Enzymatic Synthesis of Fatty Hydroxamic Acids from Palm Oil

Dedy SUHENDRA^{1*,2}, W.M.Z. Wan YUNUS¹, Md. Jelas HARON¹, Mahiran BASRI¹,
and Sidik SILONG¹

¹ Department of Chemistry, Faculty of Science and Environmental Studies Universiti Putra Malaysia
(43400 UPM Serdang Selangor, MALAYSIA)

² Department of Chemistry, FKIP Universitas Mataram NTB Indonesia
(Jl. Majapahit 62 Mataram 83125)

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Abstract: Fatty hydroxamic acids (FHA) have been successfully synthesized from palm oils by a one-step lipase catalyzed reaction. The use of immobilized lipase as the catalyst for the preparation reaction provides an easy isolation of the enzyme from the products and other components in the reaction mixture. In addition, it also allows the reaction to be carried out under mild conditions, which reduces the reaction's side products. The percentages of conversion of commercial palm olein (CPOn), RBD (refined, bleached and deodorized) palm olein (POn), RBD palm oil (PO), RBD palm stearin (PS) and RBD palm kernel olein (PKOn) into their fatty hydroxamic acids are 89, 79, 77, 90 and 98, respectively.

Key words: enzymatic synthesis, fatty hydroxamic acid, lipase, palm oil

1 Introduction

Hydroxamic acids (*N*-hydroxy carboxylic amides) may be regarded as derivatives of both hydroxylamine and carboxylic acids. The general formula of hydroxamic acid is R-CO-NHOH. In recent years, hydroxamic acids derivatives have received a lot of attention due to their biological activity as growth factors, food additives, antibiotics, antifungal agents, cell division factor and metal chelators (1), enzyme inhibitors (2), matrix metalloproteinase (MMP) inhibitor (3-5) and tumor inhibitors (6). Their complexes with various metal ions have been used for analytical chemistry as reagents for gravimetric and spectrometric metal determination (7), as collector for rare earth mineral (8), and for metal ions extraction from aqueous media (9). Simple and polymeric hydroxamic acids have also been extensively investigated for several successful analytical separations of metal ions. Besides their ability to form complex with metal ions, long-chain hydroxamic acids, by modi-

fication of the terminal OH group, are of considerable interest as efficient surfactants in the detergent industry (10).

Hydroxamic acids, in general, have been synthesized by adopting Blatt's procedure (11). In this method, the reaction of an alkyl or aryl ester (RCOOEt) with hydroxylamine in the presence of alkali and the free acid is obtained by the addition of acid in the appropriate quantity in cold solution. However, this chemical method, which is in alkali system, could evoke decomposition or degradation of the desired products due to presence of double bond.

An enzymatic route for the synthesis of hydroxamic acid, with the inherent advantages of selectivity, mild reaction conditions and enantioselection, is of considerable synthetic interest. The synthesis of alkanohydroxamic acids (*N*-acyl hydroxylamines) by *Rhizomucor miehei* lipase-catalyzed hydroxylaminolysis of fatty acids and soybean methyl ester was reported (12). Later, *Candida parapsilopsis* was also found to mediate

*Correspondence to: Dedy SUHENDRA, Department of Chemistry, Faculty of Science and Environmental Studies Universiti Putra Malaysia
43400 UPM Serdang Selangor Malaysia
E-mail: dedysuhendra@hotmail.com; fi_hendra@yahoo.com

this reaction (13, 14).

In this work we have investigated the use of an immobilized lipase as biocatalyst for hydroxylaminolysis of palm oil. This work focuses on the lipase-catalyzed synthesis of FHA from commercial palm olein. Our literature search indicates that this is first report of FHA synthesis directly from oil. The advantages of this method include ease of preparation reaction and product isolation, and commercially available of reactants and catalyst.

2 Materials and Methods

2.1 Materials

All the chemicals used were of analytical grade: hydroxylamine hydrochloride and sodium hydroxide were from Fluka (Switzerland). Methanol and hexane were from T.J. Beaker (USA). The immobilized lipase used was the product from Novo Nordisk (Denmark). RBD palm oil, RBD palm olein, RBD palm Stearin and RBD palm kernel olein were supplied by Southern Edible Oil Industry (M) Sdn. Bhd. (Malaysia). Meanwhile, the commercial palm olein was obtained from Ngo Chew Hong Oils and Fats (M) Sdn. Bhd. Malaysia.

2.2 Hydroxylaminolysis

The reaction was carried out by reacting a required amount of commercial palm olein with hydroxylamine in the present Lipozyme in a 100 mL stoppered flask. The mixture was incubated in water shaker batch at 100 rpm. The FHA formed in water-hexane interface was then separated from the water and the lipase by filtration. To obtain the solid FHA, the hexane fraction was cooled in a refrigerator ($< -5^{\circ}\text{C}$) for 5 h and then filtered and washed by hexane for several times and dried in vacuum desiccator over phosphorous pentoxide for 24 h.

2.3 Characterization

Qualitative analysis of hydroxamic acid groups on FHA was carried out by observing the colored complex formed after the methanolic solution of the FHA reacted with iron (III), copper (II) and vanadium (V) in a dilute hydrochloric acid solution. The amount of the hydroxamic acid group was estimated based on nitrogen content of the dry FHA determined by Elemental analyzer model 932 (LECO, USA). FTIR spectra of the products were recorded with Fourier Transform

Infrared Spectrophotometer (Perkin Elmer FTIR-Spectrum BX, USA) using KBr pellets. The High-Performance Liquid Chromatography (HPLC) analyses were performed according to the procedure of Gutnikov and Streng (15) with modifications using liquid chromatograph (Waters HPLC, USA) equipped with Waters Delta-600 pump, Waters 600-controller, Waters-2487 Dual λ Absorbance Detector and Shimadzu CTO-6A column oven. Separations were performed on a reversed-phase column (3.9×150 mm) obtained from Alltech Assoc. (Deerfield, IL, USA) packed with Nucleosil C₁₈, 5 μm particle size. Column temperature was maintained at 40°C . The absorbance was monitored at 213 nm, and the detector signals were fed to a Waters-2487 integrator. Samples were injected via a Waters-600 rotary injector valve fitted with a 10 μl loop. Analyses were performed using a methanol-aqueous buffer solvent system (80:20) at a flow rate 1.0 mL/min. The aqueous buffer was prepared as 20 mM sodium dihydrogenphosphate adjusted to pH 3 with concentrated phosphoric acid. A 125- μl volume of 1% (w/w) sodium nitrate per liter was added to match the absorbance of methanol at 213 nm.

3 Results and Discussion

3.1 Effect of Reaction Period

The time course is a good indicator of enzyme performance and reaction progress. It can pinpoint the shortest or adequate time necessary to obtain good yields and minimize process expenses (16). The results (Fig. 1) show that enzymatic reaction proceeded rapidly in the first 30 h. Although the maximum yield is obtained at the reaction period at around 50 h, the rate of the reaction after 30 h is small. The decrease in the reaction rate may be due to; (i) some mass-transfer limitations, which inevitably arise in a reaction mixture containing a high proportion of solid product, and (ii) the reaction reaches the equilibrium state where the rate of forward reaction was equal to the backward reaction. However, this result is faster compared with the result of Hacking *et al.*, (2000) (17), which shows that the biosynthesis of octanohydroxamic acid reaction was reaches the maximum conversion at 36 h.

3.2 Effect of Organic Solvent

Two important properties of a solvent to be used in biocatalysis are its ability to solubilize the substrate and

Fig. 1 Effect of Reaction Period on Hydroxylaminolysis of FHA. Reaction conditions: NH_2OH 10 mmol; commercial palm olein 2 g; Lipozyme 0.05 g; hexane volume 15 mL; temperature 40°C ; shaking rate 150 rpm.

the products of the reaction, and its influence on enzyme activity and stability. In addition, a solvent should also have the ability of portioning the substrates and products into different phases (18). Several organic solvent, which are widely used as solvent for palm oil were investigated for the study of the effect of organic solvent on the hydroxylaminolysis of palm oil. The results (**Table 1**) show that hexane is the best solvent for hydroxylaminolysis of the commercial palm olein. Therefore, hexane was used as solvent in the subsequent experiments.

3.3 Effect Amount of Enzyme

From an applied point of view, the substrate concentration should be as high as possible to obtain a higher degree of yield. Simultaneously, the amount of enzyme used should be as low as possible to obtain the desired result. In terms of production cost, the impact of the amount of lipase used is crucial.

Figure 2 shows that the reaction yield increases as the ratio of lipase: substrate is increased. The highest production of FHA is obtained when the ratio of lipase: substrate is 29.5 mg to 1 mmol. Further increase of lipase to substrate ratio does not significantly increase the yield. This behavior of leveling off of the reaction at higher enzyme concentrations can be explained by con-

Table 1 Effect of Solvent on Synthesis of FHA.

Solvent	Yield (mg)
Heptane	366
Hexane	270
Cyclohexane	201
Chloroform	86

Reaction conditions: NH_2OH 10 mmol; commercial palm olein 2 g; Lipase 0.05 g; hexane volume 15 mL; temperature 40°C ; shaking rate 150 rpm; reaction period 24 h.

sidering that the active sites of the enzyme molecules present in excess would not be exposed to the substrates and remain inside the bulk of enzyme particles without contributing significantly to the reaction (19).

3.4 Effect of Concentration of Hydroxylamine

The maximum mole ratio of substrates used in the preparation reaction is a challenge to be met in industrial implementation of any reaction (20). **Figure 3** shows that the yield is highest when the ratio of the hydroxylamine and the commercial palm olein was at 6 mmol to 1 mmol. This result is in agreement with Servat *et al.*

Fig. 2 Effect of Amount of Lipase on Biosynthesis of FHA; Reaction conditions: substrate (commercial palm olein) 2 g, temperature 40°C , hexane volume 15 mL, shaking rate 150 rpm, reaction period 24 h.

(12) that shows the reaction yield increases with the increase in the concentration of hydroxylamine. However, increasing further the concentration of hydroxylamine decreases the reaction yield. This phenomenon was in agreement with the results of Vaysse *et al.* (14), which shows that high concentrations of hydroxylamine were inhibitory to the reaction.

3.5 Scaling up the Reaction

The reactions were scaled-up (50 times) by using the optimum condition presented in **Table 2**. The percentages of conversion of hydroxylaminolysis of palm oils are presented in **Fig. 4**. The Figure shows that there is slight difference in the reaction yield of hydroxylaminolysis among various products of palm oil with the percentages of conversion being in the range of 77-98.

3.6 Characterization of FHA

Complexes of the FHA with vanadium, iron (III) and copper (II) are purple, dark red and green, respectively. These are the common color of the complexes observed when these metal ions are reacted with hydroxamic acids (21). FTIR spectrum of substrate (commercial palm olein, **Fig. 5a**) shows the characteristic absorption bands at 2848 cm^{-1} and 2916 cm^{-1} , which correspond to

Fig. 3 Effect of Concentration of Hydroxylamine on Biosynthesis of FHA; Reaction conditions: substrate (commercial palm olein) 2 g, Lipozyme 0.05 g, hexane volume 15 mL, shaking rate 150 rpm, reaction period 24 h

Table 2 Optimum Condition of Hydroxylaminolysis of Palm Oils.

Parameters	Condition
Lipase	Lipozyme
Solvent	Hexane
Reaction Period	30 h
Temperature	30°C
Ratio Lipozyme (mg): Substrate (mmol)	29.5: 1
Ratio Hydroxylamine (mmol): Substrate (mmol)	6: 1

C-H stretching of long alkyl chain. These absorption bands were supported by peak at 1452 cm^{-1} that corresponds to C-H bending. Additional characteristic absorption band of palm oil appear at 1740 cm^{-1} corresponding to C=O stretching. FTIR spectrum of the product (**Fig. 5b**) shows the characteristic absorption bands of hydroxamic acid at 3426 cm^{-1} and 3264 cm^{-1} , which corresponds to N-H and OH stretching respectively. These absorption bands are typical bands for amide (14). Additional characteristic absorption bands of hydroxamic acid appear at 1662 cm^{-1} that correspond to C=O in secondary amide. Fig. 4b also shows the characteristic absorption bands at 2848 cm^{-1} and 2916 cm^{-1} , which correspond to C-H stretching of long alkyl chain. Unsaturated of FHA was shown at $3010 - 3095\text{ cm}^{-1}$ that corresponds to C-H alkenes (C=C-H) and $1610 - 1680\text{ cm}^{-1}$ that corresponds to alkenes (C=C)

Fig. 4 Reaction Yield of Hydroxylaminolysis of Palm Oils. Yield (%) = (mmol product/mmol fatty acid in oil) $\times$ 100.

Fig. 5 FTIR Spectrum of (a) Commercial Palm Olein and (b) FHA from Commercial Palm Olein.

(22). Both qualitative tests support that the FHA is successfully produced from the commercial palm olein. The nitrogen content of the dry FHA analyzed by the elemental analysis is found to be 4.93%. This indicates that there was 3.52 mmol/g of hydroxamic acid groups in one gram of the FHA. HPLC analyses of the product shows that the FHA was actually a mixture of lauryl-, myristyl-, palmityl-, stearyl-, oleyl- and linoleyl-hydroxamic acid, as palm olein consist of lauric-(0.5%), myristic-(1.2%), palmitic-(38.5%), stearic-(4.4%), oleic-(41%) and linoleic acids (12.1%) (23).

4 Conclusion

The enzymatic synthesis method of fatty hydroxamic acids (FHA) described in this paper is an effective method for preparing FHA from palm oil and suggested that it could be applicable to a large-scale production of FHA. The method employed offers technical simplicity, easy isolation of the enzyme from the products and other components in the reaction mixture. Moreover, it also allows to be carried out under mild conditions, which reduce unwanted products from side-reactions. The percentages of conversion of commercial palm olein, RBD palm olein, RBD palm oil, RBD palm stearin and RBD palm kernel olein into their fatty hydroxamic acids are 89, 79, 77, 90 and 98, respectively.

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