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Enzymatic resolution of diltiazem intermediate by *Serratia marcescens* lipase: molecular mechanism of lipase secretion and its industrial application

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Abstract

A lipase from *Serratia marcescens* was selected as an asymmetric hydrolytic enzyme for *trans*-3-(4-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM], a key intermediate in the synthesis of diltiazem hydrochloride that is useful as a coronary vasodilator. This lipase has high enantioselectivity ($E = 135$) and was applied to the industrial production of the optically active intermediate of diltiazem using two-phase reaction system of organic solvent–water. Introduction of enzymatic reaction into the chemical synthetic route of diltiazem reduces the number of processes from nine to five. Analyses of the secretion mechanism of the lipase from *S. marcescens* cell membrane revealed that lipase (LipA), metalloprotease (PrtA), cell surface protein (SlaA) and flagellin are secreted via ABC-transporter, which is a common secreting mechanism in Gram-negative bacteria other than N-terminal signal peptide-dependent secreting mechanism. Molecular cloning of both the *lipA* gene, which codes the lipase protein, and *lipBCD* genes, which code the secretion device proteins, enable the production of the lipase by the self-cloning strain 140-fold as compared to the wild type strain. Immobilization of the lipase on a hollow fiber type membrane reactor contributes to the repeated use of enzyme and to efficient separation of the reaction product. Thus, enzymatic reaction and product separation are achieved simultaneously.

Keywords: Enzymatic resolution; Optically active compound; Lipase; Secretion; Hollow fiber type membrane reactor; Diltiazem

1. Introduction

Newly developed drugs have asymmetric atoms in their chemical structure, and biological effects reside in only one of its enantiomers. Another enantiomers have different biological effects or toxicity. Their action on human beings is competitive and differs in adsorption, metabolism, degradation and excretion. For the production of optically active pharmaceuticals, it is important to establish the process of optical resolution or asymmetric syntheses.

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Diltiazem is a representative calcium channel blocker and is used throughout the world to treat angina pectoris, hypertension and several other circulatory disorders [1,2].

Diltiazem has two asymmetric carbons in its chemical structure. Among the four possible stereoisomers of diltiazem, only the (+)-(2*S*,3*S*)-isomer exhibits potent coronary vasodilating activity. Therefore, diltiazem has been developed and marketed as a single isomer.

In order to obtain the desired optical isomer, various methods have been developed and the conventional chemical synthetic route is illustrated in Fig. 1. That route is composed of nine steps and chemical resolution with L-lysine was carried out at the higher molecular weight compound **6**. Thus, industrial production of diltiazem through this resolution process gave a large quantity of waste and the total processes were much fastidious. Although this resolution itself was very efficient, a simpler and more economical process was required to reduce the production cost of diltiazem.

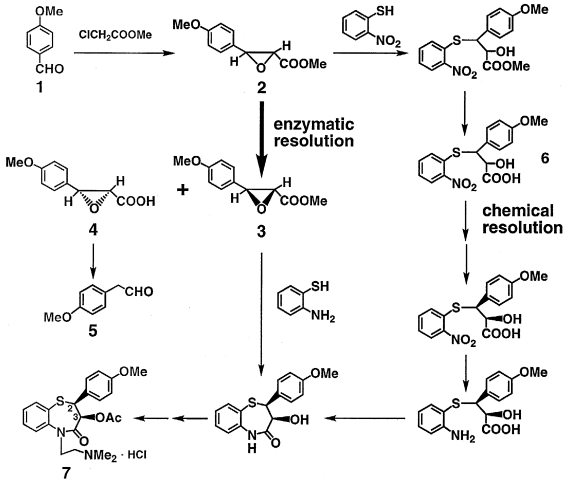

Fig. 1. The synthetic process for diltiazem. (1) 4-Anisaldehyde; (2) *trans*-3-(4-methoxyphenyl)glycidic acid methyl ester[(±)-MPGM]; (3) (2*R*, 3*S*)-3-(4-methoxyphenyl)glycidic acid methyl ester[(−)-MPGM]; (4) *threo*-2-hydroxy-3-(4-methoxyphenyl)-3-(2-nitrophenylthio)propanoic acid methyl ester; (5) *threo*-2-hydroxy-3-(4-methoxyphenyl)-3-(2-nitrophenylthio)propanoic acid; (6) (2*S*, 3*S*)-3-(2-aminophenylthio)-2-hydroxy-3-(4-methoxyphenyl)propanoic acid; (7) (2*S*, 3*S*)-2,3-dihydro-3-hydroxy-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-one; (8) (2*S*, 3*S*)-3-acetoxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)1,5-benzothiazepin-4(5*H*)-one hydrochloride [diltiazem]; (9) (2*S*, 3*R*)-3-(4-methoxyphenyl)glycidic acid; (10) 4-methoxyphenyl acetaldehyde.

We developed recently an enzymatic preparation of an optically active intermediate, methyl (−)-(2*R*,3*S*)-3-(4-methoxyphenyl)glycidate [compound 3, Fig. 1], and we utilized the enzymatic resolution process in industrial production of diltiazem [3–5]. In our studies, we found a new lipase from *Serratia marcescens*, which has a high enantioselectivity, $E = 135$, for the compound (+)-**3** [Fig. 1] and we demonstrated that the lipase from *S. marcescens* is different genetically from other known lipases [4,6]. Thus, we achieved the decrease of the number of synthetic processes from nine to five and therefore the manufacturing cost of diltiazem decreased to 2/3 compared to the original method including chemical resolution. Furthermore, overproduction of the lipase was performed since the cost of enzyme production was somewhat high.

In this review, we describe the new method for the production of optically active intermediate, glycidic ester, by the newly found lipase from *S. marcescens* [3], cloning of lipase gene [6] and also its secretion device genes [7], overproduction of lipase by self-cloning strain [8–10], and its application to the industrial scale with hollow fiber type membrane bioreactor [5].

2. Molecular mechanism of lipase secretion

2.1. Secretion of lipase by S. marcescens

N-terminal signal peptide-dependent protein secretion is abundant in procaryotes and eucaryotes. This universal mechanism consists of SecA, SecB, SecD, SecE, SecF, SecY, signal peptidase and signal peptide peptidase. Gram-negative bacteria has outer membrane and periplasm between inner and outer membrane accumulates proteins secreted through Sec system. Gram-positive bacteria has no outer mem-

brane and secretes proteins, which is secreted through N-terminal signal system into culture medium. Thus, Gram-positive bacteria are useful for industrial production of proteins, which is secreted into culture medium. Otherwise, Gram-negative bacteria also secretes some proteins into culture medium. For example, *S. marcescens* secretes lipase and proteinase into culture medium and these proteins have no N-terminal signal peptide.

From the cloning and sequence analyses of *Serratia* lipase gene (*lipA*), LipA has no N-terminal signal peptide and has short consensus sequences between some other secreting proteins, which is secreted through N-terminal signal peptide-independent secretion mechanism. These proteins are hemolysin in *Escherichia coli*, adenylcyclase in *Bordetella pertussis*, metalloprotease in *Erwinia chrysanthemi* and so on as shown in Fig. 2. In general, this N-terminal peptide-independent secretion mechanism consists of three kinds of proteins as shown in Fig. 3. Among these three proteins, the nearest protein to the cytoplasm is called ABC-protein and has ATP-binding motif. Therefore, this secretion system composed of these three proteins is classified as one of the ABC transporter families. Letoffe et al. [11,12] reported that metalloprotease and heme-binding protein are secreted into culture medium through the Has system in *S. marcescens* and expression of transporter gene is repressed by iron ions. Since lipase is secreted by *S. marcescens* in the presence of iron

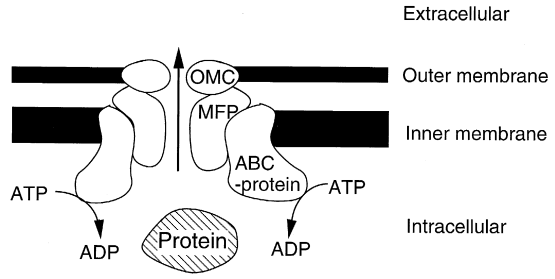

Fig. 3. ABC-transporter for Gram-negative bacteria. ABC-protein: ATP-binding cassette protein; MFP: membrane fusion protein; OMC: outer membrane component protein.

ions, secretion system other than Has system was suggested for the secretion of lipase. Cloning of the genes of secretion system was tried by the formation of hallo on tributyrin-agar and *lipB*, *lipC* and *lipD* genes, which code the proteins composed of 588, 443 and 464 amino acids, respectively, were found. These genes forms operon. As a result of homology analysis, transporter protein LipB, LipC and LipD have 55%, 46% and 42% homology with PrtD, PrtE and PrtF, which forms Prt system for the metalloprotease transporter in *E. chrysanthemi* [13] and have 54%, 45% and 26% homology with HasD, HasE and HasF, which forms Has system for the HasA protease transporter in *S. marcescens* [14].

2.2. Mechanism of Lip system

In *S. marcescens*, lipase, metalloprotease, flagellin and heme-binding protein are secreted into culture medium. On the medium for the

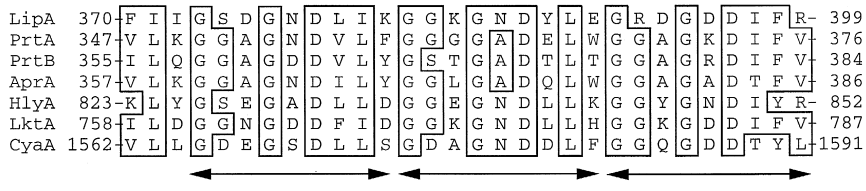

Fig. 2. Alignment of homologous regions. The nonapeptide repeat regions of the *S. marcescens* lipase are aligned with the repeats from the extracellular secreted proteins, *Serratia* sp. protease (PrtA), *E. chrysanthemi* protease (PrtB), *E. coli* hemolysin (HlyA), *P. haemolytica* leukotoxin (LktA), *B. pertussis* cyclolysin (CyaA), and *R. leguminosarum* Ca^{2+}-binding protein (NodO). The numbers refer to the positions of the amino acid residues in the protein sequences. Identical residues and alterations (I–V–M–L–F, D–E, N–Q, R–K and S–T), which are conserved in more than 60% of the proteins are boxed and nonapeptide repeat units are indicated by arrows.

production of lipase, lipase and metalloprotease are synthesized abundantly and these two proteins compete for secretion. In fact, secretion of protease is depressed under the expression of lipase gene transformed into the host *S. marcescens* with plasmid vector.

In general, genes of secreted protein forms cluster with those of transporter. However, genes of lipase and protease in *S. marcescens* does not form a cluster with those of transporters, *lipB*, *lipC* and *lipD*. On the downstream of *lipBCD*, the gene of lipopolysaccharide-related synthase is coded and on the upstream of *lipBCD*, unidentified ORF is coded in the same direction. We considered that the protein expressed from this unidentified gene might be a real substrate for Lip system and analyzed the protein expressed from this gene. As a result, this gene coded a protein composed of 1004 amino acids. Analysis of the amino acid sequence of this protein revealed that it has high homology with paracrystalline surface layer protein from *Caulobacter crescentus* [15] and it named SlaA as a homolog of cell surface protein. In fact, SlaA is secreted abundantly in *E. coli*, which is reconstructed from the vector plasmid with Lip system. In addition, in *S. marcescens* or also in *E. coli* lipase, metalloprotease and SlaA are not secreted in the deletion mutants of either *lipB*, *lipC* or *lipD* (Fig. 4). As a conclusion, *lipBCD* functions as genes of transporter system for the three proteins, lipase, metalloprotease and cell surface protein.

2.3. Recognition of secretory protein by ABC-transporter

Each ABC-transporter has its own substrate specificity for secretory proteins. In *S. marcescens*, SlaA is secreted only through Lip system and HasA is secreted only through Has system. Lipase and metalloprotease are secreted through Lip system or Prt system from *E. chrysanthemi* and also partly secreted through Has system in the reconstructed *E. coli*. Using hybrid transporter, which was reconstructed

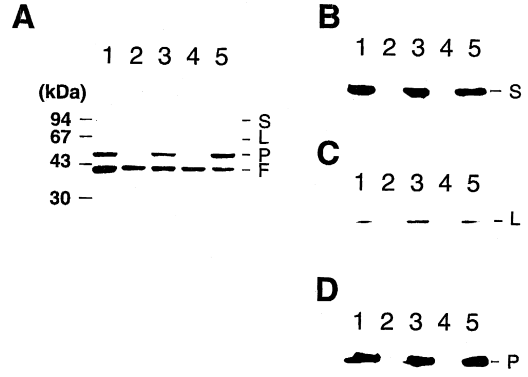

Fig. 4. Proteins in the cultured media and cell surface fraction of the wild-type strain and Lip system-dependent mutants of *S. marcescens* 176. The cells were cultured in lipase medium [10] at 30°C for 18 h. The polypeptides in the supernatant of the cultured media (0.2 OD equivalent units) were subjected to 12.5% SDS-PAGE. The gels were stained by Coomassie brilliant blue G-250 (A) and analysed by immunoblotting with antisera against SlaA (B), LipA (C) and PrtA (D). Molecular mass standards(sizes in kDa) are shown on the left. Positions of SlaA, LipA, PrtA and flagellin are shown as S, L, P and F, respectively. Lane 1: SM176; lane 2: SL2; lane 3: SL2 (pMWlipC12); lane 4: SL13; lane 5: SL13 (pMWlipB15). SM176: wild type strain of *S. marcescens*; SL2: LipC-deletion mutant of SM176; SL13: LipB-deletion mutant of SM176; pMWlipC12: vector with LipC; pMWlipB15: vector with LipB.

from Has system and Prt system, Binet and Wandersman [14] demonstrated from analyses of secretion profile of HasA and PrtC that secretory proteins are recognized by the protein, which locates nearest to cytoplasm and called as ATP-binding cassette. Furthermore, using the hybrid of Lip, Has and Prt system, we found that ABC-protein recognizes not only secretory proteins but also membrane fusion(MF)-protein [10].

3. Overproduction of *S. marcescens* lipase by cloning of *lipA* gene and its secretion device genes

The extracellular lipase from *S. marcescens* (triacylglycerol acylhydrolase [EC 3.1.1.3]) is an industrially important enzyme [3–5]. It is stable in some organic solvents and is applicable to the asymmetric synthesis of chiral com-

pounds by asymmetric hydrolysis. Previously, we cloned the *lipA* gene encoding this enzyme from *S. marcescens* and determined its nucleotide sequence [6]. The *S. marcescens* lipase has no conventional N-terminal signal sequence and is not subjected to processing at the N terminus. Thus, extracellular secretion of the *S. marcescens* lipase is not promoted by the general secretion pathway that require an N-terminal signal sequence [16]. *S. marcescens*, therefore, possesses a specific secretion system for extracellular lipase, which is a signal peptide-independent pathway [17].

The 50-kDa metalloprotease encoded by the *prtA* gene is known as one of the major secretory proteins of *S. marcescens* [17]. No overall homology was observed between the *S. marcescens* lipase and metalloprotease amino acid sequences except for a homologous region consisting of multiple repeats of nine amino acid residues (GGXGXDXXX), which is glycine and aspartic acid rich. This sequence is found in the following proteins: metalloproteases; the *prtB* and *prtC* gene products of *E. chrysanthemi* [18]; hemolysin, encoded by the *hlyA* gene of *E. coli* [19]; leukotoxin, encoded by the *lktA* gene of *Pasteurella haemolytica* [20]; cyclolysin, a multifunctional protein with adenylate cyclase activity and hemolytic activity, encoded by the *cyaA* gene of *B. pertussis* [21]; and Ca^{2+}-binding protein, encoded by the *nodO* gene of *Rhizobium leguminosarum* [22]. Colicin V, the *cyaC* gene product of *E. coli* [23], possesses a repeated glycine-rich sequence, which is not homologous to the GGXGXDXXX sequence but shares some characteristics with this consensus sequence. Although the GGXGXDXXX sequence has been proposed to be responsible for Ca-binding, the function of this region is unclear. Most of the proteins containing the above sequence have been reported to be secreted into culture medium by strains with a specialized secretion mechanism encoded by the *hlyBD/tolC* genes of *E. coli* [23] or the $prtDEF_{EC}$ genes [24] of *E. chrysanthemi* [25]. Genes encoding secretion machinery analogous to *hlyBD/tolC* and $prtDEF_{EC}$ have been reported in several bacteria: the *aprDEF* genes for *Pseudomonas aeruginosa* alkaline protease [26]; the *cyaBD* genes for cyclolysin of *B. pertussis* [21]; and the *lktBD* genes for the *P. haemolytica* leukotoxin [27]. Recently, the *hasDE* genes encoding HasD and HasE, which are two inner membrane components of the secretion mechanism for the *S. marcescens* metalloprotease and the heme-binding protein HasA [28], have been cloned from *S. marcescens* [29–31].

As the characteristics of the genes, *lipA* and *lipBCD*, became clear, we challenged to overproduce the lipase from *S. marcescens* for application to industrial production scale.

The wild type strain of *S. marcescens* expressed 30 units/ml of broth when cultured on the lipase medium. A *S. marcecsens* strain, which has *lipA* gene in the vector plasmid pUC19 and also in the chromosome gene expressed 400 units/ml of broth when cultured on the lipase medium. A *S. marcescens* strain, which has *lipA* gene and also *lipBCD* genes on the same vector cannot grow well on the lipase medium, since too much expression of many genes of *lipBCD* affects the growth of the microorganism. Therefore, we cloned *lipA* on a multicopy plasmid, pUC 19 and *lipBCD* on another small copy plasmid, pMW 121.

Self-transformation of both *lipA* gene and *lipBCD* genes resulted in the production of lipase protein at a level of 4200 units/ml of broth in the culture medium, which is 140-fold compared to the wild type *S. marcescens* strain. Details of these results will be reported in another paper, which is in preparation.

4. Industrial production of optically active diltiazem intermediate using hollow fiber type bioreactor

A lipase excreted from *S. marcescens* Sr41 8000 enantioselectively hydrolysed (+)-MPGM ($E = 135$, [1]). The reaction proceeded effi-

ciently when the enzymatic hydrolysis was carried out using a conventional emulsion reactor with toluene–aqueous biphasic system [1]. However, to achieve industrial production of (−)-MPGM using the emulsion reactor, destruction of the emulsion and separation into two phases were required for effective isolation of the product. Enzyme stability and recovery are also required for its repeated utilization. In particular, the recovery of enzyme is very important for cost reduction in industrial processes involving enzyme reactions. However, efficient recovery is difficult in the case of a stable emulsion. We proposed an immobilized enzyme system to solve these disadvantages of the emulsion reactor.

With respect to the immobilization of lipase, various methods have already been proposed [32–35]. When lipase was immobilized on various adsorbents, lipase could be efficiently immobilized on these reactors. However, it is difficult to achieve reaction and product separation simultaneously. Furthermore, since the hydrolysis of oil by lipase is an interfacial reaction involving the adsorption of enzyme on the surface of the oil droplet [36], the expressed activities of most of the immobilized lipases are low compared to those obtained with other immobilized enzyme [34]. We devised a membrane reactor based on the principle of physical adsorption of enzyme and the principle of contact between organic and aqueous phases for the purposes of efficient asymmetric hydrolysis and simplification of the product separation process. Since a pioneering work on the membrane bioreactor with liquid–liquid contact mode by Hoq et al. [37], Matson and Quinn [38] have proposed a theory of hydrophilic membrane bioreactor in which organic and aqueous phases are circulated along shell and lumen sides of the membrane, respectively. In recent years, the membrane reactor has been commercialized by Sepracor (S.L. Matson, US Pat. No. 4800162).

Flow diagram of membrane reactor is illustrated in Fig. 5 and the behavior of substrate and products in membrane reactor is illustrated

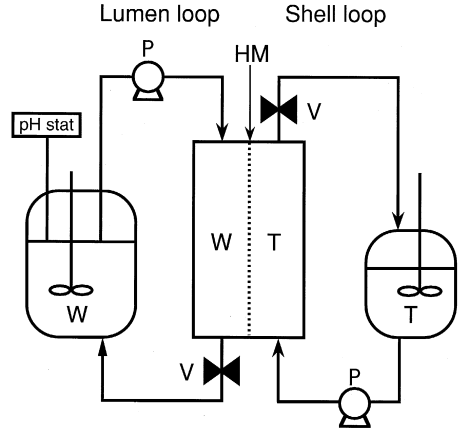

Fig. 5. Flow diagram of membrane reactor. T:1.15 l of toluene phase containing 1.15 mol of (±)-MPGM; W: 12.3 l of aqueous phase containing 1.15 mol of sodium hydrogen sulfite; HM: hydrophilic membrane; P: recycle pump; V: throttle valve.

in Fig. 6 [5]. The asymmetric hydrolysis of (±)-MPGM by the lipase from *S. marcescens* immobilized on the membrane reactor is started by circulating both (±)-MPGM solution in toluene in the shell loop and sodium hydrogen sulfite solution (pH 8.5) in the lumen loop, and (+)-MPGM is hydolyzed to (+)-(2*S*,3*R*)-3-(4-methoxyphenyl)glycidic acid and methanol. The glycidic acid is spontaneously decarboxylated to *p*-methoxyphenylacetaldehyde (RCHO) and accumulate in the toluene phase as shown in Fig. 6. Though the aldehyde acted as an inactivator of the lipase, it can be removed by transfer to the aqueous phase by formation of water-soluble adduct with sodium hydrogen sulfite added into the aqueous phase [5]. The stability of the lipase is nominally influenced when sodium hydrogen sulfite concentration is below 0.1 M at pH 8.5 and 22°C. In practice, 0.0935 M sodium hydrogen sulfite solution (pH 8.5) is applied to the aqueous phase to prevent the lowering of enzyme activity. Another by-product, methanol, is transferred from the toluene phase to the aqueous phase. As the concentration of methanol in the aqueous phase is below 0.2% (w/v), the hydrolysis of (±)-MPGM is not influenced.

The stability of the lipase on the membrane reactor is superior as compared to emulsion

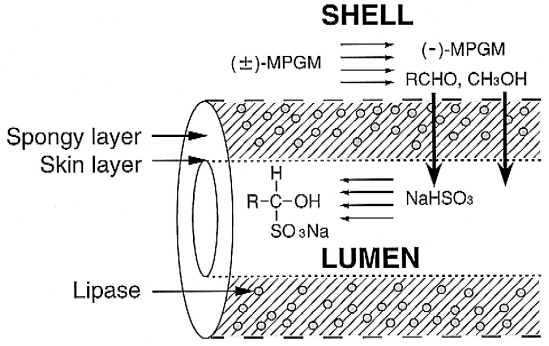

Fig. 6. Behavior of products in membrane reactor. Pressure 1 in the shell side was kept at excess pressure compared with pressure 2 in the lumen side. T: 1.15 l of toluene phase containing 1.15 mol of sodium hydrogen sulfite; RCHO: *p*-methoxyphenylacetaldehyde; $R \cdot CH \cdot OH \cdot SO_3Na$: an adduct formed between sodium hydrogen sulfite and *p*-methoxyphenylacetaldehyde.

reaction; the half-life of enzyme activity is 127 h in the membrane reactor, which is about 30 times that in the emulsion reactor. But the velocity constant on the emulsion reactor is 3.1 h^{-1} and that on the membrane reactor is 0.23 h^{-1}. In the case of emulsion reactor, a significant decrease of enzyme activity was observed. When the Sepracor membrane reactor, which immobilizes lipase on the toluene side of the membrane is utilized, the lipase is allowed to localize on the interface of the toluene solution and the aqueous solution, which permeated through the hydrophilic membrane. The density of protein at the interface of the two phases in the membrane reactor is maintained at a high value compared to that in the emulsion reactor. Therefore, by the use of the Sepracor membrane reactor, the hydrolyzing activity on (±)-MPGM is low but it is possible to stabilize the lipase. From these results, it has become possible to achieve repeated runs of the lipase.

Membrane deterioration is an important problem, which must be solved in the industrial utilization of the membrane reactor. As the filtration rate of the membrane is an important factor in the clarification of membrane deterioration, the water flux rate of the membrane before and after the regeneration of the membrane reactor is measured. After the regeneration for eight subsequent batch runs is repeated 10 times over a period of 4 months according to the Sepracor procedure (using a sodium hydrogen sulfite solution, a sodium hypochlorite one and a sodium hydroxide/isopropanol one), the water flux rate of the membrane is 4.3 ml/min $\cdot$ kPa $\cdot$ m^2, which is the same as that of a new membrane. From the result, it is thought that the membrane reactor is appropriate to achieve industrial utilization from the viewpoint of membrane regeneration.

The velocity constant of the lipase immobilized on the membrane reactor is equivalent to about 8% of that of the free lipase in the emulsion reactor. This value is higher than that of lipase immobilized on stainless steel beads [32], urethane prepolymers, duolite or alkylamine-CPG [34], [36]. The value is approximately equivalent to that of lipase immobilized on photo-cross-linkable resin prepolymer [33], polyvinylchloride, chitin, agarose, Sepharose [34] or octyl-Sepharose [35]. The hydrolysis reaction using the membrane in a biphasic system has to be carried out under excess pressure to avoid the penetration of the aqueous phase through the hydrophilic membrane into organic phase. It is therefore presumed that not only the small area of the interface but also the enlargement of diffusion resistance of molecule in the 50-μm-thick membrane cause a decreased expression of activity.

On the membrane reactor, *p*-methoxyphenylacetaldehyde, which is a spontaneously degraded derivative from (+)-(2*S*,3*R*)-3-(4-methoxyphenyl)glycidic acid, formed an adduct with sodium hydrogen sulfite, and the adduct is transferred to the aqueous phase. When the asymmetric hydrolysis of (±)-MPGM is carried out in eight subsequent batch runs using the membrane reactor with $0.80–1.6 \times 10^5$ units of lipase per m^2, (+)-MPGM is completely degraded after 23 h of reaction at the initial reaction. As the toluene phase contained only (−)-MPGM, crystalline (−)-MPGM with a high yield of 43% and optical purity of 99.9% e.e. is

obtained by concentrating the toluene solution. From the second to the sixth reaction, 0.2% to 4 % of (±)-MPGM is unreactive after 23 h of each reaction. The solubility of optically pure MPGM in toluene is nearly equal to half of the solubility of racemic (±)-MPGM in toluene. After the toluene solution containing MPGM is concentrated from 1.15 to 0.12–015 l, pure (−)-MPGM with a high yield of 40–43% and optical purity of 99.9% e.e. is obtained by the crystallization from the concentrated toluene solution at 4°C. At the seventh and eighth reactions, the yields of crystalline (−)-MPGM (99.9 % e.e.) reduced to 38% and 36%, respectively. The productivity of Sepracor membrane bioreactor is about 40 kg (−)-MPGM per m^2 per year.

In conclusion, when the lipase having a high enantioselectivity is immobilized on the membrane reactor suitable for the asymmetric hydrolysis of the key intermediate in the synthesis of diltiazem hydrochloride, the production of (−)-MPGM is achieved efficiently by simultaneous reaction and product separation, and by stabilization of the enzyme.

On the basis of these results, the pilot-plant experiments were carried out in collaboration with Sepracor, and this technique has been used in the commercial production of diltiazem hydrochloride since 1993.

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