Surfactants & Detergents Technical

Enzymatic Preparation of Monoglycerides in Microemulsion

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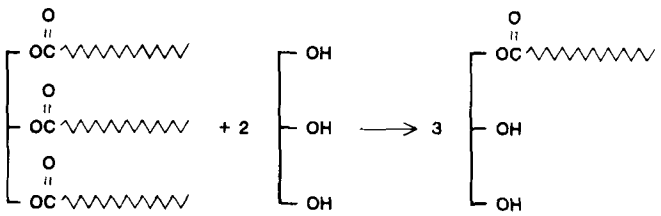
Monoglycerides have been obtained in 80% yield by enzyme catalyzed hydrolysis of the corresponding triglyceride. The reaction was carried out in an oil-rich microemulsion (L 2 phase) formulated without cosurfactant. Best results were obtained with the anionic surfactant sodium bis(2-ethylhexyl)sulfosuccinate (AOT), isooctane as hydrocarbon component and a molar ratio of aqueous buffer of pH 7 to surfactant of 12. The enzyme used was a 1,3-specific lipase which leaves the 2-position intact. However, the 2-monoglyceride formed slowly undergoes acyl migration to 1-monoglyceride which subsequently is hydrolyzed to glycerol and fatty acid. Optimal reaction time at 35 C reaction temperature was found to be three hr.

Long chain monoacylglycerols, or monoglycerides, are nonionic surfactants widely used as emulsifiers in the food and pharmaceutical areas. Normally they are produced by alcoholysis of the corresponding triglyceride with two equivalents of glycerol, as is shown in Scheme 1.



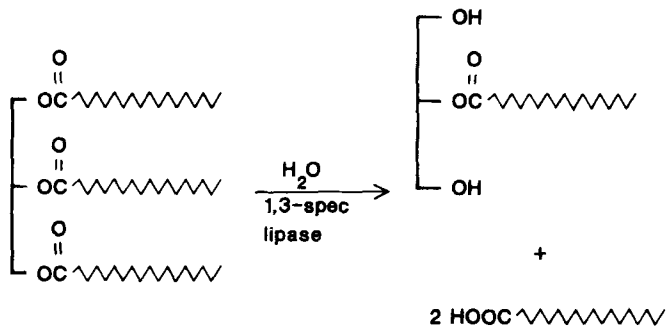
SCHEME 1. Alcoholysis of a triglyceride with glycerol giving 1-monoglyceride (the 2-monoglyceride will also form).

The reaction requires high temperatures (210-240 C) and the use of a transesterification catalyst, usually a Sn- or Pb-compound. The reaction product is an equilibrium mixture of monoglycerides, diglycerides, starting triglyceride, glycerol and free fatty acid. Furthermore, a dehydration process also takes place, leading to the formation of substituted diglycerols. After work-up the effective yield of triglyceride to monoglyceride conversion is 40-50%.

During the last decade Luisi et al. (1-3) and others (4-7) have demonstrated that many lipase catalyzed reactions can be carried out in reverse micellar systems or microemulsions. When solubilized in the small water domains of the microemulsion, the enzyme is afforded some protection from the denaturing effect of the solvent. Aliphatic hydrocarbons, such as hexane, have been used as the oil component and the lipase activity has been found to be satisfactory with anionic and nonionic, but not with cationic, surfactants. The long term effect on the enzyme function seldom has been investigated, however.

The use of a microemulsion as reaction medium eliminates the problem of insolubility frequently encountered with triglycerides and other lipophilic substrates. In addition, it opens novel synthetic possibilities. For instance, lipase catalyzed interesterification can be used to produce triglycerides, which is of interest for the production of synthetic cocoa butter (7).

The present paper describes another synthetic application of lipase catalyzed reactions in microemulsion. Using a 1,3-specific enzyme, triglycerides are converted to 2-monoglycerides in a selective manner, as illustrated in Scheme 2.



SCHEME 2. Enzymatic hydrolysis of a triglyceride to 2-monoglyceride.

EXPERIMENTAL PROCEDURES

All reactions were carried out at 35 C in 100-ml bottles using a magnetic stirrer. The enzyme was always used in 50 mg/g substrate.

The enzyme used was an extracellular microbial lipase from *Rhizopus delemar* 600 units per mg, purchased from Sigma Chemical Co., St. Louis, Missouri. Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) was from Merck, Darmstadt, W. Germany, and the nonionic surfactants were from Berol Kemi, AB, Stenungsund, Sweden. The palm oil was the standard quality purchased from Aarhus Oliefabrik, Aarhus, Denmark. No purification of the materials was made prior to use. The phosphate buffer pH 7 consisted of 0.021 M NaH_2PO_4.

After completed reaction the enzyme was denatured by heating to 90 C for 15 min. The solvent was evaporated under vacuum and the residue dissolved in 50 ml chloroform. The chloroform solution was extracted four times with 30 ml 4% aqueous $CaCl_2$ solution. To a 20-ml sample of the chloroform solution was added 0.050 ml 70% perchloric acid in order to induce acyl group migration (8). After 90 seconds stirring 50 ml periodic acid was added and analysis for vicinal diol was carried out as described in the literature (9, 10). In the calculation of total monoglyceride a correction factor of 1.15, suggested in the literature (8, 11), was used.

The combined aqueous phases from the extraction were analyzed for glycerol in the same way, using periodic acid as glycol splitting agent. No perchloric acid was added and no correction factor was used in the calculation.

Analysis for fatty acid was made by titration of a sample dissolved in ethanol with 0.1 M KOH in etha-

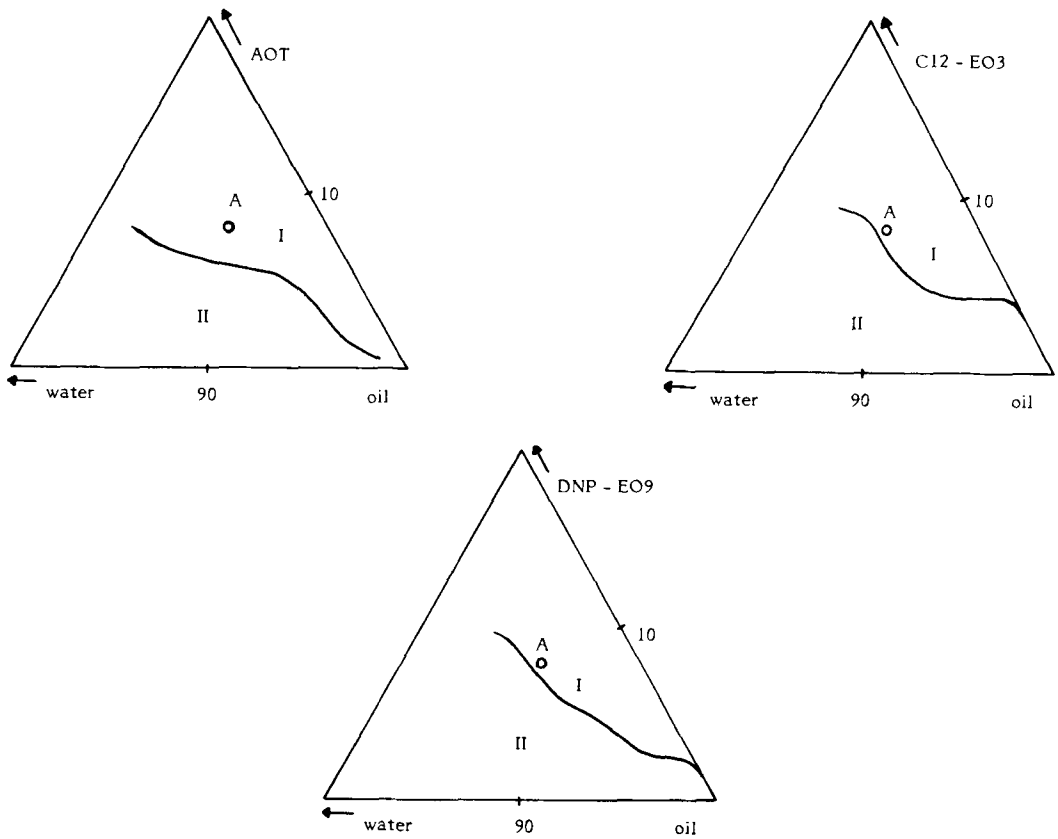


FIG. 1. Oil-rich corner of phase diagrams for the three surfactants AOT, C12-EO3 and DNP-EO9 at 35 C. The oil component consists of n-hexane:palm oil, 20:1. I and II indicate 1- and 2-phase areas, respectively.

nol. Because palm oil contains small amounts of free fatty acids, analysis must be performed on the starting material and the value of free fatty acid obtained subtracted from that of the sample.

The reactions were also monitored by thin layer chromatography (TLC) on silica gel using petroleum ether 60–70 (45 parts), diethyl ether (50 parts) and acetone (5 parts) as eluent. The plates were developed by iodine vapor.

RESULTS

Choice of surfactant. Three commercial surfactants capable of forming L 2 phases without the use of a cosurfactant were used in the investigation: sodium bis(2-ethylhexyl)sulfosuccinate (AOT), triethylene glycol monododecyl ether (C 12-EO 3) and nonaethylene glycol monodinonylphenyl ether (DNP-EO 9). Pseudo-ternary phase diagrams were constructed in which hexane:palm oil at a constant ratio of 20:1 was used as the hydrocarbon component. The diagrams are shown in Figure 1.

Reactions were carried out at the composition indicated by point A in the phase diagrams. The results are shown in Figure 2. Evidently, the microemulsion based on AOT is superior to the ones based on the nonionic surfactants. Because AOT also gives a larger isotropic phase in the water-poor region of the phase diagram (L 2 phase) than the two ethoxylates, it was considered the surfactant of choice. All subsequent work was performed with this surfactant.

Choice of hydrocarbon. n-Hexane, n-octane and isooctane were tested. As can be seen from Figure 3, isooctane gives a considerably better yield of monoglyceride than the two straight chain hydrocarbons both after one and three hr reaction time at 35 C.

Ratio of water to surfactant. The effect of the molar ratio of water to surfactant, R, on the degree of

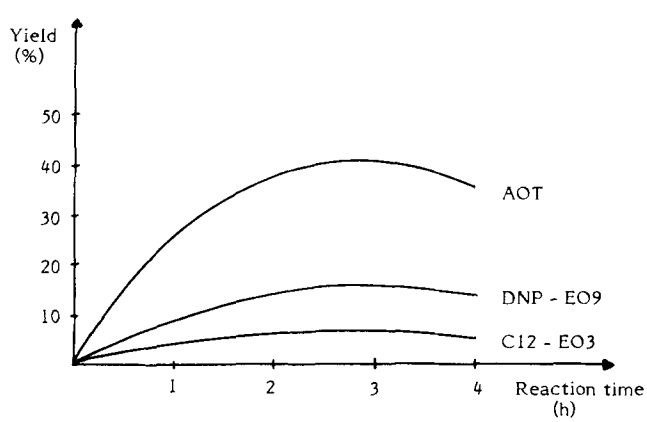


FIG. 2. Yield of monoglyceride vs reaction time for the three surfactants AOT, C12-EO3 and DNP-EO9 at 35 C. The composition used was (in weight %): n-hexane 83, palm oil 4, ph 7 phosphate buffer 5, surfactant 8.

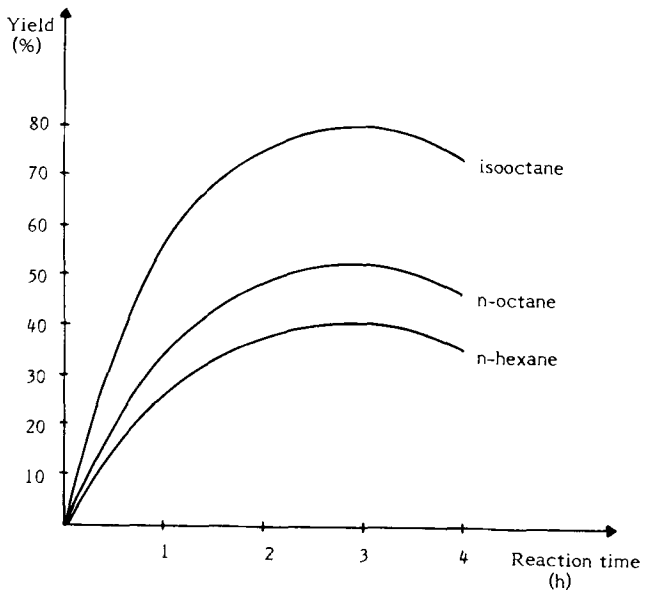


FIG. 3. Yield of monoglyceride vs reaction time for the three hydrocarbon solvents isooctane, n-octane and n-hexane at 35 C, using AOT as surfactant and the same formulation as given in Figure 2.

conversion of triglyceride to monoglyceride was studied. Figure 4 shows the results after one and three hr reaction time. A value of R around 12, corresponding to a weight ratio of water to AOT of 1.0:2.0, seems to be optimal although the variations are not very large within the interval of R studied. The radius of the water core of an AOT-based microemulsion with R = 12 is *circ.* 2 nm (4).

Influence of pH. The yield of monoglyceride as a function of pH of the phosphate buffer used in the formulation is shown in Figure 5. Optimum buffer pH was found to be around 7 but also in this case the variations in yields were moderate. However, pH 7 need not necessarily be the optimal pH of the reaction because it is known that the acidity within the water domains of reverse micelles may differ from the original pH of the buffer solution used due to an uneven distribution of protonized and deprotonized species between the phases (12). No attempt was made here to measure the actual pH of the water pools.

Influence of reaction time. Using optimal conditions (AOT as surfactant, R of 12, isooctane as hydrocarbon solvent and an aqueous buffer of pH 7) the product composition at various reaction times was studied at a reaction temperature of 35 C. As can be seen from Figure 6, the yield of monoglyceride goes through a maximum at three hr reaction time, whereas the yields of glycerol and fatty acid increase continuously.

DISCUSSION

Lipases can be divided into two groups with regard to the regiospecificity exhibited with acylglycerol substrates (13). Enzymes in the first group show no regiospecificity and catalyze hydrolysis of all ester bonds of the triglyceride. Lipases from the second group release fatty acids regiospecifically from the outer 1- and 3-positions of the substrate. The regiospecificity of the enzyme, which normally is almost absolute, results from a poor accessibility of the hindered ester of the secondary alcohol to the active site of the enzyme. The lipase used in this study belongs to the latter group and would be expected to catalyze the conversion of triglyceride to 2-monoglyceride with a high degree of specificity.

However, both 2-monoglycerides and 1,2-diglycerides are chemically unstable species and undergo acyl group migration to give 1-monoglycerides and 1,3-diglycerides, respectively. These, in turn, are good

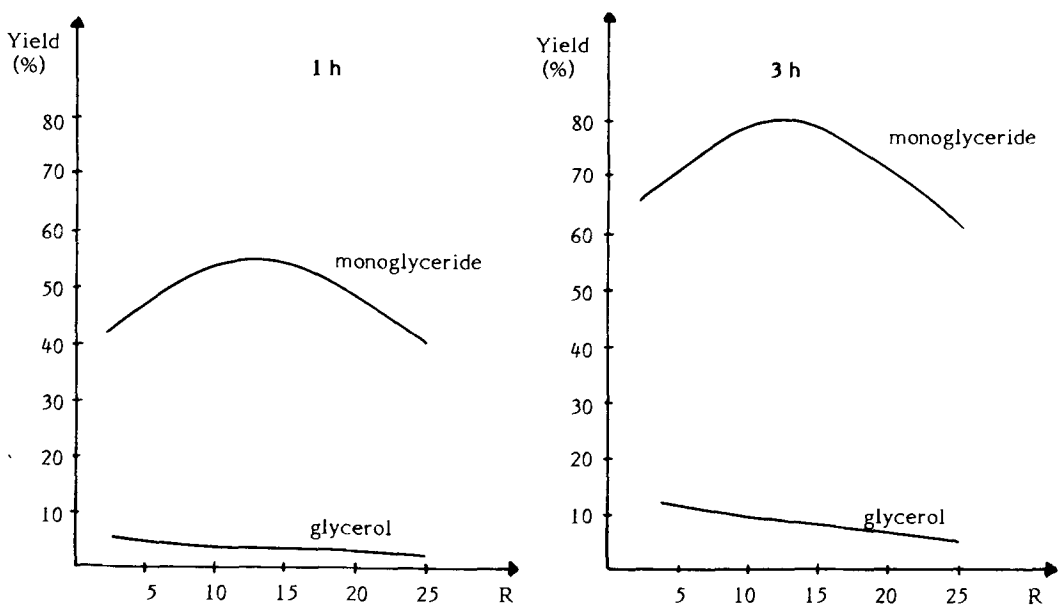


FIG. 4. Yields of monoglyceride and glycerol vs molar ratio of water to surfactant, R, at one hr and three hr reaction time at 35 C. The composition used was (in weight %): isooctane 83, palm oil 4, pH 7 phosphate buffer + AOT 13.

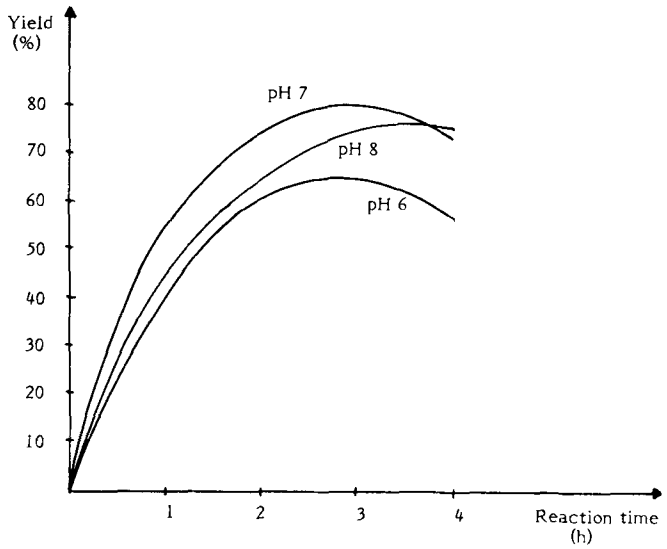


FIG. 5. Yield of monoglyceride vs reaction time for reactions carried out at 35 C using buffer pH of 6, 7 and 8. Weight ratio of phosphate buffer to AOT 2.0 and the composition was the same as that given in Figure 4.

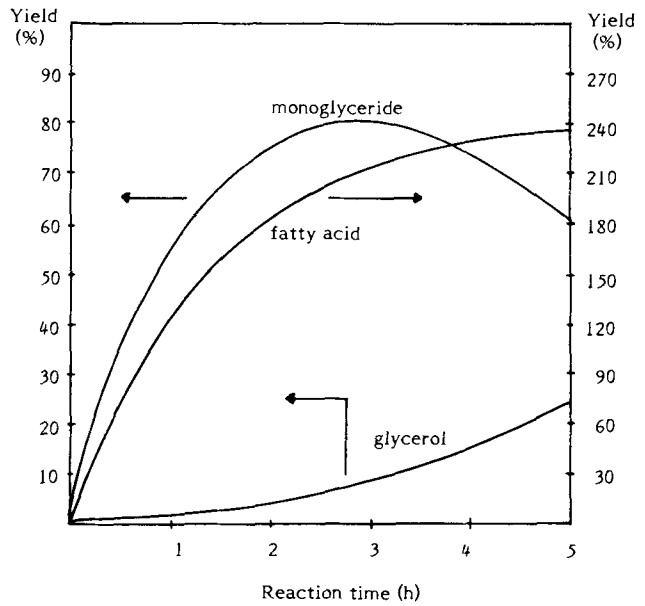


FIG. 6. Yields of monoglyceride and glycerol (left) and of fatty acid (right) in mol % based on starting triglyceride. The reaction was run at 35 C and the composition was as in Figure 4 using a weight ratio of buffer to AOT of 2.0.

substrates for the regiospecific enzyme and complete hydrolysis to fatty acids and glycerol will eventually take place. It is, therefore, important not to allow the reaction to proceed too long. Because the rate of acyl group migration is comparable with the rates of di- and monoglyceride hydrolysis, a compromise will have to be made between remaining diglyceride in the product mixture and complete hydrolysis to glycerol. In this study the maximum yield of monoglyceride is 80%, as shown in Figure 6. By optimizing the choice of enzyme and the pH of the reaction it is conceivable that the yield can be increased further.

As can be seen from Figure 2, the choice of surfactant is important for the rate of reaction. With the two nonionic surfactants a much slower conversion to monoglyceride is obtained than with AOT, although all three surfactants give relatively large L 2 phases without the use of a cosurfactant. The difference in performance may be related to differences in structure of the isotropic oil-rich phases. Whereas AOT-based microemulsions of low water content are known to have distinct water droplets, nonionic surfactants with oligo(ethylene glycol) chains have been found to possess a less ordered structure (14, 15). At low R values the water is bound to the oxyethylene groups and water pools with "free" water are formed only when the oligo(ethylene glycol) chains have been saturated with water. A parallel packing of the oligo(ethylene glycol) chains has been suggested giving rise to bilayered micelles with more or less interdigited polar groups (15). This difference in microstructure of the reaction medium may influence the rate of formation and dissolution of the activated complex, thus affecting the kinetics of the enzymatic reaction.

The R-value of a microemulsion is known to be of importance for the rate of an enzymatic reaction. It has been shown for a similar system that the effect is not associated with enzyme activity, however. The kinetic properties of a lipase catalyzed reaction, e.g. k_{cat}/K_m, were the same in AOT-based microemulsions as in bulk water (4). Instead, a change in R will affect substrate partitioning, as well as water activity, and the two effects will influence the reaction rate in opposite directions.

As shown in Figure 4, varying R between 2.5 and 25 has only a small effect on the rate of conversion of triglyceride to monoglyceride, implying that the increase in water activity on increasing R is approximately balanced by the unfavorable effect on substrate partitioning. The lipophilic substrates, tri- and diglycerides, will partition almost entirely into the oil domain. The lipase, on the other hand, is assumed to be associated exclusively with the droplets, located either in their interior or at the interface. Increasing R leads to larger droplets with a greater part of the enzyme located in the interior of the water pools, thus creating a more unfavorable situation for the formation of the enzyme-substrate complex.

From a practical point of view replacement of the water in the microemulsion formulation by glycerol would be of interest. Glycerol has been found to function as the polar component in at least some microemulsion-type systems (16-19) and would in this case also be able to participate in the reaction. Theoretically, a composition comprising glycerol and triglyceride in a molar ratio of 2:1 would give complete conversion to monoglyceride with no fatty acid formed provided the lipase was effective in catalyzing the glycerolysis reaction (See Scheme 1; a 2:1 ratio of 1- and 2-monoglyceride would form in the enzymatic process). This approach was tested, using AOT as surfactant. No monoglyceride formation could be seen, however,

indicating a lack of activity of the lipase used in this study under nonaqueous conditions.

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