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Note

Stereoselective Reduction of Carbonyl Compounds Using the Cell-Free Extract of the Earthworm, *Lumbricus rubellus*, as a Novel Source of Biocatalyst

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We found the reducing activity toward carbonyl compounds in the cell-free extract of the earthworm, *Lumbricus rubellus*. The earthworm extract had a reducing activity for keto esters in the presence of NADH or NADPH as a coenzyme. The earthworm extract reduced ethyl 3-methyl-2-oxobutanoate to the corresponding alcohol with a high enantiomeric excess (91%, *R*-form) at 50 °C in the presence of NADH. In particular, ethyl 2-oxoheptanoate was exclusively reduced to the corresponding (*R*)-hydroxyl ester with a high enantiomeric excess (> 99%).

Key words: earthworm; biocatalyst; keto ester; stereoselective reduction; cell-free extract

Biotransformations of exogenous substrates have been widely studied in order to synthesize chiral compounds.[1–3] Microbial reduction of carbonyl compounds is one of the convenient methods for obtaining optically pure alcohols. For example, bakers' yeast (*Saccharomyces cerevisiae*) has often been used for the reduction of keto esters to obtain optically active hydroxyl esters.[4,5] Furthermore, other microorganisms (such as yeast,[6] aerobic bacteria,[7–9] and microalgae[10]) or plant cultured cells[3] that can catalyze the stereoselective reduction of keto esters are also used for the preparation of chiral hydroxyl esters. As described above, the biotransformation using microorganisms or plant cells as biocatalysts has been widely investigated, however, the bioconversion using other organisms, such as invertebrates, has rarely been reported.

The earthworm belongs to the annelida phylum (in the animalia kingdom) and is a decomposer in the global ecosystem. It has long been used as a drug in Chinese medicine under the name of "*Jiryu*."[11,12] Nakajima *et al.* reported that an earthworm, *Lumbricus rubellus*, secreted alkaline serine proteases having a potent fibrinolytic activity,[13,14] and that these proteases were also useful biocatalysts.[15,16] Furthermore, they reported that one of the earthworm serine proteases acts on the hydrolysis of triacylglycerols.[17] However, little information is known about the possibility that the earthworm is a source of biocatalyst in biotransformations.

Here we report the reduction of carbonyl compounds using the cell-free extract of the earthworm, *L. rubellus*.

p-ABSF (4-(2-aminoethyl)benzenesulfonate fluoride), a trypsin inhibitor (from soybean), and ethyl pyruvate were purchased from Wako Pure Chemicals, Tokyo, Japan. Ethyl 3-methyl-2-oxobutanoate was obtained from Aldrich Chemicals, Boston, MA, USA. Ethyl benzoylformate and ethyl 2-methyl-3-oxobutanoate were purchased from Tokyo Kasei Kogyo, Tokyo, Japan. Other α-keto esters and α-hydroxy esters were synthesized according to standard methods.[4] The earthworms (*L. rubellus*, brand name "Rintarou") were purchased from Nobeoka Asahi Senni (Miyazaki, Japan; E-mail: nakamura.yr@om.asahi-kasei.co.jp). NADPH and NADH were obtained from Kohjin, Tokyo, Japan. All other chemicals used in this study were of analytical grade and are commercially available.

One hundred earthworms (42 g of fresh *L. rubellus*) were suspended in 95 ml of 50 mM potassium phosphate buffer (KPB) (pH 7.0). This suspension was cooled below 4 °C and subsequently homogenated using a household mixer for 60 s at room temperature. Cell debris in the homogenate was removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. *p*-ABSF (final concentration, 10 mM) and a soybean trypsin inhibitor (final concentration, 0.1 mM) were added as protease inhibitors. The mixture solution was stirred gently for 30 min in an ice-bath, and then the solution was centrifuged at 13,000 × *g* for 30 min at 4 °C. The supernatant (98.5 ml) served as the crude cell-free extract. The reducing activity of the cell-free extract was spectrophotometrically determined. The reaction mixture, in a total

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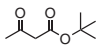
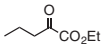
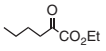
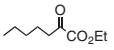
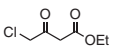
volume of 1.0 ml, contained 5.0 mM of the substrate, 0.15 mM of the coenzyme in 0.1 M KPB (pH 7.0), and a limited amount of the enzyme solution. Consumption of the reduced coenzyme was followed using a Beckman DU-640 spectrophotometer at 340 nm at 37 °C. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NAD(P)H per min under the specified conditions.

In a polypropylene tube were placed the cell-free extract (0.1 units), NAD(P)H (11 μmol), the substrate (10 μmol), and 0.1 M KPB (pH 7.0, total volume, 0.45 ml). The mixture was gently shaken at 37 °C or 50 °C. After 6 h, the mixture was filtered using an Extrelut® NT (Merck, Darmstadt, Germany) short column, extracted with ether, and then concentrated under reduced pressure. The conversions of the products and chemical yields were determined by GLC equipped with a capillary column (DB-WAX) using an internal standard. The enantiomeric excesses (e.e.) of the products were determined using a GLC equipped with an optically active capillary column (Chirasil-DEX CB, 0.25 mm × 25 m and Gamma DEX™ 225, 0.25 mm × 25 m). The absolute configuration of the isomer was determined by comparing its retention time with those of authentic samples prepared according to standard methods.[4)]

The cell-free extract of the earthworm was tested for its reducing ability toward various carbonyl compounds. The results of the reducing activities in the presence of NAD(P)H are summarized in Table 1.

Table 1. Relative Activity of the Cell-Free Extract of the Earthworm[a,b]

Substrate	Relative rate (%)[c]		Substrate	Relative rate (%)[c]	
	NADH	NADPH		NADH	NADPH
	100	46		5	3
	30	33		2	1
	10	11		0	0
	30	13		0	0
	38	26		0	0
	19	13		0	0
	10	5		0	0
	6	4		0	0
	4	2		0	0
	3	1		0	0

[a]The reducing activity (relative activity) was measured in 0.1 M KPB (pH 7.0) at 37 °C.
[b]The substrate concentration was 5 mM.
[c]Relative rates were determined by setting the activity of ethyl pyruvate in the presence of NADH to 100.

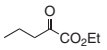
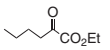
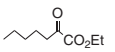
It was found that the extract of the earthworm had a reducing activity toward various α- and β-keto esters in the presence of both NADH and NADPH as the coenzyme. One hundred earthworms (42 g) gave a supernatant (98.5 ml), and the activity toward ethyl pyruvate in the presence of NADPH was 1.12 units/ml of the extract (total activity, 1.10×10^2 units). The specific activity was 1.53×10^{-1} units/mg of protein. In the reducing activity toward ethyl pyruvate, the specific activity of the earthworm extract (1.53×10^{-1} units/mg) had a higher value than that of the yeast cell-free extract (4.86×10^{-2} units/mg).[18)] This high specific activity of the earthworm extract is an advantage for the reduction of α-keto esters. As the alkyl chain length of the substrate molecule (α-keto esters) become longer, the reducing activity decreased. No catalytic activity toward alkyl phenyl ketones, methyl ketones, or other ketones (such as 1-methoxy-2-propanone, phenacyl chloride and menthone) was detected. No coenzyme regeneration (reduction of $NAD(P)^+$) was observed in the presence of D-glucose, glycerol, methanol, ethanol, 1-propanol, 2-propanol, cyclopentanol, cyclohexanol, or lactate as a substrate (data not shown). The stereoselectivity of the reduction toward α-keto esters was investigated by a GLC analysis, as shown in Table 2.

Aliphatic α-keto esters were reduced by the earthworm extract in high conversion ratios, however, the enantioselectivities of the produced hydroxyl esters were low. The selectivity in the reduction of ethyl 3-methyl-2-oxobutanoate was improved by raising the reaction temperature from 37 to 50 °C. Ethyl 2-oxoheptanoate was converted by the earthworm extract to the corresponding alcohol with a high conversion ratio and high e.e., but the yield of the alcohol was less than 60% (data not shown). To clarify the high selectivity during the reduction biotransformation of the hydroxyl ester as a substrate was then carried out (see Table 3).

The e.e. of the residual hydroxyl ester shifted to the *R*-form with the reaction time, in particular, the shift of ethyl 2-hydroxyheptanoate was remarkable. These results indicate that the enantioselective reduction of the substrate (ethyl 2-oxoheptanoate) and setereoselective hydrolysis of the produced alcohol (ethyl 2-hydroxyheptanoate) take place during the biotransformation of ethyl 2-oxoheptanoate using the earthworm extract. The stereoselective hydrolysis is probably involved in the activity of a protease, isozyme C, in the earthworm having an esterase-like activity.[16)] In fact, Ushio *et al.* reported that bakers' yeast catalyzed the enantioselective hydrolysis of α-hydroxy ester and that the hydrolysis activity was inhibited by butylsulfonyl fluoride as an esterase inhibitor.[19)]

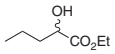
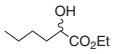
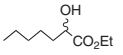
Thus, we have demonstrated the reduction of carbonyl compounds, such as keto esters, using the cell-free

Table 2. Redcution of α-Keto Esters Using the Cell-Free Extract of *Lumbricus rubellus*[a]

Substrate	37 °C						50 °C					
	NADH			NADPH			NADH			NADPH		
	Conv. (%)	e.e. (%)[b]	(*R*/*S*)[b]	Conv. (%)	e.e. (%)[b]	(*R*/*S*)[b]	Conv. (%)	e.e. (%)[b]	(*R*/*S*)[b]	Conv. (%)	e.e. (%)[b]	(*R*/*S*)[b]
	>99	45	(*S*)	>99	69	(*S*)	>99	30	(*S*)	>99	64	(*S*)
	>99	7	(*S*)	>99	29	(*S*)	>99	11	(*R*)	>99	5	(*S*)
	>99	10	(*S*)	>99	10	(*S*)	>99	7	(*R*)	>99	10	(*S*)
	>99	15	(*S*)	>99	1	(*R*)	>99	6	(*S*)	>99	21	(*R*)
	>99	>99	(*R*)	>99	>99	(*R*)	>99	50	(*R*)	>99	59	(*R*)
	>99	11	(*R*)	>99	20	(*R*)	>99	91	(*R*)	>99	86	(*R*)
	46	61	(*R*)	24	40	(*R*)	8	41	(*R*)	7	10	(*R*)

[a]The cell-free extract (0.1 units), substrate (10 µmol), coenzyme (NADH or NADPH) (11 µmol), and 0.2 M potassium phosphate buffer (pH 7.0, total volume 0.45 ml) were incubated for 6 h.
[b]Enantiomeric excesses and absolute configuration were measured by GLC analysis using optically active capillary columns.

Table 3. Biotransformation of α-Hydroxy Esters in the Earthworm Extract[a]

Substrate	After 1 h		After 2 h		After 6 h	
	e.e. (%)[b]	(*R*/*S*)[b]	e.e. (%)[b]	(*R*/*S*)[b]	e.e. (%)[b]	(*R*/*S*)[b]
	1	(*R*)	2	(*R*)	6	(*R*)
	8	(*S*)	7	(*S*)	3	(*S*)
	4	(*R*)	8	(*R*)	18	(*R*)
	17	(*R*)	31	(*R*)	60	(*R*)
	<1	(*R*)	<1	(*S*)	<1	(*S*)
	4	(*R*)	5	(*R*)	8	(*R*)

[a]The cell-free extract (0.1 units), racemic hydroxy ester (10 µmol), and 0.1 M potassium phosphate buffer (pH 7.0, total volume 0.45 ml), were incubated for 6 h at 37 °C.
[b]Enantiomeric excesses and configuration were measured by GLC analysis using optically active capillary columns.

extract of the earthworm, and indicated that the earthworm extract is a useful biocatalyst as well as microorganisms such as yeast and fungi.

To gain insight into the mechanistic interpretation of the reduction using the earthworm extract, further detailed studies, including purification of the oxidoeductase(s) and esterase(s), which contribute to the stereoselective reduction of keto esters and the stereoselective hydrolysis of hydroxy esters, are currently in progress.

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