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Journal of Molecular Catalysis B: Enzymatic 41 (2006) 55–60
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Selective hydrolysis of epoxidized soybean oil by commercially available lipases: Effects of epoxy group on the enzymatic hydrolysis

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Received 12 August 2005; received in revised form 8 April 2006; accepted 17 April 2006
Available online 23 May 2006

Abstract

Eight lipases were studied in hydrolysis reactions toward the goal of selectively removing saturated fatty acids from epoxidized soybean oil. Commercially available epoxidized soybean oil has about 18% saturated fatty acids, which manifest themselves as branches when used in polymers. The removal of these fatty acids creates a potentially valuable degree of freedom in the processing of soybean oil for polymer applications.

Hydrolysis was achieved with seven of the eight enzymes with the epoxy functionality increasing reaction rates and changing selectivities. Lipases from *Penicillium roquefortii*, *Mucor javanicus*, *Rhizomucor miehei* and *Pseudomonas* sp. showed selectivity toward diepoxy acyl moieties. *Aspergillus niger* lipase selectively hydrolyzed saturated fatty acids in soybean oil but the lipase was not selective with epoxidized soybean oil. *Penicillium camembertii* lipase was found to be an inactive enzyme for triglyceride substrates. The selectivity of *Candida rugosa* toward saturated fatty acids increased in epoxidized soybean oil. *Burkholderia cepacia* lipase had selectivity toward both palmitic acid and stearic acid but not the epoxy acyl moieties. A hypothesis is proposed that explains unexpected trends in the selectivities of the lipases.

Keywords: Lipase; Hydrolysis; Epoxidized; Selectivity; Soybean oil

1. Introduction

Natural oils and their derivatives are being researched for plastic and polymer applications due to their availability, renewability and biodegradability. Epoxidized soybean oil is a derivative of soybean oil having oxirane (epoxy) functional groups, which are products from epoxidation of carbon=carbon double bonds in soybean oil. Epoxidized soybean oils are used as plasticizers, crosslinking agents, stabilizers, pre-polymers and are intermediates for polyol production used in polyurethane, polyester and plastic resins after the oxirane ring is opened by hydroxylation or alcoholysis.

Epoxidized soybean oil triglycerides (Fig. 1) can be produced by either chemical or enzymatic oxidation of soybean oil triglycerides [1,2]. Soybean oil typically has about 85% of unsaturated fatty acids and about 15% of saturated fatty acids approximately including 11% of palmitic acid and 4% of stearic acid [3]. The saturated fatty acids cannot be oxidized because they lack carbon=carbon double bonds. These saturated moieties are unreactive, form branches when polymerized and are reported to cause poor properties in the final product of polyurethane synthesis [4–6].

Lipase, or esterase, is a renewable biocatalyst broadly employed in the transformation of lipids, especially in the production of structured lipids, which need its high stereoselectivity and regioselectivity. Generally, selectivity of lipases depends on enzyme structure, substrate structure, factors affecting binding of the enzyme to the substrate and other factors influencing the enzyme activity [7].

Researchers have reported lipase selectivity for the hydrolysis of castor oil [8,9] and the impact of epoxy groups of trivernolin on enzymes [10]. However, there is no previous work reporting the effects of epoxy groups on lipase selectivity with the reaction of epoxidized soybean oil. This investigation of lipase hydrolysis selectivity will be widely useful in the preparation of an epoxidized soybean oil that is low in saturated acid content and has high hydroxy number, which yields a better product for plastic and polymer reactions.

The mechanism of lipase binding of substrates also involves substrate configuration and conformation. Most lipases have

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doi:10.1016/j.molcatb.2006.04.008

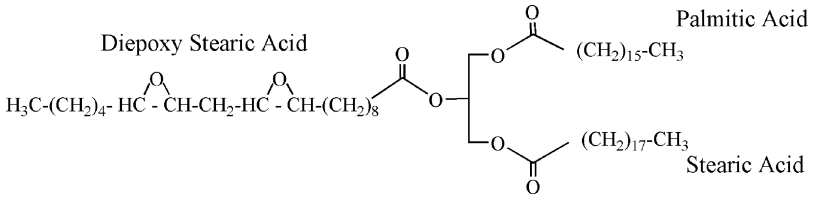

Fig. 1. An example of an epoxy acyl moiety presenting in epoxidized soybean oil triglycerides.

been determined to be 1,3-regioselective enzymes in the hydrolysis and esterificaiton of lipids. There are only a few lipases reported to be non-selective enzymes or 2-regioselective enzymes [11,12].

The regioselective composition of natural oils provides a valuable composition control to which results are compared when evaluating free fatty acid compositons. In soybean oil, more than 99% of total saturated fatty acids are located at 1- or 3-position in the triglyceride molecule [13,3]. Partial hydrolysis combined with a free fatty acid composition high in saturated fatty acids can be used as part of an argument for regioselective hydrolysis.

In this study, soybean oil triglycerides and epoxidized soybean oil triglycerides were hydrolyzed by eight lipases and their selectivities to saturated fatty acids were determined. Lipase selectivity was determined by comparing the composition of hydrolyzed fatty acids to triglyceride substrates after 5–50% hydrolysis. The objectives are to better understand the impact of epoxy groups on this discrimination and to find an enzyme, which has high selectivity toward saturated fatty acids in the hydrolysis of epoxidized soybean oil. The preferred enzyme will not only selectively cleave off saturated fatty acids from epoxidized soybean oil triglyceride but will also replace the saturated unused parts with highly reactive and useful primary hydroxy groups. The primary hydroxy group in polyols is three times more reactive with isocyanate in polyurethane synthesis than secondary hydroxy groups [14]. Commercially available epoxidized soybean oil triglycerides, containing palmitic acid (13.2%), stearic acid (5.0%), monoepoxy stearic acid (29.4%) and diepoxy stearic acid (52.4%), was the substrate with soybean oil used in control studies.

2. Experimental

2.1. Materials

Lipases from *Candida rugosa* (Lipase AY "Amano"), *Burkholderia cepacia* (Lipase PS "Amano"), *Pseudomonas* sp. (Cholesterol esterase, "Amano" 2), *Penicillium roqueforti* (Lipase R "Amano"), *Penicillium camembertii* (Lipase G "Amano"), *Aspergillus niger* (Lipase A "Amano") and *Mucor javanicus* (Lipase M "Amano") were gifts from Amano Enzyme USA, Elgin, IL. And lipase *Rhizomucor miehei* was purchased from Sigma–Aldrich, St. Louis, MO.

Epoxidized soybean oil (VIKOFLEX7170) was purchased from ATOFINA Chemicals Inc, Philadelphia, PA. Refined soybean oil (Food Club brand vegetable oil) was purchased from a local grocery store. Diazald, tetramethylammonium hydroxide (TMAH, 25% in methanol), oleic acid (90%), linolenic acid (99%), hydrogen peroxide and Novozyme 435® (lipase B from *Candida antarctica*) were purchased from Sigma–Aldrich, St. Louis, MO. Linoleic acid (90%) was purchased from City Chemical LLC, West Heaven, CT. Flax seed oil was purchased from Jedwards International, Inc., Quincy, MA. Methanol, diethyl ether, potassium bicarbonate and sulfuric acid were from Fisher, Houston, TX.

2.2. Hydrolysis of soybean oil and epoxidized soybean oil

The enzymes obtained from Amano Enzyme Inc., were studied at their optimum pH and temperature as recommended in the product specification sheets and the reactions with *R. miehei* lipase were operated at 45 °C and pH 7.0.

Table 1 shows operating conditions and enzyme activity as reported from the enzyme suppliers.

Two grams of soybean oil, or epoxidized soybean oil, and 2 g of buffer solution were mixed in a 125 mL Erlenmeyer flask. The reactions were performed in a controlled environment incubator shaker (PSYCROTHERM, New Brunswick, NJ) at the speed of 300 rpm. For a reaction at given pH, temperature and time, three replications and one control (substrate + buffer, and without enzyme) were carried out concurrently. The enzyme unit was 67.5 units per gram of substrate. The reaction was stopped by adding 20 mL of a mixture of methanol and diethyl ether (80:20).

Only in the limit of zero hydrolysis will the true, fundamental selectivity of the hydrolysis be revealed in a single concentration profile. Conversion data at 100% hydrolysis will not reveal information on selectivity. Reaction times of this investigation were selected to provide about 15% conversion since soybean oil contains about 15% saturated fatty acids. Actual conversions are reported in the results and typically varied from 5–20%.

Table 1
Operating pH and temperature for enzyme screening test

Lipase	pH	Temperature (°C)	Activity (units/gram)
C. rugosa	7.0	45	≥30000
B. cepacia	7.0	50	≥30000
Pseudomonas sp.	7.0	35	≥10000
P. camembertii	5.0	30	≥50000
P. roquefortii	7.0	40	≥10000
A. niger	6.0	45	12000–15000
M. javanicus	7.0	40	≥10000
R. miehei	7.0	45	≥20000

2.3. Fatty acids/glycerides recovery

After stopping the reaction, 80 mL of 0.5 M potassium bicarbonate and 15 mL of diethyl ether were added into the reaction product (glyceride-fatty acid mixtures). The mixture was placed in a separatory funnel. The glyceride portion (oil phase) was separated from the free fatty acid soap, which was in the lower water phase. Free fatty acid soap residues were recovered from the water phase by acidification with sulfuric acid and then by solvent extraction with diethyl ether. Eventually, the diethyl ether in both glycerides and acid residue was evaporated at 45 °C.

2.4. Analysis of reaction products

2.4.1. Analysis of methyl esters from epoxidized soybean oil

Methylation of glycerides and fatty acids were done with different catalysts before the GC analysis. Diazomethane was used for fatty acid residues and tetramethylammonium hydroxide (TMAH, 25% in methanol) was used for glycerides [15]. Constituents in fatty acid residues and glycerides were analyzed by GC, HP 6890 GC (Wilmington, DE). The column was HP MXT®WAX 70624, capillary 30.0 m × 280 μm × 0.25 μm nominal and the detector was a flame ionization detector (FID). The injection port temperature was 250 °C. The temperature program was set from 160 to 220 °C at 10 °C/min and hold at 220 °C for 12 min. Carrier gases were H_2 (40 mL/min) and make up gas was N_2 (35 mL/min). Air flow was 260 ml/min. The split ratio was 75:1.

GC retention times of monoepoxy acyl moiety and diepoxy acyl moiety were confirmed by epoxy oleate, and epoxy linoleate while the GC retention time of triepoxy acyl moiety was confirmed by epoxy linolenate and epoxy flax seed oil which contains about 54% of linolenic acid. Oleic acid, linoleic acid, linolenic acid and flax see oil were individually epoxidized by lipase B from *C. antarctica* (Novozyme 435®) and hydrogen peroxide [1,2]. After the epoxidation reaction, the epoxy fatty acids and the epoxy flax seed oil were methylated by diazomethane and analyzed by GC-FID.

2.4.2. Analysis of methyl ester from soybean oil

For the reaction products produced from the reaction of soybean oil, sodium methoxide was used to catalyze the methylation of glycerides and sulfuric acid was used for the free fatty acid residues. GC analysis was similar to that used for epoxy derivatives.

3. Results and discussion

3.1. Enzymatic hydrolysis of soybean oil

Fig. 2 presents hydrolysis conversions and compositions of the glyceride phase and fatty acid phase after product workup. Lipase from *C. rugosa* gave the highest reaction yield (24 h) that was about 25% hydrolysis. *A. niger* lipase was the second most active lipase at 12.5% hydrolysis. Lipases from *M. javanicus* and *B. cepacia* produced about 7% and 10% of hydrolysis

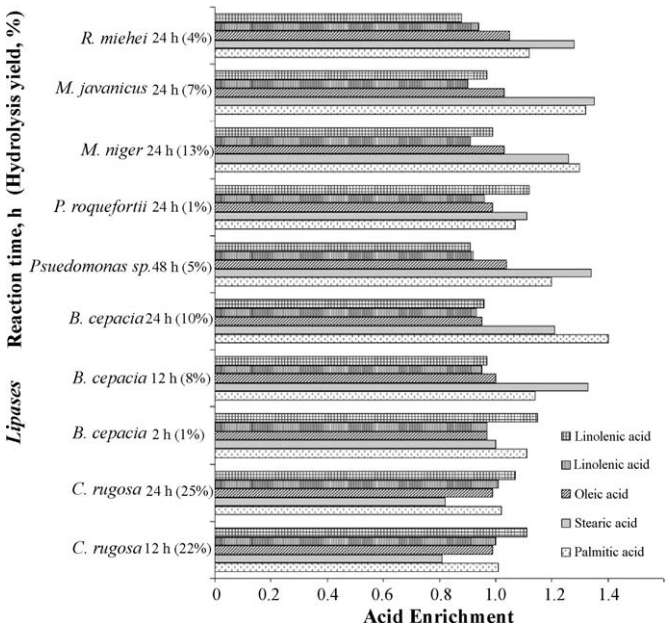

Fig. 2. Enrichment numbers in fatty acid residues after hydrolysis of soybean oil.

while lipases from *R. miehie*, *P. roquefortii* and *Pseudomonas* sp. yielded less than 5% hydrolysis.

The lipase from *P. camembertii* was ineffective for this reaction. This corresponds with the previous studies reporting that the enzyme is an ineffective catalyst for the hydrolysis of triglycerides and shows hydrolysis activity for monoglycerides and diglycerides [12,16].

3.2. Enzymatic hydrolysis of epoxidized soybean oil

Fig. 3 shows hydrolysis conversions and constituents in the glyceride phase and fatty acid phase after the enzyme hydrolysis of epoxidized soybean oil. Although soybean oil contains about 7% of linolenic acid, neither linolenic acid nor the triepoxy acyl moiety exists in the commercially available epoxidized soybean oil.

This study investigated the effects of the epoxy groups on the lipase reaction and lipase selectivity because the presenting data cannot explain lipase regioselectvity and stereoselectivity. Diepoxy stearic acids derived from both linoleic acid (fully epoxidized) and linolenic acid (not fully epoxidized) are combined and reported as one data point. Analogously, both non-fully epoxidated linoleic acid and non-fully linolenic epoxidated acid on mono epoxidated acid, and fully epoxidated oleic acid, are reported as the monoepoxy acyl moiety.

Lipases from *C. rugosa*, *B. cepecia* and *M. javanicus* were efficient biocatalysts resulting in about 20% conversion by *C. rugosa* lipase, higher than 40% conversion by *B. cepecia* lipase and about 15% by *M. javanicus* lipase (2 h). Lipases from *A. niger* slowly catalyzed the reaction and yielded 20% (24 h). Lipases from *R. miehei*, *P. roquefortii* and *Pseudomonas* sp. yielded about 3–5% hydrolysis (2 h).

Similarly to the reactions with soybean oil, *P. camembertii* was ineffective toward hydrolyzing epoxidized triglycerides.

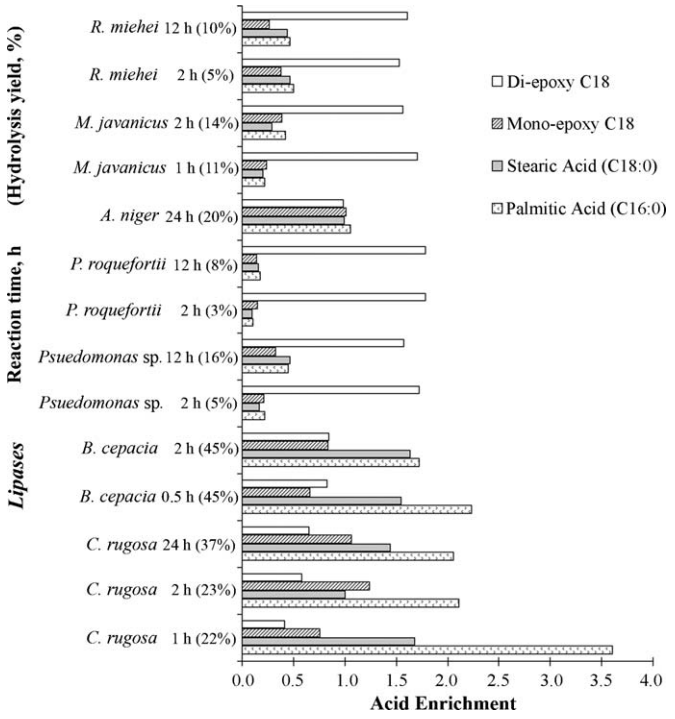

Fig. 3. Enrichment numbers in fatty acid residues after hydrolysis of epoxidized soybean oil.

3.3. Lipase selectivity of soybean oil triglycerides

After product workup, the enrichment number of each acyl moiety in fatty acid phase was calculated in order to investigate enzyme selectivity. The following equation defines the enrichment number:

$$\text{enrichment number of acyl moiety A in fatty acid residue} = \frac{\%\text{normalization of A in fatty acid phase}}{\%\text{normalization of A in triglyceride substrate}}$$

where A is palmitic acid, stearic acid or other acyl moieties

Total of every component's signal is 100 in percent normalization

The higher the enrichment number, the higher the enzyme selectivity toward hydrolyzing a particular acyl moiety. Figs. 2 and 3 show enrichment numbers from the reactions of soybean oil triglyceride and of epoxidized soybean oil triglyceride, respectively.

In view of experimental error and the need for a threshold selectivity to realize practical usefulness, a deviation of 15% was identified as a threshold value to identify a significant selectivity. Based on these criteria and the data of Fig. 2, *C. rugosa* lipase discriminated against stearic acid while it has been known to be a non-selectvie enzyme in previous work [17]. Lipases from *B. cepacia, Pseudomonas* sp., *A. niger*, *M. javanicus* and *R. miehei* promoted hydrolysis with higher enrichment numbers of saturated fatty acids than those of unsaturated fatty acids. Lipases of *P. roquefortii* seemed to show non-selective ability (24 h); however, its hydrolysis yield was too low to conclude that the enzyme was non-selective. In addition, some previous literature determined lipase from *P. roquefortii* to be a 1,3-positional and short-chain-specific lipase [12,16].

In enzymatic modification of triglycerides, most of enzymes are 1,3-selective lipases [11]. A lot of publications have determined that all of the enzymes used in this study, except lipase from *C. rugosa*, are 1,3-regioselective [11,12,16,17].

Soybean oil has <20% of saturated fatty acids where 99% of them are located at 1,3-position. It is possible that lipases either showed saturated fatty acid selectivity or 1,3-selectivity. It cannot be determined based on the available information. The present results suggest that the selectivity is dependent upon the reaction conditions and/or degree of saturation in a manner more complicated than simple 1,3-regioselectivity.

3.4. Lipase selectivity of epoxidized soybean oil triglycerides

Fig. 3 compares enrichment numbers from the hydrolysis of epoxidized soybean oil. Lipase from *B. cepacia* selectively cleaves off saturated fatty acids, both palmitic and stearic acids, when hydrolysis rates of the saturated fatty acids are two times faster than those of epoxy fatty acids (0.5 h). The rate of hydrolysis of palmitic acid was initially shown to be higher than that of stearic acid; however, they were similar at longer reaction times (2 h). There was no difference between rates of hydrolyzing the monoepoxy acyl moiety, and the diepoxy acyl moiety by *B.cepacia* lipase (similar enrichment numbers).

C. rugosa lipase selectively hydrolyzed palmitic and stearic acids from epoxidized soybean oil triglycerides with a greater selectivity to palmitic acid than stearic acid. The selective cleavage of palmitic acid was prominent at conversions up to the highest measured conversions of 37%. The greatest selectivity to cleaving palmitic acid was at lower conversions, which is consistent with the higher concentrations of bound palmitic acid at the onset of hydrolysis.

Fig. 3 shows that epoxidized soybean oil has about 52% of diepoxy acyl moieties being the prominent bound acyl moieties in epoxidized soybean oil. However, *C. rugosa* slowly hydrolyzed diepoxy acyl moieties as indicated by its lowest enrichment number in the acid residues.

The lipase from *C. rugosa* was previously reported to selectively cleave short chain fatty acids and to be a non-selective enzyme depending on substrates and reactions [9,12,18,19]. The enzyme itself likes straight chain fatty acid rather than bulky substrates due to its tunnel-like active site [18]. Shorter fatty acids tend to be straighter than longer fatty acid chains, and saturated fatty acids do not bend like unsaturated fatty acids bending at carbon double bonds. This might cause lipase *C. rugosa* to show the most selectivity to palmitic acid.

C. rugosa lipase showed a higher number of palmitic acid enrichment with the hydrolysis of epoxidized soybean oil than that of the soybean oil (Figs. 2 and 3). The enrichment numbers in excess of 3.5 (2 h) were the highest observed—it is possible that the intrinsic selectivity of *C. rugosa* lipase to short chain fatty acids was ultimately limited by thermodynamic equilibrium and non-enzymatic acyl moieties migration [19,20].

Lipases from *P. roquefortii*, *M. javanicus*, *R. miehei* and *Pseudomonas* sp. favorably hydrolyzed diepoxy acyl moieties, which on the surface appear to possibly be contrary to the previously reported 1,3-regioselective. However, these lipases could have selectivity toward diepoxy acyl moieties at position 1 or 3 in epoxidized soybean oil. These lipases discriminated against the hydrolysis of palmitic acid, stearic acid and monoepoxy stearic acid. Lipase from *P. roquefortii* catalyzed the hydrolysis reaction of diepoxy acyl moieties about 10–17 times faster than the others.

A. niger lipases showed almost identical enrichment numbers (about 1.0) for all acyl moieties (Fig. 3). From these results, *A. niger* enzyme can be either non-selective or 1,3-regioselective in the hydrolysis of epoxidized soybean oil, according to <19% of saturated fatty acids at position 1 or 3 and complicated thermodynamic equilibrium including non-enzymatic acyl migration [20].

3.5. Effect of epoxy functional group on hydrolysis conversions

Rates of hydrolysis significantly increased in the reaction of epoxidized soybean oil relative to soybean oil (see Figs. 2 and 3). The reaction conversion increased from 25% to 37% (24 h) by *C. rugosa* lipase. The hydrolysis of epoxidized soybean oil by *B. cepacia* lipase results in a 45% conversion (2 h) while the reaction with soybean oil yielded only 1% (2 h). Fig. 4 shows the comparison between the reaction of soybean oil and of epoxidized soybean oil with some lipases. It is likely due to the emulsifying characteristics of the epoxy group, which tends to increase the interface area between lipids and water.

The emulsifying nature of epoxidized soybean oil was confirmed by observations. Lipid–water mixtures during and after the hydrolysis of epoxidized soybean oil were cloudy while mixtures with soybean oil were less cloudy and more-readily separated into isolatable phases.

Epoxy acyl moieties both increased hydrolysis rates and altered lipase selectivities. It is possible that 1,3-regioselective lipases became highly selective to diepoxy acyl moieties at the 1 and 3 positions, and in such an instance, the present study would not contradict previous conclusions on 1,3-regioselectivity. One explanation for the selectivity trends exhibited by certain enzymes and not by others is the role of water in the immediate surroundings of the enzyme molecule toward promoting reaction.

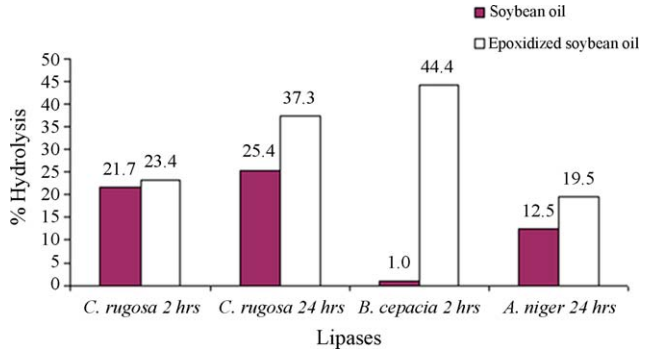

Fig. 4. Effects of the presence of epoxy acyl moieties of soybean oil triglycerides.

4. Conclusion

The presence of epoxy moieties in epoxidized soybean oil was observed to increase the lipid–water interface and enhance the rates of enzyme hydrolysis reactions. The epoxy functional group also affected the enzyme selectivity. Based on this screening of eight lipases, selective hydrolysis can be more-readily attained in epoxidized soybean oil than normal soybean oil.

Partial hydrolysis of epoxidized soybean oil triglycerides by lipases from *B. cepacia* and *C. rugosa* could selectively replace saturated fatty acid moieties with hydroxy functional groups, which are expected to be primary alcohol (high reactivity). The selectively hydrolyzed product, epoxidized soybean oil based material, has higher functionality in terms of a higher percentage of epoxy and higher hydroxy number, which is believed in some applications, to produce better performing polymers by decreasing the number of non-functional fatty acid branches on the polymer structure.

The reaction is very simple and operated at mild operating conditions where only water is needed as a reagent and no surfactant and solvent are required.

Acknowledgements

We gratefully thank the United Soybean Board for their financial support and would like to give an acknowledgement and special thanks to Amano Enzyme Inc., Elgin, IL, US, for their lipases.

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