their esters with the enzymes on the membranes of organelles are now in progress.

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