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N^5–(Carboxyethyl)ornithine Synthase in *Lactococcus lactis* IO–1 Producing a Novel Lantibiotic Nisin Z

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N^5–(carboxyethyl)ornithine synthase was successfully determined from the cell extract of *Lactococcus lactis* IO–1, which that produces an antimicrobial peptide, nisin Z. There might be the coexistence of this enzyme and nisin Z formation at molecular level since no detection of N^5–(carboxyethyl)ornithine synthase in non–nisin producing strains.

INTRODUCTION

The intracellular compounds of both N^5–(1–carboxyethyl)ornithine (Thompson *et al.*, 1986) and N^6–(1–carboxyethyl)lysine (Thompson *et al.*, 1988) were firstly detected in the cells of *Lactococcus lactis* subsp. *lactis* K1 (formerly *Streptococcus lactis* K1) as major compounds of amino acid during growth in arginine–deficient medium. High levels about 15–20 mM of N^5–(carboxyethyl)ornithine were present in several strains of *Lc. lactis* subsp. *lactis* K1 and *S. diacetylactis* (Miller and Thompson, 1987; Thompson *et al.*, 1986); however, this amino acid was not incorporated into cellular material but excreted into the culture medium during growth. Interestingly, neither compound has previously been found in either procaryotic or eucaryotic cells (Tempe, 1983). Only their corresponding regioisomers (Biellmann *et al.*, 1977; Hack and Kemp, 1977; Lioret, 1956; Lejieune, 1967; Menage, 1983; Menage, 1964; Tempe, 1982), N^2–(**D**–1–carboxyethyl)–**L**–ornithine (octopic acid) and N^6–(**D**–1–carboxyethyl)–**L**–lysine (lysopine) together with N^2–(**D**–1–carboxyethyl)–**L**–arginine (octopine) and N^2–(**D**–1–carboxyethyl)histidine (histopine), constitute the octopine family of opines (Tempe, 1983) found in crown gall tumor tissue (Hack and Kemp, 1977; Tempe, 1982). Moreover, it has been known that these opines are formed by NAD(P)H– dependent oxidoreductase enzymes (dehydrogenase) (Biellmann *et al.*, 1977; Hack, 1980), which mediate the reductive condensation between pyruvic acid and the NH_2 group of an amino acid (Thompson *et al.*, 1990). Later on, N^5–(**L**–1–carboxyethyl)–**L**–ornithine: NADP$^+$ oxidoreductase or N^5–(carboxyethyl)ornithine synthase from *Lc. lactis* subsp. *lactis* K1 (Thompson, 1989) was purified to homogeneity. This NAD(P)H–dependent enzyme mediates the reductive condensation between pyruvic acid and the δ of ξ–amino groups of **L**–ornithine and **L**–lysine to form N^5–(**L**–1–carboxyethyl)–**L**–ornithine and N^6–(**L**–1–carboxyehtyl)–**L**–lysine, respectively. Then Donkersloot and Thompson (1990) found that the spontaneous mutant of *Lc. lactis* subsp. *lactis* K1 which lacked

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N^{ω}-(carboxyethyl)ornithine synthase also had lost the abilities to ferment sucrose and to produce the antibiotic nisin A.

Since *Lc. lactis* IO-1, a novel *Lactococcus* isolated as described (Ishizaki *et al.*, 1990) was reported to produce a new peptide antibiotic nisin Z that differs from nisin A in a single amino acid residue at position 27 (Matsusaki *et al.*, 1996). Thus, the aim of this research is to determine for N^{ω}-(carboxyethyl)ornithine synthase in *Lc. lactis* IO-1 which will lead to more understandable information of the tentative coexistence of this enzyme, and sucrose-fermenting ability and nisin production among lactic streptococcus as previously reported (Donkersloot and Thompson, 1990; Gonzalez and Kunka, 1985).

MATERIALS AND METHODS

Chemicals

All chemicals used were of reagent grade and were obtained from Nacalai Tesque Inc., Kyoto, Japan, and Chameleon Reagent, Osaka, Japan. Both β-nicotinamide adenine dinucleotide (β-NADH) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH), reduced forms were supplied from Oriental Yeast Co. Ltd. Japan. Other biochemicals were obtained from elsewhere as specified.

Bacterial strains and media

The bacterial strains used in this study were as follows: *Lc. lactis* IO-1 (JCM 7638) (Ishizaki *et al.*, 1990), *Lc. lactis* subsp. *lactis* ATCC 19435^{T}, *Lc. lactis* NCDO 497, *Pediococcus* sp. ISK-1 isolated from well-aged *Nukadoko* (Herawati and Ishizaki, 1997), *Micrococcus luteus* IFO 12708 and *Lactobacillus* strain BL, a novel antimicrobial substance-producing bacterium (from Master thesis of Chulalongkorn University), isolated from Thai fermented vegetable. Recently, *P.* sp. ISK-1 was found to produce a novel lantibiotic (Kimura *et al.*, 1997 and 1998).

A Bacto thioglycollate without dextrose dehydrated, Difco Laboratories, U.S.A. designated TGC medium (Ishizaki *et al.*, 1990) was used as maintenance medium and in a liter of fermentation medium designated PGY medium containing polypeptone, glucose, yeast extract each of 10 g plus sodium chloride 5 g was used as a growth medium for all *Lactococcus* and *Micrococcus*. Purity of each strain was repeatedly checked on PGY medium containing 2% (W/V) bactoagar. Both *P.* sp. ISK-1 and *Lb.* strain BL were cultured in MRS broth from BBL Microbiology Systems (U.S.A.), and checked for their purity on the same medium containing agar 2% W/V. All organisms were cultured in two 2-liter flasks each containing 1.5 liters of medium, and were incubated at 37 °C and 30 °C for *P.* sp. ISK-1.

Preparation of cell extracts

All cultures were harvested during the exponential phase of growth, centrifuged at 16,000×g for 10 min and washed twice with distilled water containing 2 M dithiothreitol. Potassium phosphate buffer 0.1 M, pH 7.0 containing 1 mM dithiothreitol, modified KPD buffer (Thompson, 1989), was used for cell suspension.

Cell extracts for enzymatic studies were prepared by thawing frozen cells of each organism and resuspended to 3-5 ml in glass vial containing modified KPD buffer

surrounding with ice cup (of 30-ml volume) and the cells were disrupted by 4×5 min periods of sonic oscillation (operation at 75% of duty cycle, output control of 3) using a Sonifier Cell Disruption 350 manufactured by Branson Sonic Power Co., Smith Kline Company. Intact cells and cell debris were removed by centrifugation at 16,000×g for 15 min. The supernatant was stored in vial at −30°C until used. Protein was determined by the modified Lowry method (Hartree, 1972), with bovine serum albumin as the standard.

Enzyme assays

All assays were performed at ambient temperature in a UV-160 UV Visible Recording Spectrophotometer, Shimadzu Co., Kyoto, Japan. The final reaction volume for each assay was 2 ml. Specific activities were calculated from the linear part of the reaction, and values for activities were determined from a minimum of three measurements. Potassium phosphate buffer pH 6.5, 7.0, 7.5; Tris-HCl pH 8.0; and Tricine buffer pH 8.6 each of 0.1 M were prepared and used for all the enzyme assays.

N^5-(Carboxyethyl)ornithine synthase (EC 1.5.1.24) was determined by monitoring the oxidation of NADPH. The reaction mixture consisted of 0.1 M of each buffer at different pH, 2 mM dithiothreitol, 3 mM NADPH, 10 mM pyruvate and 10 mM **L**-ornithine hydrochloride or 10 mM **L**-lysine monohydrochloride. The reaction was initiated by the addition of cell extract, and the oxidation of NADPH was monitored at 340 nm.

Lactate dehydrogenase (EC 1.1.1. 27) was assayed by monitoring the oxidation of NADH. The reaction mixture consisted of 0.1 M phosphate buffer (pH 7.0 or pH 7.5), 2 mM dithiothreitol, 3 mM NADH, 10 mM pyruvate. One mM of $MgSO_4$. $7H_2O$ was added into the reaction mixture for enzymatic detection in cell extracts of all *Lactococcus*. spp. The reaction was initiated by the addition of cell extract, and the oxidation of NADH was monitored at 340 nm.

RESULTS

N^5-(Carboxyethyl)ornithine synthase activities in the cell extract of different organisms at various pHs were determined (Table 1). *Lc. lactis* IO-1, *Lc. lactis* NCDO 497, and *Pediococcus* sp. ISK-1 showed a moderate activity of the enzyme. No enzyme activity was obtained in cell extracts of *Lc. lactis* ATCC 19435^T, *Lb.* strain BL and *M. luteus* at any pH. Interestingly, the highest activity of N^5-(carboxyethyl)ornithine synthase was observed in cell extract of *P.* sp. ISK-1 which was higher than those of *Lc. lactis* IO-1 and *Lc. lactis* NCDO 497 10.6 times and 15.1 times, respectively. However, the enzyme from *Lc. lactis* IO-1 and P. sp. ISK-1 optimizedly functioned at acidic pH, whereas that of *Lc. lactis* NCDO 497 preferred neutral pH for optimal activity.

Table 2 shows lactate dehydrogenase (LDH) activity in each cell extract of five lactic acid bacteria strains at pH 7.0 and pH 7.5. Among LDH activities of *Lactococcus* the highest activity (0.815 U/mg of protein at pH 7.5) was obtained with *Lc. lactis* NCDO 497 cell extract, whereas the lowest activity was 0.411 U/mg at the same pH with *Lc. lactis* ATCC 19435^T cell extract. Obviously Mg^{2+} was essentially required as a cofactor for LDH activity in these *Lactococcus* except that of *Lc. lactis* IO-1 which can function in the absence of Mg^{2+} (0.508 U/mg) at pH 7.0. However, relatively higher activities of LDH

Table 1. N^5-(Carboxyethyl)ornithine synthase activity in cell extract of different organisms at various pH.

Organisms	Specific activity (U/mg of protein)				
	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.6
Lc. lactis IO–1	0.0030	0.0021	0.0017	0.0014	0.0014
Lc. lactis NCDO 497	0.0008	0.0011	0.0021	0.0011	0.0009
Lc. lactis ATCC 19435^T	0	0	0	0	0
Pediococcus sp. ISK–1	0.0318	0.0185	0.0061	0.0022	0.0024
Lb. strain BL	0	0	0	0	0
M. luteus	0	0	0	0	0

Table 2. Lactic acid dehydrogenase activity in cell extract of different organisms at various pH.

Organisms	Specific activity (U/mg of protein)	
	pH 7.0	pH 7.5
Lc. lactis IO–1	0.508	0.550
	0.730[a]	NT
Lc. lactis NCDO 497	0.608[a]	0.815[a]
Lc. lactis ATCC 19435^T	NT	0.411[a]
Pediococcus sp. ISK-1	NT	2.134
Lb. strain BL	3.633	4.133

[a] In the presence of $MgSO_4$. NT, not tested.

were determined in the case of *P.* sp. ISK–1 and *Lb.* strain BL, which indicates no requirement of Mg^{2+} for the LDH activities.

DISCUSSION

The high affinity for NADPH of N^5–(carboxyethyl)ornithine synthase in *Lc. lactis* IO–1 was the same as that of *Lc. lactis* K1 (Thompson, 1990). Although NADPH could not be replaced by NADH, enzyme activity was detected by replacing **L**–ornithine hydrochloride by **L**–lysine monohydrochloride (data not shown). This could be supported by the possible reductive condensation between pyruvate and the δ– or ε–amino group of **L**–ornithine and **L**–lysine to form N^5–(carboxyethyl)–**L**–ornithine and N^6–(**L**–1–carboxyethyl)–**L**–lysine, respectively (Thompson, 1990). Obviously, the subsequent survey showed that only about half of the *Lc. lactis* strains examined expressed N^5–(carboxyethyl)ornithine synthase (Thompson, 1990) and our results displayed that this enzyme was only detected in nisin– or lantibiotic–producing strains of *Lc. lactis* IO–1, *Lc. lactis* NCDO 497 and *P.* sp. ISK–1 but not in *Lc. lactis* ATCC 19435^T and *Lb.* strain BL. So, it could be possible that there is the coexistence of the enzyme and the formation of nisin or lantibiotics. Determination of N^5–(carboxyethyl)ornithine synthase as a prescreening technique of nisin–producing strains should be considered

because of its convenience, ease and simpleness. Though Donkersloot and Thompson (1990) indicated that there were linked traits of the enzyme and nisin gene encoded on the chromosome of *Lc. lactis* K1. Still, more studies at molecular level should be done to explain this coexistence since there are so many different strains among *Lactococcus* which have already been known that they harbour a wide range of large and small plasmids which encode a variety of metabolic functions (Mckay, 1983).

LDH was also determined in this work for ensuring that cells prepared were completely broken under the controlled sonication since the difficulty of disruption of lactic acid bacteria. As shown in Table 2, all strains showed LDH activity leading to confirmation of certain cell extract of each organism. However, lower LDH activities were detected than be expected. Fructose-1, 6-diphosphate was an essential activator of LDH in several genus in lactic acid bacteria (Crow and Pritchard, 1977; de Vries *et al.*, 1970; Garvie, 1980; Rengpipat *et al.*, 1989; Wolin, 1964). Thus the optimized conditions should be repeatedly studied for the precise LDH activity.

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