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Distribution and immunological characterization of microbial aldehyde reductases

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Abstract. The distribution of microbial aldo-keto reductases was examined and their immunochemical characterization was performed. *p*-Nitrobenzaldehyde, pyridine-3-aldehyde and ethyl 4-chloro-3-oxobutanoate reductase activities were found to be widely distributed in a variety of microorganisms. In immunodiffusion studies, most yeasts belonging to the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* formed precipitin bands with anti-*Sporobolomyces salmonicolor* aldehyde reductase serum. Furthermore, the results of immunotitration experiments suggested that *Sporobolomyces salmonicolor* AKU 4429 contains other enzyme(s) which can reduce *p*-nitrobenzaldehyde, pyridine-3-aldehyde and/or ethyl 4-chloro-3-oxobutanoate, and which are inactivated by anti-*Sporobolomyces salmonicolor* aldehyde reductase serum.

Key words: Aldehyde reductase – Aldo-keto reductases – *p*-Nitrobenzaldehyde reductase – Pyridine-3-aldehyde reductase – *Sporobolomyces* – *Sporidiobolus* – *Rhodotorula*

Aldehyde reductase (EC 1.1.1.2), aldose reductase (EC 1.1.1.21) and carbonyl reductase (EC 1.1.1.184) are cytosolic, monomeric oxidoreductases that catalyze the NADPH-dependent reduction of a great variety of carbonyl compounds. These enzymes have been isolated from a number of mammalian and other animal tissues, and are generally referred to as the aldo-keto reductase family (Bohren et al. 1989; Wermuth 1981; Wermuth et al. 1977, 1982, 1988; Wirth and Wermuth 1985). This grouping into an enzyme family is based on their having similar broad substrate specificities and physicochemical properties.

During the course of studies on the microbial reduction of carbonyl compounds (Hata et al. 1989a, b; Shimizu et al. 1988a, b, 1990a, b; Yamada et al. 1990), we found that *Sporobolomyces salmonicolor* AKU 4429 produces an enzyme belonging to the aldo-keto reductase family (Yamada et al. 1990). The enzyme is similar to aldehyde reductase in its substrate specificity, and it was also shown that the enzyme can catalyze the asymmetric reduction of 4-halo-3-oxobutanoate esters to the corresponding (*R*)-4-halo-3-hydroxybutanoate esters, which are promising chiral building blocks for the chemical synthesis of L-carnitine (Grunwald et al. 1986; Shieh et al. 1985; Wong et al. 1985; Zhou et al. 1983), in addition to common substrates of the aldehyde reductases. Based on this finding, we have been developed a practical procedure for the reduction of these β-keto acid esters (Shimizu et al. 1990a, b). While aldehyde reductases of animal tissues have been well characterized, the occurrence of this enzyme in microorganisms other than *Sporobolomyces salmonicolor* has not been reported. Then, we investigate the distribution and immunological characterization of microbial aldehyde reductases.

Materials and methods

Materials

Fresh beef and chicken livers were obtained from a local slaughterhouse and poultry farm, respectively. All other chemicals were of analytical grade and commercially available.

Microorganisms, cultivations and preparation of cell extracts

Microorganisms preserved in our laboratory (AKU culture collection) were used. The following media were used: medium GC for molds and yeasts, containing 5% glucose and 5% corn steep liquor, pH 6.0; medium GCC for bacteria, comprising medium GC supplemented with 1% $CaCO_3$, pH 7.0. Each microorganism was aerobically cultured at 28 °C for 2 days in a 500-ml shaking flask containing 100 ml of medium. After cultivation, the cells were harvested by centrifugation.

Fungal and bacterial cells harvested from 100 ml of culture broth, respectively, were suspended in 5–20 ml of 20 mM potassium phosphate buffer, pH 7.0, and then disrupted with an Insonater oscillator 200 M (Kubota Medical Applicance Supply Co., Tokyo, Japan) for 30–90 min at below 5 °C. Vertebrate tissue extracts were

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prepared as described by Potter (1955). Cells and debris were removed by centrifugation at 14,000 × *g* for 30 min at 4 °C. The supernatant was used as the cell-free extract.

Enzyme assay and protein determination

The enzyme activity was determined photometrically as described previously (Yamada et al. 1990). Protein concentrations were measured with a Bio-rad (Richmond, Calif., USA) protein assay kit as described previously (Yamada et al. 1990). For the purified enzyme protein concentrations were determined by measuring the absorbance at 280 nm. An absorbance value of 1.22 for 1 mg/ml and a 1-cm light path, as determined by absorbance and dry-weight measurements, was used.

Purification of aldehyde reductase from Sporobolomyces salmonicolor

Aldehyde reductase was purified from cells of *Sporobolomyces salmonicolor* AKU 4429 as described previously (Yamada et al. 1990).

Preparation of antisera

An antibody against the aldehyde reductase of *Sporobolomyces salmonicolor* AKU 4429 was raised in a rabbit by subcutaneous injection of the purified enzyme (5.0 mg) dissolved in 1.0 ml of Freund's complete adjuvant (Difco, Detroit, Mich., USA). After 4 weeks the animal received a subcutaneous booster injection in the neck of 1.0 mg of the enzyme homogenized in an equal volume of incomplete Freund's adjuvant (Difco). On the 14th day after the booster injection the animal was bled from the ear vein. The serum was centrifuged at 6,000 × *g* for 10 min and then stored at −20 °C.

Immunological procedures

Ouchterlony double-immunodiffusion tests were conducted in 1.2% Noble agar (Difco) in Tris-H_2SO_4, pH 8.0, containing 3% polyethylene glycol 6000 and 0.01% sodium azide (Ouchterlony 1949).

Immunotitration experiments were carried out as follows: a homogeneous aldehyde reductase solution or cell-free extract, containing 2.00 units of *p*-nitrobenzahldehyde reductase activity, was incubated with various concentrations of the anti-*Sporobolomyces salmonicolor* AKU 4429 aldehyde reductase serum at 37 °C for 30 min in 20 mM potassium phosphate buffer, pH 7.0, in a total volume of 1.0 ml. After brief centrifugation, the residual reductase activities toward *p*-nitrobenzaldehyde, pyridine-3-aldehyde and ethyl 4-chloro-3-oxobutanoate of each supernatant were measured.

Results

Distribution of aldehyde reductase

Using cell-free extracts of various microorganisms, and chicken and beef livers, we preliminarily surveyed NADPH-dependent *p*-nitrobenzaldehyde, pyridine-3-aldehyde and ethyl 4-chloro-3-oxobutanoate reducing activities. In all, 48 strains of yeasts (11 genera), molds (6 genera) and bacteria (12 genera), and chicken and beef livers were investigated in the survey. In general, the

Table 1. *p*-Nitrobenzaldehyde, pyridine-3-aldehyde and ethyl 4-chloro-3-oxobutanoate reductase activities of various microorganisms and animal tissues, and the immunological reactivity with the anti-*Sporobolomyces salmonicolor* aldehyde reductase serum[a]

Strain	Reductase activity (μmol/min/mg of protein)			Precipitin line[b]
	p-Nitrobenzaldehyde	Pyridine-3-aldehyde	Ethyl 4-chloro-3-oxobutanoate	
Sporobolomyces salmonicolor AKU 4429	1.69	0.62	0.21	+
Sporidiobolus salmonicolor AKU 4440	0.91	0.26	0.14	+
Saccharomyces cerevisiae AKU 4135	0.22	0.06	0.12	−
Pichia membranefaciens AKU 4252	0.09	0.02	0	−
Hansenula anomala AKU 4302	0.08	0.02	0	−
Cryptococcus albidus AKU 4507	2.09	1.12	0.03	−
Candida utilis AKU 4570	0.17	0.05	0	−
Rhodotorula glutinis AKU 4848	1.25	0.34	0.07	+
Trichosporon capitatum AKU 4869	0.15	0	0	−
Mucor ambiguus AKU 3006	0.45	0.16	0.08	−
Aspergillus niger AKU 3333	0	0	0.22	−
Penicillium chrysogenum AKU 3401	0.09	0	0.08	−
Escherichia coli AKU 8	0.22	0.16	0	−
Citrobacter freundii AKU 34	0.11	0.05	0	±
Salmonella typhimurium AKU 94	0.96	0.87	0	−
Alcaligenes faecalis AKU 103	0.01	0	0	−
Bacillus subtilis AKU 209	0.06	0	0	±
Agrobacterium radiobacter AKU 309	0.19	0.12	0.19	−
Staphylococcus aureus AKU 520	0.05	0.01	0	−
Arthrobacter oxydans AKU 623	0.10	0.09	0	−
Pseudomonas aeruginosa AKU 804	0.05	0.02	0.04	−
Beef liver	0.12	0.03	0.09	−
Chicken liver	0.07	0.03	0.08	±

[a] The assay conditions were given under "Materials and methods"
[b] Precipitin line: +, clearly formed; ±, weakly formed; −, not formed

ability to reduce *p*-nitrobenzaldehyde was widely distributed in a variety of yeasts, molds and bacteria, and chicken and beef livers (Table 1). In particular, *Sporobolomyces salmonicolor*, *Sporidiobolus salmonicolor*, *Rhodotorula glutinis*, *Mucor ambiguus* and *Salmonella typhimurium* were found to have high *p*-nitrobenzaldehyde reductase activities, and these strains also had relatively high pyridine-3-aldehyde reductase activities. There seemed to be some correlation between the distribution of *p*-nitrobenzaldehyde reducing and pyridine-3-aldehyde reducing activities. However, ethyl 4-chloro-3-oxobutanoate reducing activity was distributed differently from the *p*-nitrobenzaldehyde and pyridine-3-aldehyde reducing activities. For example, the abilities to reduce ethyl 4-chloro-3-oxobutanoate of *Rhodotorula glutinis*, *Cryptococcus albidus* and *Salmonella typhimurium*, which well catalyzed the reduction of *p*-nitrobenzaldehyde and pyridine-3-aldehyde, were relatively low or not detectable. On the contrary, *Aspergillus niger*, which possesses no *p*-nitrobenzaldehyde or pyridine-3-aldehyde reductase activity, catalyzed the reduction of ethyl 4-chloro-3-oxobutanoate at a relatively high velocity.

Immunochemical reactivity of anti-Sporobolomyces salmonicolor (AKU 4429) aldehyde reductase serum

The ability of antiserum prepared against the purified aldehyde reductase of *Sporobolomyces salmonicolor* (AKU 4429) to cross-react with cell extracts of the yeasts, molds and animals listed in Table 1 was examined by means of the immunodiffusion technique. Precipitin bands were clearly formed with the extracts derived from *Sporobolomyces salmonicolor* (AKU 4429), *Sporidiobolus salmonicolor* (AKU 4440) and *Rhodotorula glutinis* (AKU 4848), and crude extracts of *Citrobacter freundii* (AKU 34), *Bacillus subtilis* (AKU 209) and chicken liver formed weak precipitin bands.

Other yeasts belonging to the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* were also examined as to their reactivities with the anti-*Sporobolomyces salmonicolor* aldehyde reductase serum. Precipitin bands were formed with cell extracts of *Sporobolomyces salmonicolor* (AKU 4425 and AKU 4428), *Sporobolomyces holsaticus* (AKU 4437), *Sporidiobolus salmonicolor* (AKU 4441 and AKU 4443), *Sporidiobolus johnsonii* (AKU 4444), *Sporidiobolus pararoseus* (AKU 4426), *Rhodotorula glutinis* (AKU 4827 and AKU 4845), *Rhodotorula rubra* (AKU 4808, AKU 4814, AKU 4815, AKU 4816, AKU 4819, AKU 4820, AKU 4835 and AKU 4852) and *Rhodotorula* sp. (AKU 4853), while no such line was observed with the extracts of *Sporobolomyces roseus* (AKU 4431 and AKU 4442), *Rhodotorula aurantiaca* (AKU 4803), *Rhodotorula minuta* (AKU 4804 and AKU 4822) and *Rhodotorula pallida* (AKU 4810).

Cross-reactions between cell-free extracts of Sporobolomyces, Sporidiobolus and Rhodotorula yeasts

The cross-reactivity was examined between cell-free extracts of the twenty-one strains belonging to the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* that produced precipitin bands, as described above. Figure 1 summarizes the results of the cross-section experiments using cell-free extracts of the twenty-one strains. Completely fused precipitin bands were observed with the cell-free extracts of all the strains of *Sporobolomyces salmonicolor* and *Sporidiobolus salmonicolor*. The cell-free extracts of *Sporobolomyces holsaticus* (AKU 4437) and *Sporidiobolus johnsonii* (AKU 4444) produced clear spurs when the *Sporobolomyces salmonicolor* (AKU 4429) enzyme was placed in neighboring wells, while they formed fused precipitin bands with each other. The cell extract of *Sporidiobolus pararoseus* (AKU 4426) formed spurs against the cell extracts of all the other strains

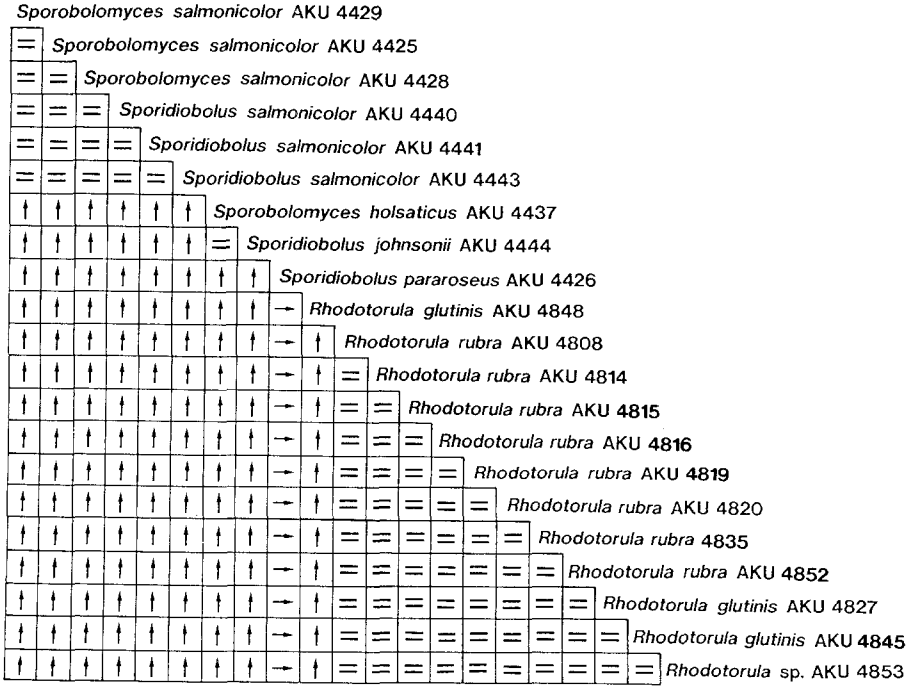

Fig. 1. Results of cross-reaction experiments with cell-free extracts of yeasts belonging to the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* exhibiting reactivity with the anti-*Sporobolomyces salmonicolor* aldehyde reductase serum. *Arrows* indicate spurred precipitin lines (reaction of partial identity) and the dominant antigen in the paired cross-reaction; = indicates fused precipitin lines (reaction of identity)

containing *Rhodotorula*. The precipitin bands formed by the cell-free extracts of *Rhodotorula* species completely fused with each other, however *Rhodotorula glutinis* (AKU 4848) dominated the other *Rhodotorula* species.

Reactivity of cell-free extracts with the anti-Sporobolomyces salmonicolor (AKU 4429) aldehyde reductase serum

Immunotitration analysis using the anti-*Sporobolomyces salmonicolor* (AKU 4429) aldehyde reductase serum was also carried out to compare the purified enzyme with the enzymes of other microorganisms. One unit of *p*-nitrobenzaldehyde reductase activity, 0.487 units of pyridine-3-aldehyde reductase activity and 0.214 units of ethyl 4-chloro-3-oxobutanoate reductase activity of the purified enzyme from *Sporobolomyces salmonicolor* (AKU 4429) can be abolished by 150 µl of the antiserum prepared in this study. The titration curves of these three activities of the purified enzyme completely coincided with each other (Fig. 2). However, when the cell-free extract of *Sporobolomyces salmonicolor* (AKU 4429), from which the enzyme was purified, was incubated with the antiserum, the titration curves of the three activities did not agree and thus the enzymes were not inactivated completely. The ratio of the decreases in the *p*-nitrobenzaldehyde, pyridine-3-aldehyde and ethyl 4-chloro-3-oxobutanoate reductase activities in the cell-free extract was about 100 : 23.6 : 35.7, while that in the case of the purified enzyme was about 100 : 48.7 : 21.4. These results suggested that the cell-free extract contained other enzyme(s), which could reduce these substrates and could be inactivated by the antiserum, other than the purified enzyme. The cell extract of *Sporobolomyces holsaticus* (AKU 4437), which formed a spur against the purified enzyme in a double immunodiffusion experiment, was titrated more extensively than the extract of *Sporobolomyces salmonicolor* (AKU 4429) for the examination of all reductase activities. On the other hand, in the case of *Rhodotorula glutinis* (AKU 4848) and *Rhodotorula* sp. (AKU 4853), only the ethyl 4-chloro-3-oxobutanoate reductase activity was affected, the other reductase activities remained unchanged. Furthermore, no change was observed in the reductase activities of *Rhodotorula rubra* (AKU 4814).

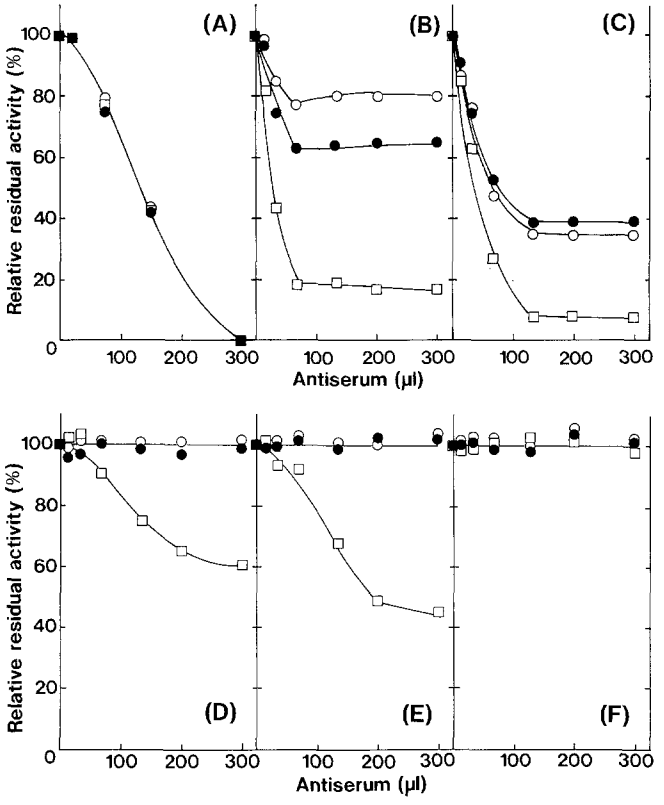

Fig. 2. Immunochemical titration of the *p*-nitrobenzaldehyde reductase (●), pyridine-3-aldehyde reductase (○) and ethyl 4-chloro-3-oxobutanoate reductase (□) activities of the purified enzyme (**A**), *Sporobolomyces salmonicolor* AKU 4429 (**B**), *Sporobolomyces holsaticus* AKU 4437 (**C**), *Rhodotorula glutinis* AKU 4848 (**D**), *Rhodotorula* sp. AKU 4853 (**E**) and *Rhodotorula rubra* AKU 4814 (**F**) with the antiserum against the purified aldehyde reductase from *Sporobolomyces salmonicolor* (AKU 4429). Immunochemical reactions and activitiy assays were carried out as described under Materials and methods. Relative residual activity was expressed as a percentage, taking 2.00 (**A**–**F**) units as being 100 for *p*-nitrobenzaldehyde reductase. The corresponding unit values for each pyridine-3-aldehyde reductase are 0.974 (**A**), 0.737 (**B**), 0.498 (**C**), 0.550 (**D**), 0.633 (**E**) and 1.14 (**F**), respectively. These values were taken as being 100 for the respective pyridine-3-aldehyde reductases. Similarly, 0.428 (**A**), 0.250 (**B**), 0.847 (**C**), 0.107 (**D**), 0.326 (**E**) and 0.0775 (**F**) units were taken as being 100 for the ethyl 4-chloro-3-oxobutanoate reductases

Discussion

The present study demonstrated that aldo-keto reductase (*p*-nitrobenzaldehyde reductase and pyridine-3-aldehyde reductase) activity occurs in a variety of microorganisms. Also, ethyl 4-chloro-3-oxobutanoate reductase was found to be widely distributed in not only microorganisms but also animal tissues. On the other hand, high immunochemical reactivity with the anti-*Sporobolomyces salmonicolor* aldehyde reductase serum was only found in the case of the yeasts of the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula*, with the use of immunodiffusion techniques. The cell extracts of *Citrobacter freundii*, *Bacillus subtilis* and chicken liver showed immunochemical reactivity with the anti-*Sporobolomyces salmonicolor* enzyme serum, however, the reactivity was low.

Although most of the yeasts belonging to the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* tested here formed precipitin bands, the immunochemical reactivity seemed to vary with the species. All strains of *Sporobolomyces salmonicolor*, *Sporidiobolus salmonicolor*, *Rhodotorula glutinis* and *Rhodotorula rubra* formed precipitin bands with the antiserum, while no such line was observed with all strains of *Sporobolomyces roseus* and *Rhodotorula minuta*. Furthermore, the results of cross-reaction experiments suggested that all strains of *Sporobolomyces salmonicolor* and *Sporidiobolus salmonicolor* formed enzymes possessing similar immunological properties, and that the other strains of *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* have enzymes that are

partially identical with the *Sporobolomyces salmonicolor* enzyme.

The immunotitration experiments suggested that the cell-free extract of *Sporobolomyces salmonicolor* AKU 4429 contained other enzyme(s), which can reduce *p*-nitrobenzaldehyde, pyridine-3-aldehyde and/or ethyl 4-chloro-3-oxobutanoate, and which is inactivated by the antiserum, similar to that of the purified aldehyde reductase. *Sporobolomyces salmonicolor* AKU 4429 might have an enzyme(s) that is identical with the reductases of other strains forming spurs in cross-reaction experiments. Furthermore, aldo-keto reductase activity was not affected on immunotitration using the cell extracts of *Rhodotorula glutinis* AKU 4848 and *Rhodotorula* sp. AKU 4853, while ethyl 4-chloro-3-oxobutanoate reductase activity was significantly affected. These results suggested that these strains might have a novel type of aldo-keto reductase which is different from aldehyde reductase and aldose reductase, but which is partially identical with aldo-keto reductase family enzymes in its primary protein structure.

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