Use of Lipases in Multiphasic Systems Solely Composed of Substrates

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Three lipase-catalyzed reactions have been investigated in relation to specificity and water dependence. The reactions in question include: the synthetic reaction between oleic acid and glycerol; the enzymatic hydrolysis of triolein; and alcoholysis/glycerolysis transesterification reactions. All reactions were carried out under solvent-free conditions. In each case, the medium composition and reaction conditions were optimized in order to work at elevated substrate concentrations and to minimize the production of by-products. Different lipase preparations have been tested in each reaction. In the synthetic reaction, the effective removal of produced water was found to be vital for the production of triolein. With water removal and glycerol amounts not higher than required by the stoichiometry of the reaction, 95% of the available oleic acid was converted to triolein in 48 hr. The production of triolein was also found to be dependent on the availability of the 1,2-diglyceride to react with oleic acid. In the hydrolysis reaction, best conversion yields of triolein towards monoolein, diolein and free fatty acid were obtained when water was considered simply as a substrate of the reaction. In glycerolysis reactions, the reaction of triolein to give monoolein and diolein followed much the same pattern as for hydrolysis, when water was replaced by glycerol. It was shown again that near stoichiometric amounts of substrates led to the best conversion to mono- and diglycerides. A small excess of glycerol was found to be very inhibitory to the reaction. All possible isomers were formed during the reaction. Conversely, in alcoholysis reactions between triolein and stearyl alcohol the specificity of the lipase was upheld. Excess alcohol in this instance was found to be beneficial.

KEY WORDS: Alcoholysis, hydrolysis, glycerolysis, lipase, synthesis, triglycerides.

In order to develop possible applications of lipases in the food industry, we have studied the main reactions dealing with triglyceride modifications as catalyzed by lipases in solvent-free systems. No organic solvent was used to solubilize the substrates, which led to the use of enzymes in multiphasic systems because without a co-solvent the different substrates are not miscible. The overall objectives of this work were to find the best conditions and define the best reaction medium to improve the efficiency of three lipase-catalyzed reactions involving oils or fats in the form of triglycerides. The reactions studied were triolein synthesis from oleic acid and glycerol, triolein hydrolysis and triolein alcoholysis, the last one involving two different alcohols, glycerol and stearyl alcohol.

Beside the fact that transformations of oils and fats without adding a solvent can find applications in the food industries, there are also other advantages in carrying out bioconversions under those conditions. In fact, when no solvent is used, it means that the whole reaction medium can be composed of only the necessary substrates and, thus, those substrates can be used at very high concentrations. So far, few processes involving lipases have been studied without organic solvent.

In a medium containing 25% water and a large excess of glycerol, few triglycerides were produced (1). Removing the produced water with a vacuum pump increased the yield of triglycerides to 43% in six weeks (2). With a microporous membrane between the phases, total conversion of fatty acids was obtained, with only 10% incorporated as triglycerides (3). Reverse micelles have been used to make triglycerides from 1,2-diglyceride and oleic acid (4). Fungal cell-bound lipases gave mono- and di-, but few triglycerides (5).

Hydrolysis has been widely studied—free lipases in biphasic media, [oil and water (6) or organic solvent and water (7)], in microemulsions (8), lipases adsorbed on membranes (9), entrapped (10) or covalently bound to supports (11). The effect of the amount of water in the reaction medium has rarely been studied. Ibrahim *et al.* (11) wrote that it is well known that the hydrolysis of fats by free lipases is a reaction requiring water. They have examined immobilized lipase systems in comparison with the free enzyme. They found that the required water content for hydrolysis for all the immobilized lipase preparations (ENTP 4000, Amberlite XAD-7 and Ca-alginate) was about 40–50% (v/v), which was similar to that for the free enzyme. Unfortunately, no data were shown. Optimal water amount has never been expressed in moles of water necessary in relation to the substrate for hydrolysis. This study examines triolein hydrolysis with different amounts of water, and compares the behavior of the same enzyme in free and immobilized states.

Transesterification is the reaction that has been studied the most without a solvent (12). Here, studies on alcoholysis of triolein with glycerol or stearyl alcohol will be presented. Glycerolysis has been widely studied for the synthesis of mono- and diglycerides (13–15). However, the effect of the ratio triglyceride/oleic acid has not been studied. Most of the studies with aliphatic alcohols deal with short-chain alcohols (16–18). We have chosen to work with long-chain alcohols because the products offer much more varied applications. Contrary to common belief (17,19), we show here that non-miscibility of the reactants does not require the use of an organic solvent.

MATERIALS AND METHODS

Materials. Lipozyme (free *Mucor miehei* lipase), Lipozyme IM-20 (*Mucor miehei* lipase immobilized on weak anion exchange resins) and SP 382 (immobilized *Candida* sp. lipase) were generous gifts from Novo Industries (Montréal, Québec, Canada). All fatty materials [substrates and high-performance liquid chromatography (HPLC) standards] were obtained from Sigma Chemical Co. (St. Louis, MO). Glycerol was purchased from Anachemia (Montréal,

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Québec, Canada). HPLC-grade acetone was purchased from Fisher Scientific (Pittsburgh, PA) and HPLC-grade acetonitrile from Caledon Laboratories (Georgetown, Ontario, Canada).

HPLC assay of fat classes. In order to follow the reactions, 5 μL of the reaction medium was diluted in 800 μL of acetone, and 15 μL of this solution was injected in the high-performance liquid chromatograph. The substrates and different products of the reactions (oleic acid, monoolein, 1,2-diolein, 1,3-diolein and triolein) were detected and quantified according to our previously described method (20), with the following modification: in order to be able to separate the two dioleins, the flow was kept at 0.8 mL/min for 8 min instead of 6 min and the increase (up to 4 mL/min) was done in 2 min instead of 4 min. This modification allows for the separation of the fat classes with the following retention times: monoolein 4.59 min, oleic acid 5.03 min, 1,3-diolein 9.2 min, 1,2-diolein 9.31 min and triolein 16.3 min. In the case of triolein alcoholysis with stearyl alcohol, a wax, stearyl oleate, is produced. It can be detected during the same HPLC analysis at a retention time of 13.6 min. The amounts of each component were calculated in percentages of oleic acid equivalents available at the beginning of the reaction, taking into account that dioleins and triolein contain two and three oleic acids moieties, respectively.

Synthesis of triglycerides. Given quantities of glycerol and oleic acid were mixed together followed by the addition of the immobilized lipase. The three-phase system was shaken vigorously at 60°C in an open Eppendorf tube on a vortex shaker for three days, and the progress of the reaction was followed by the HPLC technique just described. Water produced by the reaction freely evaporates from the tube. In order to study the specificity of the enzyme, the same reaction was carried out from either 1,2-diolein or 1,3-diolein and oleic acid, meaning that only the last step of the whole synthesis was studied.

Hydrolysis of triglycerides. Measured quantities of water and triolein were mixed followed by the addition of the lipase. Depending on the lipase used, a two- or three-phase system is operated. In both cases, the reaction was performed at 60°C in a closed Eppendorf tube to prevent any water loss. The tube was placed on a vortex shaker and the reaction was followed by the HPLC technique described above.

Alcoholysis of triglycerides. Measured quantities of triolein and alcohol (glycerol or stearyl alcohol) were mixed followed by the addition of Lipozyme. In both cases, reactions were performed in closed Eppendorf tubes at 60°C on a vortex shaker and followed by HPLC.

RESULTS AND DISCUSSION

Synthesis of triglycerides. We wished to carry out reactions in solvent-free environments, and to find the optimal substrate ratios to obtain the highest yield possible when using a solid enzyme and a medium solely composed of the substrates. Another goal was to avoid using any emulsifier or surfactant, and to perform the mixing by simple stirring without the help of any mechanical means to form emulsions or microemulsions even though the substrates are not miscible. Figure 1 shows the time course of a typical reaction when using substrates in stoichiometric amounts. Figure 2 shows the composition of the medium at equilibrium as a function of the initial ratio oleic acid/glycerol. The results presented here were obtained when using Lipozyme IM-20 as a catalyst. These results, previously published with more details (21), show that at the end of the reaction (48 hr), the medium was composed almost exclusively of triglyceride. There was little diolein remaining (less than 10% oleic acid equivalents) or oleic acid (less than 5%) and the solid enzyme was easily recovered. In order to reach 100% synthesis, water had to be removed as produced from the reaction medium. Different means of water removal have been tested—evaporation, molecular sieves, dry air and vacuum (21). These results show that the substrates had to be present in stoichiometric amount to give the maximum conversion to triolein. It is possible to work at high substrate concentrations—oleic acid was 2.94 M and glycerol was 0.98 M.

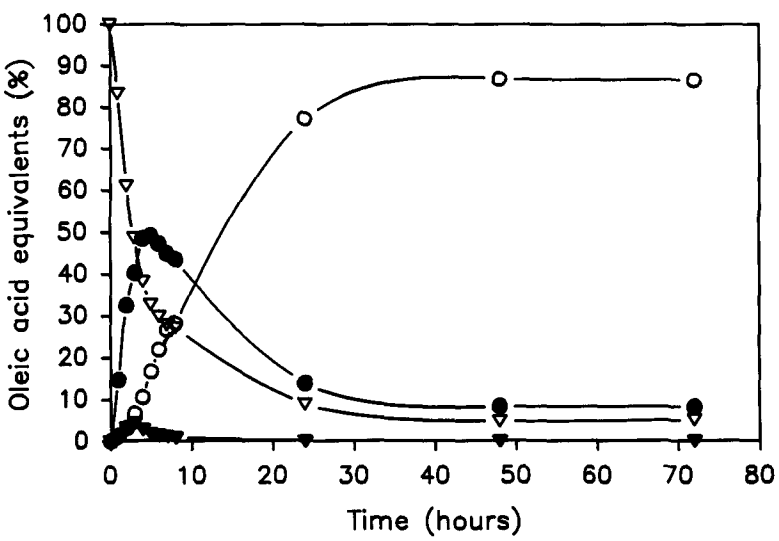

FIG. 1. Time course of triolein synthesis from 1770 μmoles of oleic acid (2.94 M) and 590 μmoles of glycerol (0.98 M) with 50 mg of Lipozyme. ○, triolein; ●, diolein; ▼, monoolein; and ▽, oleic acid.

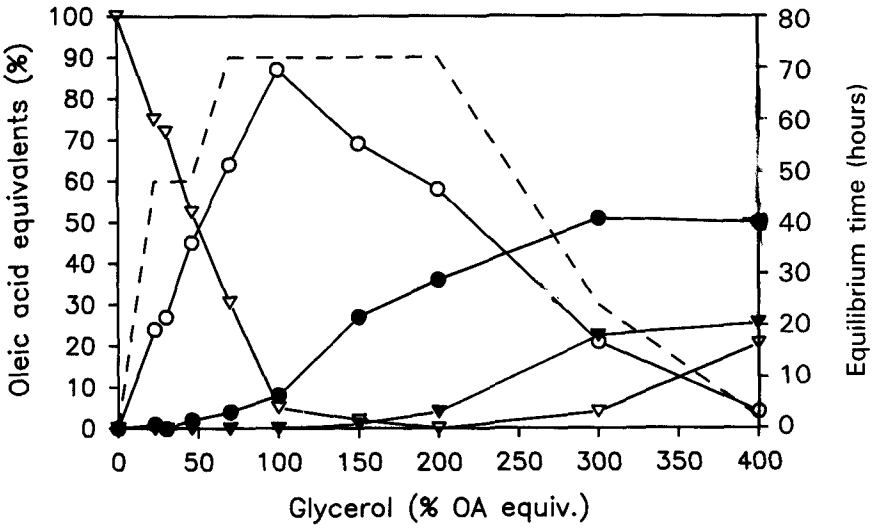

FIG. 2. Effect of the ratio oleic acid/glycerol on equilibrium of glycerides synthesis with 1770 μmoles of oleic acid, 50 mg of Lipozyme and glycerol varying from 0–2360 μmoles. Symbols are the same as in Figure 1; (----), time to reach equilibrium.

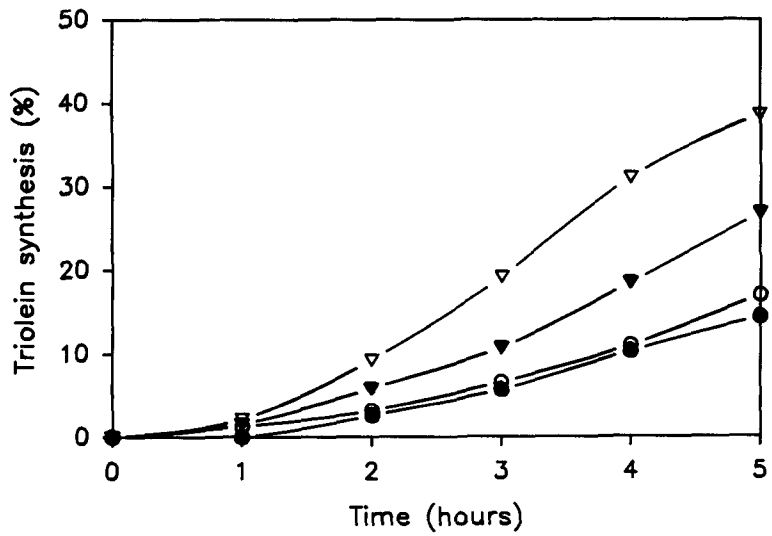

FIG. 3. Effect of lipase specificity on the rate of triolein synthesis from 1770 μmoles of oleic acid and 590 μmoles of glycerol with ●, 25 mg of Lipozyme; ○, 50 mg of Lipozyme; ▼, 25 mg of SP382; and ▽, 50 mg of SP382.

Lipozyme IM-20 is prepared with *Mucor miehei* lipase, which is well known as being 1,3-specific. Because lipase specificities have been defined in aqueous environments, we asked ourselves the question whether the specificity of the lipase had changed in this new reaction and in the new medium. In order to find an answer to that question, two sets of experiments were carried out. The first one was to compare the same reaction with a non-specific lipase (SP 382) and look at the effect of enzyme concentration on the rate of the synthesis reaction. Figure 3 shows the time course of triolein synthesis for the first 5 hr of the reaction. It shows that SP 382 was faster than the same amount of Lipozyme IM-20. It also shows that increasing the amount of the non-specific lipase (SP 382) increased the rate of triolein formation, whereas a similar increase in the 1,3-specific lipase (Lipozyme IM-20) did not affect the reaction rate. This first set of experiments indicated that the enzymatic reaction is not the limiting step in esterification when using Lipozyme IM-20 as a catalyst. The second set of experiments compared the synthesis of triolein with Lipozyme IM-20 when starting from 1,2-diolein and oleic acid or 1,3-diolein and oleic acid. The results have been plotted in Figure 4. Comparing the reaction rates of the two reactions clearly showed a preference of the enzyme towards 1,2-diolein as a substrate. That study with Lipozyme IM-20 led to the conclusion that the

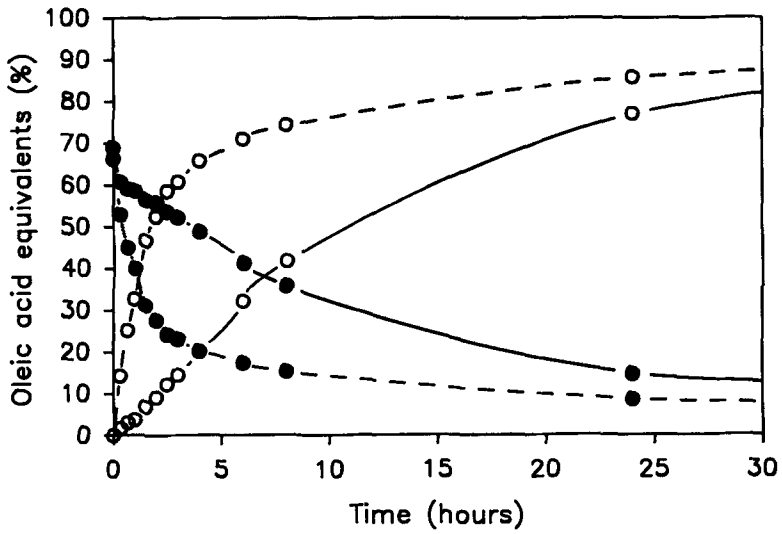

FIG. 4. Comparison of triolein synthesis with 25 mg of Lipozyme from: 295 μmoles of 1,2-diolein (----) and 295 μmoles of oleic acid; 295 μmoles of 1,3-diolein (——) and 295 μmoles of oleic acid. Symbols: ○, triolein; ●, diolein.

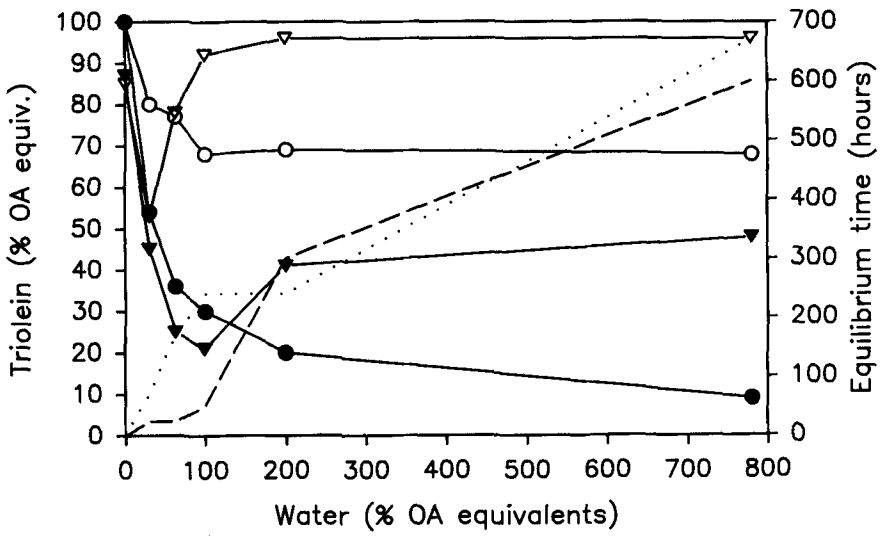

FIG. 5. Effect of the ratio triolein/water on rate and equilibrium of triolein hydrolysis with 590 μmoles of triolein, and water varying from 0–13900 μmoles: Free enzyme: ○, after 2 hr; ●, at equilibrium; ----, time to reach equilibrium. Immobilized enzyme: ▽, after 2 hr; ▼, at equilibrium; · · · ·, time to reach equilibrium.

1,3-diolein that is enzymatically produced isomerizes to 1,2-diolein, which is then used by the enzyme to form triolein. It has been shown in our laboratory that isomerization was favored by the presence of free oleic acid (data not shown). The specificity of the enzyme did not change in the oil-glycerol medium and is still 1,3-specific—isomerization from 1,3-diolein to 1,2-diolein must occur prior to triolein synthesis.

Hydrolysis of triglycerides. The effect of water on the activity (after 2 hr of reaction) of the enzyme and on the equilibrium of the reaction was investigated with free and immobilized Lipozyme. The results are presented in Figure 5. There is an optimal water amount to hydrolyze a triglyceride with the solid enzyme while there is none with the free form of the enzyme. Optimal water amount (in moles) is around 50% of oleic acid equivalents to hydrolyze, as far as activity is concerned. If one considers total hydrolysis rather than activity, then the optimal water amount (in moles) is between 80–150% of the initial triglyceride (in oleic acid equivalents). Substrate concentration can then be as high as 0.98 M for triolein with a water proportion in the medium of 1.8% (stoichiometric amounts) for optimal hydrolysis with Lipozyme IM-20. Thus, when using Lipozyme IM-20, water in hydrolysis should be considered only as a substrate of the reaction and not as a solvent. It would be interesting to study if

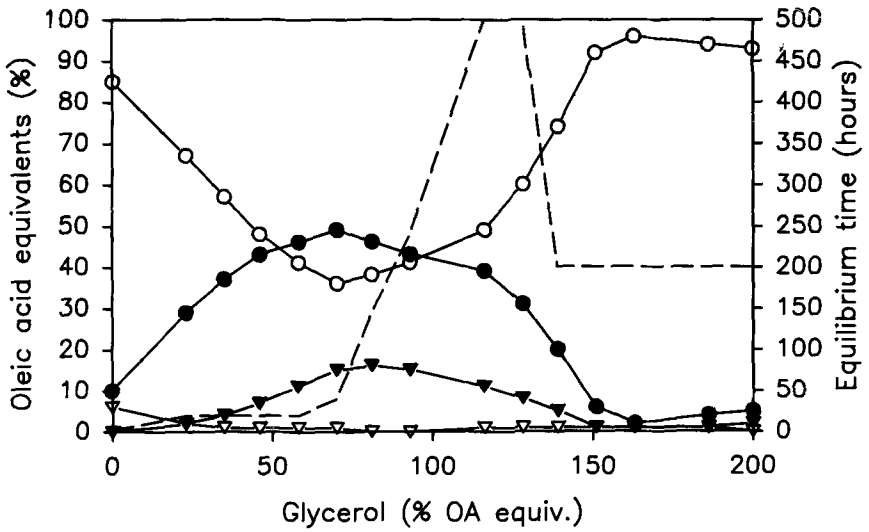

FIG. 6. Effect of the ratio triolein/glycerol on equilibrium of triglycerides glycerolysis with 590 μmoles of triolein, 50 mg of Lipozyme and glycerol varying from 0–3540 μmoles. Symbols are as in Figure 2.

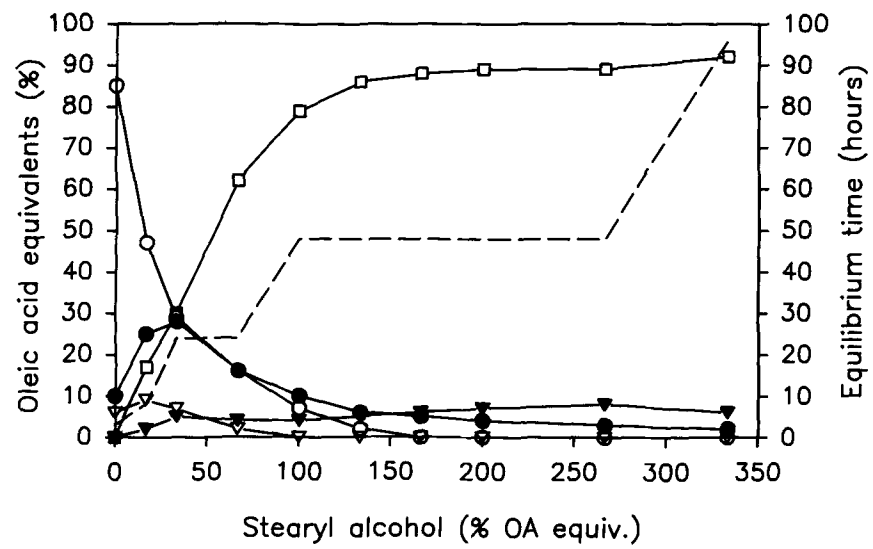

FIG. 7. Effect of the ratio triolein/stearyl alcohol on equilibrium of triglycerides alcoholysis with 590 μmoles of triolein, 50 mg of Lipozyme and alcohol varying from 0–5900 μmoles. ○, triolein; ●, diolein; ▼, monoolein; ▽, oleic acid; □, stearyl oleate; and ---, time to reach equilibrium.

that statement is true for all immobilized lipases or specific only for Lipozyme IM-20.

Alcoholysis of triglycerides. Two different alcohols, glycerol and stearyl alcohol, have been used to transesterify triolein. In both cases, the effect of the ratio of alcohol to triolein has been studied, and the composition of reaction media at equilibrium have been plotted on Figures 6 and 7. As far as glycerolysis is concerned, the conversion of triolein into monoolein and diolein followed much the same pattern as for hydrolysis, when water is replaced by glycerol. A small excess of glycerol, just above stoichiometric amount, was found to be very inhibitory to the reaction. It should be noted that all possible isomers were formed during the reaction (1,2- and 1,3-dioleins). In the case of stearyl alcohol, the main product of the reaction is the ester of oleic acid and stearyl alcohol, stearyl oleate. Excess alcohol was found to be beneficial for the formation of stearyl oleate. The 1,3-specificity of the enzyme is observed in this reaction, only 1,2-diolein could be detected.

We have shown in our work that all three lipase-catalyzed reactions are feasible without organic solvent. This implies that it is possible to work at very high substrate concentrations. Moreover, it was shown that

even without specific means of mixing, a good conversion is obtained, even from non-miscible substrates in a triphasic system. Finally, stoichiometric amounts of substrates are necessary and sufficient to achieve the best conversion.

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