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Lipase mediated simultaneous esterification and epoxidation of oleic acid for the production of alkylepoxystearates

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Abstract

Epoxy alkylstearates were synthesized by lipase catalysed esterification and perhydrolysis followed by epoxidation of oleic acid in a one-pot process. Immobilized *Candida antarctica* lipase (Novozym®435) was used as the catalyst. The esterification reaction occurred relatively quickly and was followed by epoxidation of the alkyl ester and the remaining fatty acid. Higher degree of esterification was achieved with *n*-octanol, *n*-hexanol and *n*-butanol as compared to that with ethanol and *iso*-propanol. The rate and yield of epoxidation was enhanced with *iso*-propanol but was lowered with the other alcohols. The lipase suffered significant loss in activity during the reaction primarily due to hydrogen peroxide. The presence of alcohols, in particular ethanol, further contributed to the enzyme inactivation. The epoxidation reaction could be improved by step-wise addition of the lipase.

Keywords: Lipase; Oleic acid; Alkylepoxystearate; Plasticizer

1. Introduction

Among the important applications of epoxidized vegetable oils and fatty acid esters is their function as plasticizer for polyvinyl chloride (PVC) and other plastic materials [1,2]. Plasticizers are substances that increase flexibility, workability or distensibility of plastics, hence rendering them suitable for diverse applications. The global demand for plasticizers in 1999 was estimated at 10.1 billion pounds (4.58 millions of tonnes), equivalent to about US$ 7 billion, and the current overall growth rate for production at about 2.8% per annum [3]. The epoxy esters have captured about 7% of the total plasticizer market [1].

Epoxidized soybean oil (ESBO) is commercially the most important product as plastic additive and has relative stable market of approximately 100,000 tonnes/year [4,5]. It offers more stretching ability than its petroleum based counterpart and is also capable of providing high thermal stability [3]. ESBO is a secondary plasticizer and lacks compatibility for use at high levels in typical PVC plastics [5]. It is instead used primarily as a stabilizer due to its ability to scavenge the hydrogen chloride released during decomposition of PVC when exposed to heat and light. Considerable research efforts have been made to obtain epoxy stabilizers which are also primary plasticizers, and it has been demonstrated that epoxidized esters of oleic acid, such as butyl epoxystearate and some others possess high compatibility; they impart good low temperature characteristics to PVC and are efficient plasticizers as shown by their effect on modulus, elongation, and tensile strength [1,2].

Epoxidized fatty acid esters are currently produced by a two-step process involving alcoholysis of triglycerides/fatty acid using KOH as catalyst followed by epoxidation of the esters by peroxyformic or peroxyacetic acid [1,2]. The peracid is generated *in situ* from acetic or formic acid, hydrogen peroxide and a strong mineral acid catalyst [6]. The strongly acidic conditions used make the epoxidation process less selective, leading to the formation of by-products. Furthermore, corrosion and production of large amount of salts when neutralizing the acids are other problems associated with the reaction.

Enzymatic catalysis often provides a more selective and environment-friendly alternative to the chemical catalysis.

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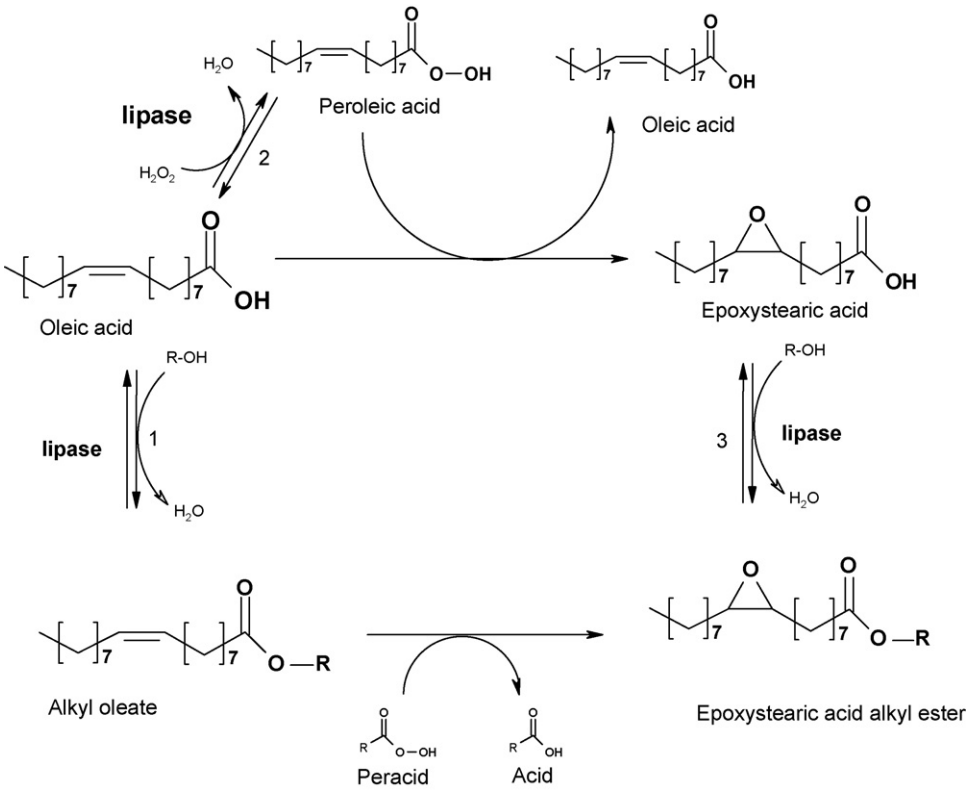

Fig. 1. Schematic presentation of the reaction pathways for lipase catalysed esterification (1) and peracid formation from oleic acid (2). The oxygen is spontaneously transferred from the peracid to the double bond to yield epoxystearic acid. In path 1, oleic acid reacts with an alcohol to yield an ester that is subsequently epoxidized. Epoxystearic acid can also serve as a substrate for lipase catalysed esterification reaction (3).

Esterification/transesterification reactions catalysed by the enzyme lipase are well established [7,8]. The efficiency of the process is dependent on the good water activity control. Even lipase mediated chemo-enzymatic epoxidation of fatty acids and oils has been demonstrated [9]. The enzyme catalyses the formation of peracid from a fatty acid and H_2O_2, which is followed by spontaneous transfer of the oxygen to the double bond in unsaturated fatty acid. We have earlier reported solvent-free chemo-enzymatic epoxidation of oleic acid and its methyl ester [10].

This paper presents a study on utilizing the lipase for catalysing simultaneous epoxidation and esterification of oleic acid in a solvent-free medium for production of alkyl epoxystearates. Fig. 1 shows the sequence of reactions that are likely to occur in such a system. Novozym®435 was used to catalyse the peracid formation using aqueous solution of hydrogen peroxide as oxidant for epoxidation, and the esterification reaction was done with different short chain and long chain alcohols.

2. Materials and methods

Oleic acid (99%) was purchased from Sigma–Aldrich, while industrial grade oleic acid (60%) was a gift from Akzo Nobel Surfactants, Stenungsund, Sweden. All the alcohols and other solvents of analytical grade were obtained from Merck (Darmstadt, Germany). Tetraethyl ammonium bromide (TEAB) and perchloric acid were from Fluka. Novozym®435 (immobilized *Candida antarctica* lipase B) was a gift from Novozymes (Bagsvaerd, Denmark).

2.1. Esterification and epoxidation of oleic acid

To the reaction mixture containing 1 ml of 0.5 M oleic acid (0.5 mmol) in toluene, 46 μl *n*-butanol (0.5 mmol), and 51 μl of 30% (w/w) H_2O_2 (0.5 mmol) in 4 ml vials was added 5 mg Novozym®435. Samples were withdrawn at regular time intervals for the analysis of the fatty acid and the various reaction products, i.e. epoxystearic acid, butyl epoxystearate, and oleic acid butyl ester.

Reactions with different alcohols were run in a 25 ml reactor using industrial grade oleic acid in a solvent-free medium. The reaction mixture comprised of oleic acid (equivalent to 27 mmol of double bonds), 27 mmol of hydrogen peroxide and 18.7 mmol of alcohol. Novozym®435 was added at a ratio of 6.7 mg/mmol of double bonds. The reactor was temperature controlled at 40 °C ± 1, and the reaction mixture was stirred at 400 rpm using a magnetic stirrer. At appropriate time intervals, samples were withdrawn from the oily phase, centrifuged for 5 min at 10 000 rpm in an eppendorf centrifuge, and then analysed for the amount of oxirane number and acid number.

2.2. HPLC analysis of fatty acid components

Quantitation of oleic acid, butyl oleate, and their epoxidized counterparts was done by HPLC analysis on a RP-C18 column,

using 95% methanol and 5% water in 0.05% acetic acid as the solvent system and a refractive index detector as described earlier [10]. Toluene eluted in the front peak, and epoxystearic acid, epoxystearic acid butyl ester, oleic acid and butyl oleate had retention times of 4, 8, 9 and 24 min, respectively. The refractive index has a linear response and calibration curves were made in a range of 0–5 mM for the different components. Butyl epoxystearate, oleic acid butyl ester and epoxystearic acid were isolated by flash chromatography following a protocol described earlier [11], and used as standards.

2.3. Product identification by mass spectrometry

Identification of the product/by-product of the epoxidation reaction was done by tandem mass spectrometry on a hybrid QSTAR Pulsar quadrupole time of flight-mass spectrometer (Applied Biosystems Sciex Instruments, Toronto, Canada), equipped with an electrospray ionization source on a positive mode. Samples for analysis were prepared by dissolving the products from each reaction in methanol and filtering through 0.2 μm filters before analysis. Initial separation was performed on a Perkin-Elmer series 200 capillary liquid chromatography system using the same method and conditions described above for the HPLC analysis.

2.4. Determination of oxirane number

The oxirane content in the epoxidized samples was measured by the method of Jay [12]. To 100 mg of sample in 10 ml chloroform was added 10 ml of 20% tetraethyl ammonium bromide in acetic acid. The mixture was titrated against 0.1 M perchloric acid in acetic acid, whereby the HBr generated reacts with the epoxide group. When all the epoxide has reacted, the equivalence point is detected potentiometrically using a 702 SM Titrino from Metrohm and Metrohm TiNet 2.5 software. The oxirane number is defined as the content of oxirane oxygen (weight percentage) in the sample.

2.5. Determination of acid number

Acid value of the fatty acids was measured according to a standard AOAC method (No. 969.17) [13]. To 100 mg of sample suspended in 20 ml of cyclohexane were added a few drops of phenolphthalein (1% in ethanol). The solution was titrated with a 0.1 M KOH solution in ethanol until a shift in colour was observed. The acid number was calculated from the amount of KOH consumed for neutralizing the acid in the sample.

2.6. Determination of residual lipase activity

Subsequent to epoxidation/esterification reaction, the residual activity of Novozym®435 was determined by esterification reaction between decanoic acid and cyclohexanol as described previously [14]. The enzyme beads were separated by filtration, washed with toluene, rinsed with diethyl ether, dried under vacuum for 1 h and weighed prior to activity determination. The reaction products were analysed by gas chromatography (GC) on a Shimadzu gas chromatograph 14 A system, equipped with flame ionization detector (FID) and a DB-5 fused silica capillary column (Supelco SPB5 15 m, 0.32 mm i.d., 25 μm film), and using helium as carrier gas. Cyclohexanol, decanoic acid and cyclohexyl decanoate had retention times of 0.95, 2.95 and 5.29, respectively. The response factor for the cyclohexyl decanoate/cyclohexanol was determined to be 2.912 (data not shown).

3. Results and discussion

3.1. One-pot esterification and epoxidation of oleic acid

Initial experiments were performed with esterification and epoxidation of 0.5 mmol oleic acid in 1 ml toluene using stoichiometric amounts of *n*-butanol and hydrogen peroxide using Novozym®435 as catalyst at 40 °C. As seen in Fig. 2, about 83% of oleic acid was used up during the first 2 h of the reaction to form about 64% butyl ester, 11% butyl epoxystearate and 6% epoxystearic acid. As the reaction continued, oleic acid was completely consumed in 15 h and the butyl oleate got epoxidized. After 24 h the principal component (about 80%) of the product was epoxystearic acid butyl ester, the butyl oleate amount was reduced to about 13% while epoxystearic acid remained steady at about 7%. The formation of these products was also observed by LC-MS as peaks in the total ion chromatogram corresponding to 298 amu (epoxystearic acid), 338 amu (butyl oleate) and 354 amu (epoxystearic acid butyl ester), respectively. The identity of the products was further confirmed by NMR (data not shown). No ring opening of the epoxide was observed. These results suggest that path 1 in Fig. 1, i.e. esterification followed by epoxidation is the preferred reaction format during lipase-catalysed synthesis of butyl epoxystearate. The epoxidation kinetics is dependent on the formation of peracid that in turn depends on the concentration of hydrogen peroxide used. The peracid is an unstable compound and is used up rapidly for epoxidation of the double bond. At the equimolar H_2O_2

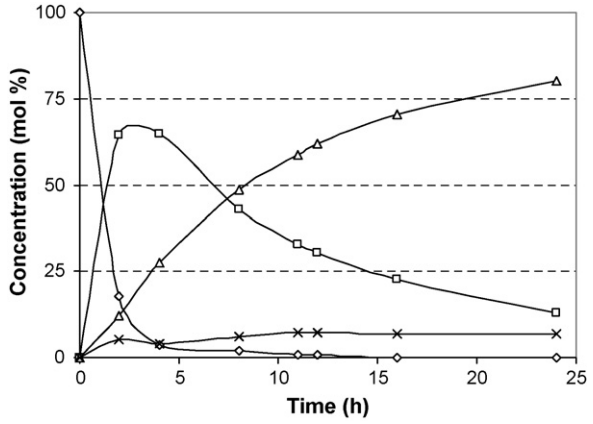

Fig. 2. Simultaneous esterification and epoxidation of 0.5 M oleic acid in toluene mediated by Novozym®435 in the presence of 46 μl *n*-butanol and 51 μl of 30% (w/w) H_2O_2 at 40 °C. The concentrations of the substrate and products were analyzed by HPLC. Symbols: oleic acid (◊), oleic acid butyl ester (□), epoxystearic acid butyl ester (△), and epoxystearic acid (×).

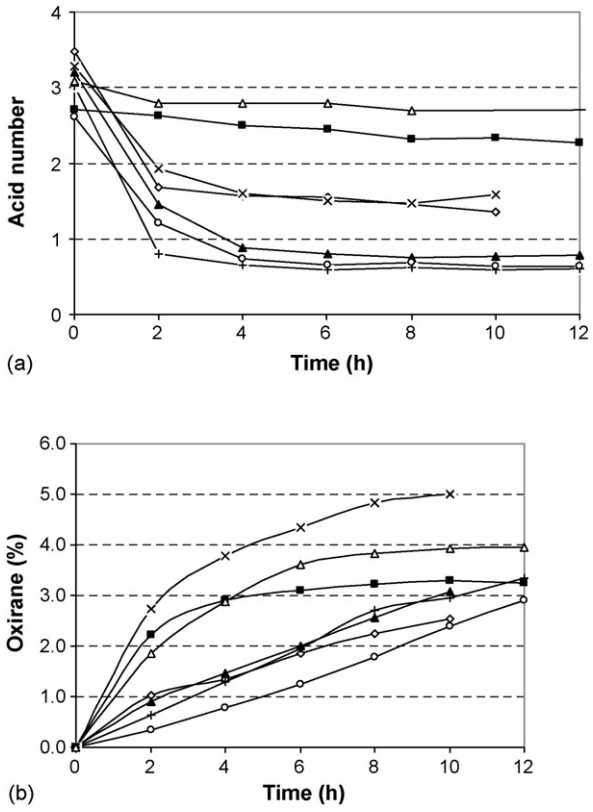

Fig. 3. Profiles of acid number (a) and oxirane number (b) during simultaneous esterification and epoxidation of industrial grade oleic acid (27 mmol of double bond) using 18.7 mmol of alcohol and 27 mmol H_2O_2, and 180 mg Novozym®435. The different alcohols used were: none (△), ethanol (◊), *iso*-propanol (×), *n*-butanol (▲), *n*-hexanol (◯), cyclohexanol (■), and *n*-octanol (+).

concentration (relative to double bonds) used in the reaction, the peracid concentration is <2% of the total fatty acid [9].

3.2. Effect of different alcohols on the epoxidation and esterification reaction

The combined epoxidation and esterification was then evaluated with different alcohols using industrial grade oleic acid (oleic acid content of 70%) as substrate. Toluene was omitted from the reaction medium. The reaction was followed by quantitation of total oxirane number and acid number due to the presence of a mixture of fatty acids. The results are shown in Fig. 3.

It was observed that esterification (monitored as a decrease in acid number) with all the alcohols except cyclohexanol took place mainly during the first 2 h of the reaction (Fig. 3a). The reaction with polar alcohols like ethanol and *iso*-propanol gave esterification yields up to 62%, while esterification with the more apolar alcohols, *n*-butanol, *n*-hexanol and *n*-octanol was relatively efficient achieving yields around 75%. On the other hand, epoxidation reaction was significantly speeded up with the use of *iso*-propanol, only slightly affected with cyclohexanol, and slowed down with ethanol, *n*-butanol and higher alcohols (Fig. 3b). When analysed after 24 h of reaction, highest ester

Table 1
Esterification and epoxidation of industrial grade oleic acid using Novozym®435 after 24 h of reaction with different alcohols[a]

Epoxidation/esterification	Ester yield (%)	Oxirane (%)[b]
No alcohol	18[c]	3.94[c]
Ethanol	62	4.25
iso-Propanol	55	5.25
n-Butanol	75	4.38
n-Hexanol	75	4.43
n-Octanol	81	5.22
Cyclohexanol	16	3.42

[a] No other solvent was used in the reaction medium.
[b] The theoretical maximum oxirane number for industrial grade oleic acid is about 6.7.
[c] The formation of ester is noted due to the ring opening of the epoxide and esterification with another fatty acid molecule. This is also reflected in lowered oxirane number.

yields (over 80%) were obtained with *n*-octanol while epoxide yields in most cases were higher than in the system without any alcohol (Table 1). *iso*-Propanol provided the highest yield of the epoxide product. The reaction with cyclohexanol yielded epoxystearic acid as the main product.

Difference in esterification reaction rates with different alcohols has been shown earlier by Zaidi et al. [15]. The reaction system studied here, for simultaneous epoxidation and esterification, is water saturated. The water is contributed not only by the H_2O_2 solution but is also generated both during peracid formation and ester synthesis. This has an unfavourable effect on the equilibrium of the esterification reaction, especially in the case of the reaction with hydrophilic short chain alcohols where hydrolysis predominates over esterification resulting in low ester yields. However, formation of even a small amount of ester (with lower melting point) provides good mass transfer conditions for the epoxidation reaction. Better availability of H_2O_2 results in faster peracid formation and epoxidation as seen in case of *iso*-propanol. It was shown in our earlier work that epoxidation of methyl oleate was much easier than that of oleic acid and resulted in high product yields even at room temperature [10]. The slower rate of epoxidation in the presence of ethanol was attributed to its denaturing effect on the enzyme. The apolar alcohols, on the other hand, partition favourably to the fatty acid phase and are hence preferentially esterified. These features, however, result in a decreased rate of peracid formation and epoxidation, the reaction being limited by the partitioning of the peroxide to the organic phase.

As can be noted from Table 1, both the ester formation and epoxidation of the fatty acid were not complete. The ester yield can be improved by removing the water in the reaction after completion of the epoxidation reaction so as to drive the esterification reaction to completion. This has been successfully demonstrated previously by applying different methods of water removal like vacuum evaporation [16], molecular sieves [17], pervaporation [18], or bubbling air through the reactor [19], etc. Epoxidation rate and yields can be improved by increasing the amount of H_2O_2 used as shown earlier for epoxidation of linoleic acid [20]. However, H_2O_2 has an adverse effect on the enzyme activity especially at elevated temperatures used for the reaction under

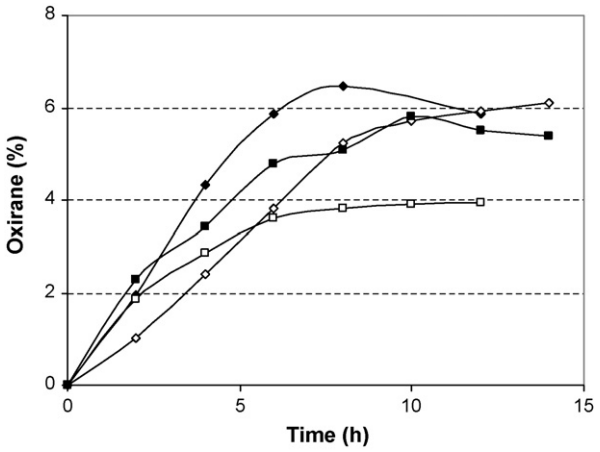

Fig. 4. Effect of mode of enzyme addition on the epoxidation of industrial grade oleic acid. Novozym®435, 180 mg, was added to 6 g of oleic acid (27 mmol double bond) and 1.2 ml of 30% (w/w) H_2O_2 in one portion at the start (0 h) of the reaction (squares), and in three portions at 0, 3 and 6 h of incubation (diamonds) in a solvent-free medium (unfilled symbols) and in a medium with 1.4 ml toluene (filled symbols). The theoretical maximum oxirane value of industrial grade oleic acid is about 6.7.

solvent-free conditions [14]. The epoxidation rate and yield were found to be enhanced if the reaction was performed by adding Novozym®435 stepwise in three portions (each after 3 h) instead of all at once at the start of the reaction (Fig. 4). Stepwise addition prevents the enzyme from getting inactivated all at once. Addition of solvent (toluene) to the reaction increased the reaction rate further and the epoxide number to 5.97, which is very near to the theoretical maximum oxirane value of 6.7 in the industrial grade oleic acid. The solvent leads to the reduction in viscosity that favoured the mass transfer in the organic phase and also prevents the formation of by-product of ring opening of the epoxide observed under solvent-free conditions [10]. Moreover, the enzyme appears to exhibit higher stability when the epoxidation is performed in toluene [21].

Inactivation of the lipase is caused also to a certain extent by the alcohols used for esterification. Ethanol is the most denaturing as it would strip away the essential water associated with the enzyme. Determination of the residual activity after a 24 h reaction in the presence of ethanol showed the lipase to be completely inactive in comparison to 26% activity remaining after the reaction involving no alcohol and 6–7% activity in the presence of other alcohols.

4. Conclusion

The enzymatic approach for the synthesis of alkyl epoxystearates is a simpler and energy efficient alternative to the chemical process, and the solvent-free conditions and good product yields further result in savings in product separation processes. The main limitation, however, is the low stability of the lipase under the reaction conditions employed. Possibility to use anhydrous reaction conditions and to minimize the exposure of the enzyme to the peroxide would be beneficial for improving product yields and performance of the biocatalyst.

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