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Preparation of Optically Active Compounds related to Pyrethroids using Biocatalysts

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Preface

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies described here have been carried out in the Biotechnology Laboratory, Takarazuka Research Center, Sumitomo Chemical Co., during 1980-1991.

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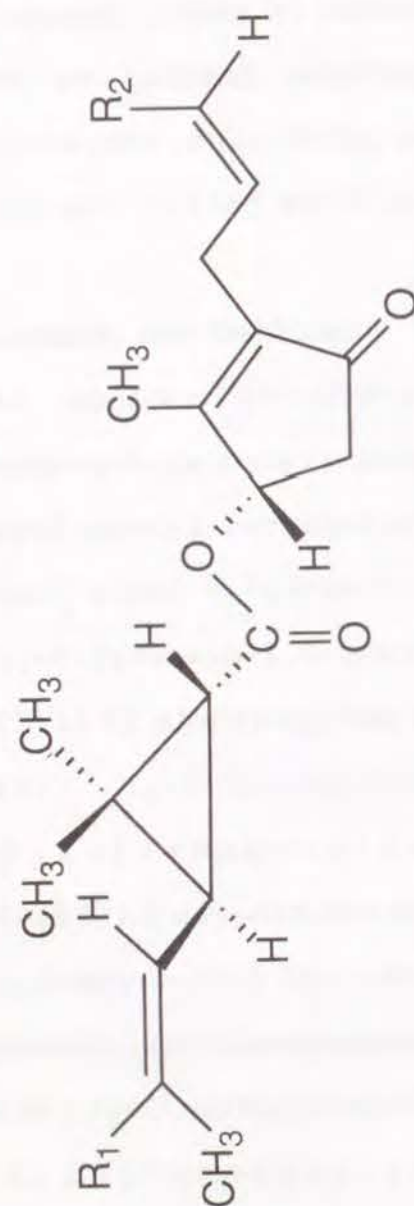
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Introduction

Pyrethroids

Pyrethrum, the powdered flower-heads of *Chrysanthemum cinerariaefolium*, and the pyrethrins extracted from these flowers have been used as insecticides for many hundreds of years, possibly since the first century AD.¹⁾ Investigations leading to the elucidation of the chemical structure of the pyrethrins, the active principles of pyrethrum, occurred during the first half of the 20th century.

Staudinger et al.²⁾ and LaForge et al.³⁾ clarified the chemical structure of the pyrethrins. The pyrethrins include six insecticidally active compounds that are substituted cyclopropane carboxylic acid esters with 4-hydroxy-2-cyclopenten-1-ones (Fig. 1). Main compounds are pyrethrin I {(1*S*)-2-methyl-4-oxo-3-[(*Z*)-2,4-pentadienyl]-2-cyclopenten-1-yl (1*R*)-*trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate} and pyrethrin II {(1*S*)-2-methyl-4-oxo-3-[(*Z*)-2,4-pentadienyl]-2-cyclopenten-1-yl (1*R*)-*trans*-3-[(*E*)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate}, and others are cinerin I {(1*S*)-3-[(*Z*)-2-butenyl]-2-methyl-4-oxo-2-cyclopenten-1-yl (1*R*)-*trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate}, cinerin II {(1*S*)-3-[(*Z*)-2-butenyl]-2-methyl-4-oxo-2-cyclopenten-1-yl (1*R*)-*trans*-3-[(*E*)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate}, jasmolin I {(1*S*)-2-methyl-4-oxo-3-[(*Z*)-2-pentenyl]-2-cyclopenten-1-yl (1*R*)-*trans*-2,2-dimethyl-3-(2-



R ₁ : -CH ₃	R ₂ : -CH=CH ₂	Pyrethrin-I (1S)-2-Methyl-4-oxo-3-[(Z)-2,4-pentadienyl]-2-cyclopenten-1-yl (1R)-trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate
R ₁ : -CH ₃	R ₂ : -CH ₃	Cinerin-I (1S)-3-[(Z)-2-Butenyl]-2-methyl-4-oxo-2-cyclopenten-1-yl (1R)-trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate
R ₁ : -CH ₃	R ₂ : -CH ₂ -CH ₃	Jasmolin-I (1S)-2-Methyl-4-oxo-3-[(Z)-2-pentenyl]-2-cyclopenten-1-yl (1R)-trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate
R ₁ : -COOCH ₃	R ₂ : -CH=CH ₂	Pyrethrin-II (1S)-2-Methyl-4-oxo-3-[(Z)-2,4-pentadienyl]-2-cyclopenten-1-yl (1R)-trans-3-[(E)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate
R ₁ : -COOCH ₃	R ₂ : -CH ₃	Cinerin-II (1S)-3-[(Z)-2-Butenyl]-2-methyl-4-oxo-2-cyclopenten-1-yl (1R)-trans-3-[(E)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate
R ₁ : -COOCH ₃	R ₂ : -CH ₂ -CH ₃	Jasmolin-II (1S)-2-Methyl-4-oxo-3-[(Z)-2-pentenyl]-2-cyclopenten-1-yl (1R)-trans-3-[(E)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate

Fig. 1. Chemical structures of pyrethrins.

Introduction

methyl-1-propenyl)cyclopropanecarboxylate} and jasmolin II {(1S)-2-methyl-4-oxo-3-[(Z)-2-pentenyl]-2-cyclopenten-1-yl (1R)-trans-3-[(E)-2-methoxycarbonyl-1-propenyl]-2,2-dimethylcyclopropanecarboxylate}. Alcohol moieties of these compounds have S configuration.⁴⁾ Both absolute configurations of C-1 and C-3 in acid moieties are R, and the stereoisomer type is generally called (+)-trans (Fig. 2).⁵⁾

The pyrethrins as insecticides have some desirable features including the exceptional knock-down effect against insect pests, the broad specificity in control of various insect pests, the low toxicity for mammals, and the rapid biodegradation in the environment.

As the knowledge progressed over this period, so also did the development of pyrethroids which are synthetic analogues of the pyrethrins. The first industrially available pyrethroid was allethrin [(1RS)-2-methyl-4-oxo-3-(2-propenyl)-2-cyclopenten-1-yl (1RS)-cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylate] which is the ester of (1RS)-cis,trans-chrysanthemic acid [(1RS)-cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylic acid] with allethrolone [(RS)-4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one] (Fig. 3).⁶⁾ One can see that pyrethrin I, cinerin I, jasmolin I, and allethrin are all closely related chemically, differing only in the nature of the side chain on their alcoholic rings.

Following the discovery of allethrin, various types of pyrethroids have been developed.⁷⁾ Some examples of pyrethroids are

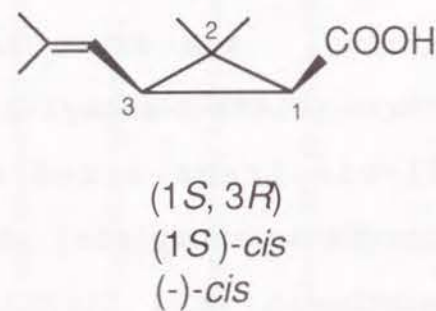
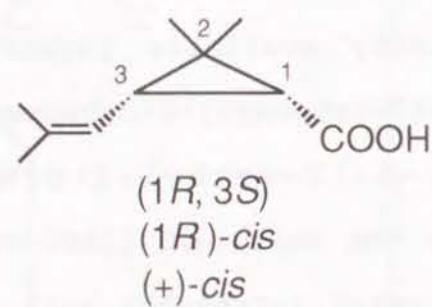
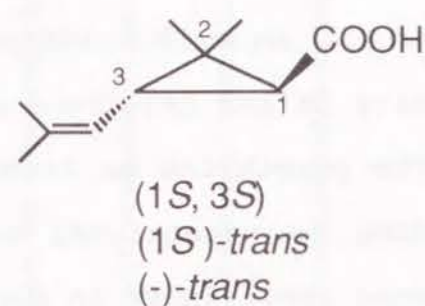
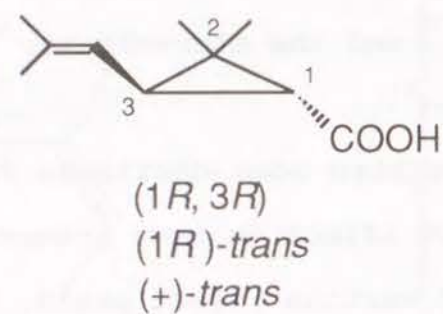
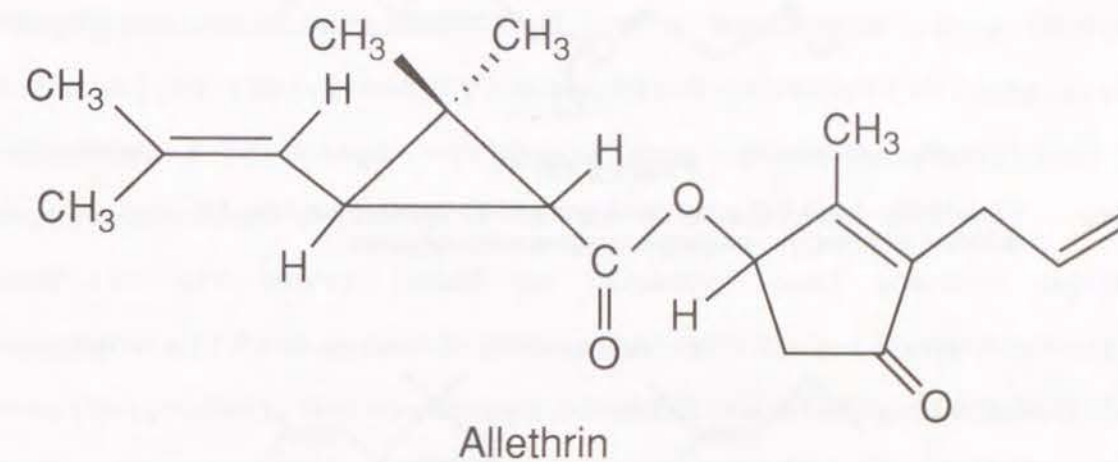
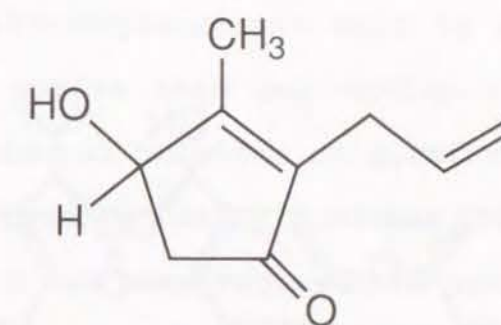


Fig. 2. Stereoisomers of chrysanthemic acid.

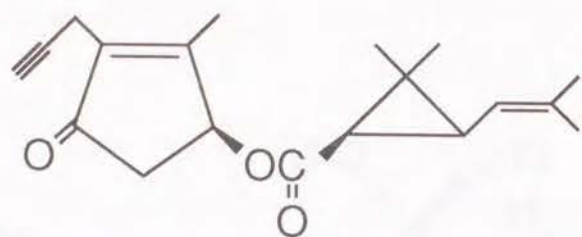


(1*RS*)-2-Methyl-4-oxo-3-(2-propenyl)-2-cyclopenten-1-yl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate



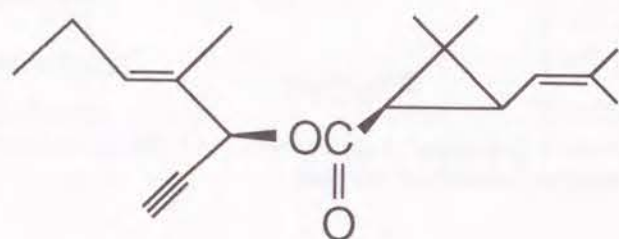
(*RS*)-4-Hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one

Fig. 3. Chemical structures of allethrin and allethrolone.



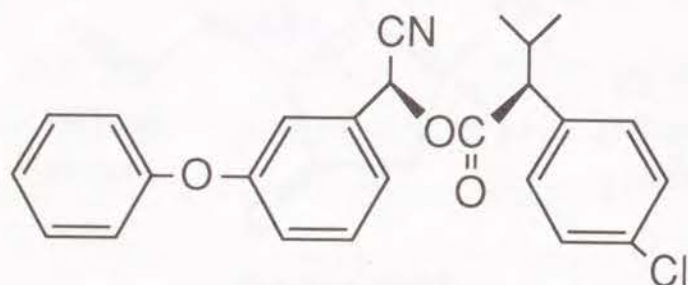
Prallethrin

(*S*)-2-Methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1*R*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate



Empenthrin (the most potent form)

(*E*)-(*S*)-1-Ethynyl-2-methyl-2-pentenyl (1*R*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate



Esfenvalerate

(*S*)- α -Cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate

shown in Fig. 4.⁸⁾ Prallethrin [(*S*)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1*R*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate] is a homologue of allethrin. Empenthrin [(*E*)-(*RS*)-1-ethynyl-2-methyl-2-pentenyl (1*R*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylate] has 1-ethynyl-2-methyl-2-penten-1-ol as its alcohol moiety. These pyrethroids are mostly used as 'indoor' pest control agents. Esfenvalerate [(*S*)- α -cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate], the structure of which is widely different from that of natural pyrethrin, has an advantage of photo-stability and is suitable for agricultural 'outdoor' use.⁹⁾

Recently, much attention has been drawn to the relationships between the absolute stereochemistry of pyrethroids and the insecticidal activity of them.^{10,11)} It is well-known that the pyrethroid having (1*R*)-chrysanthemic acid as its acid moiety is insecticidally more active than one having (1*S*)-acid moiety.¹²⁾ With regard to the alcohol moieties of pyrethroids shown in Fig. 4, (*S*)-isomers are insecticidally dominant over the respective enantiomers.⁸⁾ So, it has been required to prepare the optically active form (the insecticidally active form) of these pyrethroids.

Chemical compounds having asymmetric carbon prepared via traditional organic synthesis are usually racemic. So, various chemical methods for asymmetric synthesis and resolution of racemate have been developed to prepare the most potent form of the pyrethroid. However, in general, chemical methods are too complicated or require expensive chiral chemical reagents, so that

Fig. 4. Chemical structures of typical synthetic pyrethroids.

they are hardly applicable to practical process. Therefore, development of more efficient and practicable methods for industrial preparation of optically active pyrethroid alcohols and (1R)-chrysanthemic acid had been required.¹³⁾

Enzymes as catalysts in organic syntheses

It is well-known that many enzymes catalyze not only the conversion of their natural substrates, but also accept and convert structurally related unnatural compounds. Application of enzymatic catalyses to organic syntheses has been of interest.¹⁴⁻¹⁹⁾ It must be noticed that enzymes produced by microbial cells are substantially responsible for catalyses when microbial cells are used as catalysts in organic syntheses. The properties of the enzymes as catalysts in organic syntheses would fulfil many requirements of the organic chemist (Table 1). The most advantage of the enzymatic catalysis lies in its stereoselectivity. It was proved by the fact that most researches on application of enzymes to organic syntheses have been concentrated upon the preparation of optically active compounds which is not easily accomplished by traditional chemical methods.

On the other hand, the enzymes have some disadvantages in regard to catalysts in organic syntheses (Table 2). Recently, some works have brought good results in overcoming the disadvantages of enzymes. Use of organic media for the enzymatic reaction had been desired because most organic compounds used in organic syntheses have low solubilities in aqueous media. The techniques to use

Table 1. Advantages of enzymes as catalysts in organic syntheses.

-
- ⊙ Enzymes catalyze a wide variety of organic reactions.
 - The diversity and complexity of organic molecules in nature is a reflection of the power of enzymes as organic catalysts.
 - ⊙ Enzymes are enantioselective.
 - Enzymes usually distinguish between different stereoisomers (enantiomers, diastereomers).
 - ⊙ Enzymes are regioselective.
 - Enzymes generally attack the substrate molecule at the same site.
 - ⊙ Enzymes are chiral catalysts.
 - A new chirality center can be arisen as a result of an enzymatic reaction.
 - ⊙ Enzymes are effective under mild conditions.
 - Enzymatic reactions take place in aqueous media at low temperatures and at pH values near neutrality.
 - ⊙ Enzymes are reaction-selective.
 - The catalytic reactivity of an enzyme is usually restricted to a single reaction type, so that no side reaction takes place.
-

Table 2. Disadvantages of enzymes as catalysts in organic syntheses.

-
- Enzymes are inactivated by many organic reagents, under strong acidic or basic conditions, and at high temperatures.
 - Enzymes exhibit a limited activity in most organic solvents.
 - Enzymes are subject to product- and substrate-inhibition.
-

enzymes in organic media have been advancing remarkably for recent years.²⁰⁾ It has been found that some enzymes including lipases retained and exhibited their catalytic activities even in non-aqueous media. Chemical modification of enzymes with polyethylene glycol has enabled the dissolution of enzymes and the homogeneous reaction in organic media.²¹⁾ Furthermore, the advanced technique of protein engineering will make possible to develop the ideal enzymes desired in organic syntheses.²²⁾

Thus, it is expected that opportunities and importance of enzymatic reactions in organic syntheses will increase in future.

Application of lipase- and esterase-catalyzed reactions for optical resolution

The terminology in distinction between "lipases" and "esterases" is slightly complicated. Lipases have been the name of esterases that could hydrolyze lipids. So, the lipases can be defined as "long chain fatty acid ester hydrolases" or "any esterases capable of hydrolyzing esters of oleic acid".^{23,24)} The characteristic property of lipases is the high activity toward water-insoluble substrates. In classical experiments, Sarda and Desnuelle²⁵⁾ demonstrated that a stepwise increase in activity occurred for a "lipase", but not for an "esterase", when the solubility limit of the substrate was surpassed. Thus, the presence of an insoluble substrate-water interface is in some way affecting catalyses of lipases.

Both lipases and esterases are acceptable into routine use as

catalysts in organic synthesis because they are relatively stable, readily available and require no cofactor for their catalyses and acceptability of a broad structural range of substrates. Especially, the ability of these enzymes that discriminate between enantiomers is greatly advantageous to optical resolution and asymmetric synthesis for the preparations of optically active alcohols, carboxylic acids and carboxylic esters.²⁶⁻³⁰⁾

In recent years, the technique of using lipase and esterase in organic media has remarkably progressed. The equilibrium in the enzymatic reaction shifts to the esterification in non-aqueous media. It was found that some lipases and esterases exhibited a great stability and sufficient activity in non-aqueous organic media, contrary to the common theory that enzymes are unstable in organic media.³¹⁾ Enantioselectivity of lipase on racemic alcohols or acids enabled the asymmetric synthesis of optically active esters.^{26,32,33)} Lipase (esterase)-catalyzed esterification in organic media increased markedly the potentiality of lipase (esterase) as catalyst in organic synthesis. Now, researches in application of lipase (esterase) to organic synthesis are becoming more and more energetic.

In this thesis, applications of biocatalytic stereoselective reactions (using enzymes and microbial cells) to resolutions of secondary alcohols and chrysanthemic acid were attempted in order to prepare insecticidally active form of pyrethroids. Lipase-catalyzed enantioselective hydrolyses were examined for resolution

of 4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one and 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile.^{34,35)} Lipase-catalyzed enantioselective esterification in organic solvent was studied for resolution of (*E*)-1-ethynyl-2-methyl-2-penten-1-ol.³⁶⁾ Furthermore, efficient chemico-enzymatic processes including lipase-catalyzed hydrolyses and repeated use of lipases were presented from practical points of view. In order to prepare (1*R*)-*trans*-chrysanthemic acid, diastereoselective hydrolysis of ethyl (1*RS*)-*cis,trans*-chrysanthemate [ethyl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate] with microbial esterase (using microbial cells) was investigated, and the microorganism which hydrolyzed diastereoselectively ethyl (1*R*)-*trans*-chrysanthemate to afford (1*R*)-*trans*-chrysanthemic acid was found out.³⁷⁾

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Synopsis

Part 1. Lipase-catalyzed Enantioselective Hydrolysis of 4-Acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one

Chapter 1. Preparation of (S)-4-Hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one using *Arthrobacter* Lipase

Enzyme-catalyzed enantioselective hydrolysis of (RS)-4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one [(RS)-HMPC acetate] was examined for the preparation of the optically active alcohol moiety of synthetic pyrethroids. Through screening tests on microorganisms and commercially available lipases, it was found that several bacterial lipases hydrolyzed HMPC acetate with high enantioselectivity and reaction rate. The *Arthrobacter* lipase gave (R)-4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one [(R)-HMPC] and (S)-HMPC acetate at a hydrolysis of 50% in a two-liquid phase reaction system of water and the water-insoluble substrate. The hydrolysis proceeded even at a substrate concentration of 80w/v%. The enantioselectivity was not affected by the chain length of the acid moiety of the ester. By combination of the enzymatic resolution with a chemical inversion of (R)-HMPC into (S)-HMPC, an efficient process was developed for the total conversion of racemic HMPC to (S)-HMPC which is an important alcohol moiety of a pyrethroid that is prallethrin [(1S)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1R)-cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate].

Chapter 2. Studies on Enantioselective Hydrolysis of 4-Acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one with *Arthrobacter* Lipase

Characteristics of the enantioselective hydrolysis of HMPC acetate by the *Arthrobacter* lipase were investigated in a two-liquid phase reaction mixture of water and the water-insoluble substrate. Kinetic studies on enzymatic hydrolysis of optically active HMPC acetate revealed that the strict enantioselectivity was entirely due to a difference between the catalytic constants for the enantiomeric substrates and (S)-HMPC acetate acted as a competitive inhibitor. The comparison of enantioselectivity for acetic acid esters with analogues of HMPC indicated that hydrophobic substituents of the HMPC molecule were essential for the strict enantioselectivity.

Part 2. Enantioselective Hydrolysis of 2-Acetoxy-2-(3-phenoxyphenyl)acetonitrile with *Arthrobacter* Lipase

Lipase-catalyzed enantioselective hydrolysis of (RS)-2-acetoxy-2-(3-phenoxyphenyl)acetonitrile [(RS)-CPBA acetate] was examined to prepare (S)-CPBA. Most of lipases tested hydrolyzed (S)-CPBA acetate preferentially, while the *Candida cylindracea* lipase favored (R)-CPBA acetate. Enantioselective hydrolysis by the *Arthrobacter* lipase gave (S)-CPBA of high enantiomeric excess and (R)-CPBA acetate in a two-liquid phase reaction mixture of water and the water-insoluble substrate at pH 4.0. Kinetic studies with optically active substrate showed that (R)-CPBA acetate acted

as a competitive inhibitor. The *Arthrobacter* lipase solution in the two-liquid phase reaction system could be used repeatedly. The lipase immobilized to resins had insufficient activity or low operational stability for the repeated batch reaction. The unhydrolyzed (R)-CPBA acetate was racemized by heating with triethylamine and could be reused as the substrate of the enzymatic hydrolysis. A chemico-enzymatic process for the preparation of (S)-CPBA was presented based on these studies.

Part 3. *Pseudomonas* Lipase-catalyzed Enantioselective Transesterification of 1-Ethynyl-2-methyl-2-penten-1-ol in Organic Solvent

To obtain (S)-1-ethynyl-2-methyl-2-penten-1-ol ((S)-EMPO), lipase-catalyzed enantioselective transesterification (and esterification) of (RS)-EMPO in organic solvent was examined. Through the screening test on commercially available lipases, it was found that the *Pseudomonas* lipase catalyzed the transesterification of (RS)-EMPO with vinyl acetate in *n*-hexane to give the (R)-EMPO ester of acetic acid and (S)-EMPO with good enantioselectivity and high reaction rate. The enzymatic transesterification was accelerated by increasing the hydrophobicity of the organic solvent used as a reaction medium and decreasing the water content of the reaction mixture. Under optimum conditions, (S)-EMPO of 99% e.e. was obtained at a conversion of 68.6%. It was demonstrated that the lipase of solid powder could be recovered from the reaction mixture and reused for

the next batch reaction. Kinetic studies revealed that the enzymatic reaction proceeded under the ping-pong mechanism involving the acyl-enzyme as an intermediate.

Part 4. Diastereoselective Hydrolysis of Ethyl (1*RS*)-*cis,trans*-Chrysanthemate with Microbial Cells for Preparation of (1*R*)-*trans*-Chrysanthemic Acid

Diastereoselective hydrolysis of ethyl (1*RS*)-*cis,trans*-chrysanthemate with microbial cells was examined to prepare (1*R*)-*trans*-chrysanthemic acid. Through the screening test on 228 strains of culture collections, 31 strains were found to have hydrolytic ability. Diastereoselective hydrolysis by *Arthrobacter globiformis* IFO-12958 gave the optically pure (1*R*)-*trans*-chrysanthemic acid. The composition of cultivation media for production of the hydrolytic enzyme (the responsible esterase) was optimized. The optimum pH and temperature for hydrolysis of ethyl chrysanthemate were pH 10 and 55°C, respectively. Cells of *Arthrobacter globiformis* IFO-12958 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) that is a chemical mutagen and the advantageous mutant SC-6-98-28, whose hydrolytic activity was enhanced 2.5-fold, was isolated.

Part 1. Lipase-catalyzed Enantioselective Hydrolysis of 4-Acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one

Chapter 1. Preparation of (S)-4-Hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one using *Arthrobacter* Lipase

Summary

Enantioselective hydrolysis of 4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC acetate) with biocatalysts (enzymes and microbial cells) was examined for the preparation of the optically active alcohol moiety of a pyrethroid. Among microorganisms and lipases tested, several bacterial lipases hydrolyzed HMPC acetate with high enantioselectivity and high reaction rate. The *Arthrobacter* lipase gave (R)-4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one [(R)-HMPC] and (S)-HMPC acetate at a hydrolysis of 50%. The hydrolysis proceeded even at a substrate concentration of 80w/v%. The enantioselectivity was not affected by the chain length of the fatty acid moiety of HMPC ester. By combination of the *Arthrobacter* lipase-catalyzed enantioselective hydrolysis of racemic HMPC acetate with a chemical inversion of the (R)-HMPC to (S)-HMPC, an efficient process was developed for the total conversion of racemic HMPC to (S)-HMPC, which is an important alcohol moiety of the pyrethroid, prallethrin [(S)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1*R*)-

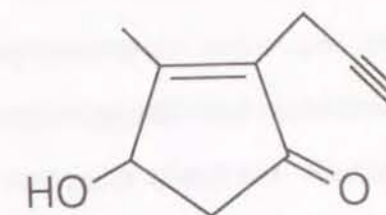
cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylate].

Introduction

4-Hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC) is one of the most promising alcohol moieties in pyrethroids (Fig. 1). (1*RS*)-*cis,trans*-Chrysanthemic acid ester with HMPC [(*RS*)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate] was found to have far better knock-down and killing activities on various household insect pests than allethrin [(*RS*)-2-methyl-4-oxo-3-(2-propenyl)-2-cyclopenten-1-yl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate] which is a well-known pyrethroid.¹⁾ Furthermore, studies on insecticidal activities of pyrethroids having optically active HMPC have revealed that the (*S*)-isomer of HMPC is responsible for the insecticidal activity of the pyrethroid.²⁾ Thus, it has been necessary to develop the technology for obtaining (*S*)-HMPC. However, known chemical methods are very complicated or require expensive chiral organic reagents for the preparation of (*S*)-HMPC.³⁾

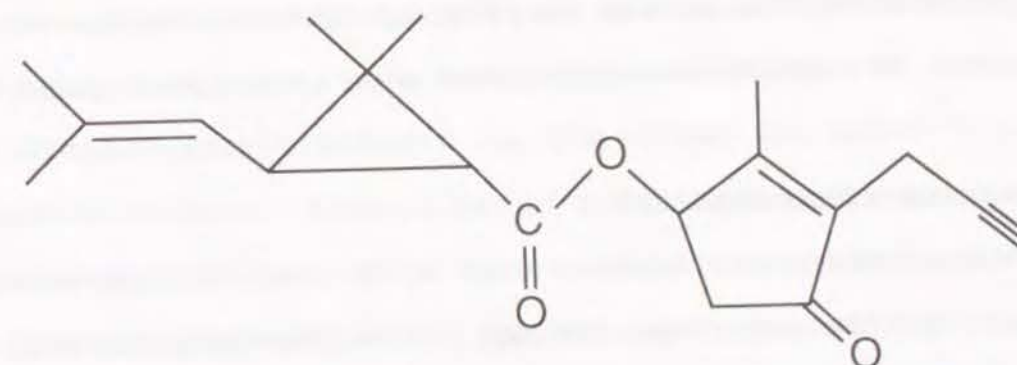
Because of the enantioselectivity of biocatalytic reactions, applications of biocatalysts (enzymes or microbial cells) are of great interest in asymmetric synthesis. Oritani and Yamashita have also reported their pioneering work on the biocatalytic resolution of some racemic monocyclic alcohols with microbial cells.⁴⁾

This chapter deals with examinations on the enantioselective



HMPC

4-Hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one



(1*RS*)-*cis,trans*-Chrysanthemic acid ester with HMPC

(1*RS*)-2-Methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate

Fig. 1. Chemical structures of HMPC and the chrysanthemic acid ester with HMPC.

hydrolysis of (RS)-4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (racemic HMPC acetate) with biocatalysts (microbial cells and enzymes). Some detailed studies were made on the *Arthrobacter* lipase which gave (R)-HMPC of high optical purity. An efficient process for the total conversion of racemic HMPC to (S)-HMPC (Fig. 5 on p. 40) is also described in this chapter.

Materials and Methods

Chemicals

Racemic HMPC was prepared from dimethyl-3-oxoglutarate and 2-propynyl chloride (Aldrich Chemical Co., Milwaukee, USA) according to the known procedure described by Matsuo et al.⁵⁾ Esterification of HMPC was carried out with the respective carboxylic anhydride and pyridine. The ester was purified by distillation under reduced pressure. All other chemicals used were of reagent grade.

Lipases and microorganisms

All of microbial lipases used were obtained from commercial sources. *Arthrobacter* species (sp.) (Sumitomo Chemical Co., Osaka, Japan), *Chromobacterium viscosum* (Toyo Jozo Co., Tokyo, Japan), *Pseudomonas* sp., *Aspergillus* sp., *Mucor* sp. and *Humicola* sp. (Amano Pharmaceutical Co., Nagoya, Japan), *Candida cylindracea*, *Alcaligenes* sp. and *Achromobacter* sp. (Meito Sangyo Co., Tokyo, Japan), *Rhizopus delemar* (Tanabe Pharmaceutical Co., Osaka, Japan) and *Rhizopus japonicus* (Osaka Saikin Kenkyusyo Co., Osaka, Japan).

Microorganisms used for the screening test were obtained from

the Institute for Fermentation, Osaka (IFO), Japan and the American Type Culture Collection (ATCC), Rockville, USA.

Screening of microorganisms

The composition of the medium for cultivation of microorganism was as follows: for bacteria, 5 g yeast extract (Difco Laboratories, Detroit, USA), 5 g polypeptone (Daigo Eiyo Co., Osaka, Japan) and 10 g soluble starch (Nichiden Kagaku Co., Osaka Japan) in 1 l distilled water, pH 6.8; for yeasts and molds, 3 g yeast extract, 3 g malt extract (Difco Laboratories, Detroit, USA), 5 g polypeptone and 10 g glucose (Nacalai Tesque Co., Kyoto, Japan) in 1 l distilled water, pH 5.5.

A loopful of each microorganism was inoculated into a 500 ml Erlenmeyer flask containing 100 ml sterilized medium and was cultured for 2 or 3 days on a rotary shaker (200 rpm) at 30°C until the stationary growth phase. To the flask was added 0.5 g of racemic HMPC acetate. After 1 day of additional cultivation on the rotary shaker (200 rpm) at 30°C, the products were extracted from the cultured broth with ethyl acetate, and the mol ratio of the liberated alcohol and the remaining ester was determined by gas-liquid chromatography (GLC).

Screening test and hydrolysis with lipases

As the first screening, the hydrolytic abilities of lipases on HMPC acetate were tested by the pH-stat method at pH 7.0 and 40°C. The reaction mixture consisted of a two-liquid phase because

the substrate was poorly miscible with water. The mixture was emulsified by stirring with a magnetic stirrer for the enzymatic hydrolysis. The lipases, which showed relatively high hydrolytic abilities, were subjected to the second screening test for the optical purity of the liberated alcohol. In the second test, the reaction with each lipase was performed up to hydrolysis degree of 10-60%. The products were extracted with ethyl acetate and subjected to analysis.

Activity measurement of lipases

Lipase activity on HMPC ester of carboxylic acid was determined by pH-stat (model PS-11, Hiranuma Co., Tokyo, Japan) measuring the initial rate of the hydrolysis under the following conditions. Eight ml of eight-fold diluted 0.1 M citric acid- Na_2HPO_4 buffer solution (McIlvaine buffer solution, pH 7.0) containing 12.5 mM CaCl_2 was mixed with 1.0 g HMPC ester and 1.0 ml lipase solution. The temperature and pH were 40°C and 7.0, respectively, unless otherwise noted. The reaction was started by stirring the mixture with a magnetic stirrer after addition of the enzyme solution and performed for 10-20 min.

Activities on olive oil emulsion were assayed titrimetrically at 40°C and pH 7.0 by the modified method of Fukumoto *et al.*⁶⁾ Protein concentration was estimated by the method of Lowry *et al.*⁷⁾ with bovine serum albumin as a standard.

Analysis

The gas-liquid chromatography (GLC) analysis was performed on a Shimadzu GC-R1A with a glass column of 10% DEGS on Chromosorb W(AW) [column size, 3.2 mm ϕ $\times$ 1.1 m; flow rate of N_2 , 50 ml/min; column temperature, 150°C; detector, flamed ionized detector (FID)] to determine the hydrolysis degree. The degree of hydrolysis was defined as the mol ratio of the liberated HMPC versus the substrate (HMPC ester) used in the enzymatic reaction. Determination of the isomer ratio of liberated HMPC was made with an enantiomer analysis using high-performance liquid chromatography (HPLC) by the method of Oi and Kitahara⁸⁾ [column, Sumipax OA-4100 (5 μm , 4 mm ϕ $\times$ 25 cm, Sumika Chemical Analysis Service Co.); mobile phase, *n*-hexane/1,2-dichloroethane/ethanol (100/20/1, 1 ml/min); detector, UV 230 nm]. *E* value calculated by the following equation was used to estimate the enantioselectivity of the enzymatic reaction.⁹⁾

$$E = \ln[1-c(1+ee(P))]/\ln[1-c(1-ee(P))]$$

where *c*, the degree of hydrolysis;

ee(P), the enantiomeric excess of the liberated HMPC.

Results and Discussion

Screening of microorganisms

Through the screening of stock cultures, nearly a hundred microorganisms, including bacteria, yeasts and molds, were found to have the hydrolytic ability on HMPC acetate. Analysis of isomer ratio of the liberated HMPC revealed that more than half of the microorganisms tested could hydrolyze HMPC acetate

enantioselectively. Examples of microorganisms having relatively high enantioselectivities are shown in Table 1, though their enantioselectivities were not sufficient. The (R)-ester was preferentially hydrolyzed by most of the microorganisms, with the exception of a few yeasts.

An addition of soybean oil to the cultivation media was found to enhance the degree of hydrolysis with some microorganisms (Table 2). This suggested that the responsible enzymes were lipases rather than esterases, since it has been known that the addition of oil or fat to the cultivation medium promoted the production of lipase occasionally.^{10,11)} Several microbial lipases are conveniently obtainable from commercial sources. So, commercially available lipases were screened next.

Screening of lipases

Eleven commercially available microbial lipases were screened for the enantioselective hydrolysis of HMPC acetate. Nine of these lipases hydrolyzed HMPC acetate and they were further tested for optical purity of the liberated alcohol at hydrolysis of 10%-60%. The results summarized in Table 3 show that all of them preferentially hydrolyzed (R)-HMPC acetate. Among the lipases tested, a lipase from *Arthrobacter* sp. gave (R)-HMPC of high enantiomeric excess at the hydrolysis of 50% with the smallest amount of the enzyme. Lipases from *Chromobacterium viscosum*, *Pseudomonas* sp. and *Alcaligenes* sp. also showed high hydrolytic activities, but their enantioselectivities were lower than that of

Table 1. Microbial hydrolysis of 4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC acetate).

Microorganism	Hydrolysis (%)	Isomer ratio of liberated HMPC (S)/(R)	E value
Bacteria			
<i>Alcaligenes faecalis</i> IFO-13111	21.5	33.3/66.7	2
<i>Bacillus badius</i> ATCC-14574	49.5	35.7/64.3	2
<i>Bacillus megaterium</i> IFO-12108	25.1	8.5/91.5	14
<i>Bacillus subtilis</i> IFO-3026	31.2	22.9/77.1	4
<i>Chromobacterium chocolateum</i> IFO-3758	11.7	4.8/95.2	22
<i>Flavobacterium arborescens</i> IFO-3750	49.9	15.8/84.2	19
<i>Streptomyces griseofuscus</i> IFO-12870	72.0	39.2/60.8	3
<i>Streptomyces panayensis</i> IFO-13576	60.8	29.1/70.9	5
Yeasts			
<i>Kloeckera corticis</i> IFO-0868	42.6	81.4/18.6	7
<i>Kluyveromyces fragilis</i> IFO-0288	46.7	79.3/20.7	6
<i>Saccharomyces uvarum</i> IFO-0565	51.5	40.2/59.8	2
<i>Schizosaccharomyces pombe</i> IFO-0346	3.8	22.7/77.3	4
Molds			
<i>Absidia hyalosporea</i> IFO-8082	10.9	13.4/86.6	7
<i>Aspergillus niger</i> IFO-6342	3.5	18.6/81.4	5
<i>Dipodascus uninucleatus</i> ATCC-14626	17.5	38.9/61.1	2
<i>Trichoderma viride</i> IFO-5720	13.7	20.7/79.3	3

Reaction conditions: substrate concentration, 0.5w/v%; reaction time, 1 day; temperature, 30°C.

Table 2. Effect of addition of soybean oil to the culture medium on microbial hydrolysis of HMPC acetate.

Microorganism	Soybean oil	Hydrolysis (%)	Isomer ratio of liberated HMPC (S)/(R)	E value
<i>Aspergillus niger</i> IFO-6342	not added	3.5	18.6/81.4	5
	added	20.5	18.7/81.3	5
<i>Mycobacterium phlei</i> IFO-3158	not added	0.0	-	-
	added	16.7	38.2/61.8	2

Amount of soybean oil added to the culture medium was 0.5w/v%.

Cultivation of each microorganism was performed for 3 days on a rotary shaker (200 rpm) at 30°C.

Table 3. Enantioselective hydrolysis of HMPC acetate with commercially available lipase.

Origin of lipase	Amount of lipase used (mg)	Hydrolysis (%)	Isomer ratio of liberated HMPC (S)/(R)	E value
<i>Achromobacter</i> sp.	100	32.9	2.4/97.6	65
<i>Alcaligenes</i> sp.	20	47.4	2.2/97.8	124
<i>Arthrobacter</i> sp.	3	50.0	0.6/99.4	850
<i>Aspergillus</i> sp.	100	13.0	39.4/60.6	2
<i>Candida cylindracea</i>	100	10.8	8.7/91.3	12
<i>Chromobacterium viscosum</i>	6	58.6	4.5/95.5	73
<i>Humicola</i> sp.	100	36.8	9.3/90.7	16
<i>Pseudomonas</i> sp.	6	47.1	2.6/97.4	101
<i>Rhizopus japonicus</i>	100	5.2	45.5/54.5	1

Reaction conditions; substrate concentration, 8.8w/v%; reaction time, 23 hr; pH, 7.0; temperature, 40°C.

the *Arthrobacter* lipase.

Hydrolytic activities of the seven lipases on HMPC acetate were compared with activities on olive oil (Table 4). Activities of these lipases on HMPC acetate were much smaller than activities on olive oil. It was noticed that substrate specificities varied with the origin of these lipases. The *Arthrobacter* lipase showed the highest specificity for HMPC acetate among the lipases tested.

Through above-mentioned screening tests on microorganisms and commercially available microbial lipases, it was found that the *Arthrobacter* lipase gave the best result in enantioselective hydrolysis of HMPC acetate. So, subsequent experiments were carried out with the *Arthrobacter* lipase.

Enantioselective hydrolysis by *Arthrobacter* lipase

Characteristics and some details of reaction aspects of the *Arthrobacter* lipase were studied. Figure 2 shows the effect of pH on the reaction rate at a substrate concentration of 10w/v%. The optimum pH was about 7, and the spontaneous hydrolysis of the substrate occurred above pH 8.

The effect of temperature expressed in terms of Arrhenius plot is given in Fig. 3. Thermal inactivation occurred above 60°C.

A time-course of the hydrolysis showed that the *Arthrobacter* lipase could hydrolyze racemic HMPC acetate even at a substrate concentration of 80w/v% without difficulty (Fig. 4). Figure 4 also shows that the reaction would not proceed above the hydrolysis of 50% where (R)-HMPC acetate was completely hydrolyzed. The

Table 4. Activities of commercial available microbial lipases on HMPC acetate and olive oil.

Origin of lipase	Activity on HMPC acetate ($\mu\text{mol}/\text{min}/\text{mg-protein}$)	Activity on olive oil ($\mu\text{mol}/\text{min}/\text{mg-protein}$)	Activity ratio of HMPC acetate/olive oil
<i>Achromobacter</i> sp.	0.8	82	0.001
<i>Alcaligenes</i> sp.	2.0	59	0.034
<i>Arthrobacter</i> sp.	17.4	230	0.076
<i>Candida cylindracea</i>	ND	110	-
<i>Chromobacterium viscosum</i>	6.7	790	0.008
<i>Humicola</i> sp.	0.2	400	0.0005
<i>Pseudomonas</i> sp.	3.9	133	0.029

Concentrations of HMPC acetate and olive oil were 10w/v% and 20w/v%, respectively. The temperature and pH were 40°C and pH 7.0, respectively.

ND means "not determined". The activity was too small to be determined.

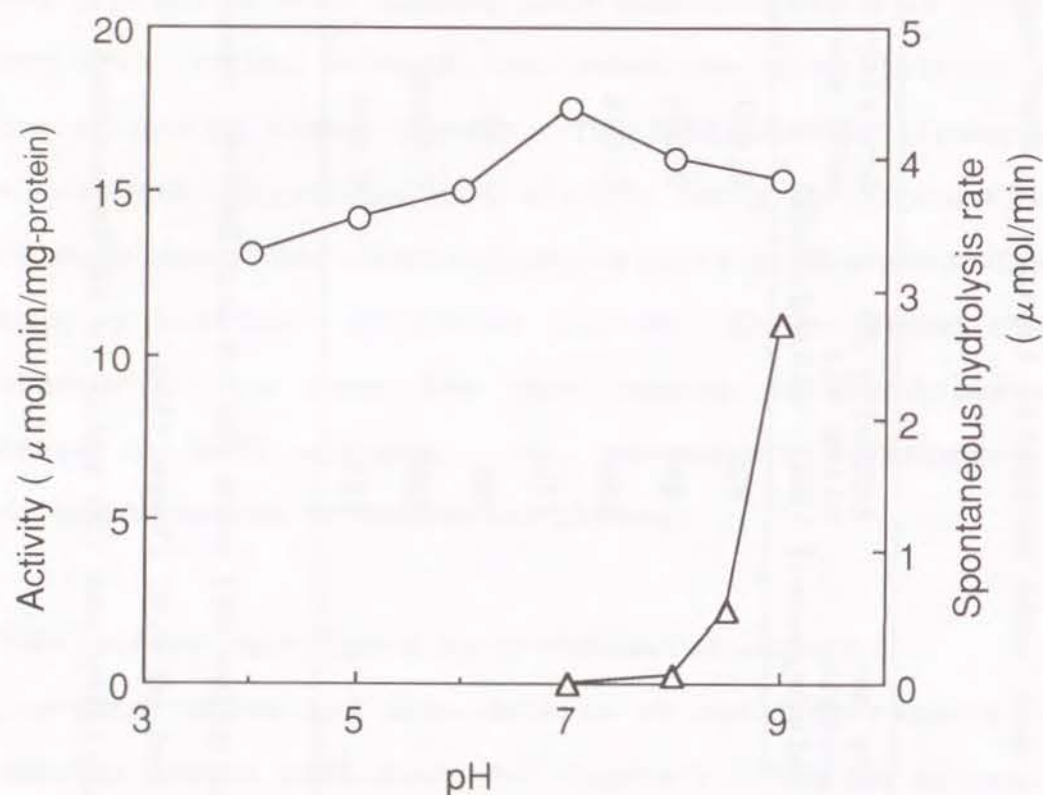


Fig. 2. Effect of pH on the hydrolysis of HMPC acetate with the *Arthrobacter* lipase. Activity of the lipase (○) and spontaneous hydrolysis rate of HMPC acetate (Δ). Buffer solutions used were as follows: pH 4.0 and pH 5.0, 0.1 M $\text{CH}_3\text{COOH}-\text{CH}_3\text{COONa}$; pH 6.0, pH 7.0 and pH 8.0, 0.1 M citric acid- Na_2HPO_4 ; pH 8.5 and pH 9.0, 0.1 M KH_2PO_4 -NaOH. The substrate concentration was 10w/v%. The activity was measured by pH-stat method at 40°C for 10-20 min as described in **Materials and Methods**.

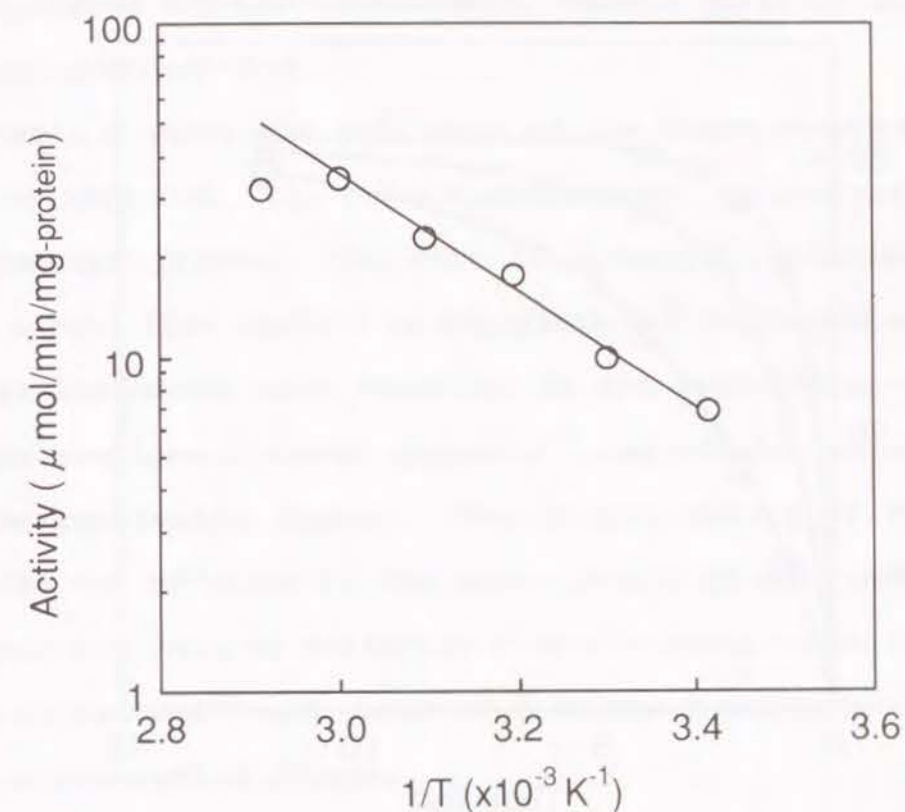


Fig. 3. Effect of temperature on the hydrolysis of HMPC acetate with the *Arthrobacter* lipase. Composition of the reaction mixture was as follows: 8.0 ml of eight-fold diluted 0.1 M citric acid- Na_2HPO_4 buffer solution (pH 7.0) containing 12.5 mM CaCl_2 , 1.0 g substrate and 1.0 ml enzyme solution containing 3.0 mg *Arthrobacter* lipase. The activity was measured by pH-stat method described in **Materials and Methods**.

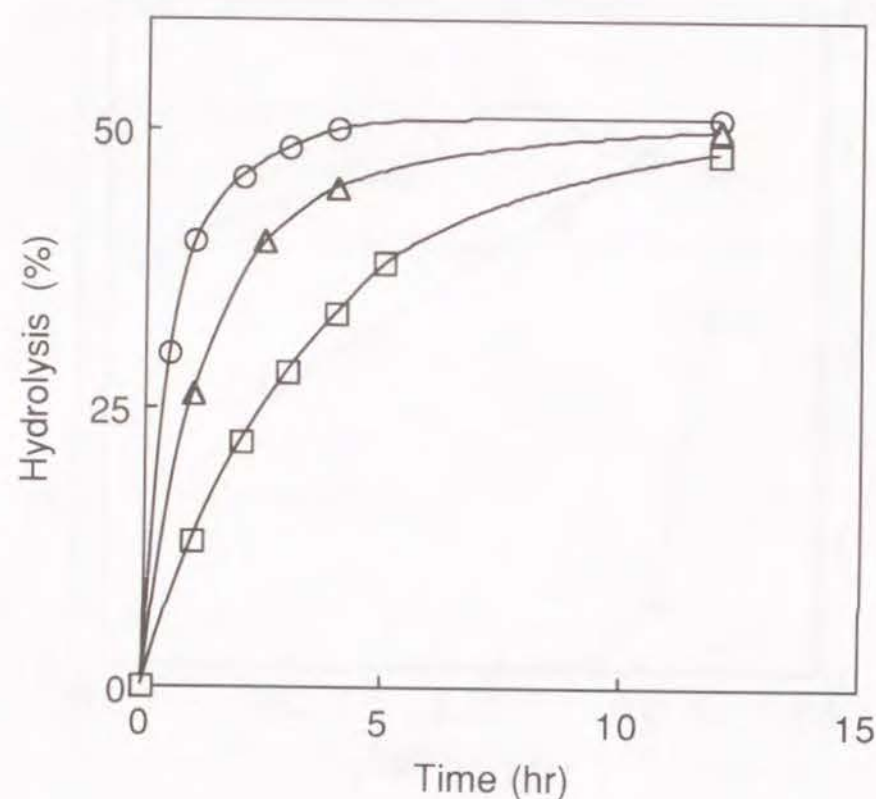


Fig. 4. Effect of the concentration of HMPC acetate on the hydrolysis by the *Arthrobacter* lipase. Substrate concentrations were 20w/v% (○), 40w/v% (△), and 80w/v% (□). HMPC acetate was added to 0.1 M citric acid- Na_2HPO_4 buffer solution (pH 7.0) containing 12 mg *Arthrobacter* lipase (total volume of the reaction mixture, 20 ml). The pH was maintained at pH 7.0 with 2.5 N NaOH and the reaction temperature was 40°C. Aliquots (50 μl) were removed from the reaction mixture at certain intervals to evaluate the degree of hydrolysis at given reaction times.

liberated HMPC was found to be almost optically pure at each experimental point in Fig. 4, irrespective of the degree of hydrolysis. Thus, it was concluded that the *Arthrobacter* lipase distinguished the two enantiomeric isomers strictly and hydrolyzed only (R)-HMPC acetate.

Table 5 shows the influence of the chain length of the fatty acid moiety on the enantioselective hydrolysis with the *Arthrobacter* lipase. The rate of hydrolysis increased with the chain length from acetate to caprylate but decreased with caprate. Similar behaviors were reported on the hydrolyses of esters of racemic 3-chloro-2-methyl propanol¹²⁾ and various monoesters¹³⁾ by a porcine pancreatic lipase. The optical purity of the liberated HMPC was not affected by the chain length of acid moiety as shown in Table 5. This is different from observations on hydrolyses of ester of menthol¹⁴⁾ and ester of 3-chloro-2-methyl propanol¹²⁾ by a porcine pancreatic lipase.

Chemico-enzymatic preparation of (S)-HMPC

The *Arthrobacter* lipase has yielded the optically pure (R)-HMPC very efficiently. This isomer is, however, less important for synthesis of the insecticidally active stereoisomer of a pyrethroid. To obtain (S)-HMPC in high yield, the unhydrolyzed (S)-HMPC acetate must be separated from (R)-HMPC and then hydrolyzed chemically. In this case, only half amount of the racemic HMPC is available for pyrethroids. Despite efficient enzymatic resolution, the total process for production of (S)-HMPC

Table 5. Effect of the fatty acid moiety on the hydrolysis of HMPC esters by the *Arthrobacter* lipase.

Fatty acid moiety	Activity ($\mu\text{mol}/\text{min}/\text{mg-protein}$)	Isomer ratio of liberated HMPC (S)/(R)
Acetate	17	0.4/99.6
Butyrate	18	0.4/99.6
Caproate	22	0.4/99.6
Caprylate	79	0.1/99.9
Caprate	65	0.4/99.6

The concentration of ester was 10w/v%. Lipase-catalyzed hydrolysis was carried out at 40°C and pH 7.0 until the degree of the hydrolysis reached 30±5%. The products were extracted at a hydrolysis of 30±5% to determine the isomer ratio of the liberated HMPC.

might not be attractive from an industrial point of view unless the (R)-isomer was utilized effectively. This problem could be solved by a Walden type chemical inversion of the (R)-HMPC as reported by Danda et al.¹⁵⁾ The liberated (R)-HMPC was sulfonated with an organic sulfonyl halide. (R)-HMPC sulfonate obtained was easily hydrolyzed by heating under acidic conditions to give the inverted (S)-HMPC. On the other hand, (S)-HMPC acetate, that had been unhydrolyzed enzymatically, was not sulfonated and retained its (S)-configuration during the chemical hydrolysis under the acidic conditions. As a result, the racemic HMPC was totally converted to (S)-HMPC which was then esterified with (1R)-*cis,trans*-chrysanthemic acid to produce the optically active pyrethroid, prallethrin [(S)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1R)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylate] (Fig. 5).

A typical experimental result is as follows.¹⁶⁾ A mixed solution of (R)-HMPC [(R)/(S)= 99.2/0.8] and (S)-HMPC acetate in dichloroethane was obtained after 50.9% hydrolysis of racemic HMPC acetate (0.15 mol). To the solution was added methanesulfonyl chloride (0.083 mol) and the mixture was stirred at a low temperature. The solution was then mixed with water and heated at 97°C for 3 hr. After concentration of the organic layer, 0.125 mol of (S)-HMPC [(R)/(S)=4.3/95.7] was obtained.

Thus, the combination of enzymatic resolution and chemical inversion has enabled racemic HMPC to convert effectively into (S)-HMPC. This process is sufficiently applicable for the industrial

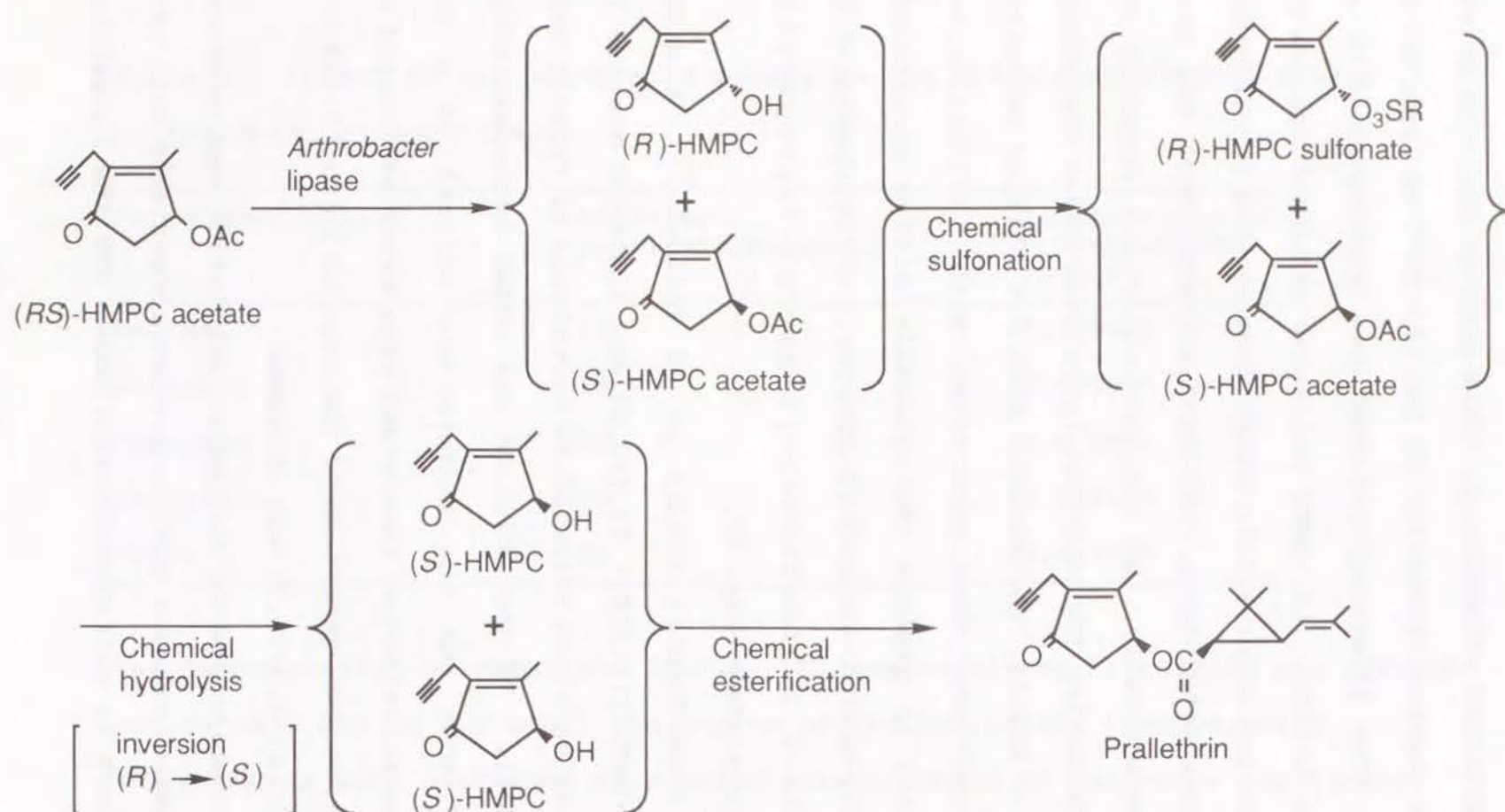


Fig. 5. Chemico-enzymatic preparation of (S)-HMPC for the optically active pyrethroid, prallethrin [(S)-2-methyl-4-oxo-3-(2-propynyl)-2-cyclopenten-1-yl (1R)-cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate].

Part 1

production of the pyrethroid, that is, prallethrin, because of the high optical and chemical yield.

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Chapter 2. Studies on Enantioselective Hydrolysis of 4-Acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one with *Arthrobacter* Lipase

Summary

Characteristics of the enantioselective hydrolysis of 4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC acetate) by the *Arthrobacter* lipase were investigated in a two-liquid phase reaction system of water and the water-insoluble substrate. Kinetic studies revealed that the strict enantioselectivity was entirely due to the difference in the catalytic constants for the enantiomeric substrates and that (S)-HMPC acetate acted as a competitive inhibitor. The comparison of enantioselectivity for the acetic acid esters with analogues of HMPC indicated that hydrophobic substituents in the HMPC molecule were essential for the strict enantioselectivity.

Introduction

Enantioselective hydrolysis with lipase is now recognized as a practical process for the synthesis of optically active compounds.¹⁾ However, few reports of kinetic studies on the enantioselective hydrolysis by lipases are known.

There are some specific problems concerning the estimation of kinetic constants in a two-liquid phase reaction system of water and the water-insoluble substrate with lipase. Benzonana and Desnuelle²⁾ suggested that the K_m of the lipase-catalyzed reaction

in the two-liquid phase system should be expressed as the interfacial area per volume, because kinetic constants vary depending on the particle size of the substrate dispersed in the emulsion. Brockerhoff³⁾ pointed out that the physical interaction between the enzyme and the surface of the substrate particle should be considered in kinetic analysis of the enzymatic biphasic reaction. In practice, it is difficult to measure the interfacial area of the insoluble substrate. However, Sugihara et al.⁴⁾ demonstrated that the apparent K_m expressed as the weight (or moles) per volume, measured under fixed condition for emulsion formation, would be usable for kinetic analysis.

In Chapter 1, it was documented that the *Arthrobacter* lipase enantioselectively hydrolyzed 4-acetoxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC acetate), and gave (R)-4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one [(R)-HMPC] and the unhydrolyzed (S)-HMPC acetate.

This chapter deals with kinetic studies on the enantioselective hydrolysis of HMPC acetate with the *Arthrobacter* lipase by the estimation of V_m and the apparent K_m expressed as moles per volume under the optimum conditions for emulsion formation.

Materials and Methods

Chemicals

Esterification of 4-hydroxy-2-cyclopenten-1-one derivatives has been performed according to the procedure described in Chapter

1. (R)-HMPC was isolated by silica gel column chromatography [solvent: *n*-hexane/diethylether (10/1 in volume)] from a mixture of (R)-HMPC and (S)-HMPC acetate obtained by the enantioselective hydrolysis of racemic HMPC acetate with the *Arthrobacter* lipase. The (S)-HMPC was prepared by the chemico-enzymatic process described in Chapter 1. Other chemicals used were of reagent grade. Synthetic non-ionic surfactants (Span 85, Tween 80, Triton X-100) were purchased from Nacalai Tesque Co.

Activity measurement

Activity of the lipase on HMPC acetate was determined by the pH-stat method (Radiometer Co., Copenhagen, Denmark) measuring the initial rate of the hydrolysis. Conditions were as described in Chapter 1.

Analytical methods

Determinations of the hydrolysis degree and the enantiomeric isomer ratio of the liberated alcohol were described in Chapter 1. The average diameter of the substrate particles in the emulsion were measured by a laser-particle-analyzer (Otsuka Electronics, Osaka, Japan) with emulsions which were prepared by using a magnetic stirrer (length of the magnet, 2 cm) at different stirring cycles. Each emulsion consisted of droplets of HMPC acetate (10w/v%) dispersed in 0.1 M citric acid- Na_2HPO_4 buffer solution containing polyvinyl alcohol (0.5w/v%) to stabilize the emulsion.

Results and Discussion

Conditions for emulsion formation

Stirring cycles and the addition of surfactants were examined in order to obtain a suitable emulsion for kinetic measurements at the maximum initial reaction rate.

At first, the effect of stirring cycles on the activity was measured with the *Arthrobacter* lipase (Fig. 1). The activity increased with the stirring cycle and levelled off at 800 rpm. The average diameters of the substrate particles in the emulsion were 68.0 μm at 400 rpm, 59.1 μm at 600 rpm, 45.9 μm at 800 rpm and 39.8 μm at 1000 rpm. Fukumoto et al.⁵⁾ demonstrated that a suitable emulsion for lipase activity measurement could be obtained by constant stirring of the biphasic reaction mixture. In author's experiments, a suitable emulsion was obtained by stirring at over 800 rpm. The average diameter of the substrate particles decreased with the increase in the stirring cycle. It was considered that the interfacial area of the substrate was sufficient for the enzyme to give maximum activity when the stirring cycle was over 800 rpm.

Next, effect of surfactants including synthetic non-ionic surfactants (Span 85, Tween 80, Triton X-100), polyvinyl alcohol and sodium deoxycholate was examined at a stirring cycle of 1000 rpm (Table 1). Span 85 (0.3w/v%) and Triton X-100 (0.4w/v%) did not affect the activity of the *Arthrobacter* lipase, but a slight decrease in the activity was observed with Tween 80 (0.3w/v%). The activity decreased significantly by addition of polyvinyl alcohol (0.4w/v%) and sodium deoxycholate (0.8w/v%). The addition of

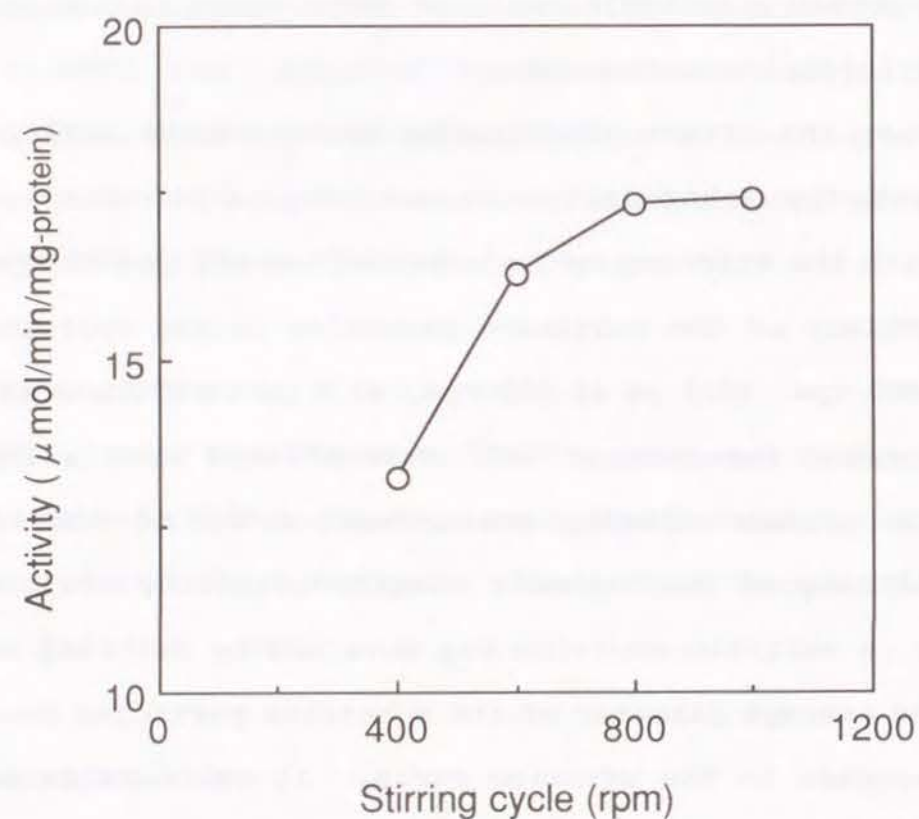


Fig. 1. Effect of the stirring cycle on hydrolytic activity. Activity was measured by pH-stat method at 40°C and pH 7.0. The substrate concentrations was 10w/v%.

Table 1. Effect of surfactant on hydrolysis of HMPC acetate with the *Arthrobacter* lipase.

Surfactant	Concentration in reaction mixture (w/v%)	Relative activity (%)
None	-	100
Span 85	0.3	93
Triton X-100	0.3	92
Tween 80	0.3	78
Polyvinyl alcohol	0.4	52
Sodium deoxycholate	0.8	51

Activity was measured by pH-stat method at 40°C and pH 7.0. The reaction mixture was stirred at 1000 rpm by a magnetic stirrer for the reaction.

surfactants did not increase the activity. It was supposed that the synthetic non-ionic surfactants and sodium deoxycholate inactivated the lipase. Polyvinyl alcohol might disturb the adsorption of enzyme at the substrate interface as reported by Borgström⁶⁾. Finally, it was decided that kinetic measurements should be carried out at a stirring cycle of 1000 rpm without any surfactant.

Kinetic studies for enantioselective hydrolysis of HMPC acetate

Figure 2 shows the initial reaction rate of enzymatic hydrolysis with mixtures of (R)-HMPC acetate and (S)-HMPC acetate at various ratios. While (R)-HMPC acetate was hydrolyzed at a high reaction rate, (S)-HMPC acetate was not hydrolyzed at all. In accordance with the kinetic model of Verger and de Haas⁷⁾, the concave shape of the plots in Fig. 2 indicated that (S)-HMPC acetate was a strong competitive inhibitor and that the K_{mR} (K_m for (R)-HMPC acetate) would be larger than K_{mS} (K_m for (S)-HMPC acetate).

Lineweaver-Burk plots were made on hydrolyses of (R)-HMPC acetate and the mixtures of (R)-HMPC acetate with a fixed amount of (S)-HMPC acetate (Fig. 3). The substrate concentration was defined as moles per volume. The linearity of the plots facilitated the determination of the values proportional to the kinetic constants. The fact that the two double reciprocal plots crossed on the ordinate confirmed that (S)-HMPC acetate was a competitive inhibitor. The apparent K_{mR} was estimated to be 100 mM

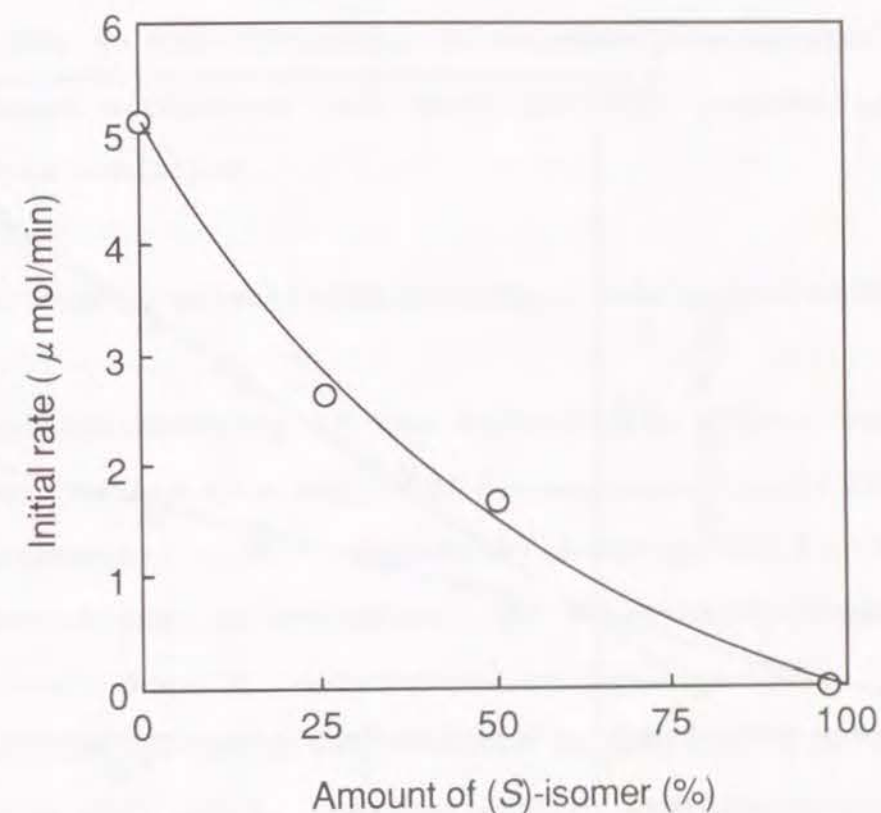


Fig. 2. Initial rate of the hydrolysis of mixtures of two enantiomeric substrates, (R)-HMPC acetate and (S)-HMPC acetate. The initial rate was measured by pH-stat method at 50°C and pH 6.0 for prevention against the crystallization and the spontaneous hydrolysis of the optically active substrate.

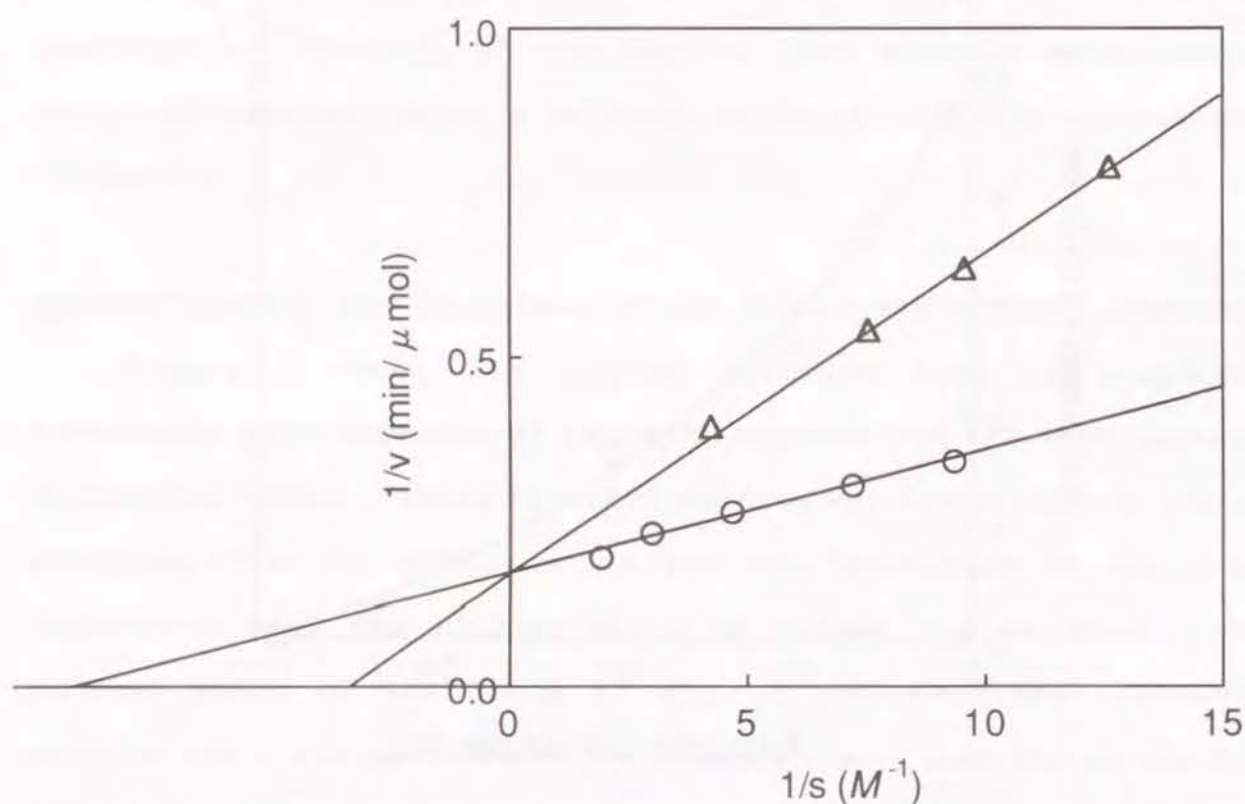


Fig. 3. Lineweaver-Burk plots for hydrolysis of (R)-HMPC acetate (○) and mixtures of (R)-HMPC acetate and a fixed amount of (S)-HMPC acetate (Δ). The concentration of (S)-HMPC acetate was fixed at 78 mM. The initial rate was measured at 50°C and pH 6.0.

and the apparent K_{ms} (the apparent K_i) was calculated to be 52 mM.

Results of kinetic studies revealed that the high enantioselectivity of the *Arthrobacter* lipase for HMPC acetate was entirely due to the difference in the catalytic constants for two enantiomeric substrates and that (S)-HMPC acetate acted as a competitive inhibitor.

Enzymatic enantioselectivity for acetic acid esters with analogues of HMPC

Enantioselectivity of the *Arthrobacter* lipase was examined with acetic esters of a series of racemic substituted 4-hydroxy-2-cyclopentenones; 1, 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one (allethrolon); 2, 4-hydroxy-2-(2-propenyl)-2-cyclopenten-1-one; 3, 4-hydroxy-2-cyclopenten-1-one (Table 2). High enantioselectivity was observed in hydrolysis of acetic acid esters with HMPC and 1, that had similar hydrophobic substituents at C-2 and C-3. The elimination of the methyl substituent at C-3 caused a slight decrease in the enantioselectivity (the acetic acid ester with 2). A significant decrease in the enantioselectivity was observed in hydrolysis of the acetic acid ester with 3, in which both substituents at C-2 and C-3 were eliminated.

The comparison of enantioselectivity for acetic acid esters with HMPC analogues indicated that the hydrophobic substituents at C-2 and C-3 of 4-hydroxy-2-cyclopenten-1-one were essential for the strict enantioselectivity in the hydrolysis of HMPC acetate by the *Arthrobacter* lipase. The substituents at C-2 and C-3 would have

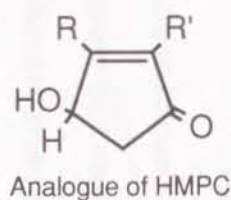
Table 2. Enantioselectivity in the hydrolysis of acetic acid esters with analogues of HMPC by the *Arthrobacter* lipase.

Analogue compound	R	R'	Hydrolysis (%)	Isomer ratio of liberated alcohol (S)/(R)	E value
HMPC	-CH ₃	-CH ₂ C≡CH	50.0	0.6/99.4	850
1	-CH ₃	-CH ₂ CH=CH ₂	48.1	1.0/99.0	315
2	-H	-CH ₂ CH=CH ₂	43.0	5.4/94.6	35
3	-H	-H	18.8	34.4/65.6	2

Enzymatic hydrolysis was carried out at 40°C and pH 7.0 for 23 hr on acetic acid esters with HMPC, 1 and 2.

The acetic acid ester with 3 was hydrolyzed enzymatically at 30°C and pH 6.0 for 10 hr to prevent spontaneous hydrolysis of the substrate.

Chemical structure of analogues of HMPC:



a hydrophobic binding property with the enzyme molecule.

Figure 4 shows the hypotheticalal configuration of the active center of the *Arthrobacter* lipase. The enzyme has two binding sites, the acyl binding site and the hydrophobic binding site, as well as one active site. When two hydrophobic substituents at C-2 and C-3 of the alcohol moiety of (R)-HMPC acetate is bound to the hydrophobic binding site, the ester bond of the substrate is positioned close to the catalytic center to be hydrolyzed. On the other hand, the ester bond of (S)-HMPC acetate is positioned out of the catalytic center.

These studies will make it possible to predict the absolute configuration of the produced alcohol and the extent of the optical purity in the enantioselective hydrolysis by the *Arthrobacter* lipase.

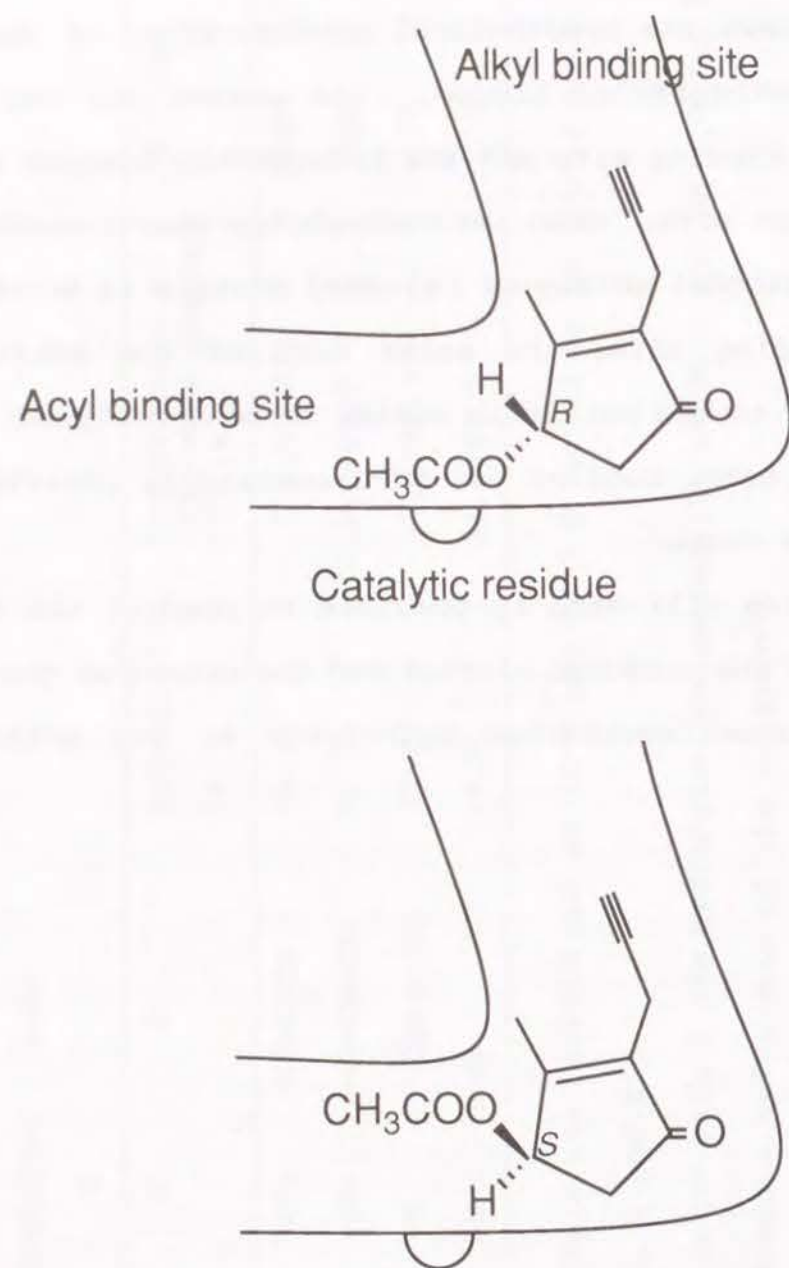


Fig. 4. Possible configuration of enzyme-substrate binding in the *Arthrobacter* lipase.

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Part 2. Enantioselective Hydrolysis of 2-Acetoxy-2-(3-phenoxyphenyl)acetonitrile with *Arthrobacter* Lipase

Summary

Lipase-catalyzed enantioselective hydrolysis of (RS)-2-acetoxy-2-(3-phenoxyphenyl)acetonitrile {(RS)- α -cyano-3-phenoxybenzyl acetate [(RS)-CPBA acetate]} was examined to prepare (S)-hydroxy-2-(3-phenoxyphenyl)acetonitrile {(S)- α -cyano-3-phenoxybenzyl alcohol [(S)-CPBA]}. Most of the lipase tested hydrolyzed (S)-CPBA acetate preferentially, while *Candida cylindracea* lipase favored (R)-CPBA acetate. Enantioselective hydrolysis by the *Arthrobacter* lipase gave (S)-CPBA of high enantiomeric excess in a two-liquid phase reaction mixture of water and water-insoluble substrate at pH 4.0. The kinetic studies showed that (R)-CPBA acetate reacted as a competitive inhibitor. The *Arthrobacter* lipase solution in the two liquid phase reaction mixture of water and water-insoluble substrate could be used repeatedly. The lipase immobilized to resins had insufficient activity or low operational stability for the repeated batch reaction. The unhydrolyzed (R)-CPBA acetate was racemized by heating with triethylamine and could be reused as the substrate of the enzymatic hydrolysis. A chemico-enzymatic process for the preparation of (S)-CPBA was developed based on these studies.

Introduction

A group of synthetic pyrethroids containing 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile [α -cyano-3-phenoxybenzyl alcohol (CPBA)] (Fig. 1) have excellent insecticidal activity for agricultural pest control.¹⁾ In a study on the relationship between the structure of the optically active pyrethroids and insecticidal activity, it was found that (S)-CPBA was the insecticidally active stereoisomer in pyrethroids.²⁾ Although several chemical methods of preparing (S)-CPBA are known, these methods are very complicated and require expensive chiral organic reagents.³⁾

A number of reports on enzymatic preparation of optically active alcohols have been published.⁴⁻¹⁰⁾ Although optically active CPBA was synthesized from 4-phenoxybenzaldehyde and cyanohydrin using the oxynitrilase, the stereoisomer given was the insecticidally inactive (R)-form.¹¹⁾ On the other hand, two examples of enantioselective hydrolysis of racemic aryloxy acetaldehyde cyanohydrin acetates with the *Pseudomonas* lipase¹²⁾ and microbial cells¹³⁾ were reported. In these studies, biocatalysts hydrolyzed (R)-isomers preferentially, and (S)-isomers were obtained as the unhydrolyzed substrate. Ohta et al.¹³⁾ reported that the yield and the optical purity of the liberated cyanohydrin were rather low because of a rapid equilibrium between the cyanohydrin and the corresponding aldehyde under their experimental conditions.

The author aimed at developing a practical process for (S)-CPBA by adopting the enzymatic enantioselective hydrolysis of

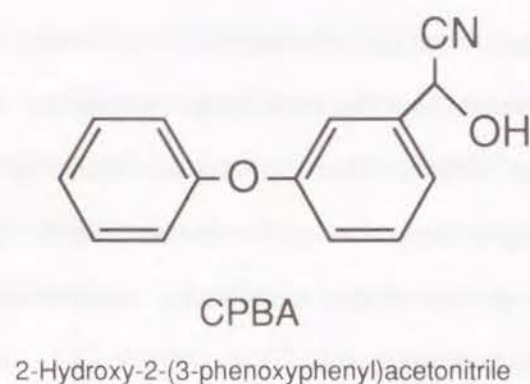


Fig. 1. Chemical structure of CPBA and esfenvalerate that is a pyrethroid having (S)-CPBA as its alcohol moiety.

(RS)-2-acetoxy-2-(3-phenoxyphenyl)acetonitrile (racemic CPBA acetate). This part deals with studies on the lipase-catalyzed enantioselective hydrolysis of racemic CPBA acetate to liberate (S)-CPBA, also describes an efficient process with the chemical racemization of the enzymatically unhydrolyzed (R)-CPBA acetate (Fig. 9 on p. 77) and the repeated use of the lipase solution for batch reactions.

Materials and Methods

Chemicals

2-Acetoxy-2-(3-phenoxyphenyl)acetonitrile [α -cyano-3-phenoxybenzyl acetate (CPBA acetate)] was prepared from 4-phenoxybenzaldehyde (PBAL), acetic anhydride and sodium cyanide by a modification of the method of Chenevert et al.¹⁴⁾ All chemicals used were of reagent grade.

Hydrolysis of CPBA acetate by lipases

Microbial lipases used for the screening test were described in Part 1. A typical method for the enzymatic hydrolysis of CPBA acetate was as follows. The reaction mixture contained CPBA acetate, the enzyme and 0.1 M acetate buffer solution to make up the total volume of 20 ml. The reaction mixture consisted of two liquid phases because the substrate was insoluble in water. The mixture was emulsified by stirring with a magnetic stirrer for the enzymatic hydrolysis. Product and the remaining substrate were extracted with toluene and were analyzed.

Immobilization of lipase

Supports for immobilization were macro-porous beads of a hydrophilic adsorbent resin having hydroxy groups (Duolite S-761) and a hydrophobic adsorbent resin having no functional group (Duolite S-861). These resins were obtained from Sumitomo Chemical Co., Osaka, Japan.

Ten ml of the dry support measured with a graduated glass cylinder was well mixed with 20 ml of lipase solution containing 300 mg lipase, the water was removed by filtration, and the lipase-adsorbed support was washed with distilled water. The lipase immobilized to the support was measured by the lipase activity remaining in the water removed and in the washing solution.

Analysis

CPBA acetate, CPBA and PBAL were detected by high-performance liquid chromatography (HPLC) [column, LiChrosorb RP-18 (7 μ m, 4 mm ϕ $\times$ 30 cm); mobile phase, methanol/H₂O (6/4, 1 ml/min); detector, UV 254 nm]. The degree of enzymatic hydrolysis was calculated from the decrease of the substrate in the reaction mixture. The isomer ratio of the liberated CPBA was measured by enantiomer analysis with HPLC on CPBA ester of lauric acid which was prepared by esterification of the liberated CPBA with lauric anhydride [column, Sumipax OA-2000I (5 μ m, 4 mm ϕ $\times$ 25 cm, Sumika Chemical Analysis Service Co.); mobile phase, *n*-hexane/ethanol (500/0.5, 1 ml/min); detector, UV 254 nm].

Results and Discussion*Screening of microbial lipases*

There is an equilibrium between cyanohydrin and the corresponding aldehyde,¹⁵⁾ and this equilibrium causes the racemization of the optically active cyanohydrin.¹³⁾ Figure 2 shows the effect of pH of the medium on the stability of CPBA. The first order rate constant for spontaneous conversion of CPBA to PBAL, which was due to the equilibrium between CPBA and PBAL, increased as the pH was raised. It was suggested that the enzymatic reaction should be done under acidic conditions to get the liberated optically active CPBA in a sufficient yield. Since it was supposed that enzymes would retain their activities in a mild acidic medium, the enzymatic reaction was done at the pH from 4 to 5. Spontaneous hydrolysis of CPBA acetate did not occurred at all below pH 8 within 24 hr.

Six microbial lipases that showed high enantioselectivity in hydrolysis of the acetic acid ester with 4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC)¹⁰⁾ were tested for the enantioselective hydrolysis of CPBA acetate in mild acidic media (Table 1).

Gradual inactivation during the enzymatic reaction was observed both on the *Alcaligenes* lipase and the *Achromobacter* lipase, so additions of a certain amount of each lipase to the reaction mixture were needed to achieve the hydrolysis of about 50%.

CPBA/PBAL mol ratios were over 50 for all enzymatic reactions

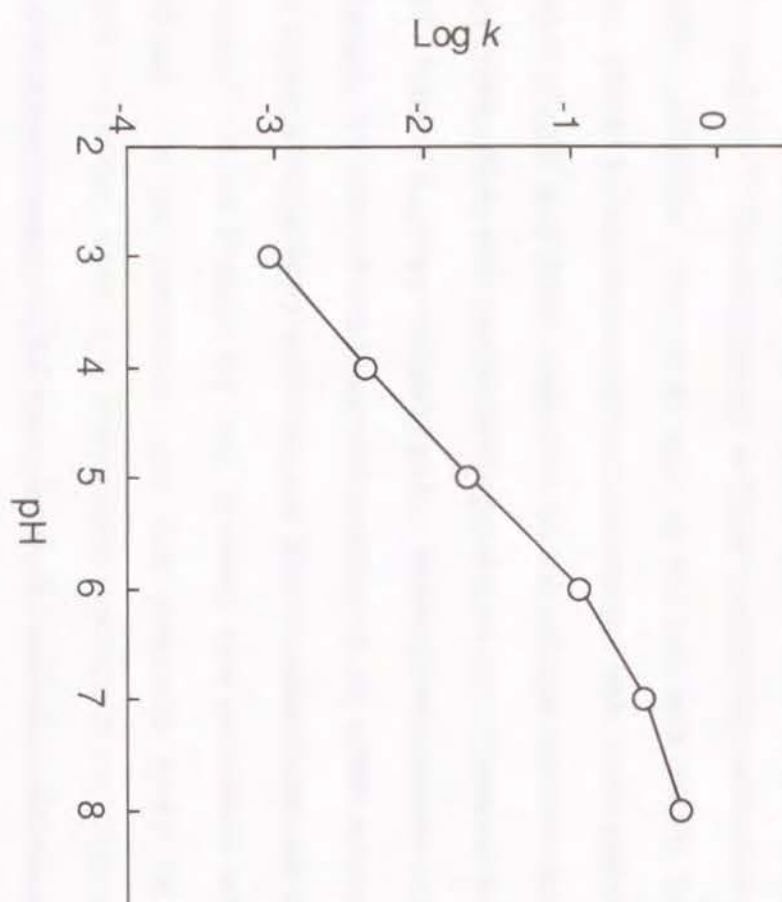


Fig. 2. Effect of pH on spontaneous conversion of CPBA to PBAL. Buffer solutions used were pH 3.0-5.0, 0.1 M acetate buffer; pH 6.0-8.0, 0.1 M phosphate buffer. Concentration of CPBA was 10w/v%. The first order rate constant of spontaneous conversion of CPBA to PBAL (k) was calculated from the amount of the PBAL produced in the mixture of CPBA and the buffer solution at 40°C for 1 hr.

Table 1. Enantioselective hydrolysis of CPBA acetate with microbial lipases^a.

Origin of lipase	Amount of lipase used ^b (mg)	Hydrolysis (%)	Isomer ratio of liberated CPBA (S)/(R)	E value
<i>Achromobacter</i> sp.	320 ^c	47.5	96.7/ 3.3	79
<i>Alcaligenes</i> sp.	80 ^d	49.8	96.4/ 3.6	88
<i>Arthrobacter</i> sp.	20	49.0	99.9/ 0.1	>3000
<i>Candida cylindracea</i>	200	50.7	14.9/85.1	12
<i>Chromobacterium</i> sp.	80	49.6	97.8/ 2.2	160
<i>Pseudomonas</i> sp.	120	49.8	96.4/ 3.6	88

^a The volume of reaction mixture was 10 ml. The reaction time was 23 hr. Reaction conditions for the *Arthrobacter* lipase and the *Chromobacterium* lipase; the substrate concentration was 20w/v%, and the reaction was carried out at 40°C and pH 4. Reaction conditions for the other lipases; the substrate concentration was 10w/v%, and the reaction was carried out at 30°C and pH 5.

^b The amount of lipase that achieved a hydrolysis of about 50% after 23 hr was used in the reaction unless otherwise stated.

^c The reaction was started with 80 mg of the lipase. After 8 hr and 20 hr, 160 mg and 80 mg of the lipase were added to the reaction mixture.

^d The reaction was started with 40 mg of the lipase. After 8 hr, 40 mg of the lipase was added to the reaction mixture.

and the liberated CPBA was highly optically active. These facts indicated that the optically active CPBA had remained stably in the enzymatic reaction mixture.

Five bacterial lipases hydrolyzed (*S*)-ester preferentially. Among these five lipases, the degree of the enantioselectivity (*E* value¹⁶⁾) on CPBA acetate was quite similar to that on HMPC acetate.¹⁰⁾ On the other hand, only the *Candida cylindracea* lipase hydrolyzed the (*R*)-ester preferentially. These results indicated that the *Candida cylindracea* lipase was distinctive in enantioselectivity from other lipases.

Among the lipases tested, the *Arthrobacter* lipase showed superior enantioselectivity and high hydrolytic activity.

Enantioselective hydrolysis by *Arthrobacter* lipase to give (*S*)-CPBA Effect of pH

Figure 3 shows the effect of pH on the hydrolytic activity of the lipase. The activity was not affected greatly by the pH from 7 to 3.5. Spontaneous conversion of CPBA to PBAL occurred at pHs higher than 5.0.

Effect of temperature

The courses of hydrolysis at different temperatures are shown in Fig. 4. The reaction did not proceed more than the hydrolysis of 50% where the (*S*)-CPBA acetate was completely hydrolyzed. The hydrolysis rate increased as the temperature was raised. Thermal inactivation of the enzyme was not observed even at 50°C (Fig. 5).

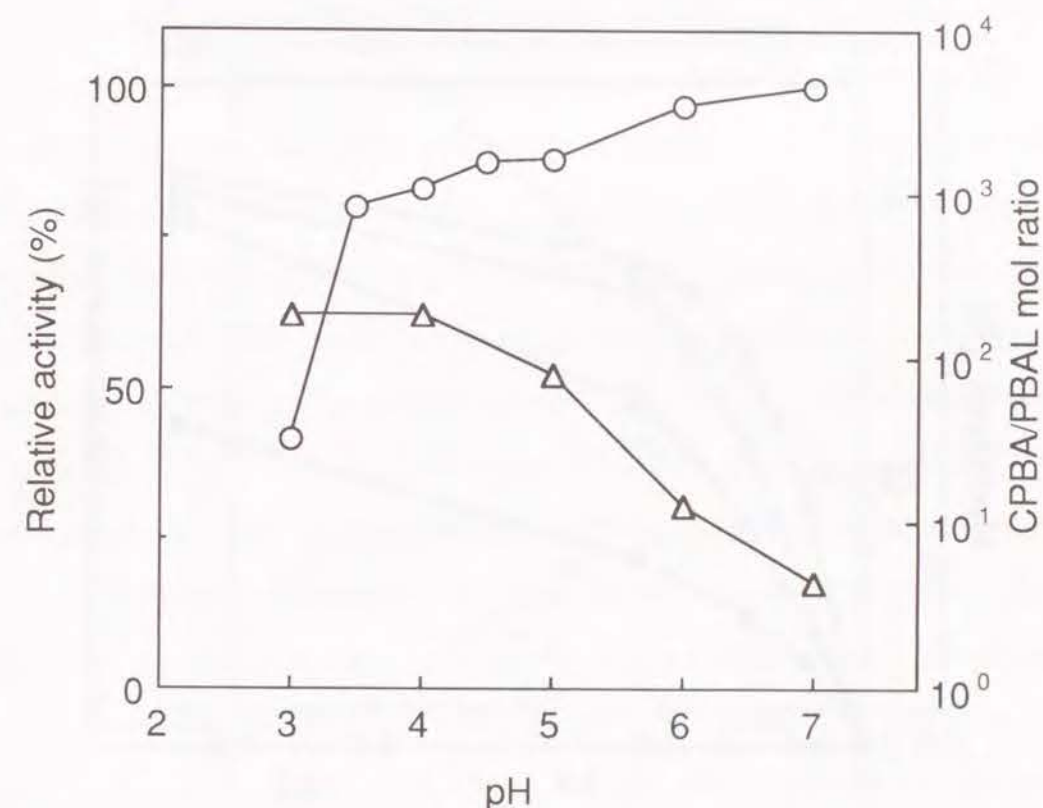


Fig. 3. Effect of pH on the hydrolysis of CPBA acetate with the *Arthrobacter* lipase. Buffer solutions used were 0.1 M acetate buffer (pH 3.0-5.0), 0.1 M phosphate buffer (pH 6.0 and pH 7.0). Concentrations of the substrate and the enzyme in the reaction mixture were 10w/v% and 0.2w/v%, respectively. The reaction temperature was 40°C. Activity (○) and CPBA/PBAL mol ratio (Δ) were measured after the reaction for 2 hr. Activity was expressed as the relative value to that at pH 7.0.

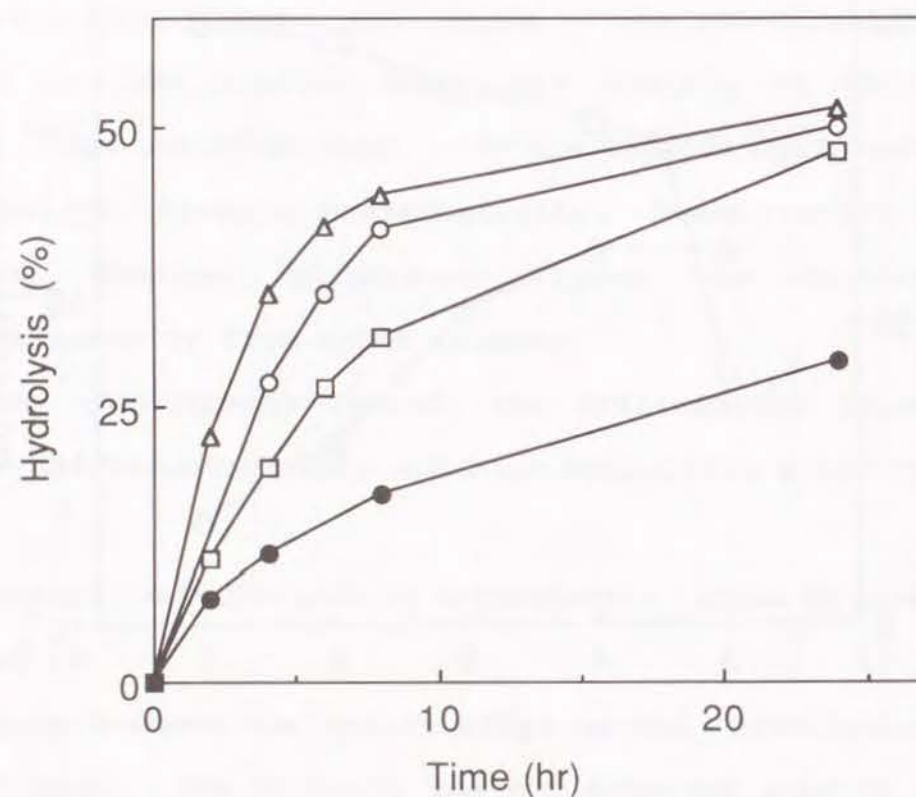


Fig. 4. Time course of lipase-catalyzed hydrolysis of CPBA acetate with the *Arthrobacter* lipase. Concentrations of the substrate and the enzyme in the reaction mixture were 20w/v% and 0.4w/v%, respectively. The pH was maintained at 4.0 with 1 N NaOH. Reaction temperatures were 20°C (●), 30°C (□), 40°C (○), and 50°C (△).

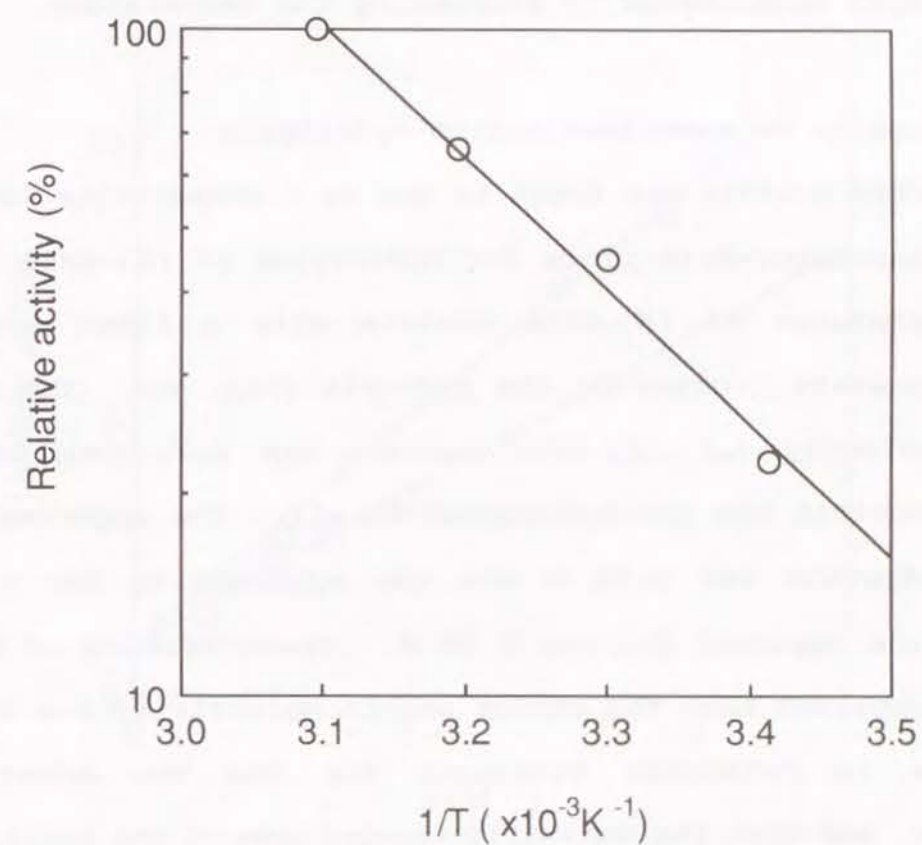


Fig. 5. Effect of temperature on the initial reaction rate of hydrolysis of CPBA acetate with the *Arthrobacter* lipase. Reaction conditions were the same as described in Fig. 3. Activity was expressed as the relative value to that at 50°C.

The CPBA/PBAL mol ratio was only 5.5 at 50°C, while the CPBA/PBAL mol ratio exceeded 50 below 40°C after the enzymatic reaction for 24 hr. This suggested that the spontaneous conversion of CPBA to PBAL was also accelerated by increasing the temperature.

Kinetic studies on enantioselective hydrolysis

(R)-CPBA acetate was found to act as a competitive inhibitor because Lineweaver-Burk plots for hydrolyses of (S)-CPBA acetate and the mixtures of (S)-CPBA acetate with a fixed amount of (R)-CPBA acetate crossed on the ordinate (Fig. 6). The maximum initial velocity for (S)-CPBA acetate was 1.21 $\mu\text{mol}/\text{min}$, and (R)-CPBA acetate was not hydrolyzed at all. The apparent K_m for (S)-CPBA acetate was 0.69 M and the apparent K_m for (R)-CPBA acetate (the apparent K_i) was 0.59 M. These results of kinetic studies suggested that the strict enantioselectivity was due to a difference in catalytic constants for the two enantiomeric substrates, and that the enzymatic recognition of the enantiomeric CPBA acetate and that of the enantiomeric HMPC acetate were the same.¹⁷⁾ Accordingly, the enantioselectivity of the *Arthrobacter* lipase for CPBA acetate can be also explained in terms of the hypothetical configuration model proposed for HMPC acetate (Fig. 7). The phenoxyphenyl group of CPBA acetate, like alkyl groups of HMPC acetate, will be bound to the alkyl binding site of the enzyme. Therefore, (S)-CPBA acetate and (R)-HMPC acetate have the same configurations at the catalytic center to be hydrolyzed while (R)-CPBA acetate and (S)-HMPC acetate have also the same

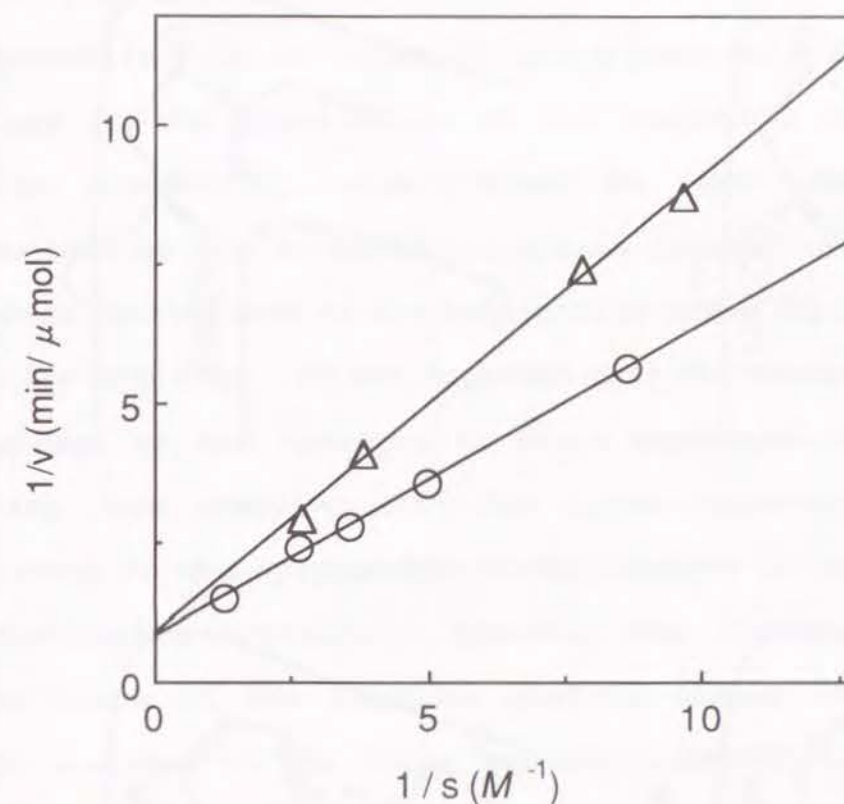


Fig. 6. Lineweaver-Burk plots for hydrolysis of (S)-CPBA acetate (○) and mixtures of (S)-CPBA acetate and a fixed amount of (R)-CPBA acetate (Δ). The concentration of (S)-CPBA acetate was expressed as s . The concentration of (R)-CPBA acetate was fixed at 0.3 M. The initial rate was measured at 40°C and pH 4.0.

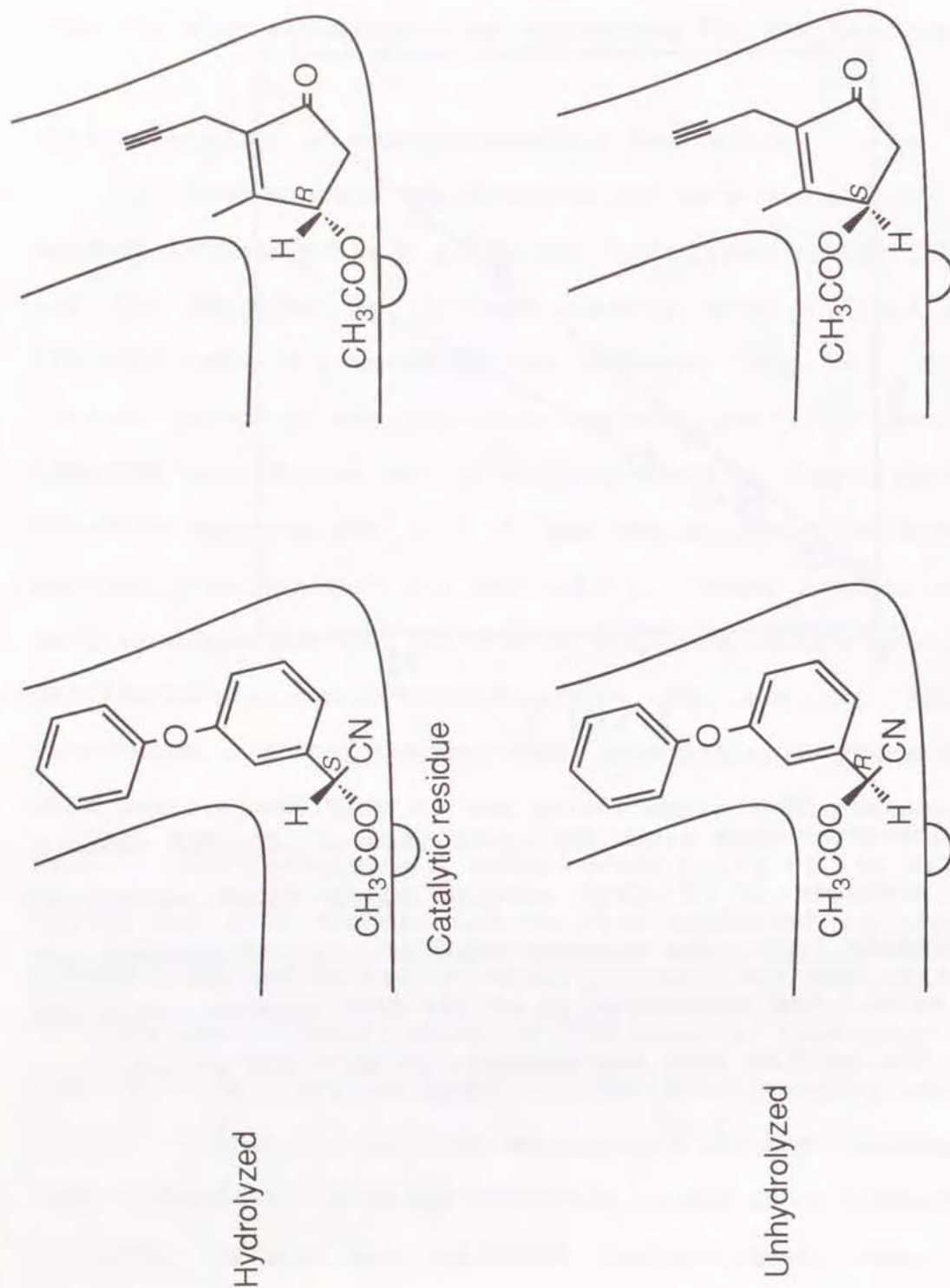


Fig. 7. Possible configuration of enzyme-substrate binding in the *Arthrobacter* lipase.

configurations not to be hydrolyzed.

Process for preparation of (S)-CPBA

Reuse of lipase

Immobilization or reuse of the enzyme will provide a great advantage in the application of the enzymatic reaction to the practical process.¹⁸⁾ Table 2 shows the result of hydrolysis of CPBA acetate by the *Arthrobacter* lipase immobilized to the resin. The lipase immobilized to the hydrophilic resin (Duolite S-761) had rather low activity. It was supposed that the water layer covering the surface of the hydrophilic resin prevented the hydrophobic substrate from reacting with the lipase molecule. The lipase immobilized to the hydrophobic resin (Duolite S-861) had a fairly high hydrolytic activity. However, the protein assay on the aqueous phase of the reaction mixture showed that 25% of the protein adsorbed to the resin had been removed from the support, and in the second use of the immobilized lipase, its activity was insufficient to complete the hydrolysis (the degree of hydrolysis was 33% after 21 hr reaction). Because the binding force was weak between the lipase and the support which had no functional group for binding, the lipase might have easily leaked from the support during vigorous stirring of the reaction mixture.

Because both CPBA acetate and the liberated CPBA were water-insoluble, the lipase solution could be recovered from the reaction mixture by water/oil phase separation, and the lipase solution could be repeatedly used for the next batch reaction by

Table 2. Hydrolysis of CPBA acetate with the immobilized *Arthrobacter* lipase.

Support	Amount of lipase used (mg)	Hydrolysis (%)	CPBA/PBAL mol ratio	Isomer ratio of liberated CPBA (S)/(R)
None	40	49.5	67	98.3/ 1.7
Duolite S-761	120	13.9	22	91.2/ 8.8
Duolite S-861	120	50.7	33	96.4/ 3.6

The volume of the reaction mixture was 20 ml and the substrate concentration was 10w/v%.

The reaction was carried out at 40°C and pH 4 for 21 hr.

addition of a fresh racemic substrate (Fig. 8). A hydrolysis of 44% was achieved even at the eighth run, though the activity of the lipase had decreased to a half of the initial activity. The enantiomeric excess of CPBA and the CPBA/PBAL mol ratio were greater than 95% e.e. and 50, respectively, for all runs.

Racemization of (R)-CPBA acetate

The unhydrolyzed (R)-CPBA acetate was separated chromatographically after the enzymatic hydrolysis of the racemic CPBA acetate by the *Arthrobacter* lipase ($[\alpha]_D^{25} = -8.8$ (c=1, benzene), 99.0% yield), and mixed with 10mol% of triethylamine in toluene at 70°C for 2 hr. The racemic CPBA acetate was obtained in a 98.9% yield after evaporation of the solvent in vacuo ($[\alpha]_D^{25} = 0$ (c=1, benzene)), and could be used for the enzymatic hydrolysis with no problem ((S)-CPBA of 98% e.e. was obtained at the hydrolysis of 50%).

Figure 9 shows a scheme of an efficient chemico-enzymatic process for an optically active pyrethroid which could be developed by combination of the enzymatic resolution with the *Arthrobacter* lipase and the chemical racemization. Repeated use of the lipase solution will provide an economical advantage for this process.

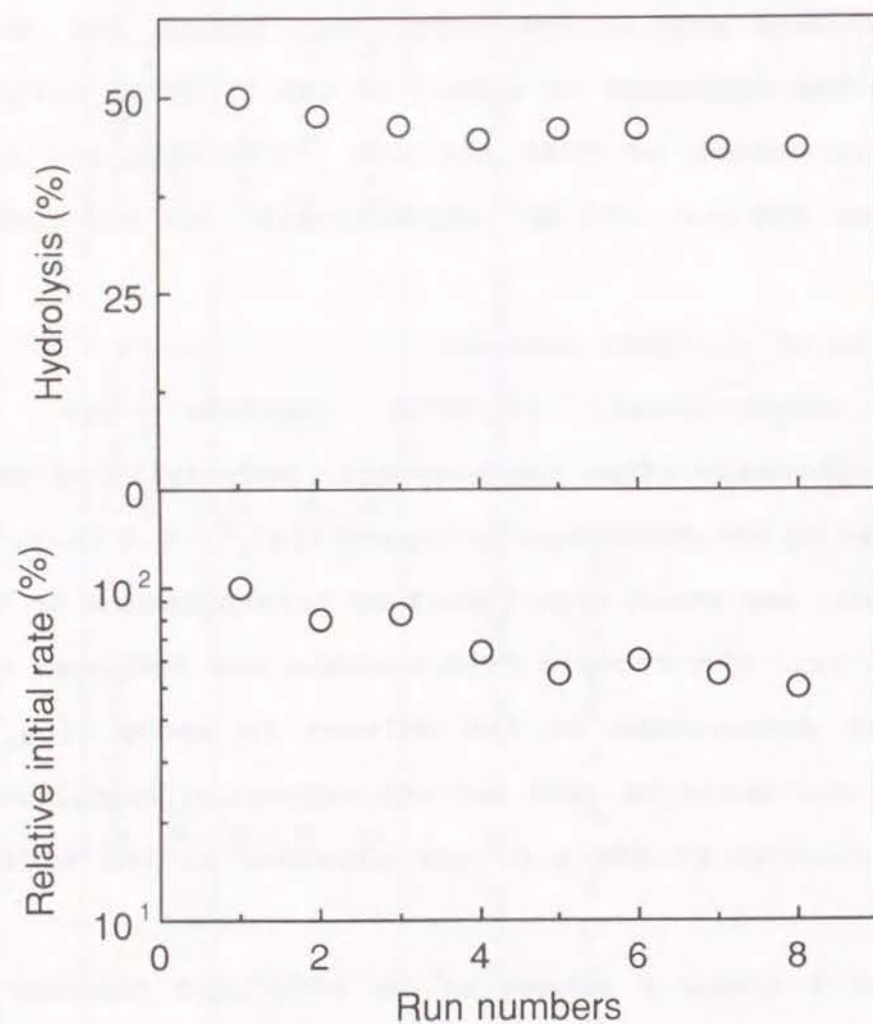


Fig. 8. Repeated use of the *Arthrobacter* lipase solution. Concentrations of the substrate and the enzyme in the reaction mixture were 20w/v% and 0.4w/v%, respectively. Each run was performed at pH 4.0 (maintained with 10% NaOH) and 40°C for 21 hr. After a run of reaction, the lipase solution (the upper phase) was separated from the oil (the lower phase) and was reused for the next run. Initial rate was expressed as the relative value to that in the first batch reaction.

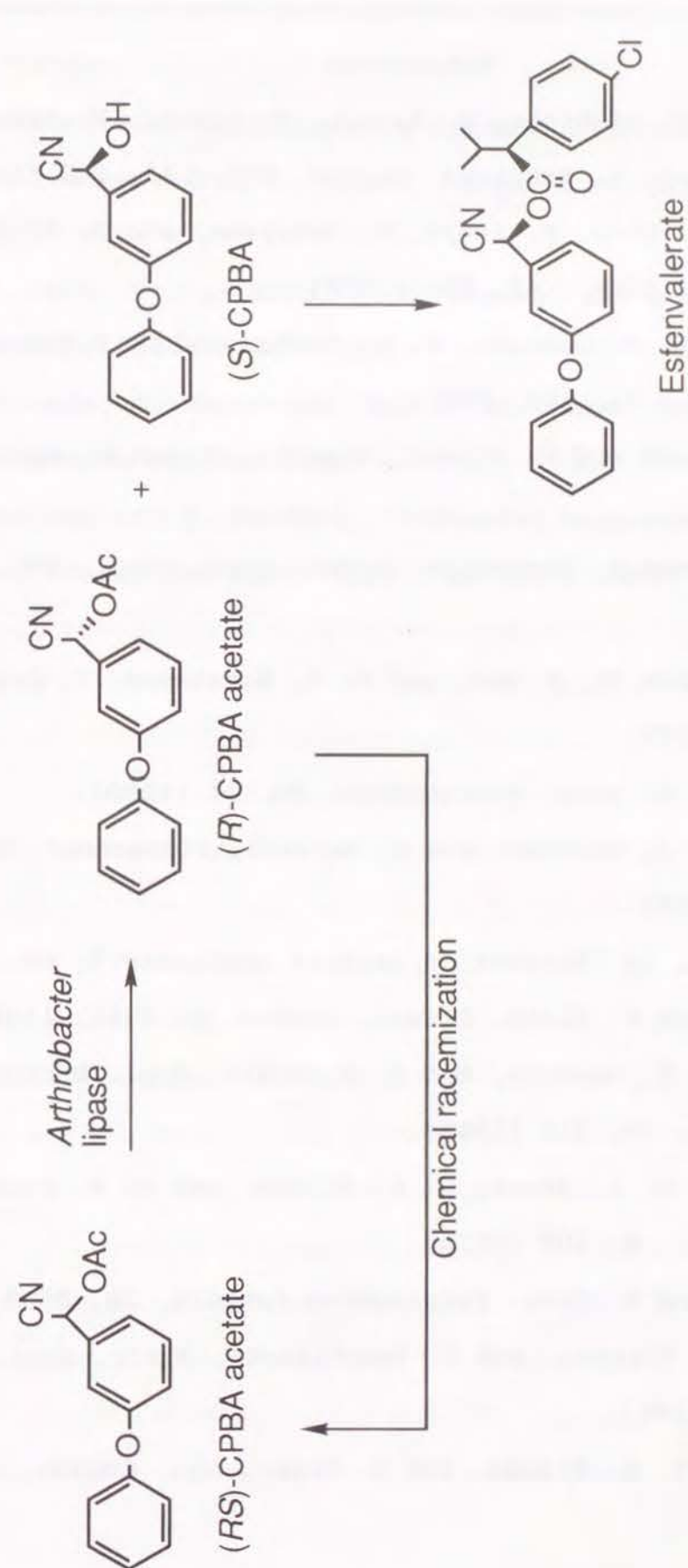


Fig. 9. Conversion of racemic CPBA acetate to (S)-CPBA for preparation of an optically active pyrethroid, esfenvalerate [(S)- α -cyano-3-phenoxybenzyl (S)-2-(4-chlorophenyl)-3-methylbutyrate].

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Part 3. *Pseudomonas* Lipase-catalyzed Enantioselective Transesterification of 1-Ethynyl-2-methyl-2-penten-1-ol in Organic Solvent

Summary

To obtain (*E*)-(*S*)-1-ethynyl-2-methyl-2-penten-1-ol [(*S*)-EMPO], lipase-catalyzed transesterification (and esterification) of (*RS*)-EMPO in organic solvent was examined. The *Pseudomonas* lipase catalyzed the enantioselective transesterification of (*RS*)-EMPO with vinyl acetate in *n*-hexane to give (*R*)-EMPO ester of acetic acid and (*S*)-EMPO with high reaction rate. Investigation on conditions of the *Pseudomonas* lipase-catalyzed transesterification revealed that the reaction was accelerated by increasing the hydrophobicity of the organic solvent used as a reaction medium and decreasing the water content of the reaction mixture. Under optimum conditions, (*S*)-EMPO of 99% e.e. was obtained at a conversion of 68.6%. Kinetic studies revealed that the lipase-catalyzed transesterification proceeded under the ping-pong mechanism involving the acyl-enzyme as an intermediate.

Introduction

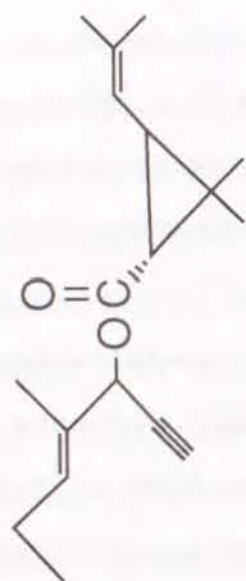
Recent extensive researches on enzymatic reactions in non-aqueous organic solvents have expanded the applicability of enzymes in organic syntheses.¹⁾ The optical resolution of racemic alcohols with hydrolytic enzymes such as lipase was achieved by enantioselective esterification (or transesterification) of the

Part 3

racemic alcohols in organic solvents,²⁻⁴⁾ as well as enantioselective hydrolysis of the corresponding racemic esters in aqueous solutions.⁵⁻⁷⁾

(*E*)-(*RS*)-1-Ethynyl-2-methyl-2-penten-1-ol [(*RS*)-EMPO] is the alcohol component of empenethrin [(*E*)-(*RS*)-1-ethynyl-2-methylpent-2-enyl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate] (Fig. 1) which is one of synthetic pyrethroid insecticides against household pests.⁸⁾ Empenethrin is especially used as a fumigant in a limited space to control hiding insects such as fabric pests because of its sufficient volatility at ambient temperature.⁹⁾ Studies on insecticidal activities of pyrethroids having optically active EMPO as alcohol components have revealed that (*S*)-EMPO was the insecticidally active enantiomer in pyrethroids.¹⁰⁾

In Part 1 and Part 2, lipase-catalyzed enantioselective hydrolyses of corresponding acetates of pyrethroid alcohols have been described. The present part deals with studies on lipase-catalyzed enantioselective transesterification (and esterification) and describes optimization of reaction conditions for the lipase-catalyzed enantioselective transesterification of (*RS*)-EMPO in organic solvent to obtain the insecticidally potent (*S*)-EMPO (Fig. 1). Kinetic studies on the lipase-catalyzed transesterification mechanism are also studied.



Empenthrin

(E)-(RS)-1-Ethynyl-2-methyl-2-pentenyl (1RS)-cis,trans-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate

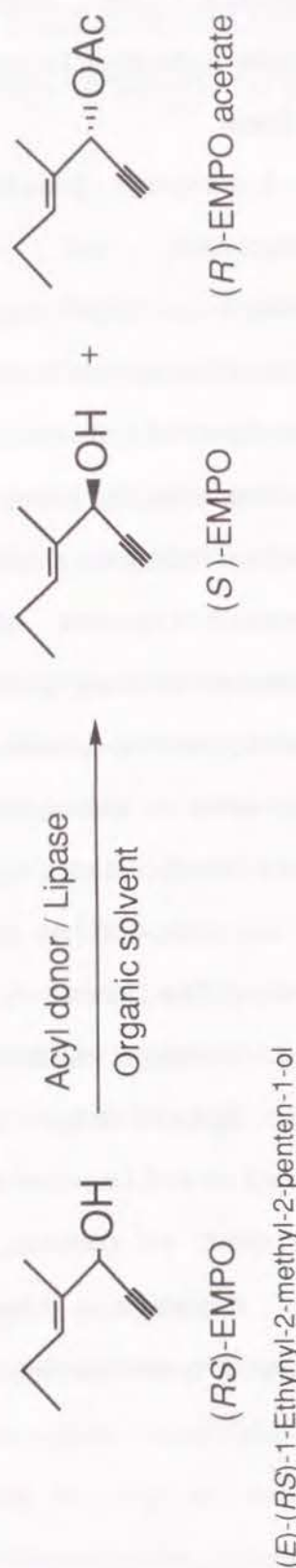


Fig. 1. Chemical structure of empenthrin and a scheme for the lipase-catalyzed enantioselective transesterification (or esterification) of (RS)-EMPO to give (S)-EMPO.

Materials and Methods

Materials

(E)-(RS)-1-Ethynyl-2-methyl-2-penten-1-ol [(RS)-EMPO] was synthesized from propionaldehyde and ethynylmagnesium bromide as described by Matsuo et al.⁸⁾ Organic solvents which had been dried with molecular sieves 4A (Nacalai Tesque Co.) were used in the reaction. Commercially available lipases used for the screening test were described in Part 1. The lipases were dried in a desiccator over P₂O₅ at room temperature.

Analysis

The gas-liquid chromatography (GLC) analysis was performed with a glass column of 10% FFAP on Chromosorb W(AW) [column size, 3.2 mmφ × 2.6 m; flow rate of N₂, 50 ml/min; column temperature, 140°C; detector, flamed ionized detector (FID)] to analyze quantitatively EMPO and (E)-1-ethynyl-2-methyl-2-pentenyl acetate (EMPO acetate) produced. Octadecane was used as an internal standard. Conversion ratio was defined as the mol ratio of EMPO acetate produced against (RS)-EMPO used as the substrate. The enantiomeric excess of EMPO was determined by high-performance liquid chromatography (HPLC) analysis on the corresponding 3,5-dinitrophenyl carbamate [column, Sumipax OA-2100 (5μ, 4 mmφ × 25 cm, Sumika Chemical Analysis Service Co.); mobile phase, n-hexane/1,2-dichloroethane/ethanol (80/10/1, 1 ml/min); detector, UV 254 nm]. Procedure to prepare the corresponding 3,5-dinitrophenyl carbamate for HPLC analysis was as follows. To

a solution containing about 5 mg of EMPO in *n*-hexane was added 3,5-dinitrophenylisocyanate (20 mg) and pyridine (0.05 ml). The mixture was heated at 70°C for 1 hr, and the supernatant solution was injected into a HPLC.

E value calculated by the following equation was used to estimate the enantioselectivity of the enzymatic reaction;¹¹⁾

$$E = \ln[(1-c)(1-ee(S))]/\ln[(1-c)(1+ee(S))]$$

where *c*, the degree of conversion;

ee(*S*), the enantiomeric excess of unreacted
(*S*)-EMPO

The water content in the organic solvent and that in the lipase were determined by using a Karl-Fischer moisture titrator (Type MKA-210, Kyoto Electronics Co., Ltd.).

Enzymatic transesterification (or esterification) of (RS)-EMPO

A general method for the enzymatic transesterification (or esterification) was as follows. The solution contained (RS)-EMPO (0.64 g, 5 mmol), an acyl donor (10 mmol) and organic solvent to make up the total volume of 25 ml in a capped 100 ml glass bottle. The reaction was started by addition of lipase (0.25 g) to the solution, and the reaction mixture was stirred with a magnetic stirrer at 600 rpm and 30°C. To estimate the initial reaction rate (lipase activity), 0.5 µl aliquot of the liquid phase was periodically withdrawn and analyzed by GLC. The reaction was stopped by separation of the lipase from the reaction mixture with a filter paper, and the filtrate was subjected to analysis. The

degree of conversion which was defined the mol ratio of EMPO acetate produced versus EMPO used as the substrate was calculated based on the quantitative analysis of the produced EMPO acetate.

Stability of lipase

The stability of the lipase in *n*-hexane was measured according to the following procedure. To *n*-hexane (20 ml) was added the *Pseudomonas* lipase (0.25 g) in a capped 100 ml glass bottle. The suspension was stirred with a magnetic stirrer (600 rpm) at a constant temperature for a certain time. Subsequently, to the suspension was added the substrate solution (5 ml) containing (RS)-EMPO (0.64 g, 5 mmol) and vinyl acetate (0.86 g, 10 mmol) in *n*-hexane. Lipase activity was estimated from the amount of EMPO acetate produced for 30 min reaction at 30°C.

The procedure for repeated use of the lipase was as follows. The solution contained (RS)-EMPO (1.28 g, 10 mmol), vinyl acetate (1.72 g, 20 mmol) and *n*-hexane to make up the total volume of 50 ml. To the solution was added the *Pseudomonas* lipase (0.5 g) and the reaction mixture was stirred under conditions mentioned above. After 21 hr, the reaction was stopped by separating the lipase from the reaction mixture with a filter paper. The recovered lipase was evaporated to dryness *in vacuo* at room temperature and used for the next run.

Results and Discussion

Screening of lipases

Table 1 shows the result of the screening test of five microbial lipases for the ability on enantioselective transesterification of (RS)-EMPO with vinyl acetate in *n*-hexane. All tested lipases transesterified (R)-EMPO preferentially to give (R)-EMPO acetate and (S)-EMPO. The *Pseudomonas* lipase showed the highest reaction rate and enantioselectivity (the highest *E* value¹¹⁾) among lipases tested. On the basis of these results, subsequent experiments were carried out with the *Pseudomonas* lipase.

Screening of acyl donors

Three acetic acid esters (for transesterification), acetic anhydride and acetic acid (for esterification) were tested as acyl donors (Table 2). High enantioselectivity was given by enol esters (vinyl acetate and isopropenyl acetate) which had been proposed as acyl donors for irreversible transesterification.^{12,13)} Vinyl acetate showed higher reaction rate than isopropenyl acetate. Acetic anhydride gave high reaction rate but poor enantioselectivity. Spontaneous esterification of EMPO by acetic anhydride was observed in an enzyme-free reaction mixture. It was considered that this spontaneous reaction lowered the apparent enantioselectivity and raised the apparent reaction rate in the reaction with the lipase.

Table 1. Enantioselective transesterification of (RS)-EMPO by microbial lipases.

Origin of lipase	Time (hr)	Conversion (%)	<i>E</i> value
<i>Alcaligenes</i> sp.	52	51.4	5
<i>Arthrobacter</i> sp.	52	23.9	<1
<i>Candida cylindracea</i>	52	20.9	<1
<i>Chromobacterium</i> sp.	52	7.4	ND
<i>Pseudomonas</i> sp.	10	48.3	16

Enzymatic reactions were done according to the general method described in **Materials and Methods**. Vinyl acetate and *n*-hexane were used as an acyl donor and organic solvent, respectively.

ND means "not determined". Conversion ratio was too small to estimate *E* value.

Table 2. Enantioselective transesterification (or esterification) of (RS)-EMPO with various acyl donors by the *Pseudomonas* lipase.

Acyl donor	Time (hr)	Conversion (%)	E value
Vinyl acetate	10	48.3	16
Isopropenyl acetate	50	45.4	18
Ethyl acetate	29	2.1	ND
Acetic anhydride	29	68.5	<5
Acetic acid	29	0.2	ND

The organic solvent was *n*-hexane.

ND means "not determined". Conversion ratio was too small to estimate *E* value.

Effect of reaction media

Eight organic solvents were tested as reaction media for the lipase-catalyzed transesterification of (RS)-EMPO with vinyl acetate. The enzymatic reaction was accelerated with increasing the hydrophobicity ($\log P$)¹⁴⁾ of organic solvent, and it was found that a linear relationship existed between the logarithm of initial reaction rate and $\log P$ (Fig. 2). Aldercreutz and Mattiason also reported that the enzyme activity was higher in organic solvent having higher $\log P$.¹⁵⁾ An appreciable dependence of enantioselectivity on $\log P$ was not observed, that is *E* values were in the range from 11 to 16 for solvents tested.

The effect of water (the total amount of the water in the lipase and the water in the liquid phase) was examined on the initial reaction rate of transesterification in *n*-hexane. The enzymatic transesterification was accelerated with decreasing the water content (Fig. 3). The highest activity was obtained at a very low water content of 0.017w/v%. At water contents above 0.634w/v%, the transesterification was entirely suppressed, and the enzymatic hydrolysis of vinyl acetate proceeded dominantly.

It is well-known that lipases have their respective optimum water content for the maximum activity in organic solvent.^{16,17)} Hirata *et al.* reported that the optimum water content existed in the range from 0.5w/v% to 1.0w/v% for the *Pseudomonas* lipase-catalyzed transesterification of tributyrin with 1-octanol (substrate was used as an organic medium without organic solvent).¹⁷⁾ Zaks and Klibanov also reported that hydrophobic

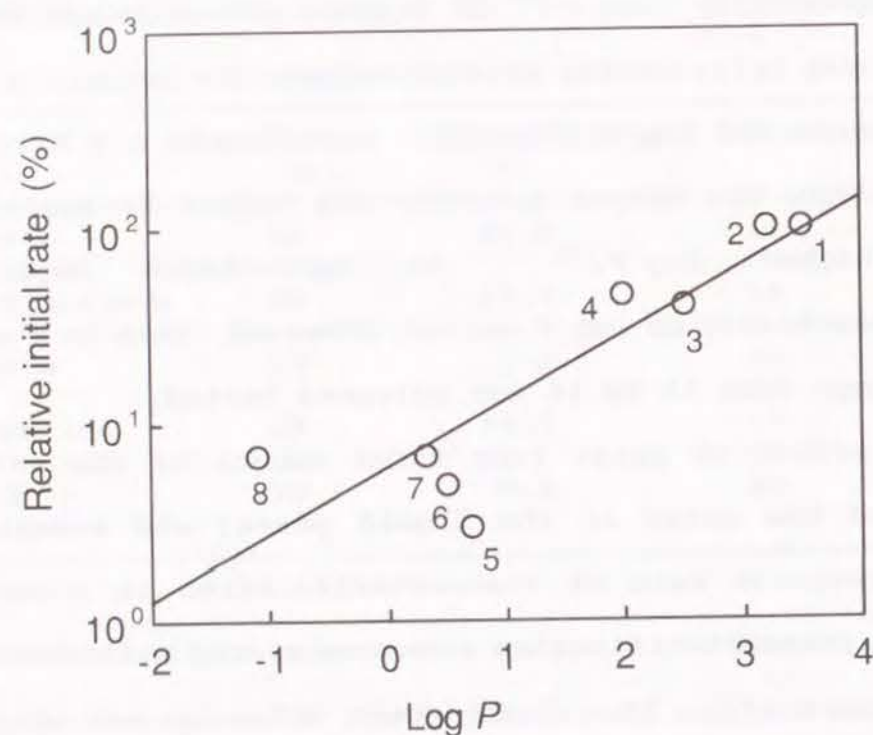


Fig. 2. Effect of organic solvents on the transesterification activity of the *Pseudomonas* lipase. Organic solvents [water content] used were *n*-hexane [0.009%] (1), cyclohexane [0.005%] (2), toluene [0.02%] (3), benzene [0.007%] (4), pyridine [0.04%] (5), tetrahydrofuran [0.04%] (6), acetonitrile [0.1%] (7), and dioxane [0.1%] (8). Enzymatic transesterification of (RS)-EMPO with vinyl acetate was performed at 30°C. Initial rate was expressed as the relative value to that in the reaction with *n*-hexane.

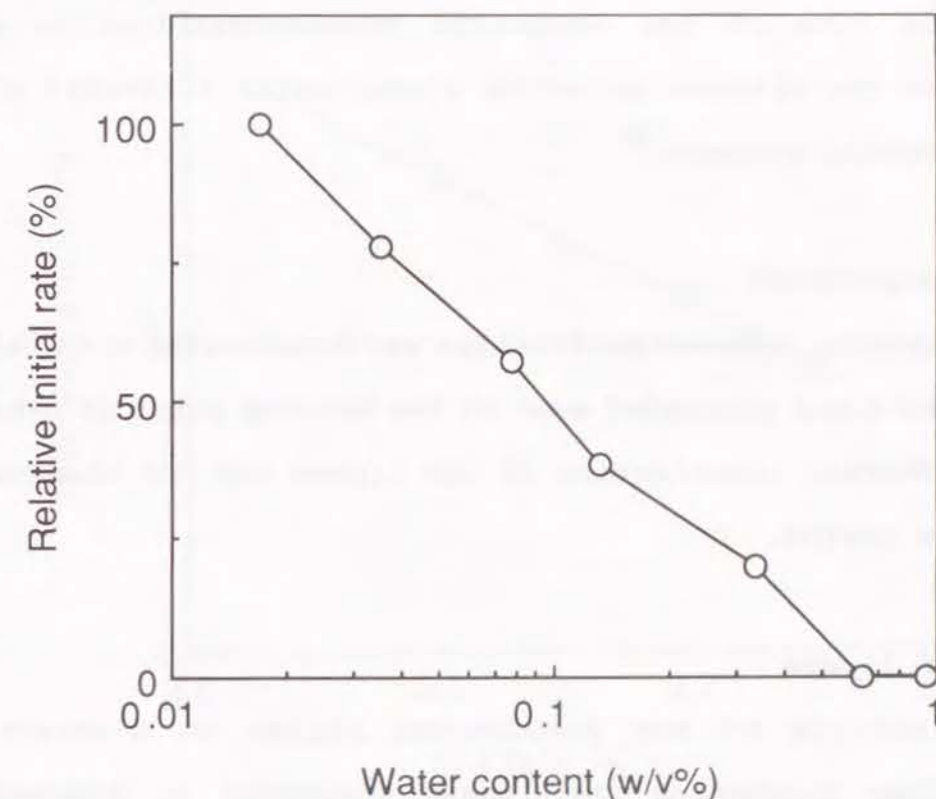


Fig. 3. Effect of water content of the reaction mixture on the transesterification activity of the *Pseudomonas* lipase. At the lowest water content of the reaction mixture (0.017w/v%), the water content of the lipase was 0.8w/w% and that of the liquid phase was 0.009w/v%. Other water contents of reaction mixture were obtained by increasing the moisture of the lipase or addition of water to the reaction mixture. Enzymatic transesterification of (RS)-EMPO with vinyl acetate in *n*-hexane was carried out at 30°C. Initial rate was expressed as the relative value to that at a water content of 0.017w/v%.

organic solvent did not strip the water on the surface of the enzyme but hydrophilic organic solvent did,¹⁸⁾ and that much less water was required to reach the maximal activity in hydrophobic solvent than hydrophilic solvent.¹⁹⁾ The optimum water content for the reaction rate of the enzymatic transesterification might correspond to the optimum hydration state (water activity) of the enzyme in organic solvent.²⁰⁾

Effect of temperature

The enzymatic transesterification was accelerated with raising the temperature and proceeded even at the boiling point of *n*-hexane (Fig. 4). Thermal inactivation of the lipase was not observed at temperatures tested.

Stability of lipase

The stability of the *Pseudomonas* lipase in *n*-hexane was examined after incubating the lipase suspended in *n*-hexane at various temperatures (Fig. 5). The lipase was quite stable at 30°C for 100 hr, but an increase in temperature lowered the stability of the lipase.

Repeated use of lipase

To examine the possibility of repeated use of the lipase, the *Pseudomonas* lipase recovered from the reaction mixture after the reaction was repeatedly used for batch reactions (Table 3). (*S*)-EMPO of 99% e.e. was obtained at a conversion of 68.6% in the

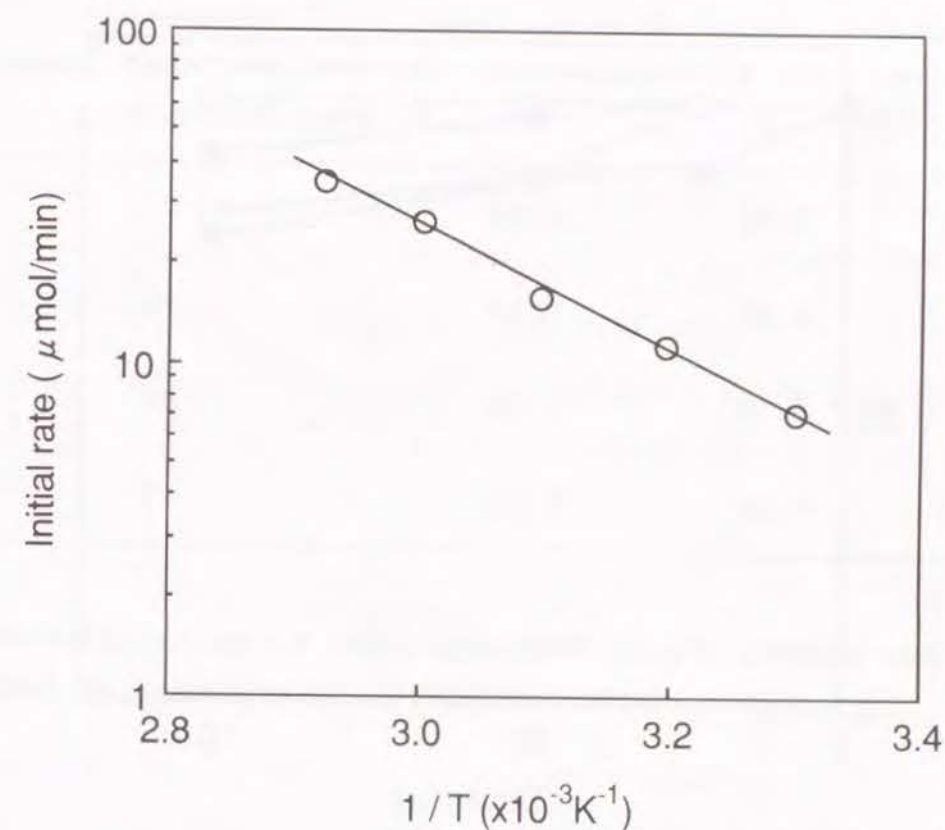


Fig. 4. Effect of temperature on enzymatic transesterification with the *Pseudomonas* lipase (Arrhenius plots). Enzymatic transesterification of (*RS*)-EMPO with vinyl acetate in *n*-hexane was performed at 30°C, 40°C, 50°C, 60°C, and 69°C (the boiling point of *n*-hexane). The water content of the reaction mixture was 0.017w/v%. Other conditions were described in **Materials and Methods**.

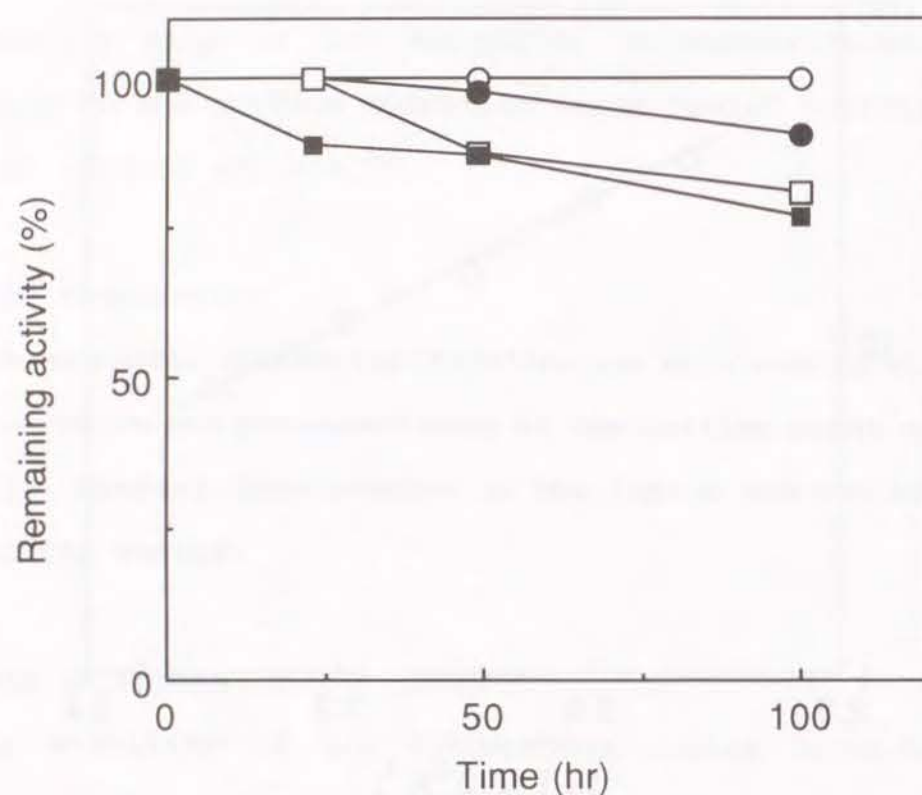


Fig. 5. Stability of the *Pseudomonas* lipase in *n*-hexane at various temperatures. The lipase was incubated in *n*-hexane at 30°C (○), 40°C (●), 50°C (□), and 60°C (■). The lipase activity was measured by transesterification of (RS)-EMPO with vinyl acetate at 30°C and was expressed as the relative value to that before incubation.

Table 3. Repeated use of the *Pseudomonas* lipase for enzymatic transesterification in organic solvent.

Run number	Relative initial reaction rate (%)	Conversion (%)	% e.e. of (S)-EMPO	E value
1	100	68.6	99.0	12
2	87	61.5	95.6	14
3	88	58.1	87.6	12
4	71	53.3	81.6	15

Transesterification of (RS)-EMPO with vinyl acetate was performed in *n*-hexane at 30°C for 21 hr.

first batch reaction. In spite of the high stability of lipase in *n*-hexane, the initial reaction rate of transesterification decreased slightly for each repeated batch reaction. It was suggested that a partial inactivation of the lipase was caused by acetaldehyde formed during the transesterification. The lipase which had been suspended in *n*-hexane containing acetaldehyde (0.4w/v%) at 30°C for 21 hr lost its activity by 25% of the initial activity. In view of the fact that the enantioselectivity of the lipase was unchanged for repeated reactions, it will be possible to use the lipase repeatedly in practical optical resolution process to prepare (*S*)-EMPO.

Kinetic studies on lipase-catalyzed transesterification

Reciprocal plots of the initial rate against the concentration of vinyl acetate at various concentrations of EMPO are given in Fig. 6. A set of parallel lines of reciprocal plots indicated the enzymatic transesterification proceeded under the ping-pong mechanism.²¹⁾ Figure 7 illustrates the ping-pong mechanism in the enzymatic transesterification taking account of the acyl-enzyme as an intermediate.²²⁾ This reaction mechanism indicates that the attack of the alcohol (EMPO) on the acyl-enzyme is competitively inhibited by the free water in the reaction mixture.

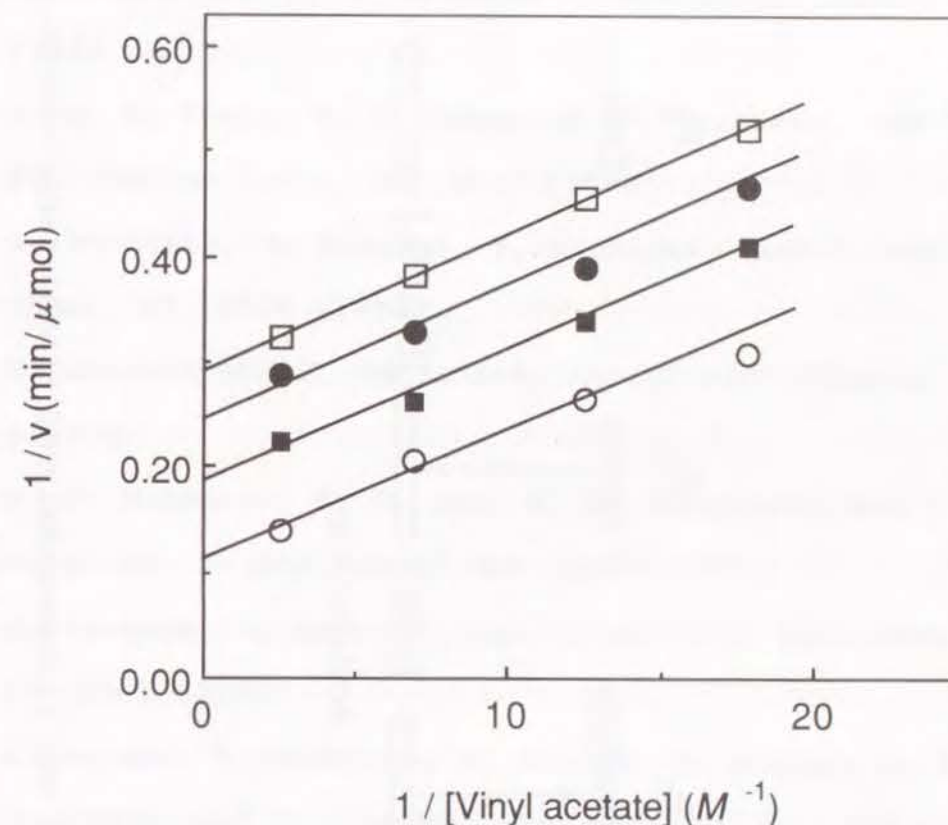


Fig. 6. Reciprocal plots of the initial rate (v) against the concentration of vinyl acetate at various concentrations of (*RS*)-EMPO. Concentration of vinyl acetate was varied at the following fixed concentrations of (*RS*)-EMPO. 0.04 M (□), 0.06 M (●), 0.1 M (■), and 0.5 M (○).

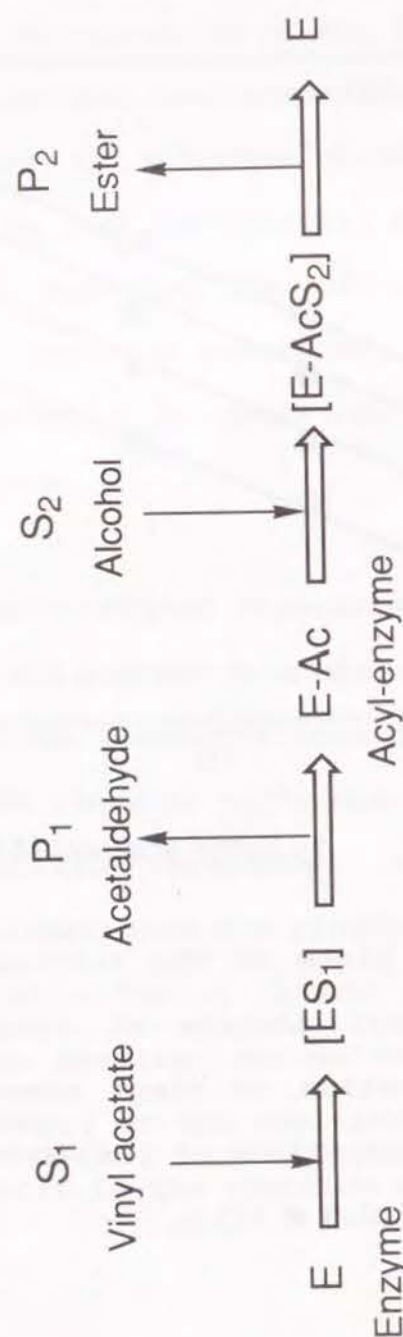


Fig. 7. Ping-pong mechanism in the lipase-catalyzed transesterification.

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Part 4. Diastereoselective Hydrolysis of Ethyl (1R)-*cis,trans*-Chrysanthemate with Microbial Cells for Preparation of (1R)-*trans*-Chrysanthemic Acid

Summary

Diastereoselective hydrolysis of ethyl (1R)-*cis,trans*-chrysanthemate with microbial cells was examined to prepare (1R)-*trans*-chrysanthemic acid that is an insecticidally potent acid moiety of pyrethroids. Through the screening test with 228 strains of culture collections, 31 strains were found to have hydrolytic ability. Diastereoselective hydrolysis of ethyl (1R)-*cis,trans*-chrysanthemate with *Arthrobacter globiformis* IFO-12958 gave (1R)-*trans*-chrysanthemic acid of excellent optical purity. The composition of cultivation medium for microbial production of the hydrolytic enzyme (the responsible esterase) was optimized. Examinations on conditions for the hydrolysis revealed that the optimum pH and temperature were pH 10 and 55°C, respectively. Cells of *Arthrobacter globiformis* IFO-12958 were treated with a chemical mutagen and the advantageous mutant SC-6-98-28, whose hydrolytic activity was enhanced 2.5-fold, was isolated.

Introduction

(1R)-*trans*-Chrysanthemic acid [(1R)-*trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylic acid] is the acidic part of the natural pyrethrin which is the insecticidally active ester compound occurring in pyrethrum flowers (*Chrysanthemum*

cinerariaefolium).¹⁾ Since the invention of allethrin [(1*RS*)-2-methyl-4-oxo-3-(2-propenyl)-2-cyclopenten-1-yl (1*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate],²⁾ various synthetic esters of chrysanthemic acid have been developed as pyrethroid insecticides.³⁾ (1*R*)-*trans*-Chrysanthemic acid for pyrethroid insecticides has been conveniently prepared by the resolution of (1*RS*)-*cis,trans*-chrysanthemic acid with chemical reagents.⁴⁾

Application of biocatalysts to the resolution of (1*RS*)-*cis,trans*-chrysanthemic acid has been attempted by some researchers, although their efforts did not succeed. Oritani et al. investigated the microbial hydrolysis of methyl (1*RS*)-*cis,trans*-chrysanthemate by *Trichoderma* sp. and found that the reaction was non-stereoselective.⁵⁾ Iriuchijima et al. reported that their attempts to hydrolyze ethyl chrysanthemate by microorganisms resulted in failure because tested microorganisms did not hydrolyze the ester.⁶⁾ On the other hand, Schneider et al. reported that the porcine liver esterase hydrolyzed methyl (1*RS*)-*cis,trans*-chrysanthemate with moderate diastereoselectivity to give (1*R*)-*trans*-chrysanthemic acid of insufficient optical purity.⁷⁾

This part deals with screening of microorganisms having the stereoselective hydrolytic ability toward ethyl (1*RS*)-*cis,trans*-chrysanthemate. It was found that *Arthrobacter globiformis* IFO-12958 could hydrolyze the (1*R*)-*trans*-chrysanthemic acid ester with strict diastereoselectivity to give optically pure (1*R*)-*trans*-chrysanthemic acid (Fig.1). Cultural conditions for production of

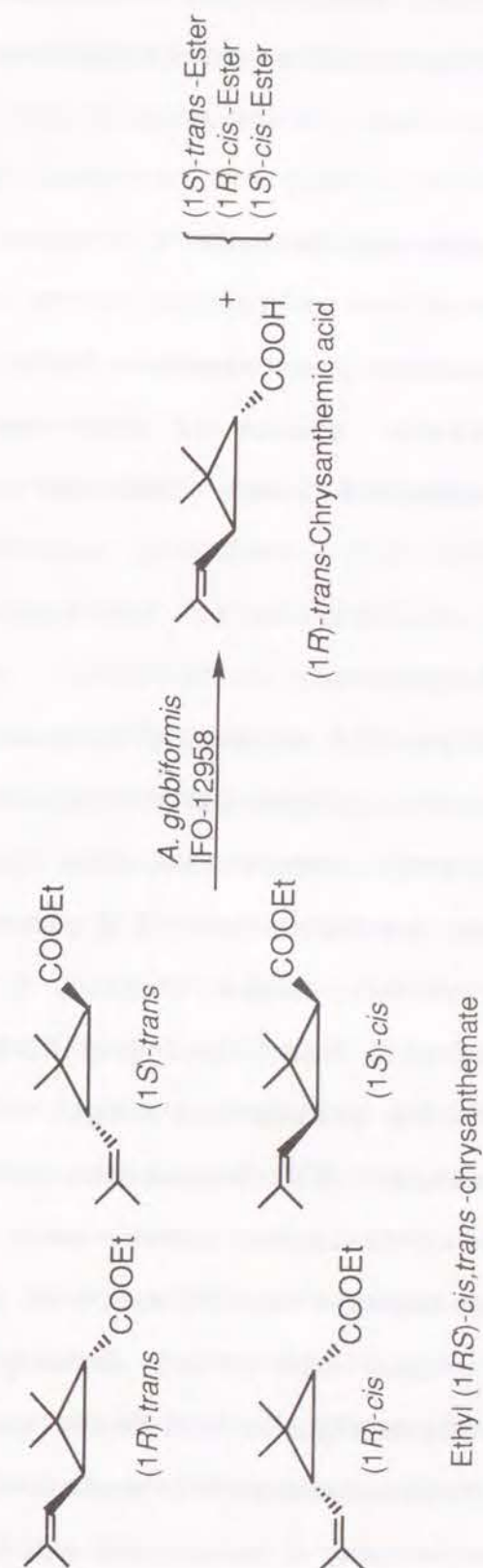


Fig. 1. Diastereoselective hydrolysis of ethyl (1*RS*)-*cis,trans*-chrysanthemate with microbial cells of *Arthrobacter globiformis* IFO-12958.

the hydrolytic enzyme (the responsible esterase) and reaction conditions for the hydrolysis of ethyl chrysanthemate were also examined in this study.

Materials and Methods

Chemicals

Ethyl (1RS)-*cis,trans*-chrysanthemate (*trans/cis*=67/33) was obtained from the industrial source of Sumitomo Chemical Co., Osaka, Japan. Other chemicals were obtained from commercial sources.

Screening test of microorganisms

Microorganisms used for the screening test were obtained from the Institute for Fermentation, Osaka (IFO), Japan and the American Type Culture Collection (ATCC), Rockville, USA. The following two cultural media were used; for bacteria, 10 g glucose, 7 g peptone ('Polypeptone', Daigo Eiyo Co., Osaka, Japan), 5 g yeast extract (Difco Laboratories, Detroit, USA), and 5 g K₂HPO₄ per liter of distilled water, pH 6.8; for yeasts and fungi, 10 g glucose, 2 g yeast extract, 3 g malt extract (Difco Laboratories, Detroit, USA), and 5 g K₂HPO₄ per liter of distilled water, pH 6.3.

The cultivation was carried out in a 500 ml Erlenmeyer flask containing 100 ml of a medium with rotary shaking (200 rpm) for 3 or 4 days at 30°C. Subsequently, to the flask was added 0.5 g of ethyl (1RS)-*cis,trans*-chrysanthemate (*trans/cis*=67/33). After 3 days of additional cultivation, 1 ml of 35% HCl aq. was added to

the flask. The liberated acid and the remaining ester were extracted with methyl isobutyl ketone and subjected to analysis. Hydrolytic ability of each strain was evaluated by the degree of hydrolysis. The degree of hydrolysis, which was defined as the mol ratio of the liberated chrysanthemic acid versus whole amount of ethyl ester used as the substrate, was determined by the gas-liquid chromatography (GLC) analysis.

Hydrolysis of ethyl (1RS)-*cis,trans*-chrysanthemate by microbial cells of *Arthrobacter globiformis* IFO-12958

Typical procedures for cultivation and hydrolytic reaction were as follows. Cultivation was carried out at 30°C for 48 hr with rotary shaking (200 rpm) in a 500 ml flask containing 100 ml of 'Medium I' which was the medium (pH 6.3) consisting of 30 g soluble starch ('Amycol', Nippon Starch Chemical Co., Osaka, Japan), 7 g peptone, 5 g yeast extract, and 5 g K₂HPO₄ per liter of distilled water. Cells were harvested by centrifugation at 10000 xg for 20 min and washed with 0.5 M sodium carbonate buffer (pH 10). Then the cells were suspended in 25 ml of the same buffer. To the suspension was added 500 mg of ethyl (1RS)-*cis,trans*-chrysanthemate (*trans/cis*=67/33), and the reaction mixture was stirred at 40°C. The reaction was terminated by adding 3 ml of 10% HCl aq. The liberated chrysanthemic acid and the remaining ester were extracted with methyl isobutyl ketone and subjected to analysis. The degree of conversion, which was defined as the mol ratio of (1R)-*trans*-chrysanthemic acid liberated versus ethyl (1R)-

trans-chrysanthemate in the substrate, was determined by a GLC analysis. The activity was evaluated from the initial reaction rate measured with the above reaction mixture. In this study, the total activity was expressed in nmol/hr per ml of the culture broth and the specific activity was expressed in nmol/hr per mg of dry cells.

Mutation

Cells of *Arthrobacter globiformis* IFO-12958 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) by the slightly modified method of Adelberg et al.⁸⁾ Mutagenized cells were cultivated with 100 ml of Medium I containing 20 mM ethyl (1*RS*)-*cis,trans*-chrysanthemate (*cis/trans*=67/33) in a flask. To isolate single colonies, small aliquot of the culture broth was spread on the agar medium plate which consisted of Medium I containing 20 mM ethyl (1*RS*)-*cis,trans*-chrysanthemate (*cis/trans*=67/33) and 2% agar. Strain of each single colony, which grew on the agar medium plate at 30°C in 48 hr, was picked up and inoculated into 100 ml of Medium I in a 500 ml flask. Cultivation was carried out at 30°C for 48 hr with rotary shaking (200 rpm). Subsequent evaluation of hydrolytic activity of each strain was carried out as above-mentioned.

Analysis

The gas-liquid chromatography (GLC) analysis was performed with a glass column of 10% Shinchrom F51 + 1% H₃PO₄ on Chromosorb

W(AW) [column size, 3.2 mmφ × 2.1 m; flow rate of N₂, 50 ml/min; column temperature, 180°C; detector, flamed ionized detector (FID)] to determine quantities of chrysanthemic acid and ethyl ester. The diastereomeric isomer ratio of chrysanthemic acid was determined by high-performance liquid chromatography (HPLC) analysis of the corresponding 3,5-dichloroanilide (column, Sumipax OA-2100 (5 μm, 4 mmφ × 25 cm, Sumika Chemical Analysis Service Co.); mobile phase, *n*-hexane/1,2-dichloroethane (17/3, 1 ml/min); detector, UV 254 nm) as described by Oi et al.⁹⁾

Results and Discussion

Screening of microorganisms

Two hundred and twenty eight strains of 43 genera from culture collections were examined as to their ability to hydrolyze ethyl chrysanthemate, and 31 strains were found to have hydrolytic ability (Table 1). Five strains that showed high ability are listed in Table 2. Three of them, *Arthrobacter globiformis* IFO-12958, *Candida humicola* IFO-0760, and *Thermomyces lanuginosus* IFO-9863, preferentially hydrolyzed ethyl (1*R*)-*trans*-chrysanthemate to give optically pure (1*R*)-*trans*-chrysanthemic acid. *Arthrobacter* sp. ATCC-21908 exhibited slight hydrolytic ability even toward *cis*-chrysanthemic acid ester. Only *Arthrobacter paraffineus* ATCC-15591 favored (1*S*)-*trans*-chrysanthemic acid ester.

Subsequent experiments were carried out with *Arthrobacter globiformis* IFO-12958 which showed the superior hydrolytic ability and the strict diastereoselectivity.

Table 1. Screening of strains having hydrolytic ability toward ethyl chrysanthemate.

Genus	Number of strains tested	Number of strains having hydrolytic ability
Bacteria		
Acetobacter	1	0
Achromobacter	2	0
Alcaligenes	3	0
Arthrobacter	17	4
Bacillus	21	0
Brevibacterium	5	0
Chromobacterium	1	0
Corynebacterium	1	0
Enterobacter	2	0
Escherichia	6	0
Flavobacterium	7	1
Klebsiella	1	0
Micrococcus	4	0
Mycobacterium	2	1
Nocardia	3	1
Pediococcus	1	0
Proteus	5	0
Pseudomonas	14	0
Rhodococcus	2	1
Serratia	2	0
Streptomyces	21	1
Yeasts		
Candida	9	3
Hansenula	8	0
Kloeckera	4	1
Pichia	2	0
Rhodosporidium	13	5
Rhodotorula	33	9
Saccharomyces	10	0
Schizosaccharomyces	1	0
Torulopsis	2	0
Fungi		
Absidia	1	0
Aspergillus	4	2
Dipodascus	1	0
Geotrichum	1	0
Gibberella	1	0
Gliocladium	5	0
Humicola	1	0
Mucor	2	0
Penicillium	2	0
Rhizopus	2	1
Thermomyces	2	1
Trichoderma	1	0
Zygorhynchus	2	0

Table 2. Strains having high hydrolytic ability toward ethyl chrysanthemate.

Strain	Hydrolysis (%)	Isomer ratio of acid liberated (1R)-trans/(1S)-trans/(1R)-cis/(1S)-cis
Arthrobacter globiformis IFO-12958	4.0	100 / 0 / 0 / 0
Arthrobacter paraffineus ATCC-15591	3.3	0 / 100 / 0 / 0
Arthrobacter sp. ATCC-21908	2.4	91 / 6 / 1.5 / 1.5
Candida humicola IFO-0760	2.1	100 / 0 / 0 / 0
Thermomyces lanuginosus IFO-9863	3.4	100 / 0 / 0 / 0

Screening test was carried out as described in Materials and Methods.

Hydrolytic activity of microbial cells of Arthrobacter globiformis IFO-12958 cultivated in various media

The effect of carbon source on the hydrolytic activity of microbial cells of *Arthrobacter globiformis* IFO-12958 was examined with glucose, glycerol and soluble starch (Table 3). Among them, soluble starch gave the best result in production of the hydrolytic enzyme (the responsible esterase). Figure 2 shows the effect of the concentration of soluble starch. The total activity increased with the concentration and leveled off above 3.0w/v%. The specific activity varied with the concentration and the maximum was obtained at 2.0w/v%.

The effect of nitrogen source was examined with peptone, corn steep liquor and NH_4NO_3 (Table 4). No appreciable difference in the hydrolytic activity (both the total activity and the specific activity) was found among these nitrogen sources. However, hydrolytic activity was greatly affected by the concentration of peptone (Fig. 3). The maximal value of the total activity and that of the specific activity were given at 0.7w/v% and 0.5w/v%, respectively.

In view of the results stated above, it was decided that the medium containing 3.0w/v% soluble starch and 0.7w/v% peptone (Medium I) was adopted as the most appropriate one. In the cultivation with Medium I, it was observed that the specific activity of cells increased with the cell growth (Fig. 4). After 24 hr cultivation, the cell concentration and the specific activity of cells attained the maximal value, and no decrease in the

Table 3. Effect of carbon sources on production of hydrolytic enzyme with *Arthrobacter globiformis* IFO-12958.

Carbon source		Relative total activity (%)	Relative specific activity (%)
Soluble starch	1.0%	85	100
	3.0%	100	94
Glucose	1.0%	20	27
	3.0%	43	36
Glycerol	1.0%	36	27
	3.0%	63	44

Experimental conditions were as described in **Materials and Methods** except that Medium I with the replaced carbon source was used for cultivation. Total activity was defined as the initial reaction rate per a definite volume of the culture broth and specific activity defined as the initial reaction rate per a definite weight of dry cells.

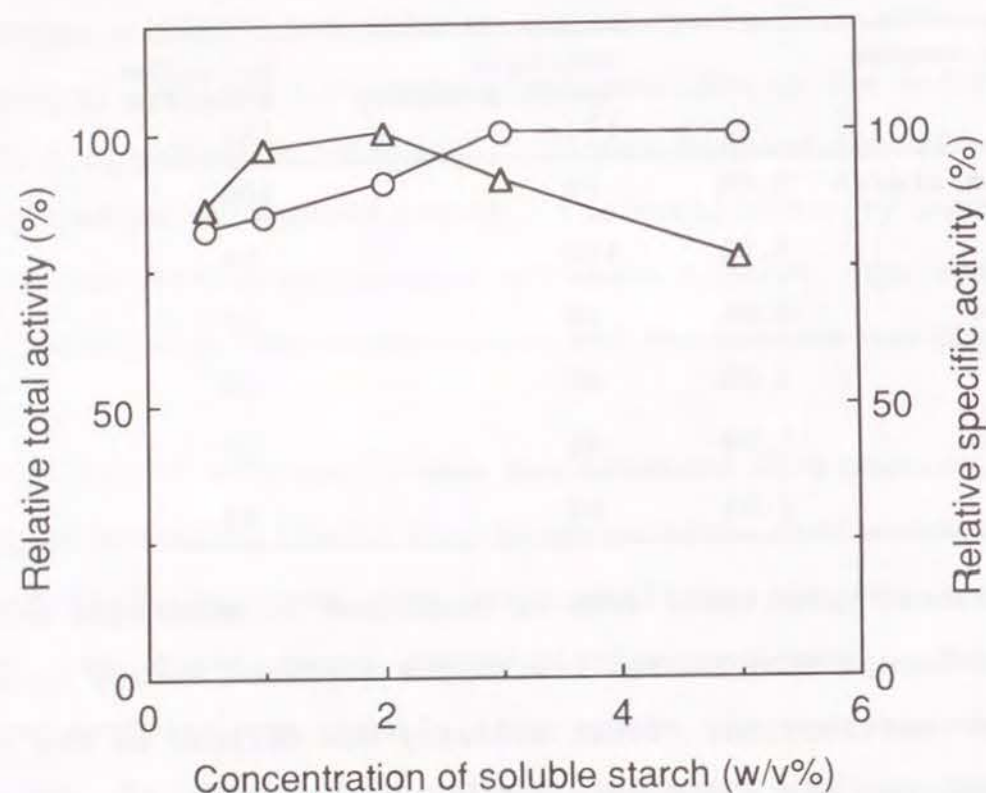


Fig. 2. Effect of the concentration of soluble starch on hydrolytic activity of *Arthrobacter globiformis* IFO-12958. Experimental conditions were as described in **Materials and Methods** except that Medium I modified for the concentration of soluble starch was used. Total activity (○) was expressed as the relative value to that at the concentration of 5.0%. Specific activity (△) was expressed as the relative value to that at the concentration of 2.0%.

Table 4. Effect of nitrogen sources on production of hydrolytic enzyme with *Arthrobacter globiformis* IFO-12958.

Nitrogen source		Relative total activity (%)	Relative specific activity (%)
Peptone	0.5%	100	100
	2.0%	74	63
C. S. L.	0.5%	85	97
	2.0%	88	66
NH ₄ NO ₃	0.5%	61	92
	2.0%	72	80

Experimental conditions were as described in **Materials and Methods** except that Medium I with the replaced nitrogen source was used for cultivation.

C.S.L. means corn steep liquor.

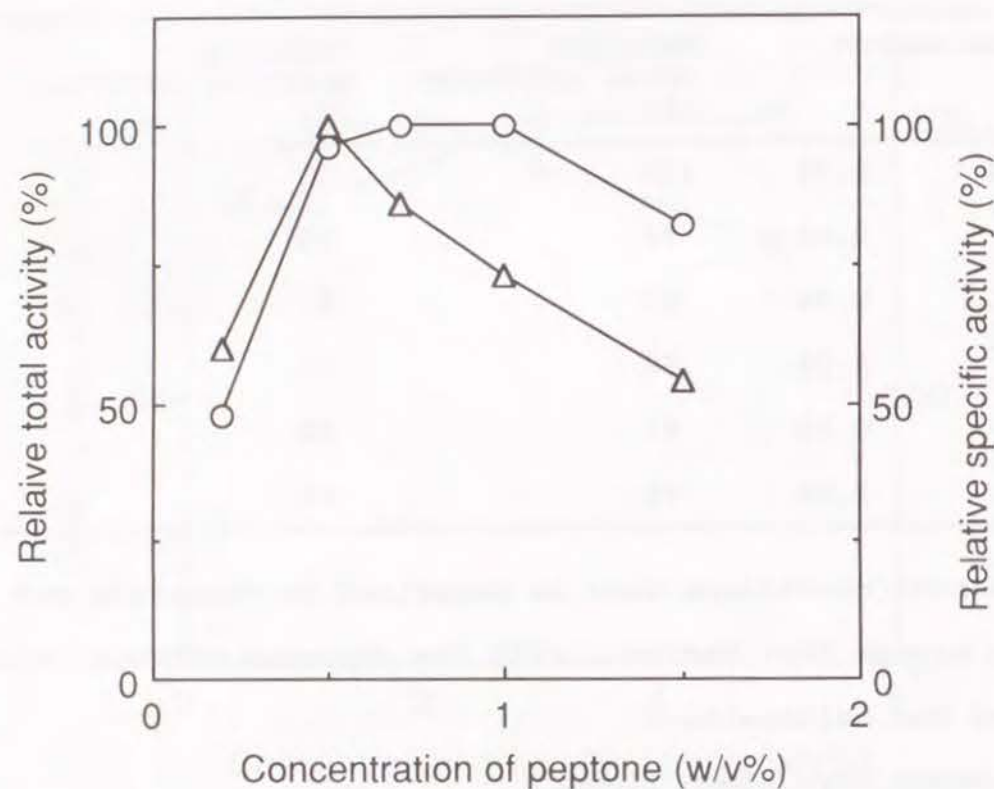


Fig. 3. Effect of the concentration of peptone on hydrolytic activity of *Arthrobacter globiformis* IFO-12958. Experimental conditions were as described in **Materials and Methods** except that Medium I modified for the concentration of peptone was used. Total activity (○) was expressed as the relative value to that at the concentration of 0.7%. Specific activity (△) was expressed as the relative value to that at the concentration of 0.5%.

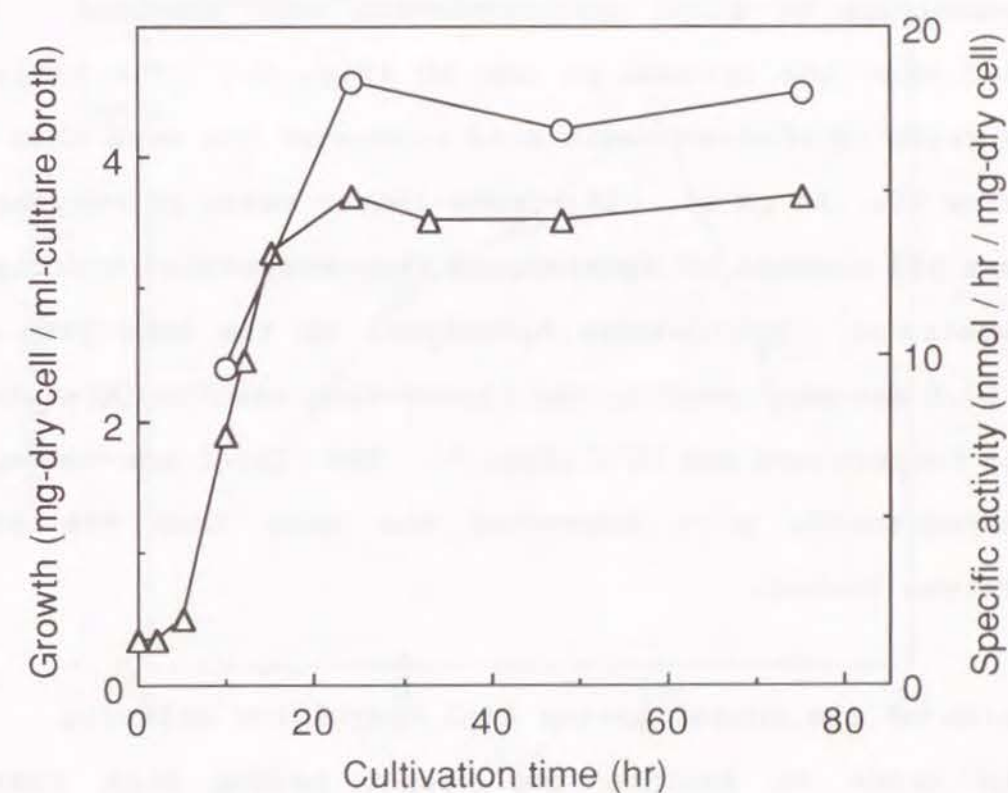


Fig. 4. Time courses of growth (△) and specific activity (○) with *Arthrobacter globiformis* IFO-12958. Cultivation was carried out with 100 ml Medium I in 500 ml flask at 30°C and 200 rpm on a rotary shaker. Specific activity was measured at 40°C and pH 10 as described in **Materials and Methods**.

specific activity occurred in the stationary phase of the growth.

Conditions for hydrolysis of ethyl chrysanthemate

Effects of pH and temperature on the initial reaction rate in the hydrolysis of ethyl chrysanthemate were examined. It was revealed that the optimum pH was 10 (Fig. 5). The (1R)-trans-isomer ratio in chrysanthemic acid liberated was more than 99% at pHs below 10. At pH 11, (1R)-trans-isomer ratio in chrysanthemic acid was 85% because of spontaneous (non-enzymatic) hydrolysis of the substrate. Spontaneous hydrolysis of the substrate at pHs above 10.5 was confirmed in the lipase-free reaction mixture. The optimum temperature was 55°C (Fig. 6). The (1R)-trans-isomer ratio in chrysanthemic acid liberated was more than 99% at each temperature tested.

Isolation of the mutant having high hydrolytic activity

In order to isolate the mutant having high hydrolytic activity, cells of *Arthrobacter globiformis* IFO-12958 was treated with NTG that is a chemical mutagen. It had been found that the growth of *Arthrobacter globiformis* IFO-12958 was inhibited by ethyl chrysanthemate at concentration above 20 mM and not inhibited by chrysanthemic acid. About 900 mutants, which grew and formed colonies on the agar medium plate consisting of Medium I with 20 mM ethyl (1RS)-cis,trans-chrysanthemate and 2% agar, were picked up. The hydrolytic activity was measured over cultured broth of each strain. The mutant SC-6-98-28 had the highest activity among

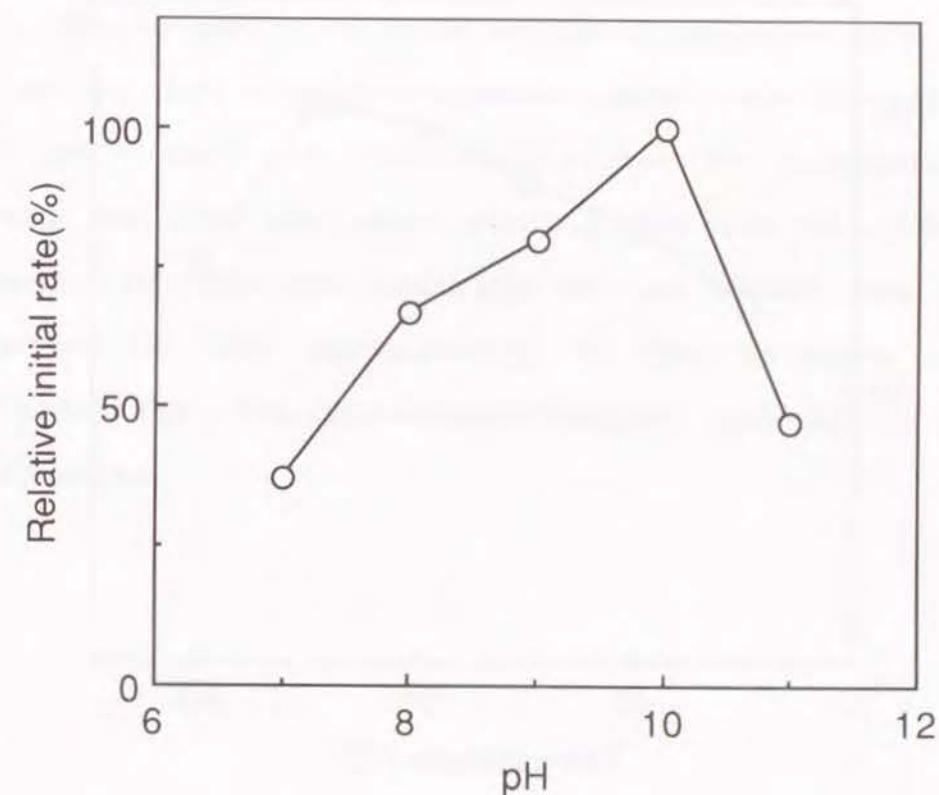


Fig. 5. Effect of pH on hydrolysis of ethyl chrysanthemate with microbial cells of *Arthrobacter globiformis* IFO-12958. Buffer solutions: 0.5 M phosphate buffer (pH 7.0, 8.0) and 0.5 M carbonate buffer (pH 9.0, 10.0, 11.0). Initial rate, which was measured at 40°C, was expressed as the relative value to that at pH 10.0.

them, and its specific activity was 2.5 times as high as that of *Arthrobacter globiformis* IFO-12958 (Table 5). Time courses of hydrolysis of ethyl (1*RS*)-*cis,trans*-chrysanthemate (*trans/cis*=67/33) by microbial cells of both strains are shown in Fig. 7. The mutant SC-6-98-28 achieved the conversion of 88% in 49 hr, whilst that with *Arthrobacter globiformis* IFO-12958 was 79% in 120 hr. Ratio of (1*R*)-*trans*-isomer in chrysanthemic acid liberated was 100% for each strain. It was thought that the enhancement of specific activity of the mutant was caused by improvement of the productivity of the esterase which was responsible for the diastereoselective hydrolysis of ethyl chrysanthemate.

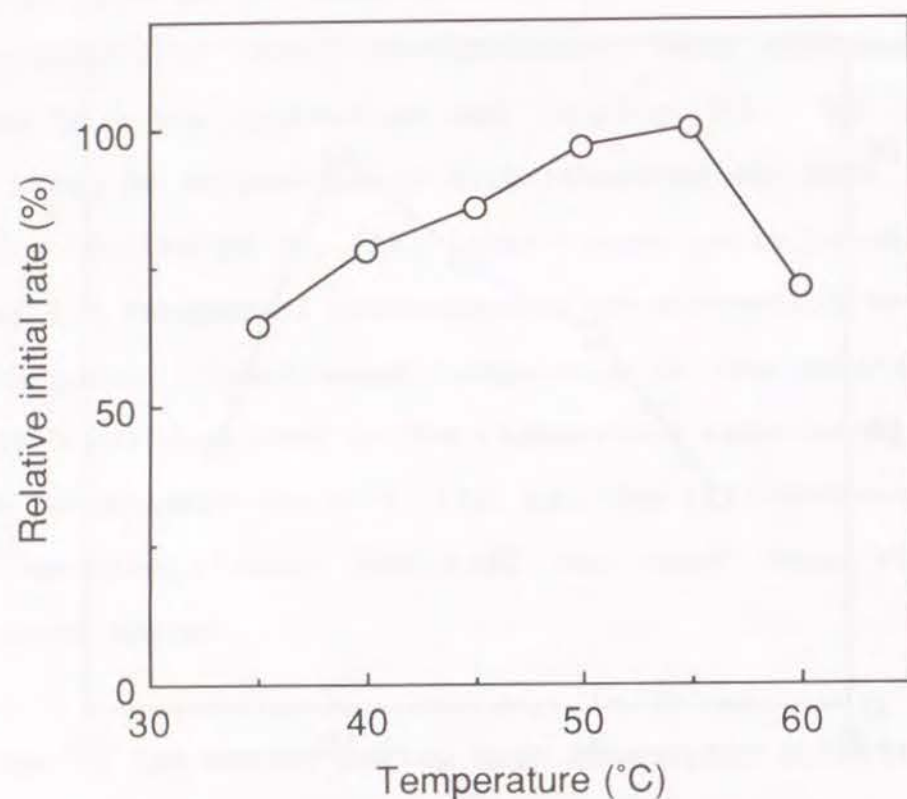


Fig. 6. Effect of temperature on hydrolysis of ethyl chrysanthemate with microbial cells of *Arthrobacter globiformis* IFO-12958. Initial rate, which was measured at pH 10.0, was expressed as the relative value to that at 55°C.

Table 5. Hydrolytic activity of microbial cells of *Arthrobacter globiformis* IFO-12958 and the mutant SC-6-98-28.

Strain	Total activity (nmol/hr/ml-cultured broth)	Specific activity (nmol/hr/mg-dry cell)
<i>A. globiformis</i> IFO-12958	58	12
Mutant SC-6-98-28	186	30

Both strains were cultivated with Medium I at 30°C for 48 hr. Other experimental conditions were as described in **Materials and Methods**.

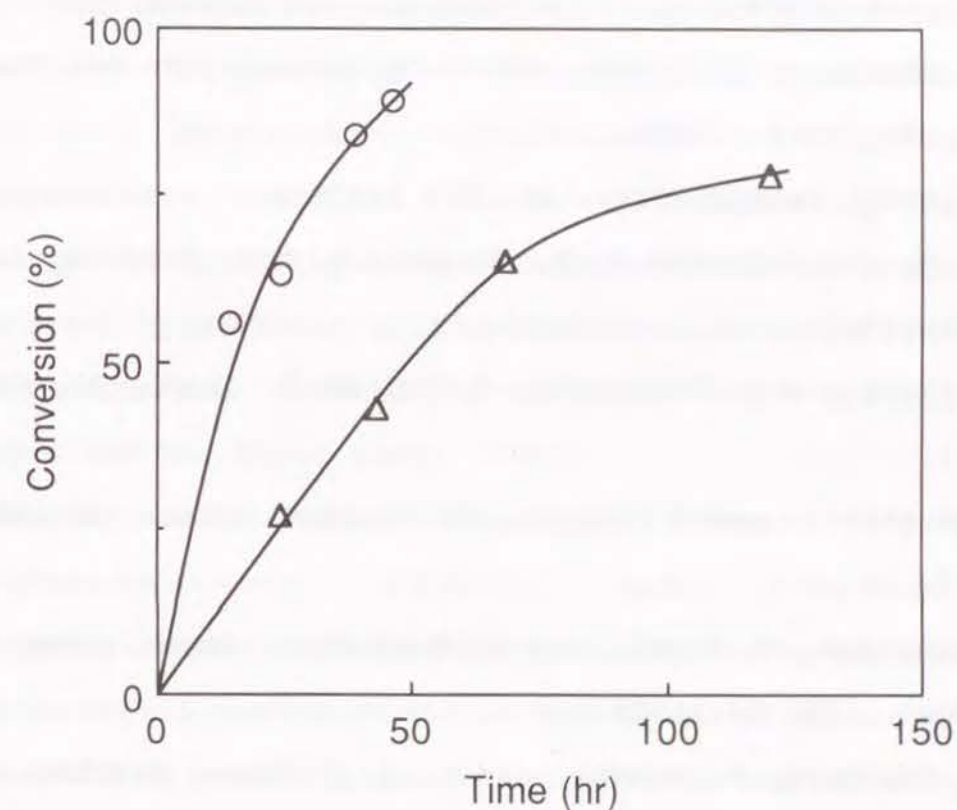


Fig. 7. Time courses of hydrolysis of ethyl chrysanthemate with microbial cells of *Arthrobacter globiformis* IFO-12958 (△) and the mutant SC-6-98-28 (○). Reaction conditions were as follows: Substrate, 500 mg ethyl (1*RS*)-*cis,trans*-chrysanthemate (*trans/cis*=67/33); intact cells, 1.25 g (as dry weight); buffer solution, 25 ml carbonate buffer (0.5 M, pH 10.0); pH, maintained at 10.0 with 10% NaOH aq.; temperature, 40°C. The degree of conversion was defined as the mol ratio (%) of (1*R*)-*trans*-chrysanthemic acid liberated versus ethyl (1*R*)-*trans*-chrysanthemate in the substrate.

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General Conclusion

Researches described in this thesis aimed at presenting biocatalytic methods for preparation of optically active components [optically active secondary alcohols and (1*R*)-*trans*-chrysanthemic acid] of pyrethroid insecticides. Lipase-catalyzed enantioselective reactions could be successfully applied to preparation of optically active alcohols. And, the preparation of (1*R*)-*trans*-chrysanthemic acid by diastereoselective hydrolysis of the corresponding racemic ethyl ester with microbial cells succeeded for the first time.

Resolutions of racemic 4-hydroxy-3-methyl-2-(2-propynyl)-2-cyclopenten-1-one (HMPC) and 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile (CPBA) were achieved by the *Arthrobacter* lipase-catalyzed enantioselective hydrolyses of acetic acid esters with respective racemic alcohols. The lipase-catalyzed hydrolysis of (RS)-HMPC ester of acetic acid proceeded efficiently at pH of neutral values to give (S)-HMPC ester of acetic acid and (R)-HMPC. The chemical inversion of (R)-HMPC to (S)-HMPC enabled the total conversion of (RS)-HMPC to (S)-HMPC in combination with the enzymatic hydrolysis. The lipase-catalyzed hydrolysis of (RS)-CPBA ester of acetic acid was carried out under acidic conditions to prevent spontaneous degradation of the liberated CPBA, and gave (R)-CPBA ester of acetic acid and (S)-CPBA. It was demonstrated that (R)-CPBA ester of acetic acid could be racemized chemically and be subjected to enzymatic hydrolysis again. Thus, chemico-

enzymatic processes for preparation of insecticidally potent (S)-HMPC and (S)-CPBA were successfully developed.

Investigation on the mechanism of enantioselectivity in lipase-catalyzed hydrolysis had been rarely made, hitherto. In order to know the mechanism of the *Arthrobacter* lipase-catalyzed enantioselective hydrolyses, kinetic studies were made on both acetic acid esters with HMPC and CPBA. It was revealed that the strict enantioselectivity of the *Arthrobacter* lipase was entirely due to the difference between the respective catalytic constants for the enantiomeric substrates.

The *Pseudomonas* lipase-catalyzed enantioselective transesterification of 1-ethynyl-2-methyl-2-penten-1-ol (EMPO) in organic solvent was studied to prepare (S)-EMPO. Transesterification with vinyl acetate in *n*-hexane gave good results in the enantioselectivity and the reaction rate to afford (R)-EMPO ester of acetic acid and (S)-EMPO. Investigation on the reaction conditions revealed that the hydrophobicity of organic media and the water content of the reaction mixture affected significantly the reaction rate. Kinetic studies elucidated that the lipase-catalyzed transesterification proceeded under the so-called ping-pong mechanism.

Finally, the biocatalytic preparation of optically active acid component of pyrethroid was tried. Diastereoselective hydrolysis of ester of (1RS)-*cis,trans*-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylic acid [(1RS)-*cis,trans*-chrysanthemic acid] with microbial cells (responsible esterase) had never known before

this study. The screening of microorganisms resulted in discovery of *Arthrobacter globiformis* IFO-12958 which could hydrolyze ethyl (1RS)-*cis,trans*-chrysanthemate to give (1R)-*trans*-chrysanthemic acid. The composition of cultivation medium was optimized for efficient production of the responsible esterase. Optimum pH and temperature for hydrolysis were pH 10 and 55°C, respectively. The advantageous mutant, SC-6-98-28, whose hydrolytic activity was enhanced 2.5-fold, was obtained by the treatment of *Arthrobacter globiformis* IFO-12958 cells with a chemical mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). It is expected that these findings will facilitate the further development of the enzymatic process for (1R)-*trans*-chrysanthemic acid.

Publication List

Part 1

- 1) Preparation of an optically pure secondary alcohol of synthetic pyrethroids using microbial lipases.
S. Mitsuda, T. Umemura, and H. Hirohara,
Appl. Microbiol. Biotechnol., 29, 310-315 (1988).
- 2) Studies on enantioselective hydrolysis of the acetic ester of a secondary alcohol with *Arthrobacter* lipase.
S. Mitsuda, S. Nabeshima, and H. Hirohara,
Appl. Microbiol. Biotechnol., 31, 334-337 (1989).

Part 2

- 3) Enantioselective hydrolysis of α -cyano-3-phenoxybenzyl acetate with *Arthrobacter* lipase.
S. Mitsuda, H. Yamamoto, T. Umemura, H. Hirohara, and S. Nabeshima,
Agric. Biol. Chem., 54, 2907-2912 (1990).

Part 3

- 4) Enzymatic optical resolution of a synthetic pyrethroid alcohol: Enantioselective transesterification by lipase in organic solvent.
S. Mitsuda and S. Nabeshima,
Recl. Trav. Chim. Pays-Bas, 110, 151-154 (1991).

Publication List

Part 4

- 5) Microbial stereoselective hydrolysis of $(\pm)$ -*cis,trans* ethyl chrysanthemate for preparation of $(+)$ -*trans* acid.
S. Mitsuda, R. Komaki, H. Hirohara, and S. Nabeshima,
Agric. Biol. Chem., 55(11), 2865-2870 (1991).

Other Publication

- 1) Enzymatic preparation of optically active alcohols related to synthetic pyrethroid insecticides.
H. Hirohara, S. Mitsuda, E. Ando, and R. Komaki,
In: *Biocatalysts in organic syntheses* edited by J. Tramper, H.C. van der Plas, and P. Linko, p.119-134 (1985).
- 2) Preparation of $(-)$ - α -ethynyl alcohol moieties of pyrethroid insecticides by lipase-catalyzed enantioselective hydrolysis.
S. Mitsuda, N. Matsuo, and S. Nabeshima,
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