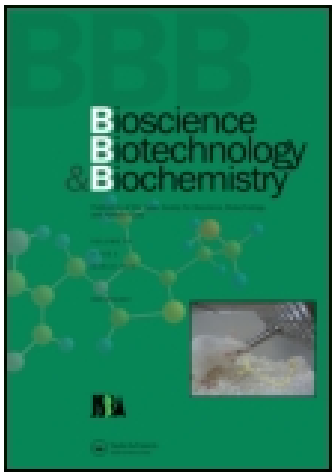


Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Conversion of a Cyanhydrin Compound into S-(-)-3-Phenyllactic Acid by Enantioselective Hydrolytic Activity of Pseudomonas sp. BC-18

Yoshihiro Hashimoto[a], Etsuko Kobayashi[a], Takakazu Endo[a], Makoto Nishiyama[ab] & Sueharu Horinouchi[ac]

[a] Central Research Laboratory, Nitto Chemical Industry Co., Ltd., 10-1 Daikoku-cho, Tsurumi-ku, Yokohama-shi, Kanagawa 230, Japan

[b] Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

[c] Department of Biotechnology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

Published online: 12 Jun 2014.

To cite this article: Yoshihiro Hashimoto, Etsuko Kobayashi, Takakazu Endo, Makoto Nishiyama & Sueharu Horinouchi (1996) Conversion of a Cyanhydrin Compound into S-(-)-3-Phenyllactic Acid by Enantioselective Hydrolytic Activity of Pseudomonas sp. BC-18, Bioscience, Biotechnology, and Biochemistry, 60:8, 1279-1283, DOI: 10.1271/bbb.60.1279

To link to this article: http://dx.doi.org/10.1271/bbb.60.1279

Biosci. Biotech. Biochem., **60** (8), 1279 1283, 1996

Conversion of a Cyanhydrin Compound into *S*-(−)-3-Phenyllactic Acid by Enantioselective Hydrolytic Activity of *Pseudomonas* sp. BC-18

Yoshihiro HASHIMOTO,† Etsuko KOBAYASHI, Takakazu ENDO, Makoto NISHIYAMA,* and Sueharu HORINOUCHI**

Central Research Laboratory, Nitto Chemical Industry Co., Ltd., 10–1 Daikoku-cho, Tsurumi-ku, Yokohama-shi, Kanagawa 230, Japan

**Biotechnology Research Center, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113, Japan*

***Department of Biotechnology, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113, Japan*

Received February 16, 1996

A *Pseudomonas* strain, named BC-18, which can convert racemic phenylacetaldehyde–cyanhydrin (3-phenyllactonitrile) enantioselectively to *S*-(−)-3-phenyllactic acid (*S*-PLA), was isolated from soil. Although PLA produced with intact cells contained the *S* enantiomers of approximately 75% enantiomeric excess (% *e.e.*), repeated crystallization gave a higher purity (99.8% *e.e.*) of the *S* configuration product. Production of *S*-PLA was significantly increased when 2.0% (w/v) of calcium chloride were added to the reaction mixture for precipitation of *S*-PLA. Chemical mutagenesis yielded a mutant strain, named BC348-9, with 16 times higher activity (40 mU/OD_{630}), compared with that of the parent strain (2.5 mU/OD_{630}). When the mutant strain BC348-9 was used, approximately 18 g/OD_{630} was produced, which is 12 times higher than that of the parent strain. The final accumulation of PLA exceeded 6.0%, 1.2 times higher than that of the parent strain.

Key words: (*S*)-3-phenyllactic acid; cyanhydrin; *Pseudomonas*; enantioselective hydrolysis

S-(−)-3-Phenyllactic acid (*S*-PLA) is a versatile precursor for the synthesis of several biologically important pharmacophores such as renin inhibitors,[1] protease inhibitors,[2] and anti-HIV reagents.[3] *S*-PLA can be synthesized from L-phenylalanine *via* diazonation followed by hydration in sulfuric acid.[4] The chemical synthesis method, however, gave a yield of about 50% due to production of a by-product, cinnamic acid, in a significant amount. Therefore, it is still difficult to synthesize *S*-PLA by the chemical method on an industrial scale. On the other hand, several microorganisms can convert nitrile compounds to the corresponding acid compounds by the action of nitrilase and/or a nitrile hydratase-amidase system. With these microorganisms, acrylamide is now produced on an industrial scale.[5,6] Furthermore, more recently, efficient processes to produce *R*-(−)-mandelic acid,[7–9] *S*-(+)-ibuprofen,[10] *O*-acetylmandelic acid,[11] and *S*-phenylglycine,[12] from the corresponding nitriles, were established using enantiospecific hydrolytic activity from various microorganisms. These findings suggested the possibility of establishing a new method for the production of *S*-PLA by using bio-transformation activity from microorganisms. Nitrilases and nitrile hydratases so far isolated, however, did not recognize α-hydroxy nitrile compounds, such as phenylacetaldehyde–cyanhydrin, which had one or more carbon atoms between the aryl group and the α-carbon. We therefore screened for a bacterial strain that could hydrolyze phenylacetaldehyde–cyanhydrin to *S*-PLA plus ammonia.

In this paper, we describe the construction of an efficient system for production of *S*-PLA from racemic phenylacetaldehyde–cyanhydrin using microorganisms. Isolation of a mutant strain that showed high-level production of *S*-PLA is also described.

Materials and Methods

Chemicals. Phenylacetaldehyde–cyanhydrin was kindly supplied by H. Sakai (The Ohtake Factory, Nitto Chemical Industry Co., Tokyo). *R*-3-Phenyllactic acid, *S*-3-phenyllactic acid, and *RS*-3-phenyllactic acid were purchased from Aldrich Chem. Co. (Milwaukee). Other nitrile compounds and their corresponding amides and acids were purchased from Tokyo Kasei Kogyo Co. (Tokyo). Yeast extract was a product of Difco Laboratory (Detroit). Polypeptone was of fine granules grade and obtained from Nihon Seiyaku Co. (Tokyo).

Bacterial strains and growth conditions. Pseudomonas sp. BC-18, which was isolated from soil,[13] was cultured aerobically with shaking at 30°C in medium I (20 g of glycerol, 2.5 g of polypeptone, 2.5 g of yeast extract, 3.6 g of Na_2HPO_4, 3.4 g of KH_2PO_4, 142 mg of Na_2SO_4, and 5 ml of metal mixture [8 g of $MgCl_2 \cdot 6H_2O$, 0.8 g of $CaCl_2$, 0.08 g of $MnSO_4 \cdot 4H_2O$, 0.013 g of $FeCl_3 \cdot 6H_2O$, and 0.009 g of $ZnSO_4 \cdot 7H_2O$ dissolved in 100 ml of distilled water] and 0.3 g of 1-pyrrolidinecarbonitrile per liter [pH 8.0]).

Assay of S-PLA-producing activity. PLA-producing activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 8.5), 15 mM phenylacetaldehyde–cyanhydrin, and an appropriate amount of the resting cells in a total volume of 1.0 ml. The reaction was done at 30°C for 2 h with moderate shaking and then stopped by centrifugation at 18,000 × *g* for 5 min. The PLA in the supernatant was measured by analytical high-pressure liquid chromatography (HPLC) (JASCO, Tokyo) with a Wakosil ODS 5C18 column (Wako Pure Chemicals, Tokyo) at a flow rate of 1.0 ml/min with the solvent system of 0.1 M phosphoric acid–acetonitrile (7 : 3, v/v), and the absorbance at 254 nm was monitored. The retention times for PLA, phenyllactamide, phenylacetaldehyde–cyanhydrin, and phenylacetaldehyde were 3.4, 2.1, 7.4, and 8.0 min, respectively. One unit of the enzyme activity was defined as the amount

† To whom correspondence should be addressed. Present address: *Nitto Chemical Industry Co., Ltd., 5–1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100, Japan.*

Abbreviations: *S*-PLA, (*S*)-3-phenyllactic acid; *R*-PLA, (*R*)-3-phenyllactic acid; PLA, 3-phenyllactic acid; HPLC, high-pressure liquid chromatography; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Tris, tris(hydroxymethyl)aminomethane; % *e.e.*, % enantiomeric excess; OD_{630}, optical density at 630 nm.

of enzyme that catalyzed the formation of 1 μmol of PLA per min. Specific activity was expressed as units per OD_{630}, where one OD_{630} unit was equivalent to 0.30 mg dry cell weight per ml.

Measurement of optical purity of S-PLA. The reaction mixture for production of *S*-PLA was the same as that described above but contained 20 mM phenylacetaldehyde–cyanhydrin in place of 15 mM phenylacetaldehyde–cyanhydrin, and the reaction was done at 30°C for 24 h with shaking. The optical purity of *S*-PLA was measured as follows. The reaction mixture was adjusted to pH 9.0 with 1 N NaOH, and unreacted substrate and phenyllactamide, which was probably produced through hydration by a nitrile hydratase, were extracted with ethyl acetate. The pH of the water layer was then adjusted to 2.0 with 1 N HCl, and PLA in the solution was extracted twice with ethyl acetate. The organic layer collected was desiccated by addition of sodium sulfate, filtered, and evaporated. The resultant solid materials were dissolved in distilled water and assayed by HPLC with a MCI gel CRS-10W column (Mitsubishi Chemical Co., Tokyo) at a flow rate of 1.0 ml/min and with a solvent system of 2 mM $CuSO_4 \cdot 5H_2O$–acetonitrile (85 : 15, v/v). *R*- and *S*-PLA were eluted at the retention times of 20.3 and 28.6 min, respectively.

Preferential crystallization of S-PLA. PLA with about 75% *e.e.* obtained in this study was dissolved in water at 50°C. The solution was then cooled gradually until white crystals emerged, and the solid material was collected by filtration and dried. Crystals were again dissolved in water and recrystallized in the same way. The optical purity of *S*-PLA thus obtained was measured by HPLC as described above.

Addition of several metal ions to precipitase S-PLA. Several mono-, di-, and trivalent metal ions (final concentration; 5% [w/v]) were added as a precipitant to the 5% of ammonium PLA salt solution that was prepared from the mixture of PLA and equimolar solution of ammonium, and the pH was adjusted to 8.5. After incubation for 10 min, the precipitate was removed by centrifugation and the PLA remaining in the supernatant was measured by HPLC as described above.

Mutagenesis. Cells of *Pseudomonas* sp. BC-18 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) by the method of Ryuno *et al.*[14] The cells in the exponential phase were harvested, washed twice with 50 mM Tris/malate buffer (pH 6.0), and then resuspended in the same buffer (10^8–10^9 cells per ml). NTG was then added to the suspension at a final concentration of 0.05% (w/v). After incubation at 30°C for 1 h, the cells were collected by centrifugation, washed twice with same buffer and then suspended in medium I. The survival rate was about 95%. The mutagenized cells were aerobically incubated at 30°C for 24 h and spread on medium II (5 g of glycerol, 1.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 142 mg of Na_2SO_4, and 5 ml metal mixture) agar plate with a nitrile compound and 10 ml of the vitamin mixture [2.0 mg of thiamin, 2.0 mg of nicotinamide, 2.0 mg of riboflavin, 2.0 mg of *p*-aminobenzoic acid, 2.0 mg of pyridoxine hydrochloride, 20.0 mg of calcium pantothenate, 0.2 mg of biotin, and 0.2 mg of folic acid dissolved in 100 ml of distilled water] per liter (pH 8.0).

Accumulation of PLA by fed-batch system. The initial reaction mixture (300 ml) consisted of 20 mM phenylacetaldehyde–cyanhydrin, 20 mM Tris–HCl (pH 8.5), 2.0% $CaCl_2$, and an appropriate amount of the resting cells. The concentration of substrate was kept at 10–20 mM in the reactor by controlling the feeding interval of the substrate, using a cyan ion sensor (PHL-20, Denkikagakukogyo, [Tokyo]). Because the cyanhydrin was partially dissociated into the corresponding aldehyde and hydrogen cyanide, therefore, the concentration of phenylacetaldehyde–cyanhydrin was estimated from the concentration of free cyan ion by using the sensor. During the reaction, the pH was controlled at 8.4 to 8.5 by the addition of 1 N NaOH or 1 N HCl using a pH controller (NPH-680D, Nissin-rika, [Tokyo]). Figure 1 illustrates the schematic diagram of the PLA fed-batch system.

Results

We found that *Pseudomonas* sp. BC-18 produced *S*-PLA with high optical specificity,[13] and to optimize the culture conditions, 0.03% (v/v) 1-pyrrolidinecarbonitrile was the most effective inducer for the production of the enzyme.

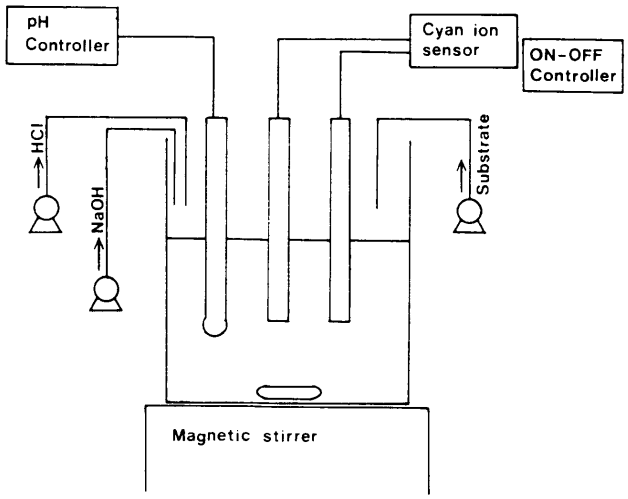


Fig. 1. Schematic Diagram of the PLA Fed-batch System.

Table I. Effects of pHs on the Optical Purity and the Enzyme Activity

pH	Reation time : 4 h		Reaction time : 21 h	
	PLA produced (mM)	Optical purity (% *e.e.*)	PLA produced (mM)	Optical purity (% *e.e.*)
6.0	6.28	63.9	12.4	21.1
6.5	5.90	67.4	11.4	42.5
7.0	5.96	66.3	11.2	48.0
7.5	5.20	68.1	9.14	63.8
8.0	4.82	71.3	9.78	66.3
8.0*	5.05	72.0	11.0	61.4
8.5*	3.91	69.6	14.1	74.1
9.0*	3.46	76.6	13.9	73.8

* 50 mM borate buffer was used for the reaction.

Sixteen mU of the resting cells and 20 mM of substrate (phenylacetaldehyde–cyanhydrin) were used in this experiment.

Reaction conditions for production of S-PLA by resting cells

Although the enzyme in strain BC-18 preferentially recognized phenylacetaldehyde–cyanhydrin with the *S*-configuration as the substrate, it was capable of hydrolyzing *R*-phenylacetaldehyde–cyanhydrin but at low efficiency. This prompted us to optimize the reaction conditions for the maximum production of *S*-PLA. When phenylacetaldehyde–cyanhydrin was incubated with intact BC-18 cells for 4 h at various pHs from 6 to 9, optical purity between 63.9% *e.e.* and 76.6% *e.e.* was obtained (Table I). Prolonged incubation for 21 h gave higher production of total PLA but with lower optical purity, especially under lower pH conditions. We chose pH 8.5 for pH to produce *S*-PLA.

Reaction temperature was also thought to be one of the important factors to produce *S*-PLA. We therefore examined the effects of temperatures from 10°C to 40°C on the production of *S*-PLA. *S*-PLA with highest optical purity (79.1% *e.e.*) was produced around at 30°C (data not shown).

We then examined the effects of the concentration of the substrate, phenylacetaldehyde–cyanhydrin, on the initial enzyme activity. After incubation of resting cells with various concentrations of the substrate for 1 h, the amount of PLA produced were measured. The enzyme showed the maximum activity in the presence of the substrate at an

initial concentration of 15 mM.

When the resting BC-18 cells were incubated with 20 mM phenylacetaldehyde for 1 h, the remaining activity was decreased by 73.6% of the original activity (data not shown). This suggested that phenylacetaldehyde, which could be released from phenylacetaldehyde–cyanhydrin through dissociation, would inactivate the enzyme. On the other hand, the dissociation of substrate was estimated to be about 10–15% on the initial reaction mixture from the concentration of free cyan ion by using a cyan ion sensor (data not shown).

We next examined the effects of several cations on the production of PLA, because cations were known to precipitate α-hydroxy acid compounds accumulated in large amounts,[15] which would inactivate the enzyme responsible for the production of PLA. For this purpose, several metal ions were added to the reaction mixture. Among 12 mono-, di-, and trivalent cations tested, the amount of PLA remaining in the supernatant was smallest when $CaCl_2$ was used as the precipitant (Table II). We added $CaCl_2$ at various concentrations to the reaction mixture and analyzed its effect on PLA production. As shown in Table III, $CaCl_2$ (1–3%) significantly increased the amount of PLA produced. Under the optimized reaction conditions, higher PLA production, up to approximately 1.5 g/OD_{630}, was obtained.

Table II. Effects of Various Mono, Di-, and Trivalent Metal Ions on 5% of Ammonium PLA Salt Solution

Metal salt	PLA remaining in the supernatant solution (%)
None	100
$CaCl_2$	4.0
$SnCl_2$	33.2
$Al_2(SO_4)_3 \cdot 16$–$18H_2O$	84.0
$MnSO_4 \cdot 4H_2O$	75.4
$FeSO_4 \cdot 7H_2O$	45.0
$Fe_2(SO_4)_3 \cdot XH_2O$	87.0
$CoSO_4 \cdot 7H_2O$	42.7
$NiSO_4 \cdot 6H_2O$	51.6
$Cu(NO_3)_2 \cdot 3H_2O$	39.6
$AgNO_3$	44.0
$Zn(NO_3)_2 \cdot 6H_2O$	45.8
$Pd(NO_3)_2$	69.5

Table III. Effects of $CaCl_2$ Concentrations on the Initial Activity and PLA Productivity of the Resting Cells

Concentration of $CaCl_2$ (%)	Relative acidity (%)	PLA productivity (g/OD_{630})
0.0	100	0.56
0.25	100	NT
0.50	108	0.84
0.75	103	NT
1	103	1.38
2	100	1.47
3	100	1.31
4	99	0.83
5	91	NT

The productivity of PLA per cells was estimated by the amount of PLA produced after 120 h of incubation in a reaction mixture containing 0.6 mg of the resting cells (OD_{630} = 2.0) (2.8 mU), 50 mM Tris–HCl (pH 8.5), and 20 mM and 10 mM phenylacetaldehyde–cyanhydrin were fed at 0 h and 48 h, respectively.

Isolation of mutants showing higher enzyme activity

For high-level *S*-PLA production, *Pseudomonas* sp. BC-18 was mutagenized with NTG as described in Materials and Methods. We chose mutant strains showing faster growth on medium II plates supplemented with a nitrile compound, adiponitrile, pimelonitrile, isobutyronitrile, cyclopropylcyanide, glutaronitrile, 3-methoxypropionitrile, 3,3-dimethoxypropionitrile, or 2-methyl-3-butenenitrile, each of which was known to give slow growth, as a sole nitrogen source. Among them, a mutant strain, BC-18-348, which was chosen as a strain showing faster growth on the 0.1% adiponitrile-containing plate, had higher specific activity (25 mU/OD_{630}) than that of the parent strain BC-18 (2.5 mU/OD_{630}).

For further improvement of PLA productivity, strain BC-18-348 was again mutagenized with NTG. Growth of the parental strain BC-18 as well as its mutant BC-18-348 was inhibited by cinnamic acid, *o*-iodobenzoic acid, and *m*-chlorobenzoic acid, all of which were analogs of *S*-PLA, when several nitrile compounds were used as nitrogen source. But these product analogs could not inhibit the growth of the parental strain when the minimum medium II containing ammonia as a nitrogen source instead of nitrile compounds was used. This suggested that these product analogs would inactivate or inhibit the enzyme. We therefore screened for mutant strains showing growth on medium II agar plate containing 0.1% pimelonitrile and the product analogs 10 mM cinnamic acid, 5 mM *o*-iodobenzoic acid, or 10 mM of *m*-chlorobenzoic acid. Only a few colonies showed both product-analog resistance and fast growth on the nitrile plate. Among them, a mutant BC348-9 showed the highest specific activity (40 mU/OD_{630}), which was 16 times higher than that of the parent strain BC-18 (2.5 mU/OD_{630}).

Accumulation of PLA with mutants

We next examined the production of PLA by fed-batch system as described in Materials and Methods (Fig. 1). When the mutant strain BC348-9 was used, approximately 18 g PLA/OD_{630} was produced, which was 12 times higher than that of the parent strain BC-18 (1.5 g PLA/OD_{630}) (Fig. 2). And by using the mutant BC 348-9, the final accumulation of PLA also exceeded 6.0%, which was 1.2 times higher than that of the parent strain BC-18 (5.0%). The optical purity of PLA produced by this mutant, BC348-9, was the same (about 75% *e.e.*) as that produced by the parent strain, BC-18.

Since the optical purity of *S*-PLA produced was still low, we tried to purify *S*-PLA from the mixture containing the *R*- and *S*-forms by preferential crystallization as described in Materials and Methods. By crystallizing the PLA twice from the water phase, the optical purity was increased to 99.8% *e.e.*

Discussion

For these ten years, microbial nitrile degradation have been extensively studied and used for industrial production of a commodity chemical, acrylamide.[5,6] Recently, a similar technique is expanded for the commercial production of

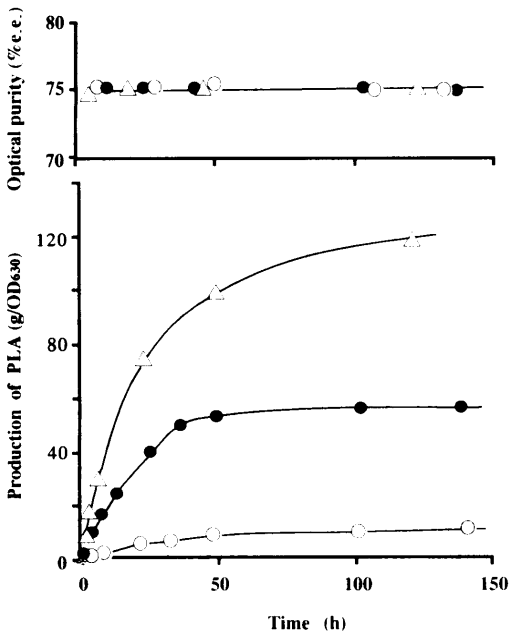
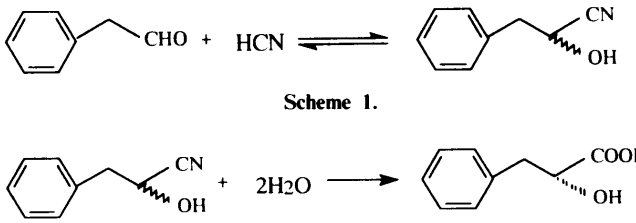


Fig. 2. Fed-batch Reaction for PLA Production.

(○), *Pseudomonas* sp. BC-18 (10.5 mg dry cell, $OD_{630}=35$); (●), mutant BC-18-348 (2.1 mg dry cell, $OD_{630}=7$); (△), mutant BC348-9 (1.05 mg dry cell, $OD_{630}=3.5$).

R-(−)-mandelic acid from the corresponding racemic benzaldehyde–cyanhydrin (mandelonitrile) in our company.[7] PLA, an α-hydroxy carboxylic acid compound with a phenyl group, had a structure identical to that of mandelic acid, although PLA has an additional carbon atom between the phenyl group and its asymmetric carbon atom. Therefore, we expected that the microorganisms used for the production of *R*-(−)-mandelic acid might be available for the production of *S*-PLA. The nitrilase and/or nitrile hydratase–amidase contained in this microorganisms, however, showed low hydrolytic activity against phenylacetaldehyde–cyanhydrin with low enantioselectivity. Although several nitrile-degrading systems in microorganisms were found to have the ability to produce *S*-(+)-ibuprofen,[10] *O*-acetylmandelic acid,[11] and *S*-phenylglycine,[12] from the corresponding nitriles, nitrilases so far isolated showed no phenylacetaldehyde–cyanhydrin hydrolyzing activities. From these points, the nitrile-degrading enzyme(s) found in this study have novel enzymatic properties. The enzymatic properties of nitrilase and nitrile hydratase in *Pseudomonas* sp. BC-18 will be characterized in future.

In this study, *S*-PLA was produced with high enantioselectivity (about 75% *e.e.*), and *S*-PLA with 99.8% *e.e.* was prepared by preferential crystallization. Since phenylacetaldehyde–cyanhydrin with the *S*-configuration was converted to *S*-PLA, one might think that the substrate with the *R*-configuration would remain in the reaction mixture. However this was not the case, because the cyanhydrin compound was partially dissociated into the corresponding aldehyde plus hydrogen cyanide and therefore phenylacetaldehyde–cyanhydrin with the *R*-configuration was racemized during incubation according to the dissociation equilibrium (Scheme 1). Dissociation of phenylacetaldehyde–cyanhydrin to the corresponding aldehyde plus hydrogen cyanide showed pH dependence and the racemic reaction itself was faster under alkaline



Scheme 1.

Scheme 2.

conditions, although the PLA-producing reaction (Scheme 2) by the responsible enzyme was accelerated under acidic conditions. As a result, *S*-PLA with highest optical purity was obtained around pH 8.5.

Metal ions are known to precipitate α-hydroxy acids by forming a complex. In this study, addition of calcium ion actually led to significant improvement of *S*-PLA production probably by decreasing the amount of *S*-PLA remaining in the reaction mixture through its precipitation. This may suggest that addition of metal ions would be effective for the microbial production of other α-hydroxy acid compounds.

Although the chemical mutagenesis gave mutants showing high *S*-PLA-producing activity, no overproduction of a specific protein was observed in the mutants, as observed by SDS–polyacrylamide gel electrophoresis of the cell lysate (data not shown). This suggests that the high *S*-PLA-producing activity was caused mainly by an increase in the enzyme activity itself. We expected that a mutant resistant to the product analogs might contain enzyme(s) more tolerant to a high concentration of PLA. But we surprised that the obtained mutant (BC-348-9) showed both product-analog resistance and fast growth on the nitrile plate, these properties accommodated the mutant to increase the final concentration of PLA, to improve the productivity per cell and to increase the specific activity.

The nitrilase genes from several microorganisms were cloned,[16-18] and their overproduction (more than 40% of the total soluble protein) was obtained under the control of the *lac* promoter in *Escherichia coli*.[19] On the other hand, using the host-vector system for a *Rhodococcus* strain we established,[20] the nitrile hydratase and the amidase from *Rhodococcus* sp. N-774 were over produced to show more than 40% and 23% of the total soluble protein, respectively.[21] Genetic engineering thus enabled us to obtain the enzymes involved in nitrile degradation in a large amount. Therefore, it would be possible to construct an efficient system for overproduction of the gene product(s) responsible for *S*-PLA production using genetic engineering techniques. At present, it is unclear which nitrile degradation system, nitrilase or nitrile hydratase–amidase, has a major role in producing *S*-PLA by *Pseudomonas* sp. BC-18. Therefore, further work is required to discover the principal enzyme responsible for *S*-PLA production.

Acknowledgments. We are grateful to Masaru Suto (Central Research Laboratory, Nitto Chemical Industry Co., Ltd.) for his kind guidance, valuable discussion, and encouragement during this study. We are grateful to Koji Tamura, Yuji Hirata, Makoto Kaneko, and Fumiaki Watanabe (Central Research Laboratory, Nitto Chemical Industry Co., Ltd.) for their kind support and helpful suggestions.

References

1) K. Hemmi, M. Neya, H. Marusawa, K. Imai, N. Kayakiri, and M. Hashimoto, U.S. Patent, 4921855 (May 1, 1990).
2) S. Kano, Y. Yuasa, T. Yokomatsu, and S. Shibuya, *J. Org. Chem.*, **53**, 3865–3868 (1988).
3) K. Budt, J. Hansen, J. Knolle, C. Meichsner, A. Paessens, D. Ruppert, B. Stowasser, and K. H. Budt, D. E. Patent, 4030350 (Apr. 11, 1991).
4) F. J. Urban and B. S. Moore, *J. Heterocyclic Chem.*, **29**, 431–438 (1992).
5) T. Nagasawa, H. Shimizu, and H. Yamada, *Appl. Microbiol. Biotechnol.*, **40**, 189–195 (1993).
6) T. Nagasawa and H. Yamada, *Trends Biotechnol.*, **7**, 153–158 (1989).
7) T. Endo, T. Yamagami, and K. Tamura, U.S. Patent 5326702 (Jul. 5, 1994).
8) K. Yamamoto, K. Oishi, I. Fujimatu, and K. Komatsu, *Appl. Environ. Microbiol.*, **57**, 3028–3032 (1991).
9) K. Yamamoto, I. Fujimatsu, and K. Komatsu, *J. Ferment. Bioeng.*, **73**, 425–430 (1992).
10) K. Yamamoto, Y. Ueno, K. Otsubo, K. Kawakami, and K. Komatsu, *Appl. Environ. Microbiol.*, **56**, 3125–3129 (1990).
11) N. Layh, A. Stolz, S. Forster, F. Effenberger, and H. Knackmuss, *Arch. Microbiol.*, **158**. 405–411 (1992).
12) S. Y. Choi and Y. M. Goo, *Arch. Pharm. Res.*, **9**, 45–47 (1986).
13) Y. Hashimoto, T. Endo, K. Tamura, and E. Kobayashi, Japan Kokai Tokkyo Koho, 6-284899 (Oct. 11, 1994).
14) K. Ryuno, T. Nagasawa, and H. Yamada, *Agric. Biol. Chem.*, **52**, 1813–1816 (1988).
15) N. Yoshida and Y. Murooka, *J. Ferment. Bioeng.*, **77**, 636–641 (1994).
16) M. Kobayashi, H. Izui, T. Nagasawa, and H. Yamada, *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 247–251 (1993).
17) M. Kobayashi, H. Komeda, N. Yanaka, T. Nagasawa, and H. Yamada, *J. Biol. Chem.*, **267**. 20746–20751 (1992).
18) M. Kobayashi, N. Yanaka, T. Nagasawa, and H. Yamada, *Biochemistry*, **31**, 9000–9007 (1992).
19) M. Kobayashi and S. Shimizu, *FEMS Microbiol. Lett.*, **120**, 217–224 (1994).
20) Y. Hashimoto, M. Nishiyama, F. Yu, I. Watanabe, S. Horinouchi, and T. Beppu, *J. Gen. Microbiol.*, **138**, 1003–1010 (1992).
21) Y. Hashimoto, M. Nishiyama, S. Horinouchi, and T. Beppu, *Biosci. Biotech. Biochem.*, **58**, 1859–1865 (1994).