Note

Distribution in Organisms and Stereospecificity of β -Hydroxyisobutyrate Dehydrogenase

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β -Hydroxyisobutyrate dehydrogenase (β -HIBA dehydrogenase, EC. 1.1.1.31) which catalyzes the dehydrogenation of β -HIBA to methylmalonate semialdehyde with NAD^+ as a coenzyme has been found in higher animals^{1,2)} and some microorganisms.^{3,4)} This enzyme was purified about 200-fold from pig kidney by Robinson *et al.*¹⁾ and 85-fold from *Pseudomonas aeruginosa* by Banerjee *et al.*³⁾ However, the author could not find any information concerning the stereospecificity of β -HIBA dehydrogenase in their reports, because these studies were carried out using racemic β -HIBA.

In the course of studies on β -HIBA metabolism in microorganisms, the author observed that the optical rotations of the extracts with ethyl acetate from the reaction mixtures containing DL- β -HIBA and the resting cells of some microorganisms were levorotatory. These findings suggested the stereospecificity of β -HIBA dehydrogenase. In this paper, the differences of β -HIBA dehydrogenation activities for the stereoisomers of β -HIBA in microorganisms which produce L- or D- β -HIBA from isobutyric acid⁵⁾ and mammalian species, and the effects of some reagents on induction of L- and D- β -HIBA dehydrogenation activities in *Candida rugosa* IFO 0750 are described.

L- and D- β -HIBA were prepared by microbial methods using *C. rugosa* IFO 1542 and IFO 0750, respectively.⁵⁾ The specific optical rotations of L- and D- β -HIBA were $[\alpha]_D^{25} +17.8^\circ$ and -17.6° ($c=1$, MeOH), respectively. The compositions of the media used were as follows: Medium A (for yeasts and molds); glucose 40 g, $(\text{NH}_4)_2\text{SO}_4$ 13 g, K_2HPO_4 7 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.8 g, NaCl 0.1 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 90 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 60 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 10 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 5 mg, and yeast extract 3 g per liter of medium, pH 7.2. Medium B (for bacteria); glucose 20 g, yeast extract 5 g, meat extract 5 g, and peptone 10 g per liter of medium, pH 6.5.

a) Preparation of cell-free extract: The yeast or mold was grown in a 2 liter-flask containing 300 ml of Medium A containing 1% of isobutyric acid at 30°C with shaking until the growth reached maximum. The bacterium was

grown in a 2 liter-flask containing 300 ml of Medium B at 30°C with shaking for 20 hr. Then, 3 g of isobutyric acid was added to the broth and incubation was continued for 6 hr under the same conditions. Washed cells were suspended in 30 ml of 0.2 M phosphate buffer (pH 6.5), and disrupted with a Braun homogenizer or ultrasonic oscillator. The supernatant solution obtained by centrifugation (15,000 rpm, 30 min) was used as the cell-free extract. In the case of mammalian tissues, about 20 g of the tissue was sliced, suspended in 50 ml of the buffer, completely disrupted in a homogenizer and centrifuged for 30 min at 15,000 rpm. The supernatant solution was used for the enzyme assay.

b) Induction test: *C. rugosa* IFO 0750 was grown in a 30 liter-jar fermentor containing 18 liters of Medium A at 30°C with agitation, 600 rpm, and aeration, 1 vvm. After 14 hr cultivation, the washed cells, with 0.9% NaCl, were suspended in 0.2 M phosphate buffer (pH 7.0) containing 1% of glucose, 0.3% of yeast extract and 1% of the organic compound to be tested. Two hundred ml of the cell suspension (dry cell weight, 2.4%) in a 2 liter-flask was incubated for 10 hr at 30°C with shaking.

c) Enzyme assay: L- and D- β -HIBA dehydrogenase activities were assayed by measurement of NADH formed at 340 nm.¹⁾ The assay system in a final volume 3.0 ml contained 120 μmol of L- or D- β -HIBA, 1.59 μmol of NAD^+ , 400 μmol of $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$ buffer (pH 9.5), and a suitable amount of cell-free extract. Incubation was carried out at 30°C in a cuvette of 1 cm light path length. One unit of the enzyme catalyzed the formation of 1 μmol of NADH per min under the standard assay conditions described above. Protein was determined by the method of Lowry *et al.*⁶⁾ with bovine serum albumin as standard.

As shown in Table I, the stereospecificity of β -HIBA dehydrogenase for the stereoisomers of β -HIBA differed depending upon the species of organism. In general, the dehydrogenation activity for L- β -HIBA was higher than that for D- β -HIBA in all cell-free extracts tested. In some cell-free extracts, for example, of *Rhodotorula*, *Trichosporon*, *Corynebacterium*, *Micrococcus* and ox liver, dehydrogenation activity for L- β -HIBA only was observed. However, the organisms which have the dehydrogenation activities for D- β -HIBA only or higher than that for L- β -HIBA were not found. A relationship between the steric configurations of β -HIBA produced from isobutyric acid and the stereospecificities of β -HIBA dehydrogenation activities in microorganisms was not found.

Induction of L- and D- β -HIBA dehydrogenation activities in *C. rugosa* IFO 0750 was studied using reagents closely related to the β -HIBA metabolism. As shown in Table II, isobutyric acid, methacrylic acid and butyric acid were the most effective inducers for the enzyme formation, and the ratio of the activity for D- β -HIBA to that for L- β -HIBA (D/L) in *C. rugosa* IFO 0750 was not affected by any of the inducers tested.

In the above studies using the optically active β -HIBA, two types of cell-free extracts were found. One type had L-

TABLE I. β -HIBA DEHYDROGENATION ACTIVITIES FOR STEREOISOMERS OF β -HIBA IN CELL-FREE EXTRACTS OF ORGANISMS

Organism	Type of β -HIBA produced ^a	Dehydrogenation activity for		D/L ^b
		L- β -HIBA (L) (U/mg protein 10 ³)	D- β -HIBA (D) (U/mg protein 10 ³)	
<i>Candida rugosa</i> IFO 0750	(D)	62.5	23.1	0.35
<i>Candida rugosa</i> IFO 1542	(L)	296	5.37	0.02
<i>Candida parapsilosis</i> IFO 0708	(D)	6.87	2.75	0.40
<i>Endomyces reessii</i> CBS 179.60	(L)	67.2	4.80	0.07
<i>Rhodotorula rubra</i>	(L)	29.2	0.00	0.00
<i>Trichosporon aculeatum</i> ATCC 22310	(L)	62.5	0.00	0.00
<i>Corynebacterium hydrocarbons</i>	(L)	149	0.00	0.00
<i>Corynebacterium paurometabolum</i>	(L)	15.4	0.00	0.00
<i>Micrococcus flavus</i>	(L)	192	0.00	0.00
<i>Micrococcus luteus</i>	(L)	84.7	0.00	0.00
<i>Pseudomonas dacunhae</i>	(L)	70.4	29.9	0.40
<i>Pseudomonas aeruginosa</i>	(L)	19.2	1.00	0.05
<i>Nocardia lyena</i>	(L)	98.4	10.9	0.11
Pig kidney	—	5.51	0.58	0.11
Rabbit liver	—	1.07	0.27	0.26
Ox liver	—	0.70	0.00	0.00

^a Steric configuration of β -HIBA produced from isobutyric acid by the microorganism.^b The ratio of the dehydrogenation activity for D- β -HIBA to that for L- β -HIBA.TABLE II. EFFECTS OF SOME REAGENTS ON INDUCTION OF L- AND D- β -HIBA DEHYDROGENATION ACTIVITIES IN *C. rugosa* IFO 0750

Reagent ^a	Dehydrogenation activity for		Induction (fold)	D/L ^b
	L- β -HIBA (L) (U/mg protein 10 ³)	D- β -HIBA (D) (U/mg protein 10 ³)		
None	5.8	2.1	(1.0)	0.36
L-Valine	22.2	7.8	3.8	0.35
Methacrylic acid	52.3	18.8	8.9	0.36
Isobutyric acid	50.2	17.6	8.5	0.35
L- β -Hydroxyisobutyric acid	17.3	6.1	2.9	0.35
D- β -Hydroxyisobutyric acid	19.1	6.9	3.2	0.36
L-Isoleucine	12.5	4.6	2.1	0.37
L-Leucine	9.8	3.5	1.7	0.36
Isocaproic acid	9.8	3.4	1.7	0.35
Isovaleric acid	11.2	4.1	1.9	0.37
Butyric acid	47.4	16.7	8.3	0.35
Ca-Pantothenate	8.1	2.9	1.4	0.36

^a 1% (w/v) reagent was added.^b See Table I.

specific β -HIBA dehydrogenation activity, and the other both L- and D- β -HIBA dehydrogenation activities. The purification of the enzymes taking part in the dehydrogenations was carried out to clarify these enzyme systems

in the cell-free extracts. As for the former type, the author purified L-specific dehydrogenase about 500-fold from *Micrococcus luteus*. This enzyme did not catalyze D- β -HIBA at all.⁷⁾ In the case of the latter type, several enzyme

systems for β -HIBA dehydrogenations will be assumed to be as follows: (a) L-specific enzyme + D-specific enzyme, (b) L-specific enzyme + racemase, (c) stereo-nonspecific enzyme with different activities for stereoisomers of β -HIBA, (d) L-specific enzyme having a racemase, (e) a mixture of the above enzymes, etc. The author succeeded in the crystallization of the enzyme of case (c) from *C. rugosa* IFO 0750.⁷⁾ This enzyme catalyzed not only L-HIBA but also D- β -HIBA dehydrogenations with the different specific activities for the stereoisomers (D/L=0.35), and racemase activity was not detected in the enzyme. While evidence of the other enzyme systems has not been found yet. In conclusion, at least, two types of β -HIBA dehydrogenase exist in organisms. One is L-specific dehydrogenase, and the other stereo-nonspecific β -HIBA dehydrogenase catalyzing more rapidly L- β -HIBA than D- β -HIBA dehydrogenation.

The difference of stereospecificity of the enzyme will be available for a new preparation of the optically active β -HIBA which is useful for synthesis of antihypertensive reagents⁸⁾ and odoriferous substances.⁹⁾ D- β -HIBA of high optical purity could be prepared from DL- β -HIBA in an appreciable amount using the resting cells of *C. rugosa* IFO 1542.

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