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Rapid and efficient gas chromatographic method for measuring the kinetics of lipase-catalyzed transesterification of phosphatidylcholine

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ABSTRACT

Both n-3 polyunsaturated fatty acids (n-3 PUFA) and phospholipids have a lot of special functions on human body. n-3 PUFA includes α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Among them, ALA is the precursor of EPA and DHA. The synthesis of ALA-containing phospholipids can solve the problems of low content of EPA and DHA in phospholipids and show an increased double effect on human body. At a water activity of 0.33, a rapid and efficient method was used for kinetics of the transesterification of phosphatidylcholine (PC) with α-linolenic acid ethyl ester (ALAEE). The rate equation was obtained using palmitic acid ethyl ester (PAEE) as index for reaction rate and the reaction proved to proceed via a Ping-Pong mechanism without inhibition by both the substrates.

1. Introduction

Both n-3 polyunsaturated fatty acids (n-3 PUFA) and phospholipids have a lot of special functions on human body [1–6]. Moreover, phospholipids containing n-3 PUFA have attracted much interest because it not only provides high digestion of n-3 PUFA to cell [7], but also has various important nutritional and medical applications [8]. For example, phospholipids containing eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) could promote cell differentiation [7], enhance survivals of tumor-bearing mice [9] and decrease plasma lipids [10]. However, when EPA or DHA was selected as n-3 PUFA sources, its incorporation rate into phospholipids was slow due to its steric hindrance of approaching to enzyme active centre (containing long carbon chain and many double bonds). In addition, fish oil as acyl donor with worry of cholesterol is needed as natural source for EPA or DHA phospholipids. Therefore, the synthesis of n-3 PUFA-containing phospholipids of EPA or DHA type is necessarily to be improved.

As one kind of n-3 PUFA and precursor of EPA and DHA, α-linolenic acid (ALA) with relatively simple molecular structure shows similar high bioactivity in vivo. Compared with EPA and DHA, the synthesis of ALA containing phospholipids has easier and securer reaction characteristics and is a hot topic [11,12].

The simplest and most direct route for the synthesis of new-type phospholipids is catalytic transesterification using lipase with the special acyl donor [13–15]. ALA-type phospholipids could be synthesized with ALA by lipases, but enzymatic reaction conditions including temperature, time and substrate concentration rate were few investigated [11,12]. Kinetic studies could provide primary parameters for elucidating transesterification mechanism [16] and designing bioreactor, but no reaction kinetics of phospholipids was reported in documents. It was difficult to obtain the kinetic parameters when the traditional procedures were performed due to the complex and time-consuming experiment procedures. However, it is easy to analyze the reaction rate using fatty acid ethyl ester as acyl donor to overcome these problems, since the initial reaction rate could be directly measured by gas chromatography, avoiding tedious derivation of free fatty acids. Two fatty acid esters were involved α-linolenic acid ethyl ester (ALAEE) and palmitic acid ethyl ester (PAEE) in the reaction, in which ALAEE content would decrease while PAEE content would increase in the solution, could be used as index for determining the initial reaction rate, but it was not clear whether their change was consistent with the transesterification rate or not.

Abbreviations: n-3 PUFA, n-3 polyunsaturated fatty acids; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PC, phosphatidylcholine; ALAEE, α-linolenic acid ethyl ester; PAEE, palmitic acid ethyl ester; SAEE, stearic acid ethyl ester.

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In this work, the transesterification of phosphatidylcholine with ALAEE was conducted with immobilized lipase Lipozyme TL IM. The differences of the two indexes were investigated, and PAEE was determined to describe the kinetics of the transesterification of phosphatidylcholine by gas chromatography and the reaction mechanism was illustrated.

2. Experimental methods

2.1. Materials

Egg-yolk phosphatidylcholine (purity 97.5%) was self-made by supercritical CO_2 extraction and purification with column chromatography as follows:

Deoiled egg phospholipids (300 g) were prepared by supercritical CO_2 extraction of egg yolk powder at 30 MPa, 45 °C for 2 h and then crude egg-yolk PC (purity 70 area%, acetone insoluble no less than 98 wt%) was obtained through 2000 ml 95% (v/v) ethanol extraction, separation of protein by filtering and vacuum drying. A glass column (30 mm i.d. × 120 cm length) packed with 80 g Al_2O_3 was used for purifying crude egg-yolk phosphatidylcholine. 5 g crude egg-yolk phosphatidylcholine was dissolved in 10 ml of chloroform and added on the column to elute with a mixture of chloroform and methanol (9:1, v/v) at 5 ml/min. Samples of known weight were withdrawn for HPLC analysis to follow phosphatidylcholine eluates. After performing column chromatography twice, the eluates (0.7 L) containing phosphatidylcholine were collected and about 2 g of high purity of 97.5% egg-yolk phosphatidylcholine was obtained through condensation and vacuum drying. Its fatty acid composition (area%) from analysis was as follows: palmitic acid (C16:0): 31.3, stearic acid (C18:0): 16.3, oleic acid (C18:1): 30.7, linoleic acid (C18:2): 13.7, and arachidonic acid (C20:4): 5.6. The average molecular weight of 779 g/mol was calculated according to this fatty acid composition.

α-Linolenic acid ethyl ester (C18:3, purity 98.5%) was purchased from Sigma–Aldrich (Shanghai, China). Immobilized lipase Lipozyme TL IM (sn-1,3 specific lipase from *Thermomyces lanuginosa*) was provided by Novoymes (Shenyang, China). Solvents used and all other chemicals were of analytical grade and obtained commercially.

2.2. Enzyme reactions

The lipase-catalyzed transesterification of phosphatidylcholine with ALAEE was performed in a 5 ml container with tight screw cap. The reaction was conducted in a water bath at 45 °C with magnetic stirring at 100 rpm and reaction was started by mixing immobilized enzyme in a total of 4 ml hexane reaction solution. After the reaction the samples were centrifuged at 5000 rpm for 5 min (this condition for the separation of immobilized lipase had been previously examined to ensure that the reaction is really stopped in the collected reaction mixtures due to a probable leak of enzyme from the support of immobilization), and some supernatants were collected to determine the kinetic parameters. Others were for the analysis of transesterified PC fatty acid and free fatty acid hydrolyzed in the reaction.

2.3. Separation of production fatty acids

The collected reaction mixtures were evaporated under reduced pressure. The dry residue was dissolved in 1 ml of toluene and 50 μl was withdrawn for TLC using chloroform–methanol–water (65:24:5, v/v) and the bands were visualized with iodine vapor and a molybdenum blue spray agent. The bands corresponding to phosphatidylcholine and free fatty acid were scraped into various tubes separately and the scrapings were extracted with 2 ml chloroform–methanol (2:1, v/v) for phosphatidylcholine and 2 ml methanol–benzene (2:1, v/v) for the FFA, respectively. The extracts were totally methylated using sodium methoxide-catalyzed method for PC and trimethylsilyldiazomethane method for free fatty acid [17].

2.4. Analysis of fatty acids by gas chromatography

Fatty acid ethyl esters were analyzed on a GC-14B gas chromatograph with flame-ionization detector (Shimadzu, Kyoto, Japan). The glass column packed with 20% DEGS on celite (3 mm i.d. × 2 m length) was employed; N_2 was used as a carrier gas; the column temperature was 190 °C and the injector and detector temperatures were 250 °C. The n-eicosane was added in reaction solution prior to reaction as internal standard to calculate the amount of various fatty acid esters in the reaction system.

2.5. Analysis of PC by HPLC

Phosphatidylcholine was analyzed using an LC-10ATVP HPLC system with SPD-10AV/VP UV detector at 205 nm and Shimpack SIL column (6 mm i.d. × 250 mm length, Shimadzu, Kyoto, Japan). The eluent used was a mixture of hexane–isopropyl alcohol–water–85% phosphoric acid (40:55:6.5:0.3, v/v), and its flow rate was 0.8 ml/min. The volume of the injected sample was 2.5 μl.

2.6. Pretreatment of lipase

Enzyme preparations and the organic phase of the reaction mixture were adjusted to the desired water activity by pre-equilibration with saturated salt solutions at 25 °C in separate containers. The salts used were LiBr (water activity, $a_w = 0.06$), LiCl ($a_w = 0.11$), $K^+CH_3COO^-$ ($a_w = 0.25$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.53$), NaCl ($a_w = 0.75$), and K_2SO_4 ($a_w = 0.97$). Equilibration was performed overnight.

3. Results and discussion

It is easily observed that the ALAEE content decreased with time if direct analysis of components in the solution was conducted by gas chromatograph. However, incorporation rate of ALA increased with reaction time, which is inconsistent with the ALAEE content in terms of curve development trend. In order to compare these changes more visually and simply, ALAEE decrease value, which was defined as the decrease of ALAEE content in the reaction solution before and after reaction, was introduced to show same trend of change with incorporation rate of ALA, since it also increased with time.

At a high water activity of 0.75 (Fig. 1), the reaction was carried out in short time. The results showed that ALAEE change (ALAEE decrease value) did not agree well with transesterification rate (ALA in phosphatidylcholine) as compared to palmitic acid ethyl ester (PAEE). The cause of these differences is the hydrolysis of esters that resulted in a large amount of free fatty acids. The free ALA increased faster than free palmitic acid released from phosphatidylcholine, indicating that hydrolysis rate of ALAEE was higher than that of phosphatidylcholine with time. Although the ALAEE content consumed decreased obviously in the solution, the ALAEE decrease per unit time could not be used as an index for the reaction rate. High water activity played a key role on this phenomenon. The influence of various water activities on the transesterification showed that both free ALA and free PA increased very rapidly with increasing water activity (Fig. 2), but the PAEE content had a same changing tendency as the ALA in phosphatidylcholine. At 0.33 of water activity, the ALA in phosphatidylcholine was the highest (24.1 μmol) and

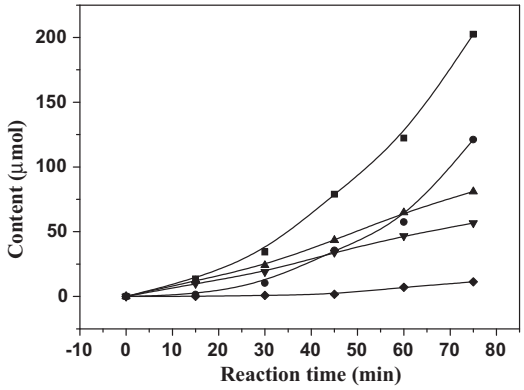

Fig. 1. Contents of the constituents in the solution as a function of reaction time at a high water activity (0.75). Reaction condition: PC (137 mg), ALAEE (214 mg), Lipozyme TL IM (200 mg). Contents of ALAEE (decrease value) (■), free ALA (●), ALA in PC (▲), PAEE (▼), and free palmitic acid (♦).

the variation (3.9 μmol) for PAEE (20.2 μmol) was lower than that (7.2 μmol) for ALAEE (31.3 μmol), showing that increased PAEE can represent reaction rate well whereas decreased ALAEE cannot describe the reaction accurately. In this case, phosphatidylcholine was utmost transesterified with ALAEE in one-step process and released very proximal PAEE to the incorporated ALA (Fig. 3). On the other hand, it is true that the sn-1 position is not always esterified with palmitic acid but also with stearic acid. However, the value of stearic acid ethyl ester (SAEE) in the solution is much lower than that of PAEE, and its change with time is also smaller. This is mainly because that stearic acid in phosphatidylcholine (16.3%) is not as high as that of palmitic acid (31.3%) and the release of SAEE is low. Another reason probably is that the selectivity of enzyme for chain length. Stearic acid belongs to 18 carbon whereas palmitic acid belongs to 16. Long carbon chain usually makes enzymatic reaction more difficult than short carbon chain. Therefore, PAEE content can be selected as an efficient index for determination of the kinetics. The increased amount of PAEE before and after reaction per unit time was thereby defined as the initial reaction rate.

By using PAEE as an index for initial reaction rate, a 100 rpm of stirring speed was adopted and the mass transfer limitations were neglected. The initial reaction rate was also increased linearly with the enzyme concentration. Thus, reaction was controlled by kinetics. When one of the substrate concentrations is kept constant, the initial reaction rate increased with the concentration of another substrate (Fig. 4). Also, no evidence for the phenomenon of

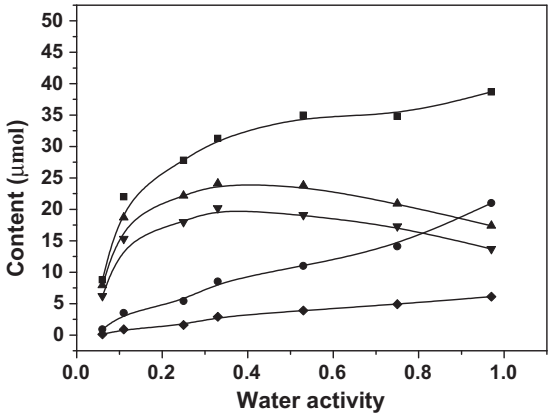

Fig. 2. Contents of the constituents in the solution as a function of water activity. Reaction condition: PC (90.5 mg), ALAEE (120 mg), reaction time (30 min), Lipozyme TL IM (150 mg). Contents of ALAEE (decrease value) (■), free ALA (●), ALA in PC (▲), PAEE (▼), and free palmitic acid (♦).

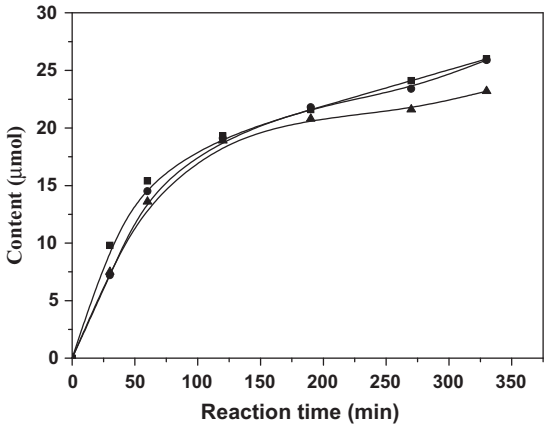

Fig. 3. Contents of the constituents in the solution as a function of reaction time at optimal water activity (0.33). Reaction condition: PC (85.0 mg), ALAEE (107 mg), Lipozyme TL IM (100 mg). Contents of ALAEE (decrease value) (■), PAEE (●), and ALA in PC (▲).

substrate inhibition can be seen. Alcohol always results in inhibition as alcohol substrate molecule can act as a dead-end inhibitor in the esterification reaction [18], but alcohol in ALAEE has been esterified with long chain fatty acid, which contributes to certain non-polar effect on protecting enzyme structure from losing water in the present reaction. On the other hand, optimal water activity provides good condition for enzyme activity, which neither makes degree of hydrolysis too high nor lowers the reaction rate.

Experimental data in Fig. 4 were converted into double-reciprocal plot of initial rate of transesterification at varying contents. The plot of $1/V$ for 1/[ALAEE] presents a group of parallel model seen in Fig. 5. This indicated that the reaction was in good agreement with Ping-Pong mechanism, which is characterized by parallel lines in the Lineweaver–Burk (double reciprocal) representation at concentrations in which there is no inhibition, as it happens in the present system.

According to the Ping-Pong mechanism above, a model of rate equation for two substrates was adopted [19,20]. The kinetic constants were obtained through further calculation involving intercepts of straight parallel lines, the reciprocal of phosphatidylcholine contents and the slope of new line ($Slope = K_{mALAEE}/V_{max}$). That is $V_{max} = 1.32 \times 10^{-2}$ μmol min^{-1} mg^{-1}; $K_{mALAEE} = 88.5$ mM; $K_{mPC} = 405$ mM. The rate equation for the mechanism as follows

$$V = \frac{1.32 \times 10^{-2}[C_{PC}][C_{ALAEE}]}{88.5[C_{PC}] + 405.2[C_{ALAEE}] + [C_{PC}][C_{ALAEE}]} \quad (1)$$

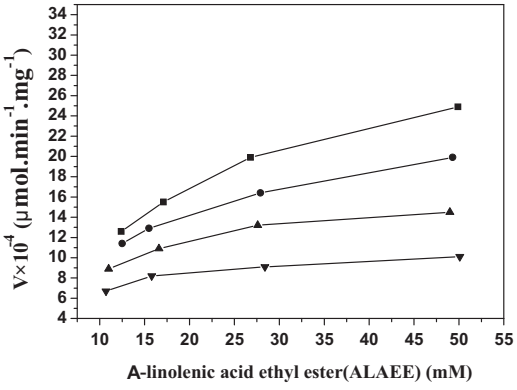

Fig. 4. Effect of substrate concentration on the initial reaction rate. Reaction condition: water activity (0.33), Lipozyme TL IM (100 mg), and time (60 min). PC concentrations: 174 mM (■), 115 mM (●), 70 mM (▲), and 39 mM (▼).

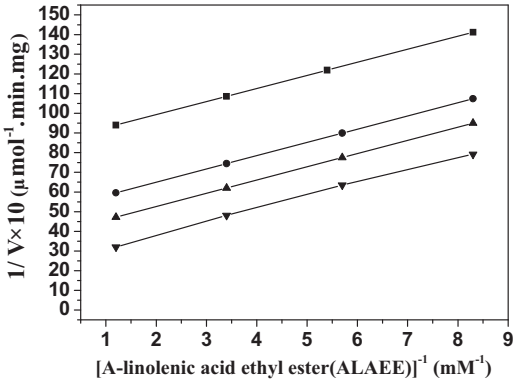

Fig. 5. Plot of reciprocal initial velocity of the reaction versus reciprocal ALAEE concentration. PC concentrations: 39 mM (■), 70 mM (●), 115 mM (▲), and 174 mM (▼).

where V is the initial reaction rate; $[C_{PC}]$ and $[C_{ALAEE}]$ are the concentrations of phosphatidylcholine and ALAEE, respectively; V_{max} is the maximum velocity or limiting rate; K_{mPC} and K_{mALAEE} are the Michaelis constants for phosphatidylcholine and ALAEE, respectively. ALAEE has K_m lower than phosphatidylcholine, which is consistent with the higher hydrolysis rate of ALAEE as compared to phosphatidylcholine, as described above. But the hydrolysis side reaction can be controlled at optimum water activity. At that time the reaction rate was dependent on the formation velocity of PAEE and the kinetics was easily and accurately investigated by gas chromatograph. Using this method, the rate equation described the appearance of PAEE and the incorporation of ALA into phosphatidylcholine, on the other hand, it illustrated very well the reaction mechanism of Ping-Pong from the PAEE point of view.

4. Conclusion

Phosphatidylcholine has lower degree of hydrolysis than that of ALAEE, and its PAEE released can be used as reliable index for determining the kinetics of the transesterification of PC with ALAEE at optimal water activity of 0.33. Under the condition, a Michaelis–Menton equation with accurate the kinetic constants and a Ping-Pong mechanism without inhibition by both substrates were obtained. The parameters were $V_{max} = 1.32 \times 10^{-2}$ μmol min^{-1} mg^{-1}; $K_{mALAEE} = 88.5$ mM; $K_{mPC} = 405$ mM.

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