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Kinetics of lipase-catalyzed de-acidification of degummed rapeseed oil utilizing monoacylglycerol as acyl-group acceptor

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ABSTRACT

Due to an increasing awareness on ecological process management the substitution of conventional processes for the de-acidification of vegetable oils has been focused by research, recently. The present study examines the mechanism of enzymatic de-acidification in degummed rapeseed oil by esterification of free fatty acids with an acyl-group acceptor (monoacylglycerol) utilizing an immobilized lipase from *Rhizomucor miehei*. As the experiments were performed in a solvent free system, first investigations comprise the exclusion of possible mass transfer limitations. The results obtained for the kinetically controlled system strongly indicate that the reaction seems to follow a multi-substrate Ping Pong mechanism. A slight competitive inhibition of the reaction by the acyl-group acceptors was observed during the determination of the effect of substrate concentration on lipase-catalyzed esterification. The free fatty acid content did not influence the activity of the biocatalyst within the ranges investigated. The kinetic parameters were determined, exhibiting that the lipase has a higher affinity towards the acyl-group acceptors ($K_{(AGA)}$ = 150 mM) than to free fatty acids ($K_{(AGD)}$ = 204 mM). The maximum reaction rate for the present reaction system was evaluated to be 41.3 U/g protein.

1. Introduction

Fats and in particular vegetable oils are essential constituents of a balanced diet as they contain several components like essential fatty acids or fat soluble vitamins with high nutritional impact. However, especially crude oils also contain accompanying substances (e.g., pigments or oxidation products) which adversely influence the sensory profile and the functional properties of the oils [1]. Their removal is not only required for food but also for technical applications and is achieved by refining of the crude oils.

The removal of free fatty acids (FFA), achieved during de-acidification, is essential in most instances, as they reduce the oxidative stability of the oils. The chemical de-acidification results in high consumption of water and chemicals, since FFA are removed as soap stock after their conversion with alkali. As a consequence, chemical neutralization contributes to high oil losses and environmental pollution [2]. Steam refining, a physical process is currently the only alternative method applicable at large scale and thus of industrial relevance [3]. Besides, high energy consumption for the supply of superheated steam, the high thermal load is a main disadvantage of physical refining as it results in trans-isomerization reaction of unsaturated fatty acids [4].

A sustainable alternative for chemical or physical de-acidification of vegetable oil could be the application of biocatalysts. It has already been adapted to industrial degumming of vegetable oils as an alternative to water degumming [5,6]. In contrast, the enzyme-assisted de-acidification has not received much attention in science and was not implemented in industry up to now. So far, only a few studies investigated the enzymatic de-acidification of different vegetable oils, focusing on oils with comparable high FFA contents [7,8]. The intended purpose rather was a pretreatment process to reduce the FFA content to a value which permits further application of conventional refining steps.

In a previous study, the general feasibility of lipase-catalyzed de-acidification of vegetable oils with low FFA content was proven. The reduction of FFA was achieved by their esterification with monoacylglycerol (MAG) as acyl-group acceptor utilizing an immobilized lipase from *Rhizomucor miehei* as biocatalyst [9]. However,

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for further improvement of the process developed and future implementation at industrial scale a more detailed insight in the reaction system is required. For this reason, a reliable and simultaneously simple kinetic model including mechanistic studies as well as the identification of any inhibitory effects on enzymatic de-acidification ought to be determined.

Commonly, lipase-catalyzed esterification is supposed to follow a non-sequential Ping Pong mechanism [10–13], but a sequential ordered mechanism is also suggested by some groups [14,15]. In many cases, the investigation of enzyme-catalyzed esterification is performed in an organic solvent like *n*-hexane or *n*-heptane applying various acids and alcohols as substrates [16,17]. The nature of the solvent is known to largely influence the kinetics of enzyme-catalyzed reactions as it affects the hydration state of the enzyme and thus the interaction with the substrate or its intrinsic activity, respectively [18,19]. Alternatively, kinetic investigations can be carried out in solvent free systems [20,21]. Inhibitory effects are observed, by alcohols or more generally by acyl-group acceptors [12,16,17,20] as well as by the acid itself acting as acyl-group donor [14,16,20].

All studies mentioned above provide a comprehensive data basis to assume a kinetic model for the present reaction system. However, observed mechanisms and inhibitory effects are mostly depending on the applied system (solvent, substrates, biocatalyst etc.). Thus, a transfer of the results to the present reaction system is not possible. Additionally, knowledge about the influence of solvent free systems on reaction kinetics is only scarcely available [12,22].

Hence, the objective of the present study is the investigation of a kinetic model for the esterification of FFA with MAG in degummed rapeseed oil catalyzed by immobilized *R. miehei* lipase. In advance, the kinetics of the reaction have to be proven and mass transfer limitations have to be excluded. Based on the obtained findings a reliable and simple kinetic model describing enzyme-assisted FFA reduction in rapeseed oil should be established.

2. Experimental procedures

2.1. Materials

A lipase (EC 3.1.1.3) from *R. miehei* immobilized on a macro porous anion exchange resin (Lipozyme© RMIM) was purchased from Novozymes (Bagsvaerd, Denmark). The particle size and bulk density of the immobilisate ranges from 0.2 to 0.6 mm and from 350 to 450 kg/m^3, respectively. Rapeseeds were purchased from BayWa AG (Nandlstadt, Germany) and screw-pressed to obtain rapeseed oil. The removal of phospholipids from the crude oil was achieved by acid degumming. The degummed oil was stored at 0 °C until use. All chemicals used were obtained from Sigma–Aldrich (Taufkirchen, Germany) and Th. Geyer (Renningen, Germany). With the exception of oleic acid and MAG (1-oleoyl-rac-glycerol), which were of technical purity, all chemicals utilized were of highest purity.

2.2. Methods

2.2.1. Enzymatic de-acidification

The FFA content of the pressed and degummed oil was determined to be 2.83 mM. The FFA contents applied in the experiments (26-34-45-84-134-279 mM) were obtained by the addition of oleic acid. For the 42 experiments – each performed in duplicate – an aliquot of 100 g of the rapeseed oil exhibiting various FFA contents was mixed with various amounts of MAG (13-52-77-258-387-516-701 mM). The reaction mixture was stirred in a glass reactor equipped with a heatable double-jacket. After the reaction temperature of 55 °C was reached, the de-acidification was initiated by the addition of 3% (w/w) of the immobilized lipase which corresponds to a protein content of 1.61 g [23]. Water, a by-product of the de-acidification reaction, was removed by constant nitrogen stripping in order to suppress enzymatic hydrolysis. For sampling the stirrer was stopped after 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 h. After sedimentation of the immobilisate a sample was taken to determine the acid number as described in paragraph 2.2.2. The initial slope of the curve (FFA content as function of time) was used to calculate the initial reaction rate.

2.2.2. Evaluation of enzymatic de-acidification

The alkalimetric titration of FFA was performed according to the DGF standard-method C-V2 (06) [24]. An aliquot of 1.0 g of the oil sample was diluted with 50 mL of ethanol/diethyl ether-solution (1:1 v/v). Phenolphthalein was added as an indicator and the sample was titrated to the transition point with 0.1 M potassium hydroxide solution (KOH). The acid number (AN) was calculated according to Eq. (1):

$$\mathrm{AN}\left[\frac{\mathrm{mg}}{\mathrm{g}}\right] = \frac{M\,(\mathrm{KOH})\left[\frac{\mathrm{mg}}{\mathrm{mmol}l}\right] \times c\,(\mathrm{KOH})\left[\frac{\mathrm{mmol}}{\mathrm{ml}}\right] \times V\,(\mathrm{KOH})\,[\mathrm{ml}]}{m\,(\mathrm{oil})\,[\mathrm{g}]} \quad (1)$$

where M (KOH) corresponds to the molar mass of potassium hydroxide, c (KOH) to the concentration of the KOH solution, V (KOH) to the volume of the KOH solution applied during titration and m (oil) to the mass of the oil sample.

The calculation of the percent mass portion of FFA converted by the reaction was done according to Eq. (2):

$$\mathrm{FFA}\left[\%\left(\mathrm{w/w}\right)\right] = \frac{M\,(\mathrm{FFA})\left[\frac{\mathrm{mg}}{\mathrm{mmol}}\right] \times \mathrm{AN}\left[\frac{\mathrm{mg}}{\mathrm{g}}\right] \times 0.1}{M\,(\mathrm{KOH})\left[\frac{\mathrm{mg}}{\mathrm{mmol}}\right]} \quad (2)$$

where M (FFA) corresponds to the average molar mass of FFA in rapeseed oil.

2.2.3. Determination of internal mass-transfer limitations

The influence of potential internal mass-transfer limitations on the present reaction system was estimated mathematically by determining the Thiele Modulus ϕ utilizing Eq. (3).

$$\phi = \frac{V_{\mathrm{obs}}}{D_{\mathrm{AB}}\,[A]}\left(\frac{R}{3}\right)^2 \quad (3)$$

where V_{obs} is the initial reaction rate, R is the particle size of the immobilisate, D_{AB} is the diffusion coefficient of MAG (A) in rapeseed oil (B) and $[A]$ is the MAG concentration. With the exception of the diffusion coefficient D_{AB} all parameters are known. However, it is possible to calculate D_{AB} by the correlation of Wilke–Chang as described in Eq. (4) [25].

$$D_{\mathrm{AB}} = 7.4 \times 10^{-12}\,\frac{(\phi \times M_{\mathrm{B}})^{1/2} \times T}{\mu_{\mathrm{B}} \times V_{\mathrm{A}}^{0.6}} \quad (4)$$

where D_{AB} is the diffusion coefficient of MAG in rapeseed oil, ϕ is the association factor for MAG, M_{B} is the middle molecular weight of rapeseed oil, T the reaction temperature, μ_{B} the viscosity of the oil and V_{A} the molar volume of MAG. Additionally, the obtained Thiele Modulus was used in order to calculate the utilization ratio η of the biocatalyst according to Eq. (5).

$$\eta = \frac{3}{\phi}\left(\frac{1}{\tanh\phi} - \frac{1}{\phi}\right) \quad (5)$$

where ϕ is the Thiele Modulus.

2.2.4. Investigation of the reaction kinetics of enzymatic de-acidification

The initial reaction rates obtained by the performance of 42 de-acidification experiments with various concentrations of acyl-group donator and acceptor (described in Section 2.2.1) were