JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Interesterification of Olive Oil with a Fully Hydrogenated Fat in a Batch Reactor Using Step Changes in Temperature

IN-HWAN KIM,*,† SUN-MI LEE,† BO-MI LEE,† HYE-KYUNG PARK,‡ JEE-YOUNG KIM,‡ KWANG-IL KWON,‡ JONG-WOOK KIM,‡ JEE-SUN LEE,‡ AND YANG-HA KIM§

Department of Food & Nutrition, Korea University, Seoul, 136-703, Nutritional Evaluation Team, Korea Food & Drug Administration, Seoul, 122-704, and Department of Food & Nutrition, Ehwa Women's University, Seoul, 120-749, Republic of Korea

Interesterification of a 60:40 (wt/wt) mixture of olive oil and fully hydrogenated canola oil was carried out in a batch reactor using a commercial immobilized lipase from *Thermomyces lanuginose* as a biocatalyst. The effects of a stepwise change of temperature on the degree of conversion, the solid fat content (SFC) of the products, and the residual activity of the enzyme were investigated. As a reference condition, an interesterification trial was conducted at a constant temperature of 70 °C for 48 h. For trials in which a temperature of 70 °C was used for the first 4 h of reaction and a temperature of 60 °C was employed for the following 44 h, there were no significant differences ($p < 0.05$) in the overall degree of conversion relative to the reference condition. Oils interesterified for only 1 or 2 h at 70 °C had melting points higher than 60 °C, whereas an oil produced by interesterification at 70 °C for only 4 h had a melting point of 58 °C. There was little difference ($p < 0.05$) between the SFC profiles of the interesterification products prepared by two different temperature protocols (70 °C for 24 h; 70 °C for 4 h followed by 60 °C for 20 h). Use of the protocol involving a step decrease in temperature significantly decreased catalyst deactivation effects, thereby increasing the residual activity of the immobilized lipase.

KEYWORDS: Fully hydrogenated canola oil; interesterfication; lipase; melting point; olive oil; residual activity; solid fat content

INTRODUCTION

Margarines and/or other plastic fats have traditionally been produced by partial hydrogenation of vegetable oils (*1*). However, partial hydrogenation of the fatty acid residues present in these oils also leads to the formation of geometric isomers with a *trans*-configuration rather than the *cis*-configuration typical of the unsaturated fatty acid residues present in naturally occurring vegetable oils (*2, 3*). Because several recent nutritional studies have suggested a direct relationship between *trans*-fatty acid consumption and increased risk for coronary heart disease (*4–6*), the edible oils industry has been vigorously investigating the possibility of producing *trans*-free products by interesterification of physical blends of oils and fats. Interesterification reactions between a fat and an oil involve the exchange of fatty acid residues between the precursor acylglycerols and are accompanied by a concomitant change in the properties of the physical mixture of the precursors (*7*). The properties of the final product are primarily governed by an appropriate choice of the precursor reagents, their respective proportions in the starting mixture, and the time that the reactants are in contact with a catalyst. Interesterification can be carried out either chemically or enzymatically. Enzymatic interesterification catalyzed by lipases offers several advantages relative to chemical interesterification. These advantages include milder reaction conditions as well as the fact that no further chemicals additions are needed. The use of 1,3-specific lipases gives a more selective reaction that is easy to control. These enzymatic reactions are accompanied by fewer side reactions than the corresponding reaction in the presence of an acid or a base catalyst; hence, there are fewer losses of the oil, and downstream processing is facilitated (*8*). In addition, in the lipase-catalyzed process, one can more easily control the degree of conversion necessary to produce an optimum product distribution of fatty acid residues on the glycerol backbone of the triacylglycerol (TAG) so as to obtain the desired physical properties (*9–11*). By contrast, chemical interesterification usually leads to a completely random distribution of residues on the backbone.

* To whom correspondence should be addressed. Tel: +822-940-2855. Fax: +822-941-7825. E-mail: k610in@korea.ac.kr.
† Korea University.
‡ Korea Food & Drug Administration.
§ Ehwa Women's University.

10.1021/jf8007585 CCC: $40.75
Published on Web 06/24/2008

These processes occur very rapidly so that it is difficult to stop the interesterification at an intermediate stage where more desirable properties might be obtained (*12*).

Palm stearins have been used as solid substrates to produce semisolid fats; some of the oils used for the exchange of acyl groups with palm stearin are coconut oil, sunflower oil, and palm kernel oil (*13–15*). Fully hydrogenated vegetable oils have also been employed both for production of fats with low to zero *trans*-fatty acid residue content and properties similar to those of partially hydrogenated fats and for production of structured lipids with low caloric contents (*16–19*).

In this study, olive oil and fully hydrogenated canola oil (FHCO) were enzymatically interesterified using a commercial immobilized lipase preparation from *Thermomyces lanuginosus* (lipozyme TL IM). The goal of this research has been to study the effects of step changes in the reaction temperature on the degree of interesterfication. The substrate consisted of a 60:40 (wt/wt) ratio of olive oil to FHCO. Four different temperature protocols were employed, namely, reaction at a constant temperature of 70 °C for 48 h and reaction at 70 °C for 1, 2, and 4 h followed by reaction at 60 °C for an additional 47, 46, and 44 h, respectively. For these trials, the degree of conversion was defined as the extent of depletion of the tristearin concentration in the reaction mixture. The melting points and solid fat contents (SFC) of the semisolid product mixtures were determined by a capillary tube method and pulsed-field NMR spectroscopy, respectively. Residual activities of the enzymes employed in trials involving different temperature protocols were also investigated.

MATERIALS AND METHODS

Materials. Olive oil and FHCO were donated from Lotte Samgang (Cheonan-Shi, Korea). Lipozyme TL IM, which is an immobilized lipase preparation from *T. lanuginose* lipase that is supported on silica granules, was supplied by Novo Nordisk Bioindustry Ltd. (Seoul, Korea). All solvents used were high-performance liquid chromatography grade (Fisher Scientific-Korea, Seoul, Korea). The TAG standards used for the gas chromatography (GC) analyses were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

Enzymatic Interesterification. All reactions were carried out in triplicate in 50 mL stoppered flasks using an enzyme loading of 1% of the total weight of substrates. A weight ratio (60:40) of olive oil to FHCO was used as the starting material for the interesterification reaction. Ten grams of this fat mixture was placed in each flask. The reaction mixture was agitated continuously using an orbital shaker water bath (model Innova 3100; New Brunswick Scientific Co. Inc., New Brunswick, NJ) operating at 300 rpm. Individual samples were removed at selected times and analyzed. The reactions were carried out either at a constant temperature of 70 °C for times up to 48 h or using protocols in which the reactions were initiated at 70 °C for short periods (1, 2, and 4 h) and then carried out at 60 °C for an additional 47, 46, and 44 h, respectively (for trials involving a change in temperature, the total time for reaction was 48 h). Prior to reaction, the water activity (a_w) of the lipozyme TL IM was adjusted to a desired level by conditioning it for at least 24 h in a constant humidity chamber containing a saturated solution of potassium acetate ($a_w = 0.2$).

Analyses of TAG by GC. Samples (20 mg) corresponding to different reaction times were dissolved in chloroform (1 mL) together with 5 mg of tripentadecanoin (an internal standard). A gas chromatograph (model 3800; Varian, Palo Alto, CA) equipped with a DB-17ht column (15 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) and flame ionization detector was used in the analysis. Initially, the column was held at 150 °C for 1 min and programmed to rise to 365 °C at a rate of 10.0 °C/min. The column was then held at 365 °C for 20 min. The carrier gas was helium, and the total gas flow rate was 50 mL/min. The injector and detector temperatures were 375 and 380 °C, respectively.

Stability of the Biocatalyst. For each type of temperature protocol employed, the residual activities of the corresponding biocatalysts were determined after each cycle of use. The first protocol employed in the stability trials involved interesterification at a constant temperature of 70 °C for 24 h. The second protocol used in the stability trials consisted of interesterification at 70 °C for the initial 4 h followed by reaction at 60 °C for an additional 20 h to obtain a constant total reaction time of 24 h.

To change the reaction temperature from 70 to 60 °C, the water in the shaking water bath was replaced by cold tap water. The temperature in the shaking water bath changed within 3 min. After each cycle, the immobilized enzymes were isolated from the reaction mixture by filtration. Then, 10 mL of chloroform was added to the stoppered flask to dissolve and remove the semisolid fat products. Then, the immobilized enzyme was recovered by filtration and dried under vacuum. To readjust the water activity of the enzyme to the desired level prior to subsequent use, the dried enzymes were conditioned for 24 h in a constant humidity chamber containing a saturated solution of potassium acetate (a_w, 0.2) before each reuse. The recovered enzyme was then reused multiple times in several subsequent trials employing the same temperature protocol. The residual activity of the enzyme (in percent) after each cycle was calculated on the basis of the consumption of tristearin relative to its consumption in the initial trial.

Melting Point. Melting points were determined in accordance with the AOCS *Cc* 3−-25 open tube melting point method (*20*). Capillary tubes with a 1 cm high column of fat were chilled at 6 ± 1 °C for 16 h before being immersed in a beaker of boiled distilled water. The water bath was stirred and heated, and the temperature was noted where the column of fat rises in the tube.

Determination of SFC. SFC values for the interesterified products were determined using a Bruker Minispec Solid Fat Analyzer (Bruker, Canada). NMR tubes (10 mm in diameter) were filled with 1 mL of fat mixture and capped. Tempering pretreatment of all samples was carried out using IUPAC Method 2.150 (*21*). SFC values were determined at 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C.

Statistical Analysis. If not otherwise specified, experiments were carried out at least in triplicate. The Statistical Analysis Systems was used to analyze data (*22*). Significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

An enzyme's thermostability is a major consideration for its industrial use, primarily because of the necessity for minimizing the thermal degradation of the enzyme's activity. The temperature at which a reaction is carried out can affect a variety of reaction parameters such as enzyme activity, selectivity, and stability, as well as the affinity of the enzyme for the substrate (*23*). In this study, the interesterification reactions were conducted using either a constant temperature (70 °C) protocol or a stepwise procedure involving two different temperatures, namely, 60 and 70 °C. The trials at constant temperature were used as a reference condition; trials were conducted at 70 °C for 48 h. For subsequent experimental trials, interesterification was first carried out at 70 °C for periods of 1, 2, and 4 h from initiation of the reaction, while the remainder of the reaction was carried out at 60 °C for an additional 47, 46, and 44 h to obtain a constant total time of reaction equal to 48 h. For these trials, the enzyme loading was held constant at 1% of the total weight of substrate. The time course of the reaction was studied for each temperature protocol. Samples taken at selected times were analyzed to monitor changes in the degree of conversion (see **Figure 1**). Depletion of the primary TAG species present in the original substrates, specifically tristearin (SSS), has previously been used as a direct indicator of the extent of interesterification (*24*). Degrees of conversion are calculated as the ratio of the depletion in the concentration of tristearin relative to its corresponding initial concentration: conversion $= 100 \times \{[(SSS)_0 - (SSS)]/(SSS)_0\}$ where the conversion is expressed

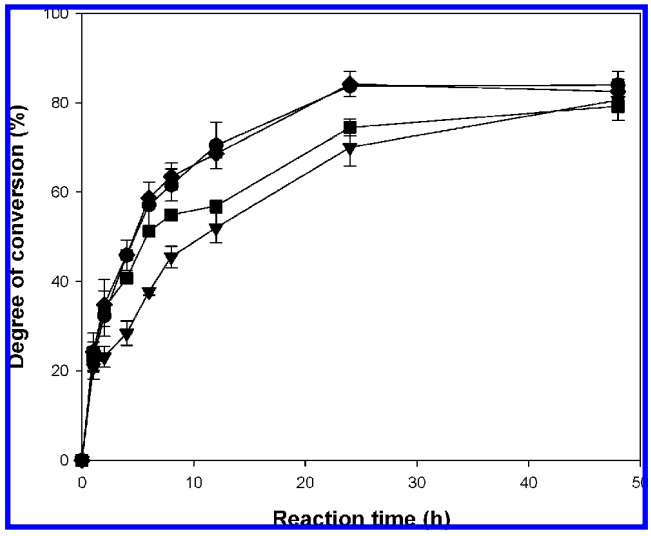

Figure 1. Effect of a step decrease in reaction temperature during interesterification of olive oil and FHCO. Reaction conditions: 6 g of olive oil, 4 g of FHCO, and 0.05 g of lipozyme TLIM (1% of substrate) were incubated for 48 h in an orbital shaker operating at 300 rpm and 70 °C or combinations of 70 and 60 °C (●, at 70 °C for 48 h; ▼, at 70 °C for 1 h and then 60 °C for 47 h; ■, at 70 °C for 2 h and then 60 °C for 46 h; and □, at 70 °C for 4 h and then 60 °C for 44 h).

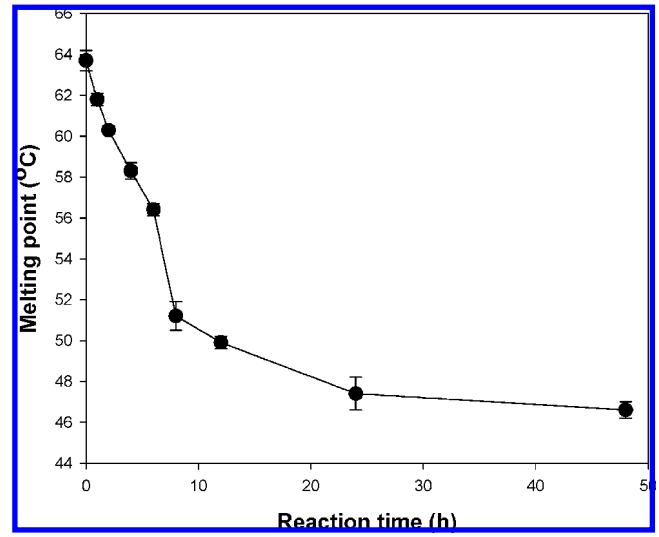

Figure 2. Effect of reaction time on the melting points of products of interesterification at 70 °C for 48 h. Reaction conditions: 6 g of olive oil, 4 g of FHCO, and 0.05 g of lipozyme TLIM (1% of substrate) were incubated in an orbital shaker operating at 300 rpm.

in percent and where (SSS) and $(SSS)_0$ refer to the concentrations of tristearin in the product mixture (mg/g) at times t and zero, respectively.

For the reference trials in which the reaction temperature was held constant at 70 °C for 48 h, the degree of conversion increased rapidly in the first 6 h (**Figure 1**). During the next 18 h, there was a slow but steady increase in the degree of conversion. However, for times greater than 24 h, further increases in degree of conversion were negligible. Hence, the time required to approach reaction equilibrium for this temperature protocol was essentially 24 h. For those trials in which the temperature was 70 °C for the first 1 h of reaction and then decreased to 60 °C, subsequent degrees of conversion during the next 23 h were significantly ($p < 0.05$) lower than the corresponding conversions for the reference trial. However, when the total reaction time was further increased to 48 h, there was no significant difference ($p < 0.05$) between the degrees of conversion for the reference trial and the trial involving a temperature decrease after 1 h. For the trials in which the initial reaction temperature of 70 °C was employed for 2 h, followed by a decrease to 60 °C for the subsequent 46 h, similar trends were observed in the conversion relative to those observed when the temperature decrease was imposed after 1 h. The observed overall degrees of conversion were higher than those in trial involving only 1 h at 70 °C. However, for those trials in which the initial temperature of 70 °C was maintained for 4 h, there were no significant differences ($p < 0.05$) in the degrees of conversion relative to those for the constant temperature reference trial.

For the trial involving a constant temperature of 70 °C for 48 h, samples were taken at selected times and subjected to analysis to determine any corresponding changes in the melting point of the semisolid product. Inspection of **Figure 2** reveals that as the reaction time increases to 8 h, significant decreases are observed in the melting point. Less marked changes occur as the reaction time increases to 24 h, but further increases in reaction time do not markedly affect the melting point. To conduct the interesterification reaction effectively, the temperature needs to be above the melting point of the substrate (*25*). However, in our work, the melting points of the mixtures interesterified for 1 and 2 h at 70 °C exceeded 60 °C, whereas the melting point of a sample that was interesterified at 70 °C for 4 h was 58 °C. These results suggest that the rate of interesterification reaction may have been retarded by precipitation of solids on the surface of the immobilized enzyme when the reaction temperature was lower than the melting point of the reaction mixture or substrate. Moreover, Rønnte et al. (*26*) have reported that for the interesterification of rapeseed oil and butterfat in the presence of lipozyme TL IM, an increase in temperature from 60 to 70 °C had minimal effect on the observed degree on conversion, all other conditions for the continuous flow reactor being held constant. Our results also showed that no significant differences ($p < 0.05$) were observed in conversion between a reaction held at a constant temperature of 70 °C for 48 h and stepwise variation of the temperature from 70 °C for 4 h followed by 60 °C for 44 h. These results should make it possible to employ a lower reaction temperature after the first 4 h of reaction, thereby decreasing the possibility of thermal deactivation of the enzyme and producing a corresponding reduction in energy requirements for the process. The period of reduced temperature may also decrease the effects of undesirable side reactions such as hydrolysis and oxidation. Consequently, 24 h was selected as the optimum total reaction time for two different temperature protocols employed, namely, operation at a constant temperature of 70 °C for 24 h or initiation of the reaction at 70 °C for 4 h followed by reaction at 60 °C to bring the total reaction time to 24 h.

The SFC profile of a specific mixture of TAG is primarily responsible for many of the important functional properties of fats, such as physical appearance, sensory properties, melting and softening points, and spreadability (*27, 28*). SFC values of two interesterification products prepared using the two temperature protocols described earlier, as well as that of a physical blend of the starting materials, were determined (see **Figure 3**). The first interesterification product was for 24 h of reaction at a constant temperature of 70 °C. For the second product, the reaction was initially carried out at 70 °C for 4 h and subsequently at 60 °C for 20 h. The SFC values of these two products decreased sharply as the temperature of each sample was increased from 25 to 65 °C. By contrast, the SFC value of the physical mixture decreased much more gradually as the

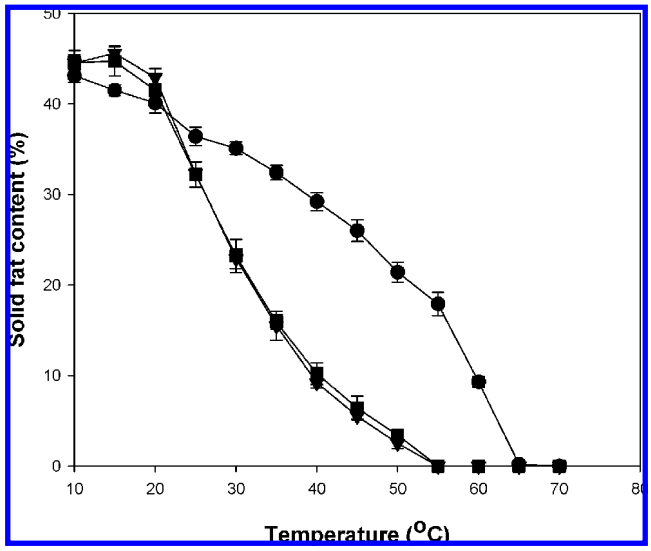

Figure 3. SFCs of products of interesterification of olive oil and FHCO at 70 °C or at a combination of temperature of 60 and 70 °C for 24 h. Other reaction conditions were the same as those for the trials in **Figure 1** (●, physical blend; ▼, at 70 °C for 24 h; and ■, at 70 °C for 4 h and then 60 °C for 20 h).

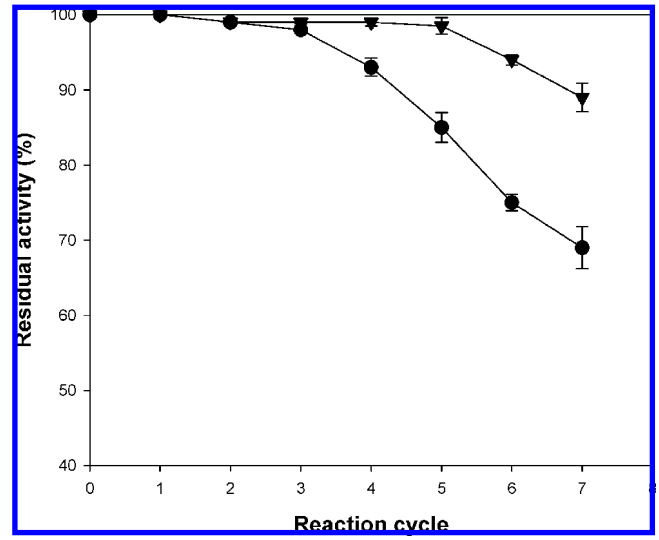

Figure 4. Residual activity of enzyme after use in several consecutive cycles of the interesterification of olive oil and FHCO (60:40, wt/wt). The reaction conditions were the same as those for the trials in **Figure 3** (●, at 70 °C for 24 h; and ▼, at 70 °C for 4 h and then 60 °C for 20 h).

temperature was increased from 25 to 65 °C. However, for the interesterified products, the SFC values at 10 and 15 °C were slightly higher than that of the physical blend. A similar result was obtained by Zhang et al. (*29*) for the enzymatic interesterification of palm stearin with coconut oil. They explained their results that after interesterification, the fatty acid residues at the *sn*-1,3 positions in the two substrates had been exchanged to produce new TAG species. The new species synthesized in the interesterification reaction could affect the SFC profile of the product at both high and low temperatures.

The stability of enzyme is another important characteristic of biocatalysts used in industrial applications. During use, enzymes can undergo deactivation due to the effects of several different factors, including high temperatures, shear stresses, and exposure to interfaces. Thermal deactivation of enzymes usually occurs as a result of unfolding of the protein molecule. At elevated temperatures, the various forces maintaining the structure of these molecules (hydrogen bonding, ionic and van der Waals interactions, hydrophobic interactions, etc.) diminish in importance relative to the thermal forces responsible for the various modes of molecular motion (translation, rotation, and vibration) and the attendant collisions, thereby leading to unfolding of the enzyme. Consequently, we determined the residual activities of the same batch of immobilized lipases following use in a series of trials (**Figure 4**). The stabilities of two different batches of enzyme were investigated. For the first batch of immobilized enzyme, the interesterification reactions were carried out at a constant temperature of 70 °C for 24 h. For the second batch, the reactions were initiated at 70 °C, and this temperature was maintained for 4 h; the temperature was then changed to 60 °C for the remaining 20 h of reaction. The other experimental conditions for these stability trials were an enzyme loading of 1% of the total weight of substrates and agitation of the shaker at 300 rpm. The reaction time employed for each cycle was 24 h. Seven cycles were conducted for each trial involving a different temperature protocol. For both batches of enzyme, no significant reductions ($p < 0.05$) in residual activity were observed during the first three reaction/cleaning cycles. However, for batch employed in the constant temperature (70 °C) trial, significant decreases ($p < 0.05$) in the residual activity were observed, beginning with the fourth cycle. For this batch of biocatalyst, the activity of enzyme was maintained at ca. 85% of the original activity for up to five cycles, after which a more marked decline in activity is observed. By contrast, the batch of enzyme employed in the trials involving a temperature decrease after 4 h of reaction retained ca. 90% of its original activity for seven cycles. This result implies that the costs of the enzyme per unit of product will be significantly lower if this protocol is employed in industrial situations.

In summary, the use of a step decrease in temperature part way through the lipase-catalyzed interesterification of olive oil and FHCO has minimal effect on either the conversion achieved or the SFC profile of the interesterified products. Moreover, use of this procedure leads to a significant increase in enzyme stability, will reduce energy requirements for the process, and reduce the potential for lipid oxidation with adverse effects on product quality.

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Received for review March 12, 2008. Revised manuscript received May 11, 2008. Accepted May 12, 2008. This work was supported by the Korea Food & Drug Administration (KFDA).

JF8007585