Substrate Specificity of Lipases in Protease Preparations

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Commercial protease preparations have been screened for their biocatalytic activity in the esterification of various fatty acids with 1-butanol. Of all protease preparations tested, only those from pineapple (bromelain) and *Rhizopus* sp. were found to contain active lipases. Similar to lipases from microorganisms, animals, and plants, such as papaya (*Carica papaya*) latex, lipases in the protease preparations of bromelain and *Rhizopus* sp. strongly discriminated against fatty acids having a *cis*-4 unsaturation, for example, *all-cis*-4,7,10,13,16,19-docosahexaenoic acid, *cis*-6 unsaturation, for example, petroselinic (*cis*-6-octadecenoic), γ-linolenic (*all-cis*-6,9,12-octadecatrienoic), and stearidonic (*all-cis*-6,9,12,15-octadecatetraenoic) acid, as well as *cis*-8 unsaturation, for example, dihomo-γ-linolenic (*all-cis*-8,11,14-eicosatrienoic) acid. Fatty acids having a *cis*-9 unsaturation, for example, oleic (*cis*-9-octadecenoic) and α-linolenic (*all-cis*-9,12,15-octadecatrienoic) acids, were very well accepted as substrates by both protease preparations. Fatty acids having hydroxy groups, for example, ricinoleic (12-hydroxy-*cis*-9-octadecenoic) and 12-hydroxystearic acid, epoxy groups, for example, *trans*-9,10-epoxystearic acid, and cyclopentenyl groups, for example, hydnocarpic [(11-(2′-cyclopentenyl)undecanoic] and chaulmoogric [13-(2′-cyclopentenyl)tridecanoic] acid, were also well accepted as substrates by both enzyme preparations.

Keywords: *Protease; lipase, biocatalyst; enzymatic esterification; fatty acid specificity*

INTRODUCTION

Latex from papaya (*Carica papaya*) is a commercially available bulk scale enzyme preparation containing the protease papain which is widely used in the food and beverage industries. *C. papaya* latex has also been employed as a biocatalyst in esterification reactions (Dordick, 1989; Stevenson and Storer, 1991). Recently, *C. papaya* latex has been shown to have a good activity in the hydrolysis of tributyroylglycerol (Giordani et al., 1991) and long-chain triacylglycerols (Villeneuve et al., 1995) as well as in the interesterification of definite triacylglycerols (Villeneuve et al., 1997). We have shown recently that the lipase in *C. papaya* latex also catalyzes the esterification of various fatty acids with 1-butanol, thereby exhibiting a similar substrate selectivity (Mukherjee and Kiewitt, 1996) as observed with lipase preparations from microorganisms, animals, and plants (Hills et al., 1989, 1990a,b; Mukherjee and Kiewitt, 1991; Mukherjee et al., 1993; Syed Rahmatullah et al., 1994a,b; Jachmanián and Mukherjee, 1995; Jachmanián et al., 1996). Apparently, lipase in the commercially available protease preparation of *C. papaya* latex has great potential as a biocatalyst for the modification of fats and other lipids for use in food and related areas (Mukherjee and Kiewitt, 1996).

As a continuation of the above studies, we report here the activity and substrate selectivity of lipases in commercial protease preparations in the esterification of 1-butanol with various unsaturated fatty acids and fatty acids having hydroxy, epoxy, and cyclopentenyl groups. The reactions were carried out under competetive conditions in the presence of myristic acid as the reference standard.

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MATERIALS AND METHODS

Materials. The following enzyme preparations were obtained from Sigma, Deisenhofen, Germany: bromelain (B-2252, EC 3.4.22.32, from pineapple stem, 1430 units/g), chymopapain (C-9007, EC 3.4.22.6, from papaya latex, 2.6 units/mg), α-chymotrypsin (C-4129, EC 3.4.21.1, from bovine pancreas, 52 units/mg), ficin (F-4165, EC 3.4.22.3, from fig tree latex, 0.2 unit/mg), papain (P-4762, EC 3.4.22.2, from papaya latex, 14 units/mg), pepsin A (P-7000, EC 3.4.23.1, from porcine stomach mucosa, 437 units/mg), protease type II (P-4755, EC 3.4.21.4 from *Aspergillus oryzae*, 0.12 unit/mg), protease type IV (P-0384 from *Streptomyces caespitosus*, 0.7 unit/mg), protease type VIII (P-5380, subtilisin Carlsberg, EC 3.4.21.62, from *Bacillus licheniformis*, 12.5 units/mg), protease type XVIII (P-5027, Newlase from *Rhizopus* sp., 0.26 unit/mg), and trypsin [T-7409, EC 3.4.21.4, from porcine pancreas, 1300 (*N*α-benzoyl-L-arginine ethyl ester) (BAEE) units/mg]. All chemicals of analytical grade were from E. Merck, Darmstadt, Germany. Pure fatty acids were purchased from Sigma, and the cyclopentenyl fatty acids were a generous gift from Prof. Helmut K. Mangold, Münster, Germany.

Esterification. In control experiments myristic acid (50 mM) and 1-butanol (100 mM) dissolved in a total volume of 250 μL of hexane were reacted in the presence of 100 mg of enzyme preparation by magnetic stirring at 30 °C for various periods.

For the determination of specificity constants, esterification reactions were carried out as described above using 25 mM concentrations of each of the fatty acids, individually, together with 25 mM myristic acid, the reference standard, and 100 mM 1-butanol dissolved in a total volume of 250 μL of hexane in the presence of 100 mg of enzyme preparation. The esterification of 12-hydroxystearic acid and *cis*-9,10-epoxystearic acid was carried out using 250 μL of methyl *tert*-butyl ether and chloroform/hexane (1:1 v/v), respectively, as reaction medium.

Lipid Extraction and Analysis. Lipids were extracted from the reaction products, the unreacted fatty acids present

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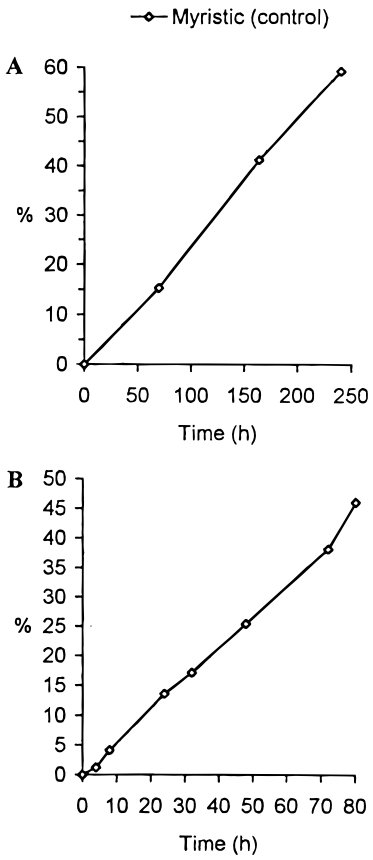

Figure 1. Formation (percent) of butyl esters during esterification of myristic acid (50 mM) with 100 mM 1-butanol in hexane using bromelain (A) and *Rhizopus* protease XVIII (B) as biocatalyst.

in the reaction products were converted to methyl esters using diazomethane, and the resulting mixtures of methyl esters and butyl esters were analyzed by gas chromatography as described earlier (Mukherjee and Kiewitt, 1996).

Kinetic Analysis. Specificity constants were calculated for each fatty acid according to the method of Rangheard et al. (1989) from the extent (micromoles) of formation of butyl ester of the fatty acid examined and that of myristic acid by reaction with 1-butanol under competetive conditions (Mukherjee and Kiewitt, 1996).

RESULTS

Of all the enzyme preparations tested for reaction periods of up to 24 h, only bromelain and *Rhizopus* protease XVIII were able to catalyze the esterification of myristic acid with 1-butanol to a measurable extent (Figure 1). Although crude preparation of *C. papaya* latex was shown to efficiently catalyze the esterification of myristic acid and several other long-chain fatty acids with 1-butanol (Mukherjee and Kiewitt, 1996), purified papain and chymopapain, both from *C. papaya* latex, were unable to catalyze such a reaction, apparently because the lipase contained in the crude enzyme preparations was eliminated during purification of the papain. The ability of the preparations of bromelain and *Rhizopus* protease XVIII to catalyze the esterification reaction is attributed to the presence of lipase in these protease preparations.

Figure 1 shows the time course of esterification of the reference compound, myristic acid, with 1-butanol catalyzed by bromelain and *Rhizopus* protease XVIII. It is evident that *Rhizopus* protease XVIII is far more effective than bromelain in the conversion of myristic acid to its butyl ester (Figure 1); however, both enzyme preparations were far less effective than crude *C. papaya* latex, which was able to esterify >70% of the myristic acid within a reaction time of 1 h (Mukherjee and Kiewitt, 1996).

The time course of esterification of 1-butanol with individual unsaturated fatty acids in the presence of the reference compound, myristic acid, is given in Figures 2 and 4 for the reactions catalyzed by bromelain and *Rhizopus* protease XVIII, respectively. Similarly, Figures 3 and 5 show for the reactions catalyzed by bromelain and *Rhizopus* protease XVIII, respectively, the time course of esterification of 1-butanol with individual fatty acids having hydroxy, epoxy, and cyclopentenyl groups in the presence of the reference compound, myristic acid.

Figure 6 shows the specificity constants obtained in the esterification of the individual fatty acids with 1-butanol catalyzed by bromelain and *Rhizopus* protease XVIII. For all the fatty acids the specificity constants were calculated from the composition of the reaction products at the end of the time course studies (Figures 2−5).

Low overall reaction rates (Figures 2 and 4) and low specificity constants (Figure 6) were observed in the esterification of fatty acids having a *cis*-4 unsaturation, for example, docosahexaenoic acid, a *cis*-6 unsaturation, for example, petroselinic, γ-linolenic, and stearidonic acid, as well as a *cis*-8 unsaturation, for example, dihomo-γ-linolenic acid. Obviously, the above groups of fatty acids were discriminated against as substrates in esterification with 1-butanol, catalyzed by either bromelain or *Rhizopus* protease XVIII. In contrast, oleic and α-linolenic acids having a *cis*-9 unsaturation gave good overall rates of esterification (Figures 2 and 4) and specificity constants close to 1 (Figure 6), showing that these two fatty acids are well accepted as substrates by both enzyme preparations. Eicosapentaenoic acid having a *cis*-5 unsaturation was well accepted as a substrate by bromelain (Figure 2) but not by the *Rhizopus* protease XVIII (Figure 4).

Most of the other less common fatty acids were found to be excellent substrates in the esterification with 1-butanol, catalyzed by either bromelain or *Rhizopus* protease XVIII, as evident from good overall reaction rates (Figures 3 and 5) and the specificity constants, which were similar to that of myristic acid or even substantially higher (Figure 6).

Thus, with both ricinoleic acid and 12-hydroxystearic acid as substrate, relatively high overall rates of esterification as compared to myristic acid were observed with both enzyme preparations (Figures 3 and 5).

In the reaction of the mixture of *cis*-9,10-epoxystearic and myristic acids with 1-butanol, catalyzed by either bromelain or *Rhizopus* protease XVIII, very low rates of esterification were observed (Figures 3 and 5); however, the specificity constant (Figure 6) shows discrimination against *cis*-9,10-epoxystearic acid over myristic acid by the *Rhizopus* protease XVIII but not by bromelain. With *trans*-9,10-epoxystearic acid as substrate, the overall rates of esterification were dis-

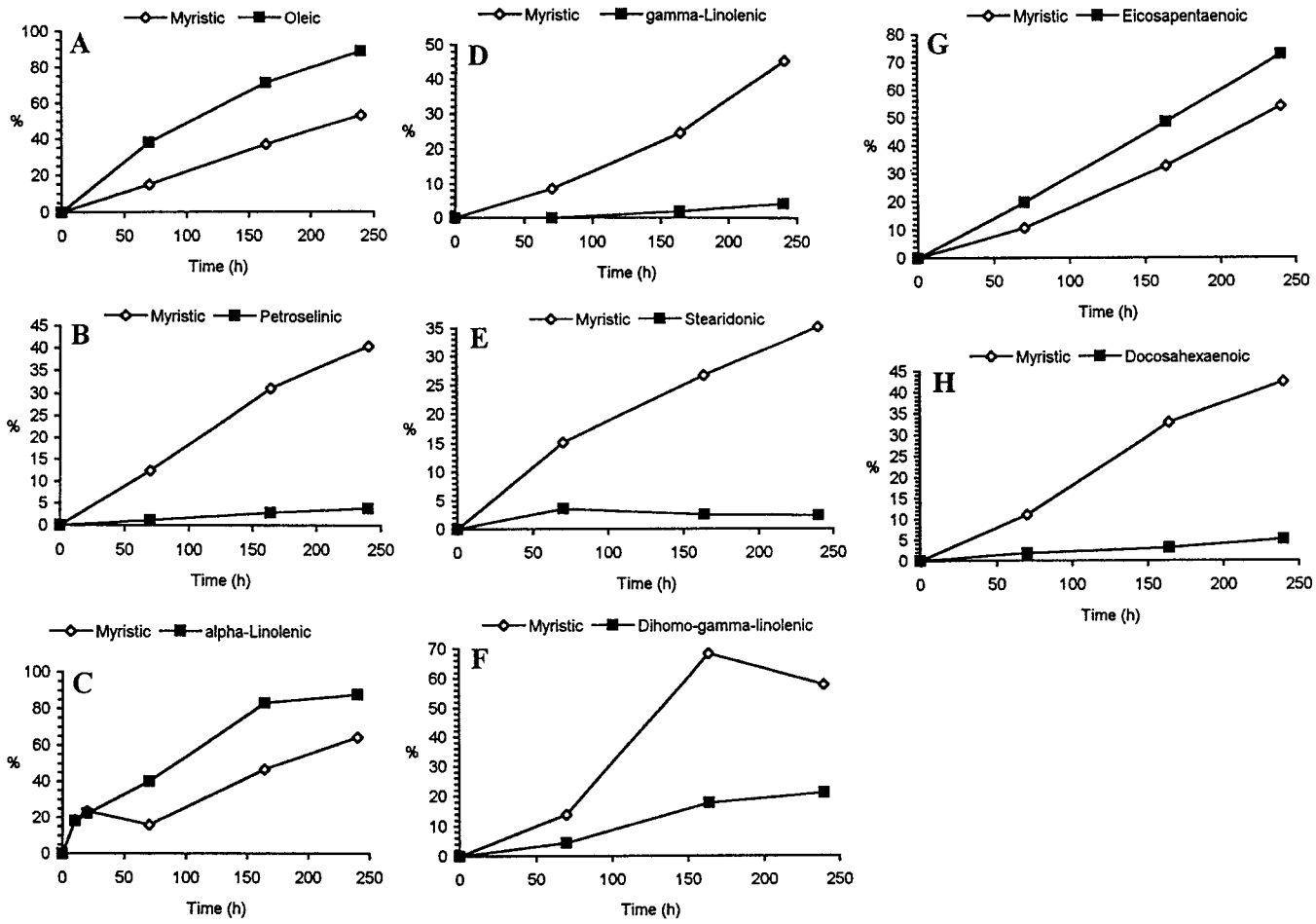

Figure 2. Formation (percent) of butyl esters during esterification of mixtures (25 mM each) of myristic acid and individual unsaturated fatty acids with 100 mM 1-butanol in hexane using bromelain as biocatalyst: (A) oleic (B); petroselinic; (C) α-linolenic; (D) γ-linolenic; (E) stearidonic; (F) dihomo-γ-linolenic; (G) eicosapentaenoic; (H) docosahexaenoic.

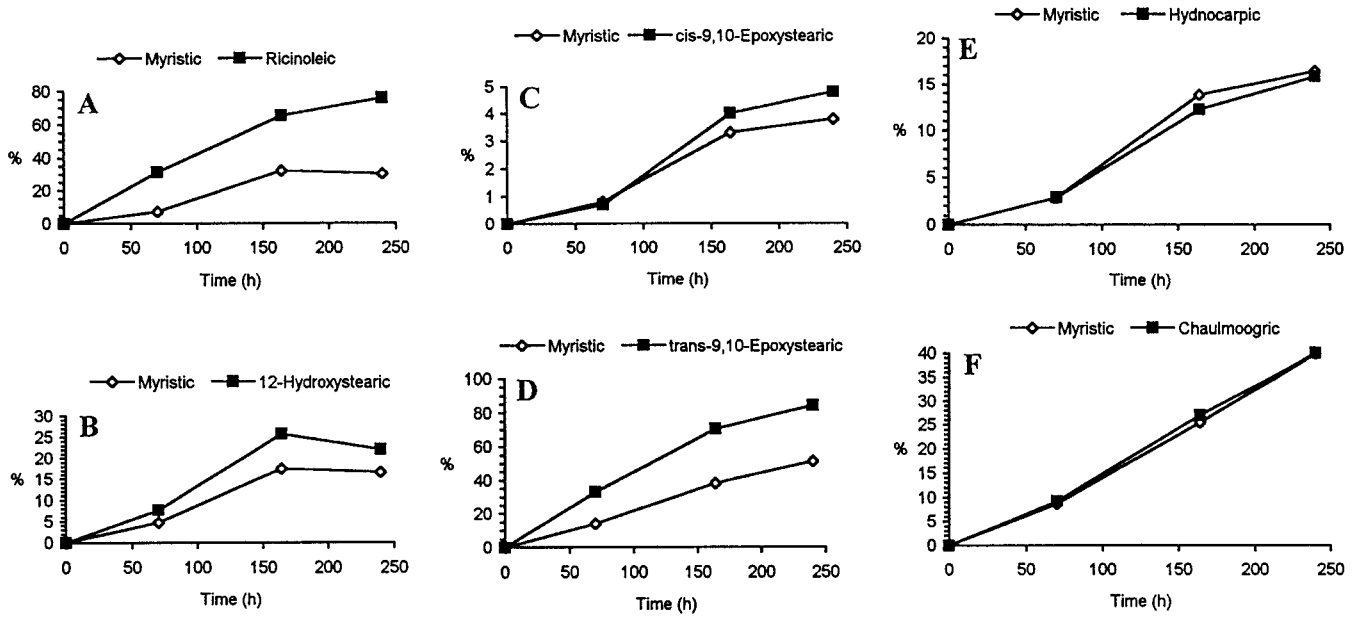

Figure 3. Formation (percent) of butyl esters during esterification of mixtures (25 mM each) of myristic acid and individual hydroxy, epoxy, and cyclopentenyl fatty acids with 100 mM 1-butanol in hexane using bromelain as biocatalyst: (A) ricinoleic; (B) 12-hydroxystearic; (C) *cis*-9,10-epoxystearic; (D) *trans*-9,10-epoxystearic; (E) hydnocarpic; (F) chaulmoogric.

tinctly higher than with the corresponding cis isomer (Figures 3 and 5) and the specificity constant was close to 3 (Figure 6), indicating that *trans*-9,10-epoxystearic acid is a much better substrate than myristic acid. Similar behaviors of *cis*- and *trans*-9,10-epoxystearic acids in lipase-catalyzed esterification reactions were observed earlier with other lipases (Jachmanián et al., 1996; Mukherjee and Kiewitt, 1996).

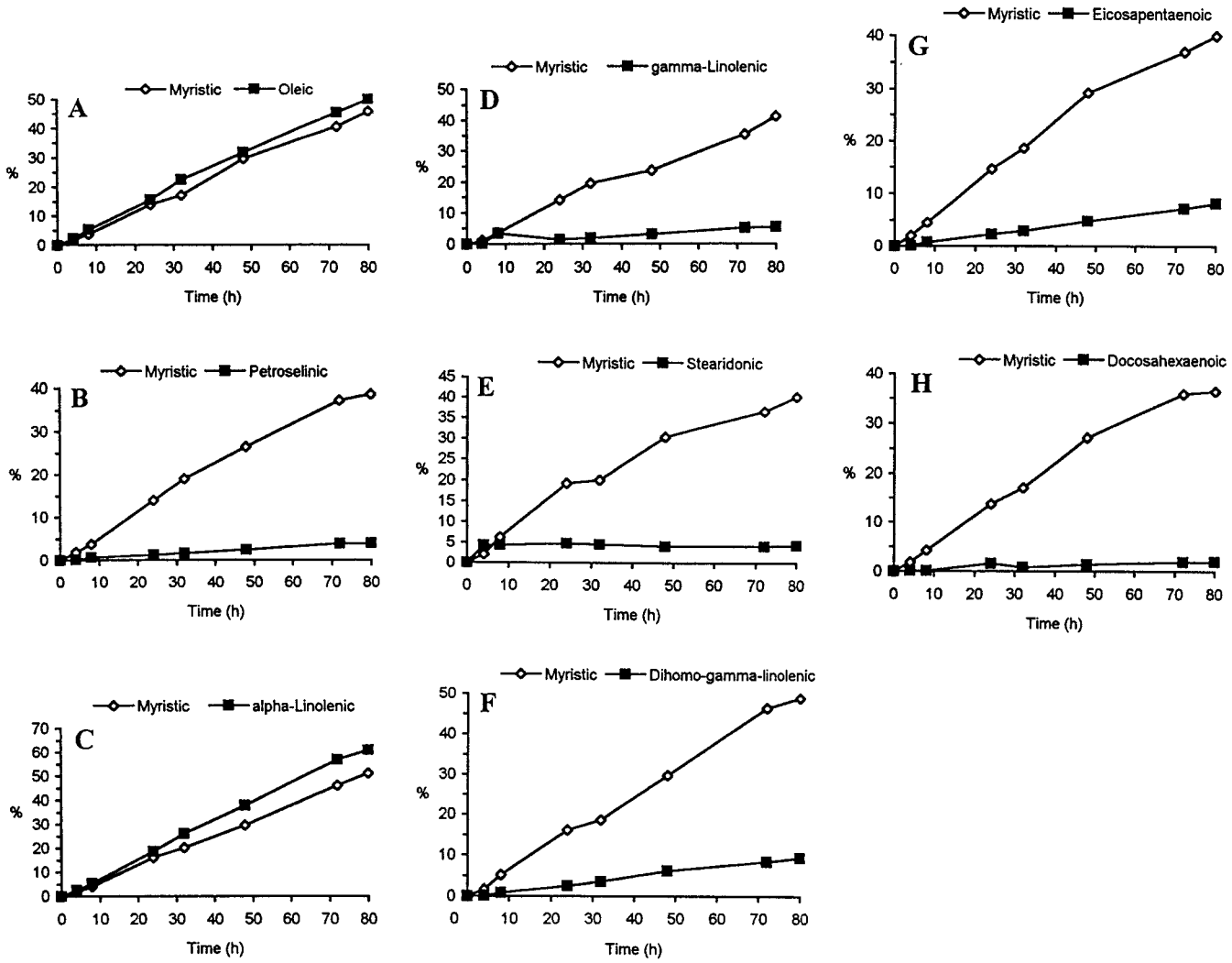

Figure 4. Formation (percent) of butyl esters during esterification of mixtures (25 mM each) of myristic acid and individual unsaturated fatty acids with 100 mM 1-butanol in hexane using *Rhizopus* protease XVIII as biocatalyst: (A) oleic; (B) petroselinic; (C) α-linolenic; (D) γ-linolenic; (E) stearidonic; (F) dihomo-γ-linolenic; (G) eicosapentaenoic; (H) docosahexaenoic.

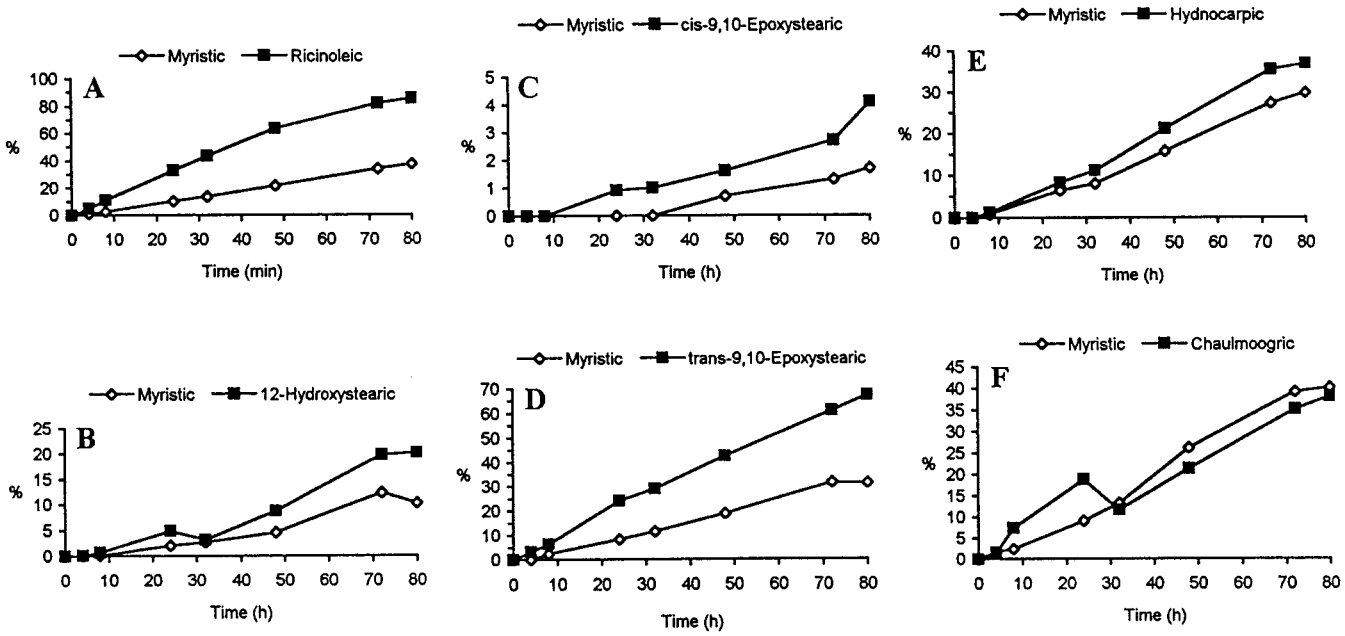

Figure 5. Formation (percent) of butyl esters during esterification of mixtures (25 mM each) of myristic acid and individual hydroxy, epoxy, and cyclopentenyl fatty acids with 100 mM 1-butanol in hexane using *Rhizopus* protease XVIII as biocatalyst: (A) ricinoleic; (B) 12-hydroxystearic; (C) *cis*-9,10-epoxystearic; (D) *trans*-9,10-epoxystearic; (E) hydnocarpic; (F) chaulmoogric.

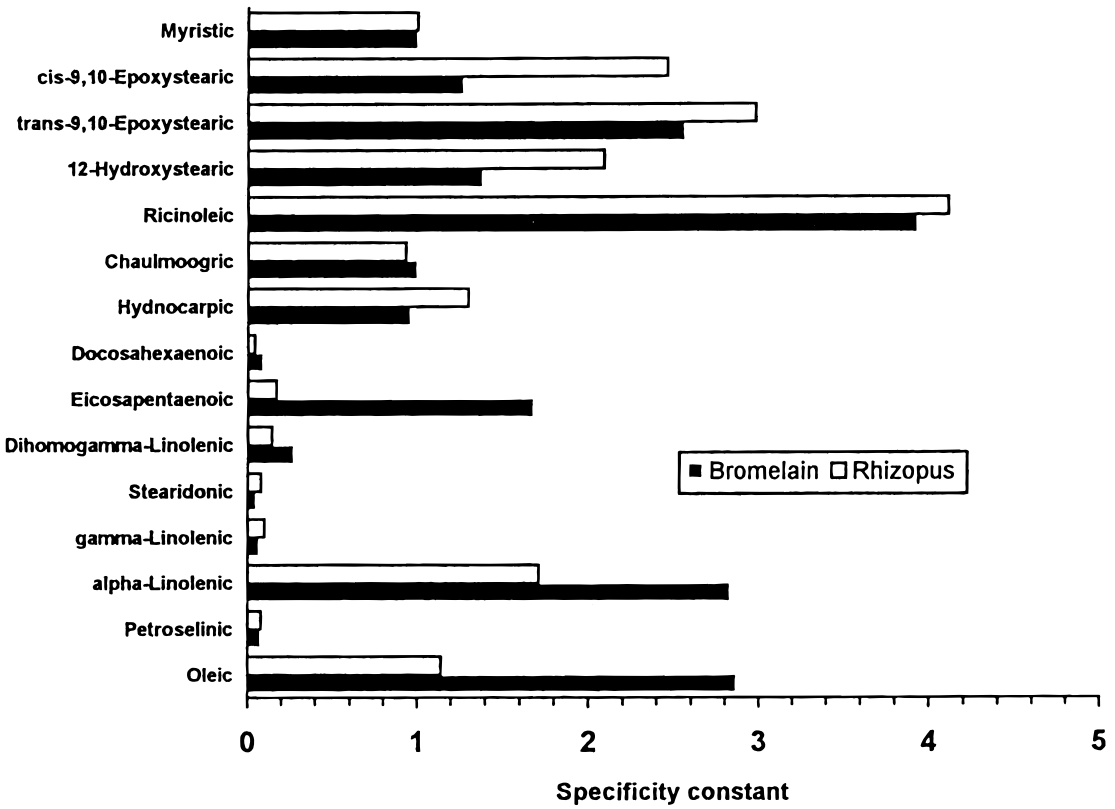

Figure 6. Specificity constant in the esterification of mixtures (25 mM each) of myristic acid and individual unsaturated, hydroxy, epoxy, and cyclopentenyl fatty acids with 100 mM 1-butanol in hexane using bromelain and *Rhizopus* protease XVIII as biocatalysts.

The specificity constants obtained for the cyclopentenyl fatty acids having saturated alkyl chains, for example, hydnocarpic acid and chaulmoogric acid, were close to 1 or higher (Figure 6), which shows that these two fatty acids are well accepted as substrates by both enzyme preparations.

DISCUSSION

In agreement with earlier findings using lipases from microbial, animal, and plant origin (Hills et al., 1989, 1990a,b; Mukherjee and Kiewitt, 1991, 1996; Mukherjee et al., 1993; Syed Rahmatullah et al., 1994a,b; Jachmanián and Mukherjee, 1995; Jachmanián et al., 1996), the enzyme preparations bromelain and *Rhizopus* protease XVIII discriminate against straight-chain fatty acids having a *cis*-4 unsaturation (docosahexaenoic), a *cis*-6 unsaturation (petroselinic, γ-linolenic, and stearidonic), or a *cis*-8 unsaturation (dihomo-γ-linolenic) in the esterification with 1-butanol (Figures 2 and 4). These data show that most triacylglycerol lipases irrespective of their origin discriminate against fatty acid substrates having the first double bond after the carboxyl group as *cis*-4, *cis*-6, or *cis*-8. In the case of the lipase from *Brassica napus*, a discrimination against fatty acids with a double bond at even-number carbons was attributed to the anti orientation of such double bonds with respect to the carboxyl group (Hills et al., 1990b). The same is probably the case with most triacylglycerol lipases, as is evident from the present data and results of recent studies (Jachmanián et al., 1996; Mukherjee and Kiewitt, 1996).

Interestingly most of the fatty acids with less common structures, such as hydroxy, *trans*-epoxy, and cyclopentenyl acids having saturated alkyl chains, are generally favored by the enzyme preparations bromelain and *Rhizopus* protease XVIII as substrates for esterification as compared to a straight-chain fatty acid, such as myristic acid (Figures 3, 5, and 6). Several lipases of microbial, animal, and plant origin (Jachmanián et al., 1996) as well as crude *C. papaya* latex (Mukherjee and Kiewitt, 1996) have also been shown to favor the above fatty acids with less common structures as substrates for esterification. It appears from these data that substrate preference for fatty acids having hydroxy, *trans*-epoxy, and cyclopentenyl groups is a common feature of most triacylglycerol lipases irrespective of their origin. A very low rate of esterification of *cis*-9,-10-epoxystearic acid, compared to its trans isomer as reported here (Figures 3 and 5) and in earlier studies (Jachmanián et al., 1996; Mukherjee and Kiewitt, 1996), is obviously due to the configuration of the epoxy group.

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