Enzyme and Microbial Technology 82 (2016) 164–172

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Study of microwave effects on the lipase-catalyzed hydrolysis

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ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form 6 October 2015
Accepted 8 October 2015
Available online 22 October 2015

Keywords:
Lipase-catalyzed reaction
Microwave
Mass spectrometry
Triolein
Quantitative analysis

ABSTRACT

The effect of microwave heating on lipase-catalyzed reaction remains controversial. It is not clear whether the reaction rate enhancements are purely due to thermal/heating effects or to non-thermal effects. Therefore, quantitative mass spectrometry was used to conduct accurate kinetic analysis of lipase-catalyzed hydrolysis of triolein by microwave and conventional heating. Commercial lipases from *Candida rugosa* (CRL), *Porcine Pancreas* (PPL), and *Burkholderia cepacia* (BCL) were used. Hydrolysis reactions were performed at various temperatures and pH levels, along with various amounts of buffer and enzymes. Hydrolysis product yields at each time point using an internal-standard method showed no significant difference between microwave and conventional heating conditions when the reaction was carried out at the same temperature. CRL showed optimum catalytic activity at 37 °C, while PPL and BCL had better activities at 50 °C. The phosphate buffer was found to give a better hydrolysis yield than the Tris–HCl buffer. Overall results prove that a non-thermal effect does not exist in microwave-assisted lipase hydrolysis of triolein. Therefore, conventional heating at high temperatures (e.g., 50 °C) can be also used to accelerate hydrolysis reactions.

1. Introduction

Microwave irradiation has emerged as a clean, economic and convenient tool for heating. In synthetic organic chemistry, microwave-assisted reactions afford higher yields and increasing reaction rates in shorter reaction times [1–6]. However, it is not very clear whether the rate enhancements are purely due to thermal/heating effects or to non-thermal effects. The two contradictory viewpoints have been well-summarized in literatures [2,7]. Several researchers believe that the rate of reaction in various syntheses is so high that it cannot be accounted for by the heating effect alone [7,8]. In contrast, other researchers emphasize that there are no general non-thermal effects, and that the so-called non-thermal effect is actually due to the superheating of solvents above their boiling points [9,10]. Nevertheless, this continuing controversy has not limited an increasing number of applications for microwave heating.

The microwave heating technique has also attracted increase attention in the biosciences, such as enzymology for the induction and acceleration of enzymatic digestions [6,11–13], peptide synthesis, and DNA amplification by polymerase chain reaction [14]. Several studies have reported that enzyme activity can be enhanced by using controlled microwave irradiation and suggested that these effects are non-thermal in origin [15]. Tajchakavit and Ramaswamy [16] reported that inactivation of a pectin methyl esterase was faster in the microwave heating mode than in the conventional thermal heating mode, suggesting the presence of some contributory non-thermal effects under microwave radiation. Porcelli et al. [15] also found that microwave radiation caused a non-thermal, irreversible and time-dependent inactivation of thermophilic enzymes, and they suggested that microwave-induced protein structural rearrangements (conformational changes) were not related to temperature. Young et al. [6] have presented evidence that microwave irradiation could activate hyperthermophilic enzymes in aqueous buffer solutions at temperatures far below their optimum conditions. Such a microwave effect was rationalized by suggesting that the microwave induced the conformational flexibility of the enzymes.

Lipases are hydrolytic enzymes which play a vital role in a wide range of industries including detergents, food, leather, textiles, cosmetics, paper, and pharmaceuticals [3,4,17,18]. Microwaves are also used to assist hydrolysis, esterification and transesterification reactions using lipases [4,19–29]. Bradoo et al. [30] used various commercial lipases from *Porcine Pancreas*, *Mucor*

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http://dx.doi.org/10.1016/j.enzmictec.2015.10.003
0141-0229/

miehei, *Candida rugosa*, *Pseudomonas cepacia* as well as lipases from laboratory isolates (*Bacillus stearothermophilus* and *Burkholderia cepacia*) for triolein hydrolysis and the esterification of sucrose/methanol/ascorbic acid with different fatty acids. The hydrolysis rates increased by 7–12 fold and esterification was achieved within 30 s in a microwave oven using lipases. Similarly, hydrolysis of triolein using *Aspergillus carneus* lipase was carried out both under normal conditions and microwave irradiation at low (175 W) and high (800 W) power levels. The complete hydrolysis of triolein took place in 24 h under normal conditions, 160 s at the low power microwave level and 75 s at the high power level [4]. The authors concluded that the microwave treatment dynamically sped up the enzyme catalytic reactions without negatively impacting enzyme properties such as stability and selectivity [4,30]. In these cases, no direct comparisons with reference to conventional heating were provided, because no strict temperature control was applied during microwave irradiation [4,30]. Furthermore, several published results suggest that enzyme activity and product yields are significantly higher under microwave heating than classical thermal heating [21,22,25,26,28,29]. Most such reports demonstrate that microwaves are capable of causing non-thermal effects [2,6,15,29,31]. On the other hand, lipase-catalyzed transesterification reactions have been studied under controlled conditions, with identical reaction rates and product conversions obtained under microwave and conventional heating [23,24].

To clarify the controversy over the effect of microwave on rate enhancements, we have designed an accurate and quantitative approach for the analysis of lipase catalyzed reactions. A critical comparison of conventional and microwave-assisted hydrolysis of triolein was carried out using an internal-standard method [31–33]. The yields of oleic acid (analyte) obtained from the triolein hydrolysis under various conditions including variation of solvents, temperatures, pH values and lipases, with and without microwave irradiation were determined quantitatively using an internal standard (linoleic acid) and mass spectrometry.

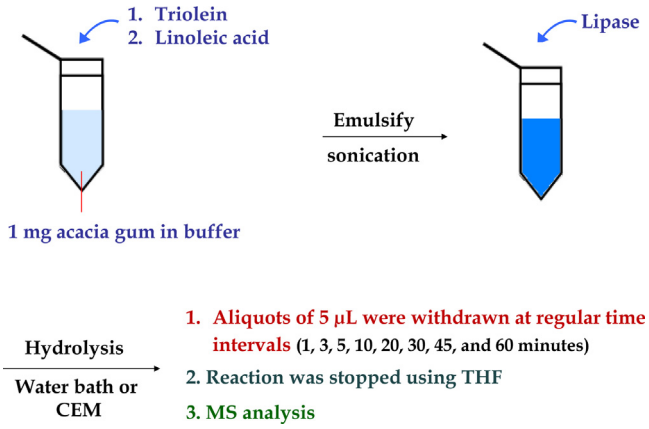
2. Experimental

2.1. Enzymes and chemicals

Lipases from *C. rugosa* (CRL), *Porcine Pancreas* (PPL), and *B. cepacia* (BCL) were purchased from Sigma (St. Louis, MO, USA). The lipases were used without further isolation and their activities determined by the manufacturer were indicated in Supporting information (Table S1). Glyceryl trioleate (triolein, $C_{57}H_{104}O_6$), linoleic acid ($C_{18}H_{32}O_2$), acacia gum and dihydroxybenzoic acid were purchased from Sigma (St. Louis, MO, USA). Oleic acid ($C_{18}H_{34}O_2$), potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Tris–HCl (pH 7.5) was obtained from Affymetrix (Cleveland, OH, USA). Tetrahydrofuran (THF) was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). All the reagents were of analytical grade and used as received. Ultrapure Milli-Q water was produced in the laboratory with a Millipore system (Bedford, MA, USA).

2.2. Conventional/microwave-assisted hydrolysis of triolein

The hydrolysis of triolein was carried out under conventional heating and microwave irradiation. The conventional heating experiments were performed in a water bath with reaction temperature internally measured using a fiber-optic thermometer. For microwave experiments we used a monomode CEM apparatus, equipped with a fiber-optic probe sensor. The quantities of reactant and enzyme in the microwave-assisted reaction were identical to those used in the reaction under conventional heating. Because the heating rates for conventional and microwave experiments differ, the reaction mixtures without the lipase were heated to the desired temperature. The lipase was added to the reaction vessels to initiate the hydrolysis after the reaction temperature was stabilized.



Scheme 1. General experimental procedures for conventional or microwave-assisted hydrolysis of triolein, followed by MS characterization.

2.3. Triolein quantification by MS

In quantification experiments, 10 μL triolein (density 0.91 g/cm^3) and 10 μL of linoleic acid (density 0.914 g/cm^3, as internal standard for quantification) were dissolved in 200 μL of buffer solution containing 1 mg acacia gum using sonication. A known quantity of the lipase (0.05–0.3 mg of CRL, 0.3–0.7 mg of BCL or 10–20 mg of PPL) was then added to initiate the reaction. Aliquots of 5 μL were withdrawn at various times up to 1 h (1, 3, 5, 10, 20, 30, 45, and 60 min) and the reactions in the withdrawn solutions were terminated using 15 μL THF. A volume of 0.5 μL was applied on a sample plate and the products formed were analyzed using laser desorption ionization mass spectrometry (LDI-MS). The sample solutions for the calibration curves were prepared by mixing the stock of oleic acid in different volumes (i.e., 0.5, 1.0, 2.5, 5.0, 7.5, 10, and 15 μL) with 10 μL of linoleic acid (internal standard) in 200 μL of buffer solution containing 1 mg acacia gum using sonication. Each sample (5 μL) was then mixed with 15 μL THF. A volume of 0.5 μL was applied on a sample plate and the peak ratios between oleic acid and linoleic acid were determined using LDI-MS. A calibration curve was constructed by plotting the peak ratios of the oleic acid and linoleic acid as a function of their mole ratios. The amount of oleic acid produced through triolein hydrolysis was determined using the calibration curves. The procedures for the conventional or microwave-assisted hydrolysis of triolein are summarized in Scheme 1.

2.4. Instrumentation

All the mass spectra were obtained in the negative ion mode using an Autoflex time-of-flight mass spectrometer (Bruker Daltonic, Bremen, Germany) equipped with a 337-nm nitrogen laser. The mass spectrometer was operated in the reflectron mode with an acceleration voltage of −19.2 kV (reverse potential of −18.2 kV). Each mass spectrum was derived from 100 summed scans. The laser energy was carefully adjusted (maximum energy of 120 μJ/pulse, frequency of 6.7 Hz/s) to obtain MS spectra with the best S/N ratio and the smallest interference. All experiments were performed in triplicate. Flex Control (v2.0) and Flex Analysis (v2.4) softwares were used for data acquisition. All microwave irradiation experiments described herein were performed using a CEM Discover