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NOVEL ESTERASE AND PROCESS FOR PREPARING THE SAME

This invention relates to novel esterase and process for preparing the same.

Recently, attempts have been made to use esterase for organic synthetic reactions (e.g., hydrolysis reaction). For example, esterase derived from pig liver is often used for this purpose. However, this esterase is disadvantageous for industrial use because it is expensive. On the other hand, there have been known esterases derived from microorganisms such as Arthrobacter globiformis IFO 12985 [Patent Publication (unexamined) No. 181788/1989] (Molecular Weight: 43,000), Bacillus stearothermophilus [Archiv. Biochem. Biophys. 160, 504-513 (1974)] (Molecular Weight: 47,000), Geotrichum candidum [Agric. Biol. Chem. 37 (6), 1457-1464 (1973)] (Molecular Weight: 53,000-55,000), Pseudomonas aeruginosa [J. Biochem. 86, 643-656 (1979)] (Molecular Weight: 55,000), Pseudomonas fluorescens [J. Biochem. 95, 1047-1054 (1984)] (Molecular Weight: 48,000).

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However, these esterases have difficulties that they can not be widely applied because of their narrow substrate specificities.

The object of the present invention is to provide a novel esterase which is derived from a microorganism and can be applied for organic synthetic reactions as widely as pig liver esterase.

Other object of the present invention is to provide a process for preparing said esterase.

Another object of the present invention is to provide a process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate by using said esterase.

As a result of various investigations, we have now found a potent esterase which is derived from a microorganism belonging to genus Serratia.

The esterase of the present invention is a novel esterase having the following physico-chmical properties and enzymatic characteristics:

(1) Activity

It (i.e., said esterase) hydrolyzes an ester bond of an organic carboxylates.

(2) Substrate specificity

It acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters. For example, the esterase of the present invention has ability to hydrolyze a lower or higher alkyl esters of organic carboxylic acids (e.g., a lower fatty acid, a higher fatty acid, a lower alkoxy-substituted-phenylglycidic acid and the like). Moreover, it also has potent ability to hydrolyze triglycerides composed of glycerol and

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water-soluble lower fatty acids or water-insoluble higher fatty acids. Further, it also has ability to hydrolyze thiol esters such as dimercaprol tributyrate and the like. But it has no ability to hydrolyze casein.

(3) Optimum pH

It shows optimum pH of about 7.5-9.0 when olive oil is used as the substrate.

(4) pH stability

It remains more than 95% of its activity when it is stored at a pH 5-9 at 30 °C for 60 minutes.

(5) Optimum temperature

It shows optimum temperature of about 40-50 °C when the enzymatic reaction is carried out in 100 mM Tris-HCl buffer (pH 8.0) by using olive oil as the substrate.

(6) Heat stability

It remains 100% of its activity when it is stored at a temperature not higher than 50 °C in 100 mM Tris-HCl buffer (pH 8.0) for 30 minutes.

(7) Assay of enzyme activity

(a) activity on olive oil

The enzyme reaction is carried out at pH 8.0 at 37 °C for 20 minutes by using olive oil as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 1)

(b) activity on triglycerides or fatty acid esters

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The enzyme reaction is carried out at pH 8.0 at 37 °C for 10 minutes by using triglycerides or fatty acid esters as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 3)

(c) activity on thiol esters

The enzyme reaction is carried out at pH 8.5 at 30 °C for 10 minutes by using dimercaprol tributylate as the substrate. After the reaction, 5,5'-dithiobis(2-nitrobenzoic acid) (color-producing reagent) is added to the reaction mixture. The amount of the liberated 5-mercapto-2-nitrobenzoic acid is measured by spectrophotometer. The activity of the esterase which produces one μ mole of 5-mercapto-2-nitrobenzoic acid per minute is defined as one unit. (cf., Example 4)

(8) Molecular weight

62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis)

(9) Isoelectric point

4.6 ± 0.1

(10) Effect of metal ions

It is activated in the presence of 1 mM calcium ion. On the other hand, its activity is inhibited by 40-90% in the presence of 1 mM zinc ion, copper ion, manganese ion, and completely inhibited in the presence of 1 mM cobalt ion, nickel ion, iron (II) ion, iron (III) ion or ethylenediaminetetraacetic acid.

The above-mentioned esterase of the present invention is obviously different from known esterases in that it has molecular

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weight of 62,000 ± 2,000 (measured by SDS-polyacrylamide gel electrophoresis) and isoelectric point of 4.6 ± 0.1.

According to the present invention, the above-mentioned esterase can be prepared by cultivating a microorganism belonging to the genus Serratia in a medium, accumulating said esterase inside or outside of the microorganism, and recovering the accumulated esterase therefrom.

Any microorganism belonging to the genus Serratia which can produce the above-mentioned esterase may be used as the esterase-producing microorganism of the present invention. Examples of such microorganism include Serratia marcescens Sr41 (FERM-BP No. 487), a mutant thereof and the like. But, the microorganism used in the present invention are not limited to those mentioned-above. For example, a recombinant which is produced from the above mentioned microoganisms according to the bioengineering methods may be also used.

Any medium in which the esterase-producing microorganisms of the present invention can grow and proliferate may be used as the medium. Examples of the carbon source include, for example, sugars such as glucose, sucrose, molasses and the like, organic acids such as fumaric acid, citric acid and the like, alcohols such as glycerol and the like, amino acids such as alanine, glutamine, asparagine and the like and so forth. Inorganic ammonium salts such as ammonium sulfate, ammonium chloride and the like, urea, peptone, corn steep liquor, yeast extract, casein hydrolysate and the like can be used as the

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nitrogen source. It is preferred to use 1 - 15 w/w% of the carbon source and 0.1 - 2.0 w/w% of the nitrogen source. If required, inorganic salts such as phosphates, magnesium salts, potassium salts, calcium salts and the like, or metal ions such as iron ions, manganese ions, copper ions, zinc ions and the like may be added to the medium. When a synthetic medium is used, vitamins such as biotin, thiamin and the like, or growth-promoting substances such as carnitine may, if required, be added to the medium. Moreover, inducers such as vegetable oils or surfactants may, if necessary, be added to the medium. It is preferred to adjust the pH of the medium to a pH of about 5 - 8.

The cultivation can be carried out in a conventional manner after inoculating the microorganism into the medium. For example, any method such as shake culture, aeration spinner culture, stationary culture or continuous culture may be used for this purpose.

The condition of cultivation may vary depending on a kind of the medium, a cultivation method and the like. Any condition under which the microorganism of the present invention can grow, proliferate and produce the esterase is appropriate for this purpose. But it is usually preferred to begin the cultivation at a pH of about 5 - 8, and then carry it out at room temperature or under warming, for example, at a temperature between 20 and 40 °C for one to 2 days.

The esterase accumlated inside or outside of the cultivated microorganism can be recovered and purified in a conventional manner. For example, the esterase accumlated in the culture broth

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can be rcovered by a combination of known methods such as salting-out with inorganic salt (e.g., ammonium sulfate, an alkali metal sulfate or an alkali metal halide), differential precipitation with hydrophilic organic solvent (e.g., an alcohol or acetone), column chromatography by ion exchange resin or hydrophobic resin, gel filtration and protein precipitation with nucleic acid, tannin or the like. The thus-obtained esterase can be further purified by a combination of known purification methods such as isoelectric precipitation, dialysis, electrodialysis, electrophoresis and the like. For example, the purification is carried out by;

(1) removing microbial cells from the culture broth by centrifugation,

(2) treating the supernatant with a 45% saturated ammonium sulfate solution,

(3) subjecting the resulting precipitate to an anion exchange resin (DEAE-TOYOPEARL 650M, MonoQ) chromatography after dialysis thereof,

(4) subjecting active fractions to hydrophobic resin (Butyl-TOYOPEARL 650S) chromatography after dialysis thereof, and

(5) subjecting the obtained active fractions to gel filtration (Superose 6) after dialysis and condensation thereof. The thus-obtained purified esterase is a polypeptide showing a single band with a molecular weight of 62,000 ± 2,000 by SDS-polyacrylamide gel electrophoresis.

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As mentioned before, the esterase of the present invention have potent ability to hydrolyze wide range of substrate such as alkyl esters of organic carboxylic acids, triglycerides or thiol esters. Therefore, it can be applied for organic synthetic reactions as widely as pig liver esterase. For example, it can be applied for preparation of an optically active isomer from a racemic mixture thereof, or preparation of a chiral compound from a prochiral compound thereof. In particular, the esterase of the present invention is characterized in that it has strong ability to hydrolyze a (lower)alkyl (lower)alkoxyphenyl-glycidate. For example, when it is used for hydrolysis of a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate, a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate can be prepared in good yield. The thus-obtained lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate is useful as a synthetic intermediate of pharmaceuticals such as diltiazem hydrochloride.

In the following Examples, "%" means "w/v%" unless otherwise prescribed.

Examples

Example 1

A medium (pH 7.0, 20 liters) containing dextrin (1%), ammonium sulfate (0.2%), meast-S (2%), potassium dihydrogenphosphate (0.1%), magnesium sulfate (0.05%), calcium chloride (0.01%), ferrous sulfate (0.001%), Tween80 (0.5 v/v%) and polyalkylene glycol derivative-type surfactant (trade name: KARARIN 102, manufactured by Sanyo Chemical Industries, Ltd., 0.1 v/v%) was placed in a 30-liter jar-

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fermenter and sterillized by autoclaving. A broth (200 ml) of Serratia marcescens Sr41 which was obtained by reciprocal shaking at 30 °C for 20 hours in the same medium as above was inoculated into the sterillized medium. The cultivations was carried out by aeration and agitation (200 rpm, 0.5vvm) at 30 °C for 18 hours. The culture broth was centrifuged, and the supernatant (4.5 liters) was salted out with 45% saturated ammonium sulfate solution. The precipitate was collected by filtration with celite, eluted with water. The eluate was dialyzed and lyophilized. 4.1 g of esterase (18,600 unit/g) were obtained as crude enzyme powder.

(Assay of enzyme activity)

The enzyme activity was estimated according to the following method.

A mixture of 225 ml of 2% polyvinyl alcohol (Poval 117, manufactured by Kurare Co., Ltd.) and 75 ml of olive oil was emulsified by stirring at 14,500 rpm at 5-10 °C for 10 minutes. 5.0 ml of the olive oil-emulsion thus-obtained and 4.0 ml of 0.25M Tris-HCl buffer (pH 8.0, containing 2.5 mM calcium chloride) were preincubated at 37 °C for 10 minutes. One ml of an enzyme solution was added thereto to initiate enzymatic reaction. After the mixture was incubated at 37 °C for 20 minutes, 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) is added

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to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which liberated one μmole of fatty acid per minute was defined as one unit (U).

Example 2

10 liters of the supernatant obtained in the same manner as described in Example 1 were salted out with 45% saturated ammonium sulfate solution. The precipitate (esterase) was dissolved in 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEARL 650M, Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 1.0 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.27 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a strong anion exchange resin (MonoQ, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.6 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.35 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 5% saturated ammonium sulfate solution

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containing 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Butyl-TOYOPEAL 650S, manufactured by Toyo Soda Co. Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 5 to 0 % saturated ammonium sulfate solution containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5) and condensed. The residue was subjected to gel filtration of molecular sieve resin (Superose 6, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. 56.8 mg of purified esterase protein were obtained.

The purified esterase showed molecular weight of about 590,000 by gel filtration and about 62,000 by SDS-polyacrylamide gel electrophoresis.

The specific activities and yields of esterases obtained in each of the above purification steps are shown in the following Table 1 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.)

The esterase obtained above showed no protease activity when casein was used as the substrate.

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Table 1

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	16470	385400	23.4	100
45% ammonium sulfate	1529	341000	223	88.5
DEAE-TOYOPEAL 650M	247	227500	921	59.0
MonoQ	143	141300	988	36.7
Butyl-TOYOPEAL 650S	84.5	85350	1010	22.1
Superose 6	56.8	58500	1030	15.2

Example 3

The substrate specificity on various kinds of triglyderides and fatty acid esters was investigated with respect to the esterase obtained in Example 2.

(Assay of hydrolyzing activity on triglycerides and fatty acid esters)

0.2 g of each substrate, 4 ml of 0.2 M Tris-HCl buffer (pH 8.0) and one ml of 6 mM calcium chloride solution were placed in 100 ml flask. After a ten minute-preincubation, one ml of enzyme solution was added thereto to initiate enzymatic reaction. The mixture was incubated at 37 °C under 80 rpm for 10 minutes. 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop

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the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) was added to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which can liberate one μmole of fatty acid per minute is defined as one unit.

(Results)

The result are shown in the following Tables 2 and 3. The enzyme activity is shown as relative activity (%) [methyl n-caprylate (Table 2), tricaprylin (Table 3) = 100].

Table 2

Substrate	Relative activity (%)
methyl acetate	9.7
methyl n-butyrate	72.8
methyl n-valerate	38.9
methyl n-caproate	14.6
methyl n-caprylate	100 1)
methyl n-caprate	92.2
methyl laurate	61.2
methyl myristate	34.0
methyl palmitate	14.6
methyl linolenate	38.9

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methyl linolate	51.5
methyl oleate	2.9

1): 204 unit/mg protein

Table 3

Substrate	Relative activity (%)
triacetin	7.6
tributylin	93.2
tricaproin	70.3
tricaprylin	100 2)
tricaprin	42.4
trilaurin	29.7
trimyristin	33.9
tripalmitin	1.6
Tristearin	1.6

2): 1169 unit/mg protein

Example 4

The substrate specificity on dimercaprol tributyrate was investigated with respect to the purified esterase obtained in Example 2.

The esterase was added to a substrate solution of Lipase Kit S [Substrate: dimercaprol tributyrate, manufactured by Dainippon Pharmaceutical Co., Ltd.]. After the hydrolysis was carried out, 5,5-dithiobis(2-nitrobenzoic acid) (color-producing agent) was added to

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the reaction mixture. Enzyme activity was estimated by measuring the amount of the liberated 5-mercapto-2-nitrobenzoic acid at 412 nm.

The esterase showed the enzyme activity of 1.7×10^5 units/mg protein.

Example 5

1.5 mg of the purified esterase obtained in Example 2 were added to 75 ml of an aqueous 1mM calcium chloride solution. 75 ml of toluene solution containing 1M racemic methyl trans-3-(4-methoxyphenyl)glycidate were added thereto. The mixture was adjusted to pH 8.0 and stirred at 30 °C for 4 hours. After the reaction, the ratio of (+)-isomer [(2S, 3R)-isomer] and (-)-isomer [(2R, 3S)-isomer] of methyl trans-3-(4-methoxyphenyl)glycidate in toluene layer was measured by High Performance Liquid Chromatography (column: Chiralcel OJ, manufactured by Daicel Chemical Industries, Ltd., mobile phase: n-hexane : isopropyl alcohol = 9:1).

The results are shown in the following Table 4

Table 4

The ratio of optically active isomers (%)		Optical yield of (-)-trans isomer (%)
(+)-trans-isomer	(-)-trans-isomer	
0.4	99.6	94

Example 6

Optimum pH and pH stability were investigated with respect to the purified esterase obtained in Example 2.

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Optimum pH was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction was carried out at various pH. The each activity was shown as relative activity (%) (the activity at pH 8 = 100).

On the other hand, pH stability was examined by adjusting the enzyme solution at a specified pH, incubating at 30 °C for one hour and then estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100), and the pH adjustment was carried out by using McIlvaine buffer (pH 3.0-7.0), 100 mM Tris-HCl buffer (pH 8.0 - 9.0) and 100 mM glycine buffer (pH 9.0 - 11.0).

(Results)

The results are shown in the following Table 5 and 6. It is clear from these Tables that the optimum pH of the esterase is about 7.5 - 9.0, and the esterase is stable at a pH of about 5 - 9.

Table 5 (Relative activities at each pH)

pH	3	4	5	6	7	8	9	10	11
Relative activity (%)	0	5	20	39	69	100	80	2	0

Table 6 (pH stability)

pH	3	4	5	6	7	8	9	10	11
Residual activity (%)	0	5	95	100	100	100	100	65	0

Example 7

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Optimum temperature and heat stability were investigated with respect to the purified esterase obtained in Example 2.

The optimum temperature was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction is carried out at various temperature. The enzyme activity was shown as relative activity (%) (the activity at 45 °C = 100).

On the other hand, the heat stability was examined by incubating the enzyme in 100 mM Tris-HCl buffer for 30 minutes at a specified temperature, and estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100).

(Results)

The results are shown in the following Table 7 and 8. It is clear from Tables that the optimum temperature is about 40 to 50 °C, and the esterase is stable at a temperature not higher than 50 °C.

Table 7

Temperature (°C)	10	15	20	30	35	40	45	50	55
Relative activity (%)	10	14	19	39	51	72	100	64	4

Table 8

Temperature (°C)	4	30	40	50	55	60
Residual acitivity (%)	100	100	100	100	2	0

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Example 8

The isoelectric point of the purified esterase obtained in Example 2 was examined by means of electrophoresis under the following conditions:

carrier: Ampholine (pH 3-10)

density gradient of 0 to 50 % sucrose

voltage: at 400V for 26 hours from the beginning and then at 800V for 14 hours

temperature: 2 °C

110 ml column was used.

As a result, the isoelectric point of said esterase was found to be 4.6 ± 0.1

Example 9

The purified esterase obtained in Example 2 (protein: 95 μg) was dialyzed against distilled water, and the dialyzed solution was evaporated to dryness. The residue was subjected to an amino acid sequencer. As a result, the amino acid sequence from N-terminal to 12th was found to be as follows:

N-terminal-(X)-Ile^{2}-Phe-Ser-Tyr^{5}-Lys-Asp-Leu-Asp-Glu^{10}-Asn-Ala

wherein X is an amino acid which has not yet been identified.

Example 10

The effect of metal ions or known enzyme-inhibitors on the purified esterase obtained in Example 2 was investigated as follows:

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1mM each metal ion or 1mM each inhibitor was added to the enzyme solution, and the enzyme activity was estimated in the same manner as described in Example 1.

The activity was shown as relative activity (%) (the activity estimated by no addition of metal ion or inhibitor = 100).

(Results)

The results are shown in the following Table 9 and 10.

Table 9 (The effect of metal ions)

Metal ion (1mM)	Relative activity (%)
no addition	100
Co^{2+}	0
Li^{+}	143
Zn^{2+}	29
Cu^{2+}	57
Mg^{2+}	123
Ni^{2+}	0
Mn^{2+}	11
Hg^{2+}	86
Fe^{2+}	0
Fe^{3+}	0
Ca^{2+}	285
Pb^{2+}	86
K^{+}	94

Table 10 (The effect of inhibitors)

Inhibitors(1mM)	Relative activity
without reagent	100
Ethylenediaminetetraacetic acid	0
p-Chloromercury benzoate	86

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Sodium dodecyl sulfate	157
Phenylmethylsulfonyl fluoride	100

Example 11

12 liters of the supernatant obtained from the culture broth in the same manner as described in Example 1 were concentrated by using ultrafiltration membrane (SIP-3013, Asahi Chemical Industries Co., Ltd.). The concentrate was salted out with 35% saturated ammonium sulfate solution containing 10 mM Tris-HCl buffer (pH 7.5). The precipitate (esterase) was dissolved in 10 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 10 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.8 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.3 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 10 mM Tris-HCl buffer (pH 7.5). The solution (101 ml) was concentrated to 8 ml by ultrafiltration (Diaflo ultrafilter PM-10, manufactured by Amicon Co., Ltd.). The concentrate was subjected to gel filtration of a molecular sieve resin (Sephacryl S-300 HR, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The active fractions were collected and dialyzed against 2% saturated ammonium sulfate solution containing 10 mM Tris-HCl

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buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Phenyl-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer. Enzyme was eluted from the resin by a stepwise gradient of 2% to 0% saturated ammonium salfate solutions containing 10 mM Tris-HCl buffer (pH 7.5). 133mg of purified esterase protein were obtained.

Physico-chmical properties and enzymatic characteristics of this purified esterase are identical to those obtained in Example 2.

The specific activities and yields of esterase obtained in each of the above-purification steps are shown in the following Table 11 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.). The esterase obtained above showed no protease activity when casein was used as the substrate.

All of purification steps above was carried out below 5 °C

Table 11

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	12000	13900	1416000	102	100
Concentration by UF membrane	1000	1920	1343000	699	95

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35% ammonium sulfate	175	645	1269000	1967	90
DEAE-TOYOPEAL 650M	101	298	656000	2201	46
Sephacryl S-300 HR	45	194	590000	3041	42
Phenyl-TOYOPEAL 650M	18	133	414000	3113	29

Example 12

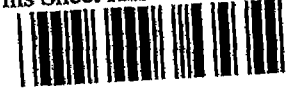
The amino acid composition of purified esterase obtained in Example 11 was examined by using an amino acid analyzer under the following conditions.

Each enzyme solution in 6M hydrochloride (2.0 mg/ml) was sealed in a glass tube under vacuum and then hydrolyzed for 20, 40 or 70 hours at 110 °C. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in 0.02N hydrochloride solution and analyzed with an amino acid analyzer (Hitachi model L-8500). Cystein and cystine were determined as cysteic acid after performic acid oxidation of the sample according to the method of Moore[1]. Tryptophan was determined according to the method of Simpson et al.[2]

References: 1) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

2) Simpson, R.J., Neuberger, M.R., and Lin, T.-Y. (1976) J. Biol. Chem. 251, 1936-1940.

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Table 12

Amino acid	Residues / molecule[a)]
Asx[b)]	89
Thr[c)]	40
Ser[c)]	43
Glx[b)]	40
Pro	15
Gly	78
Ala	56
Val	28
Ile	31
Leu	58
Tyr	23
Phe	28
Lys	20
His	14
Arg	14
Cys	0 - 1
Met	2
Trp	7

a): Based on an Molecular weight of 62,000.

b): Sum of acid and amide forms.

c): The serine and threonine values were obtained by extrapolation to time zero, assuming first-order decay.

Example 13

The purified esterase obtained in Example 11 was dialyzed against water, and its calcium content was determined by using an atomic absoption spectrometry (Hitachi model Z-9000).

As a result, the calcium content in the purified esterase was one mole per an enzyme molecular mass of 62,000.

Example 14

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The substrate specificity of the purified esterase obtained in Example 11 on dimercaprol tributyrate was investigated in the same manner as described in Example 4.

The esterase showed the enzyme activity of 5.2×10^5 units/mg protein.

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* C L M S *

What is claimed is:

1. An esterase having the following physico-chemical properties and enzymatic characteristics:

(1) Activity: it (i.e., said esterase) hydrolyzes an ester bond of organic carboxylates,

(2) Substrate specificity: it acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters,

(3) Optimum pH: its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate,

(4) pH stability: it is stable at pH 5.0 - 9.0 when it is stored at 30 °C for one hour,

(5) Optimum temperature: its optimum temperature is 40 - 50 °C when the hydrolysis is carried out by using olive oil as the substrate,

(6) Heat stability: it is stable at a temperature of not higher than 50°C when it is stored at pH 8.0 for 30 minutes,

(7) Molecular weight: 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis),

(8) Isoelectric point: 4.6 ± 0.1,

(9) Effect of metal ions: it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid.

2. The esterase according to Claim 1 which is derived from a microorganism belonging to the genus Serratia.

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3. The esterase according to Claim 2 in which the microorganism is Serratia marcescens.

4. A process for preparing novel esterase which comprises

(a) cultivating a microorganism having ability to produce the esterase claimed in Claim 1, and

(b) recovering said esterase from the culture broth.

5 A process according to Claim 4 in which the microorganism belong to the genus Serratia.

6. The process according to Claim 5 in which the microorganism is Serratia marcescens.

7. A process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate which comprises the steps of:

(a) treating a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate with the esterase claimed in Claim 1 thereby stereoselectively hydrolyzing 2S, 3R-isomer thereof, and

(b) recovering unreacted 2R, 3S-isomer from the reaction mixture.

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ABSTRACT

A novel esterase which is derived from Serratia marcescens is disclosed. Said esterase has the following physico-chemical properties and enzymatic characteristics:

(1) Activity; it (i.e., said esterase) hydrolyzes an ester bond of organic carboxylates, (2) Substrate specificity; it acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters, (3) Optimum pH; its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate, (4) pH stability; it is stable at pH 5.0 - 9.0 when it is stored at 30 °C for one hour, (5) Optimum temperature; its optimum temperature is 40 - 50 °C when the hydrolysis is carried out by using olive oil as the substrate, (6) Heat stability; it is stable at a temperature of not higher than 50°C when it is stored at pH 8.0 for 30 minutes, (7) Molecular weight; 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis), (8) Isoelectric point; 4.6 ± 0.1, (9) Effect of metal ions; it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid. Said esterase can widely be applied for organic synthetic reactions.

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NOVEL ESTERASE AND PROCESS FOR PREPARING THE SAME

This invention relates to novel esterase and process for preparing the same.

Recently, attempts have been made to use esterase for organic synthetic reactions (e.g., hydrolysis reaction). For example, esterase derived from pig liver is often used for this purpose. However, this esterase is disadvantageous for industrial use because it is expensive. On the other hand, there have been known esterases derived from microorganisms such as Arthrobacter globiformis IFO 12985 [Patent Publication (unexamined) No. 181788/1989] (Molecular Weight: 43,000), Bacillus stearothermophilus [Archiv. Biochem. Biophys. 160, 504-513 (1974)] (Molecular Weight: 47,000), Geotrichum candidum [Agric. Biol. Chem. 37 (6), 1457-1464 (1973)] (Molecular Weight: 53,000-55,000), Pseudomonas aeruginosa [J. Biochem. 86, 643-656 (1979)] (Molecular Weight: 55,000), Pseudomonas fluorescens [J. Biochem. 95, 1047-1054 (1984)] (Molecular Weight: 48,000).

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However, these esterases have difficulties that they can not be widely applied because of their narrow substrate specificities.

The object of the present invention is to provide a novel esterase which is derived from a microorganism and can be applied for organic synthetic reactions as widely as pig liver esterase.

Other object of the present invention is to provide a process for preparing said esterase.

Another object of the present invention is to provide a process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate by using said esterase.

As a result of various investigations, we have now found a potent esterase which is derived from a microorganism belonging to genus Serratia.

The esterase of the present invention is a novel esterase having the following physico-chmical properties and enzymatic characteristics:

(1) Activity

It (i.e., said esterase) hydrolyzes an ester bond of an organic carboxylates.

(2) Substrate specificity

It acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters. For example, the esterase of the present invention has ability to hydrolyze a lower or higher alkyl esters of organic carboxylic acids (e.g., a lower fatty acid, a higher fatty acid, a lower alkoxy-substituted-phenylglycidic acid and the like). Moreover, it also has potent ability to hydrolyze triglycerides composed of glycerol and

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water-soluble lower fatty acids or water-insoluble higher fatty acids. Further, it also has ability to hydrolyze thiol esters such as dimercaprol tributyrate and the like. But it has no ability to hydrolyze casein.

(3) Optimum pH

It shows optimum pH of about 7.5-9.0 when olive oil is used as the substrate.

(4) pH stability

It remains more than 95% of its activity when it is stored at a pH 5-9 at 30 °C for 60 minutes.

(5) Optimum temperature

It shows optimum temperature of about 40-50 °C when the enzymatic reaction is carried out in 100 mM Tris-HCl buffer (pH 8.0) by using olive oil as the substrate.

(6) Heat stability

It remains 100% of its activity when it is stored at a temperature not higher than 50 °C in 100 mM Tris-HCl buffer (pH 8.0) for 30 minutes.

(7) Assay of enzyme activity

(a) activity on olive oil

The enzyme reaction is carried out at pH 8.0 at 37 °C for 20 minutes by using olive oil as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 1)

(b) activity on triglycerides or fatty acid esters

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The enzyme reaction is carried out at pH 8.0 at 37 °C for 10 minutes by using triglycerides or fatty acid esters as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 3)

(c) activity on thiol esters

The enzyme reaction is carried out at pH 8.5 at 30 °C for 10 minutes by using dimercaprol tributylate as the substrate. After the reaction, 5,5'-dithiobis(2-nitrobenzoic acid) (color-producing reagent) is added to the reaction mixture. The amount of the liberated 5-mercapto 2-nitrobenzoic acid is measured by spectrophotometer. The activity of the esterase which produces one μ mole of 5-mercapto-2-nitrobenzoic acid per minute is defined as one unit. (cf., Example 4)

(8) Molecular weight

62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis)

(9) Isoelectric point

4.6 ± 0.1

(10) Effect of metal ions

It is activated in the presence of 1 mM calcium ion. On the other hand, its activity is inhibited by 40-90% in the presence of 1 mM zinc ion, copper ion, manganese ion, and completely inhibited in the presence of 1 mM cobalt ion, nickel ion, iron (II) ion, iron (III) ion or ethylenediaminetetraacetic acid.

The above-mentioned esterase of the present invention is obviously different from known esterases in that it has molecular

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weight of 62,000 ± 2,000 (measured by SDS-polyacrylamide gel electrophoresis) and isoelectric point of 4.6 ± 0.1.

According to the present invention, the above-mentioned esterase can be prepared by cultivating a microorganism belonging to the genus Serratia in a medium, accumulating said esterase inside or outside of the microorganism, and recovering the accumulated esterase therefrom.

Any microorganism belonging to the genus Serratia which can produce the above-mentioned esterase may be used as the esterase-producing microorganism of the present invention. Examples of such microorganism include Serratia marcescens Sr41 (FERM-BP No. 487), a mutant thereof and the like. But, the microorganism used in the present invention are not limited to those mentioned-above. For example, a recombinant which is produced from the above mentioned microoganisms according to the bioengineering methods may be also used.

Any medium in which the esterase-producing microorganisms of the present invention can grow and proliferate may be used as the medium. Examples of the carbon source include, for example, sugars such as glucose, sucrose, molasses and the like, organic acids such as fumaric acid, citric acid and the like, alcohols such as glycerol and the like, amino acids such as alanine, glutamine, asparagine and the like and so forth. Inorganic ammonium salts such as ammonium sulfate, ammonium chloride and the like, urea, peptone, corn steep liquor, yeast extract, casein hydrolysate and the like can be used as the

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nitrogen source. It is preferred to use 1 - 15 w/w% of the carbon source and 0.1 - 2.0 w/w% of the nitrogen source. If required, inorganic salts such as phosphates, magnesium salts, potassium salts, calcium salts and the like, or metal ions such as iron ions, manganese ions, copper ions, zinc ions and the like may be added to the medium. When a synthetic medium is used, vitamins such as biotin, thiamin and the like, or growth-promoting substances such as carnitine may, if required, be added to the medium. Moreover, inducers such as vegetable oils or surfactants may, if necessary, be added to the medium. It is preferred to adjust the pH of the medium to a pH of about 5 - 8.

The cultivation can be carried out in a conventional manner after inoculating the microorganism into the medium. For example, any method such as shake culture, aeration spinner culture, stationary culture or continuous culture may be used for this purpose.

The condition of cultivation may vary depending on a kind of the medium, a cultivation method and the like. Any condition under which the microorganism of the present invention can grow, proliferate and produce the esterase is appropriate for this purpose. But it is usually preferred to begin the cultivation at a pH of about 5 - 8, and then carry it out at room temperature or under warming, for example, at a temperature between 20 and 40 °C for one to 2 days.

The esterase accumlated inside or outside of the cultivated microorganism can be recovered and purified in a conventional manner. For example, the esterase accumlated in the culture broth

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can be rcovered by a combination of known methods such as salting-out with inorganic salt (e.g., ammonium sulfate, an alkali metal sulfate or an alkali metal halide), differential precipitation with hydrophilic organic solvent (e.g., an alcohol or acetone), column chromatography by ion exchange resin or hydrophobic resin, gel filtration and protein precipitation with nucleic acid, tannin or the like. The thus-obtained esterase can be further purified by a combination of known purification methods such as isoelectric precipitation, dialysis, electrodialysis, electrophoresis and the like. For example, the purification is carried out by;

(1) removing microbial cells from the culture broth by centrifugation,

(2) treating the supernatant with a 45% saturated ammonium sulfate solution,

(3) subjecting the resulting precipitate to an anion exchange resin (DEAE-TOYOPEARL 650M, MonoQ) chromatography after dialysis thereof,

(4) subjecting active fractions to hydrophobic resin (Butyl-TOYOPEARL 650S) chromatography after dialysis thereof, and

(5) subjecting the obtained active fractions to gel filtration (Superose 6) after dialysis and condensation thereof. The thus-obtained purified esterase is a polypeptide showing a single band with a molecular weight of 62,000 ± 2,000 by SDS-polyacrylamide gel electrophoresis.

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As mentioned before, the esterase of the present invention have potent ability to hydrolyze wide range of substrate such as alkyl esters of organic carboxylic acids, triglycerides or thiol esters. Therefore, it can be applied for organic synthetic reactions as widely as pig liver esterase. For example, it can be applied for preparation of an optically active isomer from a racemic mixture thereof, or preparation of a chiral compound from a prochiral compound thereof. In particular, the esterase of the present invention is characterized in that it has strong ability to hydrolyze a (lower)alkyl (lower)alkoxyphenyl-glycidate. For example, when it is used for hydrolysis of a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate, a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate can be prepared in good yield. The thus-obtained lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate is useful as a synthetic intermediate of pharmaceuticals such as diltiazem hydrochloride.

In the following Examples, "%" means "w/v%" unless otherwise prescribed.

Examples

Example 1

A medium (pH 7.0, 20 liters) containing dextrin (1%), ammonium sulfate (0.2%), meast-S (2%), potassium dihydrogenphosphate (0.1%), magnesium sulfate (0.05%), calcium chloride (0.01%), ferrous sulfate (0.001%), Tween80 (0.5 v/v%) and polyalkylene glycol derivative-type surfactant (trade name: KARARIN 102, manufactured by Sanyo Chemical Industries, Ltd., 0.1 v/v%) was placed in a 30-liter jar-

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fermenter and sterillized by autoclaving. A broth (200 ml) of Serratia marcescens Sr41 which was obtained by reciprocal shaking at 30 °C for 20 hours in the same medium as above was inoculated into the sterillized medium. The cultivations was carried out by aeration and agitation (200 rpm, 0.5vvm) at 30 °C for 18 hours. The culture broth was centrifuged, and the supernatant (4.5 liters) was salted out with 45% saturated ammonium sulfate solution. The precipitate was collected by filtration with celite, eluted with water. The eluate was dialyzed and lyophilized. 4.1 g of esterase (18,600 unit/g) were obtained as crude enzyme powder.

(Assay of enzyme activity)

The enzyme activity was estimated according to the following method.

A mixture of 225 ml of 2% polyvinyl alcohol (Poval 117, manufactured by Kurare Co., Ltd.) and 75 ml of olive oil was emulsified by stirring at 14,500 rpm at 5-10 °C for 10 minutes. 5.0 ml of the olive oil-emulsion thus-obtained and 4.0 ml of 0.25M Tris-HCl buffer (pH 8.0, containing 2.5 mM calcium chloride) were preincubated at 37 °C for 10 minutes. One ml of an enzyme solution was added thereto to initiate enzymatic reaction. After the mixture was incubated at 37 °C for 20 minutes, 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) is added

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to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which liberated one μmole of fatty acid per minute was defined as one unit (U).

Example 2

10 liters of the supernatant obtained in the same manner as described in Example 1 were salted out with 45% saturated ammonium sulfate solution. The precipitate (esterase) was dissolved in 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEARL 650M, Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 1.0 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.27 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a strong anion exchange resin (MonoQ, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.6 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.35 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 5% saturated ammonium sulfate solution

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containing 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Butyl-TOYOPEAL 650S, manufactured by Toyo Soda Co. Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 5 to 0 % saturated ammonium sulfate solution containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5) and condensed. The residue was subjected to gel filtration of molecular sieve resin (Superose 6, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. 56.8 mg of purified esterase protein were obtained.

The purified esterase showed molecular weight of about 590,000 by gel filtration and about 62,000 by SDS-polyacrylamide gel electrophoresis.

The specific activities and yields of esterases obtained in each of the above purification steps are shown in the following Table 1 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.)

The esterase obtained above showed no protease activity when casein was used as the substrate.

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Table 1

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	16470	385400	23.4	100
45% ammonium sulfate	1529	341000	223	88.5
DEAE-TOYOPEAL 650M	247	227500	921	59.0
MonoQ	143	141300	988	36.7
Butyl-TOYOPEAL 650S	84.5	85350	1010	22.1
Superose 6	56.8	58500	1030	15.2

Example 3

The substrate specificity on various kinds of triglyderides and fatty acid esters was investigated with respect to the esterase obtained in Example 2.

(Assay of hydrolyzing activity on triglycerides and fatty acid esters)

0.2 g of each substrate, 4 ml of 0.2 M Tris-HCl buffer (pH 8.0) and one ml of 6 mM calcium chloride solution were placed in 100 ml flask. After a ten minute-preincubation, one ml of enzyme solution was added thereto to initiate enzymatic reaction. The mixture was incubated at 37 °C under 80 rpm for 10 minutes. 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop

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the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) was added to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which can liberate one μmole of fatty acid per minute is defined as one unit.

(Results)

The result are shown in the following Tables 2 and 3. The enzyme activity is shown as relative activity (%) [methyl n-caprylate (Table 2), tricaprylin (Table 3) = 100].

Table 2

Substrate	Relative activity (%)
methyl acetate	9.7
methyl n-butyrate	72.8
methyl n-valerate	38.9
methyl n-caproate	14.6
methyl n-caprylate	100 1)
methyl n-caprate	92.2
methyl laurate	61.2
methyl myristate	34.0
methyl palmitate	14.6
methyl linolenate	38.9

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methyl linolate	51.5
methyl oleate	2.9

1): 204 unit/mg protein

Table 3

Substrate	Relative activity (%)
triacetin	7.6
tributylin	93.2
tricaproin	70.3
tricaprylin	100 2)
tricaprin	42.4
trilaurin	29.7
trimyristin	33.9
tripalmitin	1.6
Tristearin	1.6

2): 1169 unit/mg protein

Example 4

The substrate specificity on dimercaprol tributyrate was investigated with respect to the purified esterase obtained in Example 2.

The esterase was added to a substrate solution of Lipase Kit S [Substrate: dimercaprol tributyrate, manufactured by Dainippon Pharmaceutical Co., Ltd.]. After the hydrolysis was carried out, 5,5-dithiobis(2-nitrobenzoic acid) (color-producing agent) was added to

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the reaction mixture. Enzyme activity was estimated by measuring the amount of the liberated 5-mercapto-2-nitrobenzoic acid at 412 nm.

The esterase showed the enzyme activity of 1.7×10^5 units/mg protein.

Example 5

1.5 mg of the purified esterase obtained in Example 2 were added to 75 ml of an aqueous 1mM calcium chloride solution. 75 ml of toluene solution containing 1M racemic methyl trans-3-(4-methoxyphenyl)glycidate were added thereto. The mixture was adjusted to pH 8.0 and stirred at 30 °C for 4 hours. After the reaction, the ratio of (+)-isomer [(2S, 3R)-isomer] and (-)-isomer [(2R, 3S)-isomer] of methyl trans-3-(4-methoxyphenyl)glycidate in toluene layer was measured by High Performance Liquid Chromatography (column: Chiralcel OJ, manufactured by Daicel Chemical Industries, Ltd., mobile phase: n-hexane : isopropyl alcohol = 9:1).

The results are shown in the following Table 4

Table 4

The ratio of optically active isomers (%)		Optical yield of (-)-trans isomer (%)
(+)-trans-isomer	(-)-trans-isomer	
0.4	99.6	94

Example 6

Optimum pH and pH stability were investigated with respect to the purified esterase obtained in Example 2.

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Optimum pH was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction was carried out at various pH. The each activity was shown as relative activity (%) (the activity at pH 8 = 100).

On the other hand, pH stability was examined by adjusting the enzyme solution at a specified pH, incubating at 30 °C for one hour and then estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100), and the pH adjustment was carried out by using McIlvaine buffer (pH 3.0-7.0), 100 mM Tris-HCl buffer (pH 8.0 - 9.0) and 100 mM glycine buffer (pH 9.0 - 11.0).

(Results)

The results are shown in the following Table 5 and 6. It is clear from these Tables that the optimum pH of the esterase is about 7.5 - 9.0, and the esterase is stable at a pH of about 5 - 9.

Table 5 (Relative activities at each pH)

pH	3	4	5	6	7	8	9	10	11
Relative activity (%)	0	5	20	39	69	100	80	2	0

Table 6 (pH stability)

pH	3	4	5	6	7	8	9	10	11
Residual activity (%)	0	5	95	100	100	100	100	65	0

Example 7

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Optimum temperature and heat stability were investigated with respect to the purified esterase obtained in Example 2.

The optimum temperature was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction is carried out at various temperature. The enzyme activity was shown as relative activity (%) (the activity at 45 °C = 100).

On the other hand, the heat stability was examined by incubating the enzyme in 100 mM Tris-HCl buffer for 30 minutes at a specified temperature, and estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100).

(Results)

The results are shown in the following Table 7 and 8. It is clear from Tables that the optimum temperature is about 40 to 50 °C, and the esterase is stable at a temperature not higher than 50 °C.

Table 7

Temperature (°C)	10	15	20	30	35	40	45	50	55
Relative activity (%)	10	14	19	39	51	72	100	64	4

Table 8

Temperature (°C)	4	30	40	50	55	60
Residual acitivity (%)	100	100	-100	100	2	0

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Example 8

The isoelectric point of the purified esterase obtained in Example 2 was examined by means of electrophoresis under the following conditions:

carrier: Ampholine (pH 3-10)

density gradient of 0 to 50 % sucrose

voltage: at 400V for 26 hours from the beginning and then at 800V for14 hours

temperature: 2 °C

110 ml column was used.

As a result, the isoelectric point of said esterase was found to be 4.6 ± 0.1

Example 9

The purified esterase obtained in Example 2 (protein: 95 μg) was dialyzed against distilled water, and the dialyzed solution was evaporated to dryness. The residue was subjected to an amino acid sequencer. As a result, the amino acid sequence from N-terminal to 12th was found to be as follows:

N-terminal-(X)-Ile^{2}-Phe-Ser-Tyr^{5}-Lys-Asp-Leu-Asp-Glu^{10}-Asn-Ala

wherein X is an amino acid which has not yet been identified.

Example 10

The effect of metal ions or known enzyme-inhibitors on the purified esterase obtained in Example 2 was investigated as follows:

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1mM each metal ion or 1mM each inhibitor was added to the enzyme solution, and the enzyme activity was estimated in the same manner as described in Example 1.

The activity was shown as relative activity (%) (the activity estimated by no addition of metal ion or inhibitor = 100).

(Results)

The results are shown in the following Table 9 and 10.

Table 9 (The effect of metal ions)

Metal ion (1mM)	Relative activity (%)
no addition	100
Co^{2+}	0
Li^{+}	143
Zn^{2+}	29
Cu^{2+}	57
Mg^{2+}	123
Ni^{2+}	0
Mn^{2+}	11
Hg^{2+}	86
Fe^{2+}	0
Fe^{3+}	0
Ca^{2+}	285
Pb^{2+}	86
K^{+}	94

Table 10 (The effect of inhibitors)

Inhibitors(1mM)	Relative activity
without reagent	100
Ethylenediaminetetraacetic acid	0
p-Chloromercury benzoate	86

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Sodium dodecyl sulfate	157
Phenylmethylsulfonyl fluoride	100

Example 11

12 liters of the supernatant obtained from the culture broth in the same manner as described in Example 1 were concentrated by using ultrafiltration membrane (SIP-3013, Asahi Chemical Industries Co., Ltd.). The concentrate was salted out with 35% saturated ammonium sulfate solution containing 10 mM Tris-HCl buffer (pH 7.5). The precipitate (esterase) was dissolved in 10 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 10 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.8 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.3 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 10 mM Tris-HCl buffer (pH 7.5). The solution (101 ml) was concentrated to 8 ml by ultrafiltration (Diaflo ultrafilter PM-10, manufactured by Amicon Co., Ltd.). The concentrate was subjected to gel filtration of a molecular sieve resin (Sephacryl S-300 HR, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The active fractions were collected and dialyzed against 2% saturated ammonium sulfate solution containing 10 mM Tris-HCl

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buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Phenyl-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer. Enzyme was eluted from the resin by a stepwise gradient of 2% to 0% saturated ammonium salfate solutions containing 10 mM Tris-HCl buffer (pH 7.5). 133mg of purified esterase protein were obtained.

Physico-chmical properties and enzymatic characteristics of this purified esterase are identical to those obtained in Example 2.

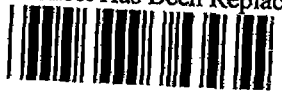
The specific activities and yields of esterase obtained in each of the above-purification steps are shown in the following Table 11 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.). The esterase obtained above showed no protease activity when casein was used as the substrate.

All of purification steps above was carried out below 5 °C

Table 11

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	12000	13900	1416000	102	100
Concentration by UF membrane	1000	1920	1343000	699	95

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35% ammonium sulfate	175	645	1269000	1967	90
DEAE-TOYOPEAL 650M	101	298	656000	2201	46
Sephacryl S-300 HR	45	194	590000	3041	42
Phenyl-TOYOPEAL 650M	18	133	414000	3113	29

Example 12

The amino acid composition of purified esterase obtained in Example 11 was examined by using an amino acid analyzer under the following conditions.

Each enzyme solution in 6M hydrochloride (2.0 mg/ml) was sealed in a glass tube under vacuum and then hydrolyzed for 20, 40 or 70 hours at 110 °C. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in 0.02N hydrochloride solution and analyzed with an amino acid analyzer (Hitachi model L-8500). Cystein and cystine were determined as cysteic acid after performic acid oxidation of the sample according to the method of Moore[1]. Tryptophan was determined according to the method of Simpson et al.[2]

References: 1) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

2) Simpson, R.J., Neuberger, M.R., and Lin, T.-Y. (1976) J. Biol. Chem. 251, 1936-1940.

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Table 12

Amino acid	Residues / molecule[a)]
Asx[b)]	89
Thr[c)]	40
Ser[c)]	43
Glx[b)]	40
Pro	15
Gly	78
Ala	56
Val	28
Ile	31
Leu	58
Tyr	23
Phe	28
Lys	20
His	14
Arg	14
Cys	0 - 1
Met	2
Trp	7

a): Based on an Molecular weight of 62,000.

b): Sum of acid and amide forms.

c): The serine and threonine values were obtained by extrapolation to time zero, assuming first-order decay.

Example 13

The purified esterase obtained in Example 11 was dialyzed against water, and its calcium content was determined by using an atomic absoption spectrometry (Hitachi model Z-9000).

As a result, the calcium content in the purified esterase was one mole per an enzyme molecular mass of 62,000.

Example 14

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The substrate specificity of the purified esterase obtained in Example 11 on dimercaprol tributyrate was investigated in the same manner as described in Example 4.

The esterase showed the enzyme activity of 5.2×10^5 units/mg protein.

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What is claimed is:

1. An esterase having the following physico-chemical properties and enzymatic characteristics:

(1) Activity: it (i.e., said esterase) hydrolyzes an ester bond of organic carboxylates,

(2) Substrate specificity: it acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters,

(3) Optimum pH: its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate,

(4) pH stability: it is stable at pH 5.0 - 9.0 when it is stored at 30 °C for one hour,

(5) Optimum temperature: its optimum temperature is 40 - 50 °C when the hydrolysis is carried out by using olive oil as the substrate,

(6) Heat stability: it is stable at a temperature of not higher than 50°C when it is stored at pH 8.0 for 30 minutes,

(7) Molecular weight: 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis),

(8) Isoelectric point: 4.6 ± 0.1,

(9) Effect of metal ions: it is activated in the presence of 1mM calcium ion, and inhibited in the presence of 1mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid.

2. The esterase according to Claim 1 which is derived from a microorganism belonging to the genus Serratia.

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3. The esterase according to Claim 2 in which the microorganism is Serratia marcescens.

4. A process for preparing novel esterase which comprises

(a) cultivating a microorganism having ability to produce the esterase claimed in Claim 1, and

(b) recovering said esterase from the culture broth.

5 A process according to Claim 4 in which the microorganism belong to the genus Serratia.

6. The process according to Claim 5 in which the microorganism is Serratia marcescens.

7. A process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate which comprises the steps of:

(a) treating a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate with the esterase claimed in Claim 1 thereby stereoselectively hydrolyzing 2S, 3R-isomer thereof, and

(b) recovering unreacted 2R, 3S-isomer from the reaction mixture.

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NOVEL ESTERASE AND PROCESS FOR PREPARING THE SAME

ABSTRACT

A novel esterase which is derived from Serratia marcescens is disclosed. Said esterase has the following physico-chemical properties and enzymatic charactaristics:

(1) Activity; it (i.e., said esterase) hydrolyzes an ester bond of organic carboxylates, (2) Substrate specificity; it acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters, (3) Optimum pH; its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate, (4) pH stability; it is stable at pH 5.0 - 9.0 when it is stored at 30 °C for one hour, (5) Optimum temperature; its optimum temperature is 40 - 50 °C when the hydrolysis is carried out by using olive oil as the substrate, (6) Heat stability; it is stable at a temperature of not higher than 50°C when it is stored at pH 8.0 for 30 minutes, (7) Molecular weight; 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis), (8) Isoelectric point; 4.6 ± 0.1, (9) Effect of metal ions; it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid. Said esterase can widely be applied for organic synthetic reactions.

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NOVEL ESTERASE AND PROCESS FOR PREPARING THE SAME

This invention relates to novel esterase and process for preparing the same.

Recently, attempts have been made to use esterase for organic synthetic reactions (e.g., hydrolysis reaction). For example, esterase derived from pig liver is often used for this purpose. However, this esterase is disadvantageous for industrial use because it is expensive. On the other hand, there have been known esterases derived from microorganisms such as Arthrobacter globiformis IFO 12985 [Patent Publication (unexamined) No. 181788/1989] (Molecular Weight: 43,000), Bacillus stearothermophilus [Archiv. Biochem. Biophys. 160, 504-513 (1974)] (Molecular Weight: 47,000), Geotrichum candidum [Agric. Biol. Chem. 37 (6), 1457-1464 (1973)] (Molecular Weight: 53,000-55,000), Pseudomonas aeruginosa [J. Biochem. 86, 643-656 (1979)] (Molecular Weight: 55,000), Pseudomonas fluorescens [J. Biochem. 95, 1047-1054 (1984)] (Molecular Weight: 48,000).

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However, these esterases have difficulties that they can not be widely applied because of their narrow substrate specificities.

The object of the present invention is to provide a novel esterase which is derived from a microorganism and can be applied for organic synthetic reactions as widely as pig liver esterase.

Other object of the present invention is to provide a process for preparing said esterase.

Another object of the present invention is to provide a process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate by using said esterase.

As a result of various investigations, we have now found a potent esterase which is derived from a microorganism belonging to genus Serratia.

The esterase of the present invention is a novel esterase having the following physico-chmical properties and enzymatic characteristics:

(1) Activity

It (i.e., said esterase) hydrolyzes an ester bond of an organic carboxylates.

(2) Substrate specificity

It acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters. For example, the esterase of the present invention has ability to hydrolyze a lower or higher alkyl esters of organic carboxylic acids (e.g., a lower fatty acid, a higher fatty acid, a lower alkoxy-substituted-phenylglycidic acid and the like). Moreover, it also has potent ability to hydrolyze triglycerides composed of glycerol and

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water-soluble lower fatty acids or water-insoluble higher fatty acids. Further, it also has ability to hydrolyze thiol esters such as dimercaprol tributyrate and the like. But it has no ability to hydrolyze casein.

(3) Optimum pH

It shows optimum pH of about 7.5-9.0 when olive oil is used as the substrate.

(4) pH stability

~~It remains more than~~ It retains at least 95% of its activity when it is stored at a pH 5-9 at 30 °C for 60 minutes.

(5) Optimum temperature

It shows optimum temperature of about 40-50 °C when the enzymatic reaction is carried out in 100 mM Tris-HCl buffer (pH 8.0) by using olive oil as the substrate.

(6) Heat stability

It remains 100% of its activity when it is stored at a temperature not higher than 50 °C in 100 mM Tris-HCl buffer (pH 8.0) for 30 minutes.

(7) Assay of enzyme activity

(a) activity on olive oil

The enzyme reaction is carried out at pH 8.0 at 37 °C for 20 minutes by using olive oil as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 1)

(b) activity on triglycerides or fatty acid esters

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The enzyme reaction is carried out at pH 8.0 at 37 °C for 10 minutes by using triglycerides or fatty acid esters as the substrate. The activity of the esterase which produces one μ mole of fatty acid per minute is defined as one unit. (cf., Example 3)

(c) activity on thiol esters

The enzyme reaction is carried out at pH 8.5 at 30 °C for 10 minutes by using dimercaprol tributylate as the substrate. After the reaction, 5,5'-dithiobis(2-nitrobenzoic acid) (color-producing reagent) is added to the reaction mixture. The amount of the liberated 5-mercapto-2-nitrobenzoic acid is measured by spectrophotometer. The activity of the esterase which produces one μ mole of 5-mercapto-2-nitrobenzoic acid per minute is defined as one unit. (cf., Example 4)

(8) Molecular weight

62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis)

(9) Isoelectric point

4.6 ± 0.1

(10) Effect of metal ions

It is activated in the presence of 1 mM calcium ion. On the other hand, its activity is inhibited by 40-90% in the presence of 1 mM zinc ion, copper ion, manganese ion, and completely inhibited in the presence of 1 mM cobalt ion, nickel ion, iron (II) ion, iron (III) ion or ethylenediaminetetraacetic acid.

The above-mentioned esterase of the present invention is obviously different from known esterases in that it has molecular

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weight of 62,000 ± 2,000 (measured by SDS-polyacrylamide gel electrophoresis) and isoelectric point of 4.6 ± 0.1.

According to the present invention, the above-mentioned esterase can be prepared by cultivating a microorganism belonging to the genus Serratia in a medium, accumulating said esterase inside or outside of the microorgansim, and recovering the accumulated esterase therefrom.

Any microorganism belonging to the genus Serratia which can produce the above-mentioned esterase may be used as the esterase-producing microorganism of the present invention. Examples of such microorganism include Serratia marcescens Sr41 (deposited at Fermentation Research Institute Agency of Industrial Science and Technology on 26 March, 1983 and accorded the accession number FERM-BP No. 487), a mutant thereof and the like. But, the microorganism used in the present invention are not limited to those mentioned-above. For example, a recombinant which is produced from the above mentioned microorgansims according to the bioengineering methods may be also used.

Any medium in which the esterase-producing microorganisms of the present invention can grow and proliferate may be used as the medium. Examples of the carbon source include, for example, sugars such as glucose, sucrose, molasses and the like, organic acids such as fumaric acid, citric acid and the like, alcohols such as glycerol and the like, amino acids such as alanine, glutamine, asparagine and the like and so forth. Inorganic ammonim salts such as ammonium sulfate, ammonium chloride and the like, urea, peptone, corn steep liquor, yeast extract, casein hydrolysate and the like can be used as the

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nitrogen source. It is preferred to use 1 - 15 w/w% of the carbon source and 0.1 - 2.0 w/w% of the nitrogen source. If required, inorganic salts such as phosphates, magnesium salts, potassium salts, calcium salts and the like, or metal ions such as iron ions, manganese ions, copper ions, zinc ions and the like may be added to the medium. When a synthetic medium is used, vitamins such as biotin, thiamin and the like, or growth-promoting substances such as carnitine may, if required, be added to the medium. Moreover, inducers such as vegetable oils or surfactants may, if necessary, be added to the medium. It is preferred to adjust the pH of the medium to a pH of about 5 - 8.

The cultivation can be carried out in a conventional manner after inoculating the microorganism into the medium. For example, any method such as shake culture, aeration spinner culture, stationary culture or continuous culture may be used for this purpose.

The condition of cultivation may vary depending on a kind of the medium, a cultivation method and the like. Any condition under which the microorganism of the present invention can grow, proliferate and produce the esterase is appropriate for this purpose. But it is usually preferred to begin the cultivation at a pH of about 5 - 8, and then carry it out at room temperature or under warming, for example, at a temperature between 20 and 40 °C for one to 2 days.

The esterase accumlated inside or outside of the cultivated microorganism can be recovered and purified in a conventional manner. For example, the esterase accumlated in the culture broth

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can be rcovered by a combination of known methods such as salting-out with inorganic salt (e.g., ammonium sulfate, an alkali metal sulfate or an alkali metal halide), differential precipitation with hydrophilic organic solvent (e.g., an alcohol or acetone), column chromatography by ion exchange resin or hydrophobic resin, gel filtration and protein precipitation with nucleic acid, tannin or the like. The thus-obtained esterase can be further purified by a combination of known purification methods such as isoelectric precipitation, dialysis, electrodialysis, electrophoresis and the like. For example, the purification is carried out by;

(1) removing microbial cells from the culture broth by centrifugation,

(2) treating the supernatant with a 45% saturated ammonium sulfate solution,

(3) subjecting the resulting precipitate to an anion exchange resin (DEAE-TOYOPEARL 650M, MonoQ) chromatography after dialysis thereof,

(4) subjecting active fractions to hydrophobic resin (Butyl-TOYOPEARL 650S) chromatography after dialysis thereof, and

(5) subjecting the obtained active fractions to gel filtration (Superose 6) after dialysis and condensation thereof. The thus-obtained purified esterase is a polypeptide showing a single band with a molecular weight of 62,000 ± 2,000 by SDS-polyacrylamide gel electrophoresis.

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As mentioned before, the esterase of the present invention have potent ability to hydrolyze wide range of substrate such as alkyl esters of organic carboxylic acids, triglycerides or thiol esters. Therefore, it can be applied for organic synthetic reactions as widely as pig liver esterase. For example, it can be applied for preparation of an optically active isomer from a racemic mixture thereof, or preparation of a chiral compound from a prochiral compound thereof. In particular, the esterase of the present invention is characterized in that it has strong ability to hydrolyze a (lower)alkyl (lower)alkoxyphenyl-glycidate. For example, when it is used for hydrolysis of a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate, a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate can be prepared in good yield. The thus-obtained lower alkyl (2R, 3S)-3-(4-lower alkoxy-phenyl)glycidate is useful as a synthetic intermediate of pharmaceuticals such as diltiazem hydrochloride.

In the following Examples, "%" means "w/v%" unless otherwise prescribed.

Examples

Example 1

A medium (pH 7.0, 20 liters) containing dextrin (1%), ammonium sulfate (0.2%), meast-S (2%), potassium dihydrogenphosphate (0.1%), magnesium sulfate (0.05%), calcium chloride (0.01%), ferrous sulfate (0.001%), Tween80 (0.5 v/v%) and polyalkylene glycol derivative-type surfactant (trade name: KARARIN 102, manufactured by Sanyo Chemical Industries, Ltd., 0.1 v/v%) was placed in a 30-liter jar-

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fermenter and sterillized by autoclaving. A broth (200 ml) of Serratia marcescens Sr41 which was obtained by reciprocal shaking at 30 °C for 20 hours in the same medium as above was inoculated into the sterillized medium. The cultivations was carried out by aeration and agitation (200 rpm, 0.5vvm) at 30 °C for 18 hours. The culture broth was centrifuged, and the supernatant (4.5 liters) was salted out with 45% saturated ammonium sulfate solution. The precipitate was collected by filtration with celite, eluted with water. The eluate was dialyzed and lyophilized. 4.1 g of esterase (18,600 unit/g) were obtained as crude enzyme powder.

(Assay of enzyme activity)

The enzyme activity was estimated according to the following method.

A mixture of 225 ml of 2% polyvinyl alcohol (Poval 117, manufactured by Kurare Co., Ltd.) and 75 ml of olive oil was emulsified by stirring at 14,500 rpm at 5-10 °C for 10 minutes. 5.0 ml of the olive oil-emulsion thus-obtained and 4.0 ml of 0.25M Tris-HCl buffer (pH 8.0, containing 2.5 mM calcium chloride) were preincubated at 37 °C for 10 minutes. One ml of an enzyme solution was added thereto to initiate enzymatic reaction. After the mixture was incubated at 37 °C for 20 minutes, 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) is added

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to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which liberated one μmole of fatty acid per minute was defined as one unit (U).

Example 2

10 liters of the supernatant obtained in the same manner as described in Example 1 were salted out with 45% saturated ammonium sulfate solution. The precipitate (esterase) was dissolved in 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEARL 650M, Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 1.0 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.27 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a strong anion exchange resin (MonoQ, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.6 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.35 M sodium chloride solutions containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 5% saturated ammonium sulfate solution

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containing 20 mM Tris-HCl buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Butyl-TOYOPEAL 650S, manufactured by Toyo Soda Co. Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 5 to 0 % saturated ammonium sulfate solution containing 20 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 20 mM Tris-HCl buffer (pH 7.5) and condensed. The residue was subjected to gel filtration of molecular sieve resin (Superose 6, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. 56.8 mg of purified esterase protein were obtained.

The purified esterase showed molecular weight of about 590,000 by gel filtration and about 62,000 by SDS-polyacrylamide gel electrophoresis.

The specific activities and yields of esterases obtained in each of the above purification steps are shown in the following Table 1 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.)

The esterase obtained above showed no protease activity when casein was used as the substrate.

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Table 1

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	16470	385400	23.4	100
45% ammonium sulfate	1529	341000	223	88.5
DEAE-TOYOPEAL 650M	247	227500	921	59.0
MonoQ	143	141300	988	36.7
Butyl-TOYOPEAL 650S	84.5	85350	1010	22.1
Superose 6	56.8	58500	1030	15.2

Example 3

The substrate specificity on various kinds of triglyderides and fatty acid esters was investigated with respect to the esterase obtained in Example 2.

(Assay of hydrolyzing activity on triglycerides and fatty acid esters)

0.2 g of each substrate, 4 ml of 0.2 M Tris-HCl buffer (pH 8.0) and one ml of 6 mM calcium chloride solution were placed in 100 ml flask. After a ten minute-preincubation, one ml of enzyme solution was added thereto to initiate enzymatic reaction. The mixture was incubated at 37 °C under 80 rpm for 10 minutes. 20 ml of a mixture of acetone - ethanol (1:1) were added to the reaction mixture to stop

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the enzymatic reaction. The mixture was titrated with 0.05 N sodium hydroxide solution by using phenolphthalein as the indicator. A blank solution was prepared in the same manner as above except that acetone - ethanol (1:1) was added to the substrate solution before addition of the enzyme solution. Said blank solution was titrated in the same manner as above. The amount of enzyme which can liberate one μmole of fatty acid per minute is defined as one unit.

(Results)

The result are shown in the following Tables 2 and 3. The enzyme activity is shown as relative activity (%) [methyl n-caprylate (Table 2), tricaprylin (Table 3) = 100].

Table 2

Substrate	Relative activity (%)
methyl acetate	9.7
methyl n-butyrate	72.8
methyl n-valerate	38.9
methyl n-caproate	14.6
methyl n-caprylate	100 1)
methyl n-caprate	92.2
methyl laurate	61.2
methyl myristate	34.0
methyl palmitate	14.6
methyl linolenate	38.9

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methyl linolate	51.5
methyl oleate	2.9

1): 204 unit/mg protein

Table 3

Substrate	Relative activity (%)
triacetin	7.6
tributylin	93.2
tricaproin	70.3
tricaprylin	100 2)
tricaprin	42.4
trilaurin	29.7
trimyristin	33.9
tripalmitin	1.6
Tristearin	1.6

2): 1169 unit/mg protein

Example 4

The substrate specificity on dimercaprol tributyrate was investigated with respect to the purified esterase obtained in Example 2.

The esterase was added to a substrate solution of Lipase Kit S [Substrate: dimercaprol tributyrate, manufactured by Dainippon Pharmaceutical Co., Ltd.]. After the hydrolysis was carried out, 5,5-dithiobis(2-nitrobenzoic acid) (color-producing agent) was added to

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the reaction mixture. Enzyme activity was estimated by measuring the amount of the liberated 5-mercapto-2-nitrobenzoic acid at 412 nm.

The esterase showed the enzyme activity of 1.7×10^5 units/mg protein.

Example 5

1.5 mg of the purified esterase obtained in Example 2 were added to 75 ml of an aqueous 1mM calcium chloride solution. 75 ml of toluene solution containing 1M racemic methyl trans-3-(4-methoxyphenyl)glycidate were added thereto. The mixture was adjusted to pH 8.0 and stirred at 30 °C for 4 hours. After the reaction, the ratio of (+)-isomer [(2S, 3R)-isomer] and (-)-isomer [(2R, 3S)-isomer] of methyl trans-3-(4-methoxyphenyl)glycidate in toluene layer was measured by High Performance Liquid Chromatography (column: Chiralcel OJ, manufactured by Daicel Chemical Industries, Ltd., mobile phase: n-hexane : isopropyl alcohol = 9:1).

The results are shown in the following Table 4

Table 4

The ratio of optically active isomers (%)		Optical yield of (-)-trans isomer (%)
(+)-trans-isomer	(-)-trans-isomer	
0.4	99.6	94

Example 6

Optimum pH and pH stability were investigated with respect to the purified esterase obtained in Example 2.

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Optimum pH was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction was carried out at various pH. The each activity was shown as relative activity (%) (the activity at pH 8 = 100).

On the other hand, pH stability was examined by adjusting the enzyme solution at a specified pH, incubating at 30 °C for one hour and then estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100), and the pH adjustment was carried out by using McIlvaine buffer (pH 3.0-7.0), 100 mM Tris-HCl buffer (pH 8.0 - 9.0) and 100 mM glycine buffer (pH 9.0 - 11.0).

(Results)

The results are shown in the following Table 5 and 6. It is clear from these Tables that the optimum pH of the esterase is about 7.5 - 9.0, and the esterase is stable at a pH of about 5 - 9.

Table 5 (Relative activities at each pH)

pH	3	4	5	6	7	8	9	10	11
Relative activity (%)	0	5	20	39	69	100	80	2	0

Table 6 (pH stability)

pH	3	4	5	6	7	8	9	10	11
Residual activity (%)	0	5	95	100	100	100	100	65	0

Example 7

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Optimum temperature and heat stability were investigated with respect to the purified esterase obtained in Example 2.

The optimum temperature was determined by measuring the enzyme activity in the same manner as described in Example 1 except that the enzymatic reaction is carried out at various temperature. The enzyme activity was shown as relative activity (%) (the activity at 45 °C = 100).

On the other hand, the heat stability was examined by incubating the enzyme in 100 mM Tris-HCl buffer for 30 minutes at a specified temperature, and estimating the enzyme activity in the same manner as described in Example 1. The enzyme activity was shown as the relative activity (%) of residual activity (the activity measured immediately before incubation = 100).

(Results)

The results are shown in the following Table 7 and 8. It is clear from Tables that the optimum temperature is about 40 to 50 °C, and the esterase is stable at a temperature not higher than 50 °C.

Table 7

Temperature (°C)	10	15	20	30	35	40	45	50	55
Relative activity (%)	10	14	19	39	51	72	100	64	4

Table 8

Temperature (°C)	4	30	40	50	55	60
Residual acitivity (%)	100	100	100	100	2	0

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Example 8

The isoelectric point of the purified esterase obtained in Example 2 was examined by means of electrophoresis under the following conditions:

carrier: Ampholine (pH 3-10)

density gradient of 0 to 50 % sucrose

voltage: at 400V for 26 hours from the beginning and then at 800V for 14 hours

temperature: 2 °C

110 ml column was used.

As a result, the isoelectric point of said esterase was found to be 4.6 ± 0.1

Example 9

The purified esterase obtained in Example 2 (protein: 95 μg) was dialyzed against distilled water, and the dialyzed solution was evaporated to dryness. The residue was subjected to an amino acid sequencer. As a result, the amino acid sequence from N-terminal to 12th was found to be as follows:

N-terminal-(X)-Ile^{2}-Phe-Ser-Tyr^{5}-Lys-Asp-Leu-Asp-Glu^{10}-Asn-Ala

wherein X is an amino acid which has not yet been identified.

Example 10

The effect of metal ions or known enzyme-inhibitors on the purified esterase obtained in Example 2 was investigated as follows:

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1 mM each metal ion or 1 mM each inhibitor was added to the enzyme solution, and the enzyme activity was estimated in the same manner as described in Example 1.

The activity was shown as relative activity (%) (the activity estimated by no addition of metal ion or inhibitor = 100).

(Results)

The results are shown in the following Table 9 and 10.

Table 9 (The effect of metal ions)

Metal ion (1 mM)	Relative activity (%)
no addition	100
Co^{2+}	0
Li^{+}	143
Zn^{2+}	29
Cu^{2+}	57
Mg^{2+}	123
Ni^{2+}	0
Mn^{2+}	11
Hg^{2+}	86
Fe^{2+}	0
Fe^{3+}	0
Ca^{2+}	285
Pb^{2+}	86
K^{+}	94

Table 10 (The effect of inhibitors)

Inhibitors(1 mM)	Relative activity
without reagent	100
Ethylenediaminetetraacetic acid	0
p-Chloromercury benzoate	86

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Sodium dodecyl sulfate	157
Phenylmethylsulfonyl fluoride	100

Example 11

12 liters of the supernatant obtained from the culture broth in the same manner as described in Example 1 were concentrated by using ultrafiltration membrane (SIP-3013, Asahi Chemical Industries Co., Ltd.). The concentrate was salted out with 35% saturated ammonium sulfate solution containing 10 mM Tris-HCl buffer (pH 7.5). The precipitate (esterase) was dissolved in 10 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed and subjected to a column of an anion exchange resin (DEAE-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with 10 mM Tris-HCl buffer (pH 7.5). The elution of enzyme was carried out by a liner gradient of 0 to 0.8 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). Esterase was eluted with about 0.3 M sodium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.5). The active fractions were collected and dialyzed against 0.15 M sodium chloride solution containing 10 mM Tris-HCl buffer (pH 7.5). The solution (101 ml) was concentrated to 8 ml by ultrafiltration (Diaflo ultrafilter PM-10, manufactured by Amicon Co., Ltd.). The concentrate was subjected to gel filtration of a molecular sieve resin (Sephacryl S-300 HR, manufactured by Pharmacia LKB Biotechnology) which was pre-equilibrated with the same buffer. The active fractions were collected and dialyzed against 2% saturated ammonium sulfate solution containing 10 mM Tris-HCl

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buffer (pH 7.5) and subjected to a column of a hydrophobic resin (Phenyl-TOYOPEAL 650M, manufactured by Toyo Soda Co., Ltd.) which was pre-equilibrated with the same buffer. The column was washed with the same buffer. Enzyme was eluted from the resin by a stepwise gradient of 2% to 0% saturated ammonium salfate solutions containing 10 mM Tris-HCl buffer (pH 7.5). 133mg of purified esterase protein were obtained.

Physico-chmical properties and enzymatic characteristics of this purified esterase are identical to those obtained in Example 2.

The specific activities and yields of esterase obtained in each of the above-purification steps are shown in the following Table 11 (The enzyme activity was estimated in the same manner as described in Example 1 and the amount of protein was measured by Lowry method.). The esterase obtained above showed no protease activity when casein was used as the substrate.

All of purification steps above was carried out below 5 °C

Table 11

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Supernatant of culture	12000	13900	1416000	102	100
Concentration by UF membrane	1000	1920	1343000	699	95

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35% ammonium sulfate	175	645	1269000	1967	90
DEAE-TOYOPEAL 650M	101	298	656000	2201	46
Sephacryl S-300 HR	45	194	590000	3041	42
Phenyl-TOYOPEAL 650M	18	133	414000	3113	29

Example 12

The amino acid composition of purified esterase obtained in Example 11 was examined by using an amino acid analyzer under the following conditions.

Each enzyme solution in 6M hydrochloride (2.0 mg/ml) was sealed in a glass tube under vacuum and then hydrolyzed for 20, 40 or 70 hours at 110 °C. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in 0.02N hydrochloride solution and analyzed with an amino acid analyzer (Hitachi model L-8500). Cystein and cystine were determined as cysteic acid after performic acid oxidation of the sample according to the method of Moore[1]. Tryptophan was determined according to the method of Simpson et al.[2]

References: 1) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

2) Simpson, R.J., Neuberger, M.R., and Lin, T.-Y. (1976) J. Biol. Chem. 251, 1936-1940.

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Table 12

Amino acid	Residues / molecule[a)]
Asx[b)]	89
Thr[c)]	40
Ser[c)]	43
Glx[b)]	40
Pro	15
Gly	78
Ala	56
Val	28
Ile	31
Leu	58
Tyr	23
Phe	28
Lys	20
His	14
Arg	14
Cys	0 - 1
Met	2
Trp	7

a): Based on an Molecular weight of 62,000.

b): Sum of acid and amide forms.

c): The serine and threonine values were obtained by extrapolation to time zero, assuming first-order decay.

Example 13

The purified esterase obtained in Example 11 was dialyzed against water; and its calcium content was determined by using an atomic absoption spectrometry (Hitachi model Z-9000).

As a result, the calcium content in the purified esterase was one mole per an enzyme molecular mass of 62,000.

Example 14

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The substrate specificity of the purified esterase obtained in Example 11 on dimercaprol tributyrate was investigated in the same manner as described in Example 4.

The esterase showed the enzyme activity of 5.2×10^5 units/mg protein.

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COMPARATIVE EXPERIMENTS

Example 15

Stereoselectivity for substrate

Stereoselectivity of the esterase of the present invention and the enzyme disclosed in the reference AU40977/89 was determined.

Enzymes used

The esterase of the present invention: Serratia marcescens Sr41 (FERM BP-487) was cultivated in 60 ml of nutrient broth (dextrin 1%, ammonium sulfate 0.2%, meast S 2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, $FeSO_4 \cdot 7H_2O$ 0.001%, Tween 80 0.5% and Colorin 102 0.1%, pH 7.0) at 30°C for 18 hours. The culture broth was centrifuged to obtain a supernatant. The supernatant was used as an enzyme solution of the present invention.

The enzyme of the reference: According to the same manner as that described above, Serratia marcescens ATCC 27117 strain was cultivated and the resulting supernatant was used as an enzyme solution of the reference.

Method

3.75 ml of 0.4 M Tris-HCl buffer (pH 8) containing 2mM $CaCl_2$ was mixed with 7.5 ml of a toluene solution of 1.5 g of racemic methyl trans-3-(4-methoxylphenyl)glycidate and

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the mixture was pre-incubated at 30°C for 10 minutes. To this mixture was added 3.75 ml of the above supernatant (enzyme solution) and the resulting mixture was stirred at 1400 rpm for 3 hours to carried out the hydrolysis reaction.

The enzyme was used in such amount that the extent of hydrolysis of the substrate was 40 to 50% after the reaction for 3 hours.

After completion of the reaction, methyl trans-3-(4-methoxyphenyl)glycidate recovered was analyzed by high performance liquid chromatography (HPLC) using a Daicel Chiralcel OJ column to determine the extent of hydrolysis and optical purity of methyl (2R, 3S)-3-(4-methoxyphenyl)-glycidate. Based on these values, E-value was calculated according to the following formula [Journal of American Chemical Society, 104, pp7294-7299(1982)]. The stereoselectivity of each enzyme was evaluated by E-value.

$$E=\frac{\ln[(1-C)(1-e.e.)]}{\ln[(1-C)(1+e.e.)]}$$

Note) C: Extent of hydrolysis (%)/100

e.e.: Optical purity

$$e.e.=\frac{\text{Amount of remaining (2R, 3S)-isomer} - \text{Amount of remaining (2S, 3R)-isomer}}{\text{Amount of remaining (2R, 3S)-isomer} + \text{Amount of remaining (2S, 3R)-isomer}}$$

Results

The results are shown in Table 13

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Table 13

Enzyme	C(%)	e.e.(%)	E-value
Present invention	43.7	73.7	85
Reference	49.7	94.3	155

Consideration

As seen from the above results, the E-value of the enzyme of the present invention is about one half of that of the reference enzyme. That is, stereoselectivity of the enzyme of the present invention is about twice higher than that of the reference enzyme. In view of this, it is clear that, when racemic glycidate is subjected to asymmetric hydrolysis, the desired optically active glycidate having high optical purity can be obtained by using the esterase of the present invention.

Example 16

pH Stability of substrate and enzyme

pH stability of the substrate, the enzyme of the present invention and the enzyme of the reference were evaluated.

(1) pH Stability of substrate

Method

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7.5 ml of a toluene solution containing 0.75 mg of racemic methyl trans-3-(4-methoxyphenyl)glycidate was added to 7.5 ml of each of various 0.2 M buffer solutions and the mixture was stirred at 1400 rpm at 30°C for 6 hours. Then, the ratio (%) of methyl trans-3-(4-methoxyphenyl)glycidate remaining in the reaction mixture was determined by HPLC. As the buffer solutions, McIlvain buffer solution (pH 4-7), Tris-HCl buffer solution (pH 8-9) and glycine-NaOH buffer solution (pH 10) were used.

Results

The results are shown in Table 14.

Table 14

pH	Remaining ratio of substrate(%)
4	18
5	55
6	69
7	87
8	96
9	97
10	95

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(2) pH Stability of enzymes

Method

5 ml of Tris-HCl buffer solution (pH 8) was added to 5 ml of each enzyme solution and the mixture was subjected to heat treatment at 50°C for 0 to 6 hours. The enzyme solution thus treated was diluted (100 times in the case of the present invention and 25 times in the case of the reference). To 50 µl of the diluted solution were added 1 ml of the color developing agent, 5,5'-dithiobis(2-nitrobenzoic acid), and 20 µl of phenylmethylsulfonyl fluoride and the mixture was pre-incubated at 30°C for 5 hours. Then, 100 µl of a substrate solution (aqueous dimercaprol tributylate solution: Lipase Kits manufactured by Dainippon Seiyaku K.K.) was added thereto and the mixture was reacted at 30°C for 10 minutes.

After termination of the reaction, absorbance at 412 nm was measured to determine the amount of the released 5-thio-2-nitrobenzoic acid. The enzyme activity was evaluated by taking the amount of the enzyme which release 1 µmol of 5-thio-2-nitrobenzoic acid per 1 minute as 1 unit. The residual activity of the reaction mixture was shown by the relative activity (%) by taking the enzyme activity of the reaction mixture without heat treatment as 100 %. The amount of protein in the reaction mixture (mg/ml) was measured according to Lowry method.

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Results

The results are shown in Table 15

Table 15

Heating time (hrs)	Present invention		Reference	
	Residual activity (%)	Amount of protein (mg/ml)	Residual activity (%)	Amount of protein (mg/ml)
0	100 *	1.20	100 **	1.00
1.5	99	1.30	82	1.00
3.5	101	1.30	31	1.00
6.0	107	1.30	10	1.05

Note *: The enzymatic acitivity of the reaction mixture was 1.6×10^4 unit/ml.

**: The enzymatic activity of the reaction mixture was 1.6×10^3 units/ml.

Consideration

As seen from the results of the above Example 15, stability of the substrate [racemic methyl trans-3-(4-methoxyphenyl)-glycidate] becomes hightest at pH 8-9. And, according to the results of the above Example 16, the enzyme activity of the esterase of the present invention is not decreased even subjected to heat treatment at 50°C for 6 hours where the substrate is stable and it is clear that the esterase of the present invention is much more stable than the enzyme of the refer-

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ence. Therefore, the esterase of the present invention is remarkably superior over the enzyme of the reference.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An esterase derived from Serratia marcescens Sr41 (Ferm BP-487) having the following physico-chemical properties and enzymatic characteristics:

(1) Activity: it (i.e. said esterase) hydrolyzes an ester bond of organic carboxylates;

(2) Substrate specificity: it acts on alkyl esters of organic carboxylic acids, triglyceridees or thiol esters;

(3) Optimum pH: its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate;

(4) pH stability: it is stable at pH 5.0 - 9.0 when it is stored at 30°C for one hour;

(5) Optimum temperature: its optimum temperature is 40 - 50 °C when the hydrolysis is carried out in 100 mM Tris-HCI buffer (pH 8.0) by using olive oil as the substrate;

(6) Heat stability: it is stable at a temperature of not higher than 50 °C when it is stored in 100 mM Tris-HCI buffer (pH 8.0) for 30 minutes;

(7) Molecular weight: 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis);

(8) Isoelectric point: 4.6 ± 0.1;

(9) Effect of metal ions: it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid.

2. A process for preparing novel esterase which comprises

(a) cultivating serratia marcescens Sr41 (FERM BP-487); and

(b) recovering the esterase claimed in claim 1 from the culture broth.

3. A process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate which comprises the steps of:

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(a) treating a racemic lower alkyl trans-3-(4-lower alkoxyphenyl)glycidate with the esterase claimed in claim 1 thereby stereoselectively hydrolyzing 2S, 3R-isomer thereof, and;

(b) recovering unreacted 2R, 3S-isomer from the reaction mixture.

4. An esterase according to claim 1 substantially as hereinbefore described with reference to any one of the examples.

5. A process substantially as hereinbefore described with reference to any one of examples 1, 2, 5, 11 and 12.

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Table 13

Enzyme	C(%)	e.e.(%)	E-value
Reference	43.7	73.7	85
Present invention	49.7	94.3	155

Consideration

As seen from the above results, the E-value of the enzyme of the present invention is about twice of that of the reference enzyme. That is, stereoselectivity of the enzyme of the present invention is about twice higher than that of the reference enzyme. In view of this, it is clear that, when racemic glycidate is subjected to asymmetric hydrolysis, the desired optically active glycidate having high optical purity can be obtained by using the esterase of the present invention.

Example 16

pH Stability of substrate and enzyme

pH stability of the substrate, the enzyme of the present invention and the enzyme of the reference were evaluated.

(1) pH Stability of substrate

Method

* C L M S *

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An esterase derived from Serratia marcescens Sr41 (FERM BP-487) having the following physico-chemical properties and enzymatic characteristics:

(1) Activity: it (i.e. said esterase) hydrolyzes an ester bond of organic carboxylates;

(2) Substrate specificity: it acts on alkyl esters of organic carboxylic acids, triglyceridees or thiol esters;

(3) Optimum pH: its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate;

(4) pH stability: it is stable at pH 5.0 - 9.0 when it is stored at 30°C for one hour;

(5) Optimum temperature: its optimum temperature is 40 - 50 °C when the hydrolysis is carried out in 100 mM Tris-HCl buffer (pH 8.0) by using olive oil as the substrate;

(6) Heat stability: it is stable at a temperature of not higher than 50 °C when it is stored in 100 mM Tris-HCl buffer (pH 8.0) for 30 minutes;

(7) Molecular weight: 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis);

(8) Isoelectric point: 4.6 ± 0.1;

(9) Effect of metal ions: it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid.

2. A process for preparing novel esterase which comprises

(a) cultivating serratia marcescens Sr41 (FERM BP-487); and

(b) recovering the esterase claimed in claim 1 from the culture broth.

3. A process for preparing a lower alkyl (2R, 3S)-3-(4-lower alkoxyphenyl)glycidate which comprises the steps of:

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NOVEL ESTERASE AND PROCESS FOR PREPARING THE SAME

ABSTRACT

A novel esterase which is derived from Serratia marcescens is disclosed. Said esterase has the following physico-chemical properties and enzymatic charactaristics:
(1) Activity; it (i.e., said esterase) hydrolyzes an ester bond of organic carboxylates, (2) Substrate specificity; it acts on alkyl esters of organic carboxylic acids, triglycerides or thiol esters, (3) Optimum pH; its optimum pH is 7.5 - 9.0 when the hydrolysis is carried out by using olive oil as the substrate, (4) pH stability; it is stable at pH 5.0 - 9.0 when it is stored at 30 °C for one hour, (5) Optimum temperature; its optimum temperature is 40 - 50 °C when the hydrolysis is carried out by using olive oil as the substrate, (6) Heat stability; it is stable at a temperature of not higher than 50°C when it is stored at pH 8.0 for 30 minutes, (7) Molecular weight; 62,000 ± 2,000 (SDS-polyacrylamide gel electrophoresis), (8) Isoelectric point; 4.6 ± 0.1, (9) Effect of metal ions; it is activated in the presence of 1 mM calcium ion, and inhibited in the presence of 1 mM cobalt ion, nickel ion, iron ion or ethylenediaminetetraacetic acid. Said esterase can widely be applied for organic synthetic reactions.