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LETTER TO THE EDITOR

Chemo-enzymatic Synthesis of Novel β-Hydroxy-β-methylbutryric Acid (HMB)–Medium Chain Triacylglycerol (MCT) Complexes

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Abstract β-Hydroxy-β-methylbutyrate (HMB) is reported to have ergogenic benefits such as reducing muscle wasting, promoting exercise performance and skeletal muscle hypertrophy. When used as a dietary feed, it has been found to improve colostral milk fat and the growth of piglets. Medium chain triacylglycerols (MCT) are a good energy source as they can be subjected to β-oxidation in the liver without being stored as fats. When used as animal feed, HMB–MCT complexes are reported to stimulate growth and produce leaner meat product. A high yield two-steps chemo-enzymatic pathway for synthesis of HMB–MCT complexes has been developed. Ethyl HMB ester was first synthesized using a chemical catalyst. Following that, it was transesterified with MCT by using a biocatalyst. Approximately 85 wt% of HMB–MCT complexes can be achieved using this two-step synthetic pathway. The reaction mechanism and some of the reaction parameters were also briefly elucidated.

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Dear Editor,

β-Hydroxy-β-methylbutyrate (HMB) is a metabolite of the essential branched-chain amino acid leucine, which has been found to reduce muscle wasting in patients associated with trauma and cancer cachexia by reducing muscle atrophy and increasing muscle hypertrophy [1, 2]. In addition, HMB has also been reported to have ergogenic benefits including anticatabolic, anabolic and lipolytic effects. As a result, HMB in the form of the calcium salt has been used extensively by bodybuilders and athletes as an ergogenic acid to promote exercise performance and skeletal muscle hypertrophy [3]. Although not studied extensively yet, HMB when incorporated into dietary feed was found to result in improved colostral milk fat and growth of piglets [4]. Medium chain triacylglycerols (MCT) such as tricaprylin and tricaprin are reported to be readily hydrolyzed by lingual and gastric lipases. The medium chain fatty acids formed are then subjected predominantly to β-oxidation in the liver without being stored as fats. Thus, MCT is a good energy source especially for patients with pancreatic insufficiency and fat malabsorption [5]. In addition, MCT improves colostrum yield of sows and thus ensure a higher transfer of nutrients to the piglets immediately after birth [6]. Taking these beneficial effects into consideration, HMB if incorporated into MCT is postulated to have combined beneficial effects of an increased muscle growth rate and decreased accumulation of fat. This may present an interesting opportunity to the animal feed industry as such structured lipids are expected to improve the animal growth rate and meet the current demand of health-conscious consumers for leaner meat products. In addition, a structured lipid is regarded as a fat source and can be added to animal feed and does not require approval for inclusion in animal feeds. To date,

there are no, or only limited, studies that examine either the benefits effects of or the synthetic pathway of HMB–MCT complexes. The present study was aimed at developing an efficient pathway for a high yield of HMB–MCT complexes.

A one-step enzymatic acidolysis of HMB and tricaprylin was first attempted to produce HMB–MCT complexes. HMB purchased from Th. Geyer Danmark (Roskilde, Denmark) and tricaprylin obtained from Cognis Care Chemicals, Dusseldorf, Germany) were weighed accurately at different substrate molar ratios [3:1 (2.67:3.53 mg), 2:1 (1.78:3.53 mg), 1:1 (0.89:3.53 mg), 1:2 (0.89:7.06 mg) and 1:3 (0.89:10.59 mg)] into jacketed glass reactors and stirred at 250 rpm. The jacketed glass reactors were then heated to a constant 65 °C using a circulating water bath. The acidolysis reactions were initiated by addition of Novozym 435 (10 wt% of total substrate weight). Novozym 435 (lipase from *Candida antarctica* B) was kindly donated by Novozymes A/S (Bagsværd, Denmark). The reactions were carried out in duplicate. Reactions were monitored by withdrawing the reaction mixtures periodically for detection of changes in acylglycerol composition throughout the duration of the reaction. Analysis was carried out using a high performance liquid chromatograph (HPLC) (Thermo Fisher Scientific Inc., Roskilde, Denmark). Separation of the different components was performed using a reverse phase Supelcosil LC-18, 5 µm column (250 mm x 4.6 mm) (Supelcosil Inc., Bellefonte, PA). A binary solvent system of acetonitrile (solvent A) and isopropanol: hexane (2:1) (solvent B) under gradient elution was used. Beginning with 70 % solvent A and 30 % solvent B, solvent A was reduced to 40 % and solvent B was increased to 60 % at 40 min. The composition of 40 % solvent A and 60 % solvent B was maintained for 10 min before reverting back to 70 % solvent A and 30 % solvent B for another 6 min. The flow rate of the solvent was at a constant 1.0 ml/min. A Sedex (S.E.D.E.R.E., Alfortville, France) model 75 ELSD was used for detection; the pressure of the nebulizer gas (air) was maintained at 3.2 bar and the drift tube temperature was set at 40 °C. The identity of each peak was identified by determining its molecular mass using a Dionex Ultimate 3000 HPLC system coupled through an electrospray ionization (ESI) inlet to a qTOF mass spectrometer (microTOFq) (Bruker Daltonic GmbH, Bremen, Germany). Data were acquired and processed using Bruker Compass software including Bruker Daltonics Hystar, MicroTOF control and Dataanalysis. Samples were analyzed using both negative and positive ionization mode to ensure detection of all relevant compounds. The ESI-qTOF was operated under the following conditions: nebulizer pressure at 3.4 bar, dry gas flow 10 L/min, source voltage 4.0 kV and transfer time 120 ms. The acylglycerol composition was expressed as the wt% of the total weight of the sample. Standard curves were constructed using pure reaction substrates (ethyl HMB esters and tricaprylin) and purified reaction products.

The one-step enzymatic acidolysis of HMB and tricaprylin was carried out for 5 days. Nevertheless, no enzymatic activity or product formation was observed in the one-step enzymatic acidolysis of HMB and tricaprylin. This is mainly due to the strong acidity of HMB which disrupts the enzyme structure and causes it to lose its catalytic activity. Numerous studies have reported that the presence of strong acid such as acetic acid will affect the aqueous microenvironment of the biocatalyst resulted in inactivation of lipase. Novozym 435; for example, was found to be most catalytically active at neutral pH of 7 to 8. It was shown that activity of Novozym 435 dropped tremendously in both acidic and alkaline microenvironments [7]. The reaction mixture containing both HMB and tricaprylin was rather acidic with a pH of approximately 3 to 4. Thus, this may have resulted in inactivation of Novozym 435.

Following the ineffective single-step synthetic pathway, we proposed a two-step chemo-enzymatic pathway to synthesize HMB–MCT complexes. In the first step, the acidity of HMB was reduced by converting HMB into ethyl HMB ester through esterification (Fig. 1a). In the second step, ethyl HMB ester was transesterified with tricaprylin to produce HMB–MCT complexes (Fig. 1b).

Esterification of HMB was conducted by using chemical catalysts. Two chemical catalysts were studied namely sulfuric acid and p-toluenesulfonic acid anhydrous. Sulfuric acid was obtained from Sigma Aldrich (Brøndby, Denmark); meanwhile, *p*-toluenesulfonic acid anhydrous was purchased from Eurolabs Ltd. (Cheshire, United Kingdom). Firstly, HMB (8.85 g), absolute ethanol (6.9 g) [molar ratio of HMB:ethanol, 1:2] and chemical catalyst (5 wt% of HMB) were weighed accurately into a 100-ml round-bottom flask. In the case where *p*-toluenesulfonic acid anhydrous was used, 45 ml of cyclohexane and 70 g of molecular sieves were also added to the reaction flask. The reaction was initiated by heating the mixture constantly under reflux at 85 °C by using an oil bath. After 5 h of reaction, the reaction was stopped by cooling the reaction mixture to room temperature and adding anhydrous sodium sulfate. The reaction mixture was then distilled at 150 mbar and 50 °C to remove the cyclohexane and excess ethanol. Finally, the resulting distillate was washed several times using a mixture of dichloromethane and saturated aqueous sodium bicarbonate (1:1/v:v). Following the washing steps, the mixture was centrifuged to obtain the organic and aqueous phases. The organic phase was pooled and distilled to obtain ethyl HMB ester. The purity and molecular structure of the ethyl HMB ester was identified by ^{1}H NMR performed on a Varian Mercury 400 MHz NMR spectrometer (Palo Alto, CA, USA).

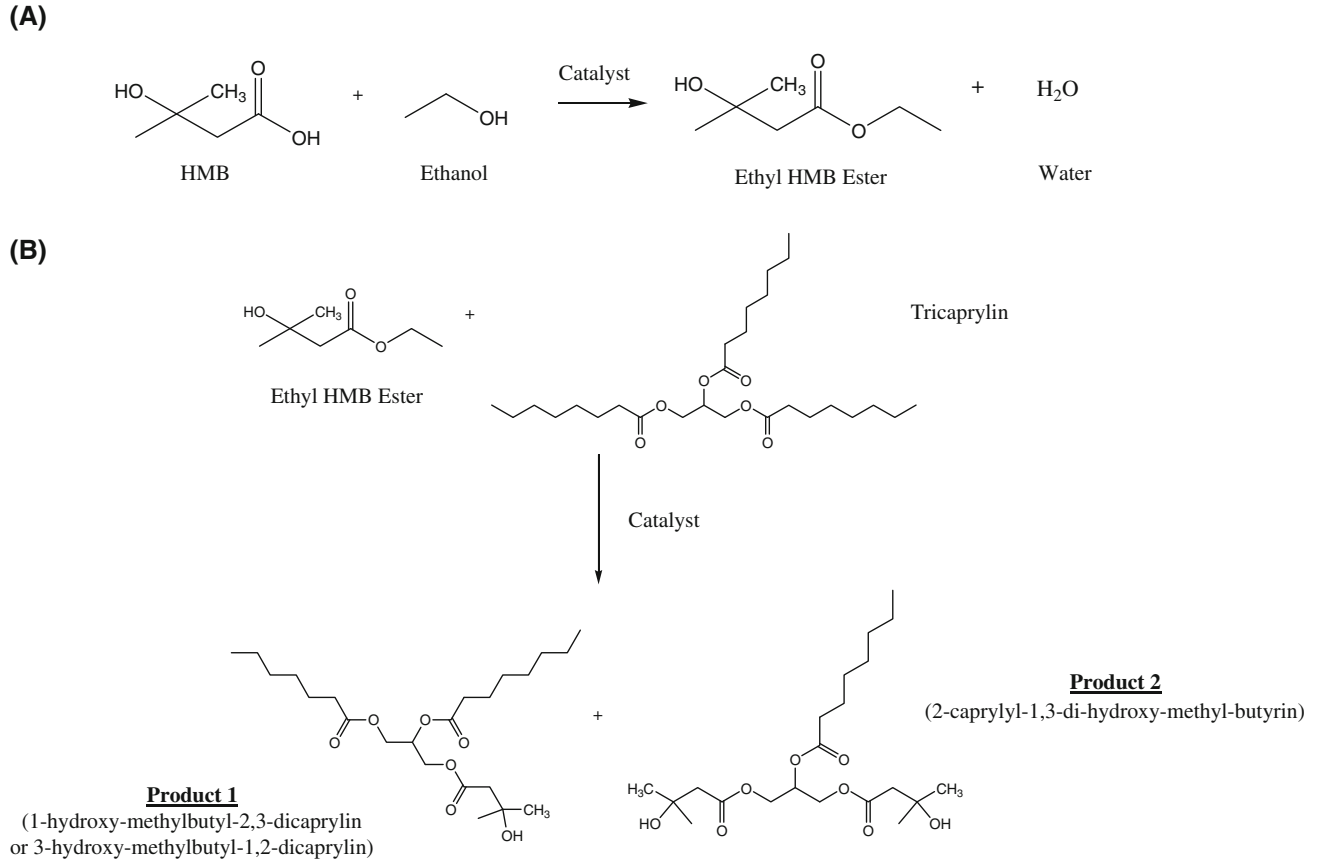

Fig. 1 The two-step chemoenzymatic pathway for high yield synthesis of HMB–MCT complexes. **a** Step 1: esterification of HMB to form ethyl HMB esters. **b** Step 2: transesterification of ethyl HMB ester with tricaprylin to form HMB–MCT complexes

In the esterification reaction when sulfuric acid was used as the catalyst, a very low yield of ethyl HMB ester (10 %) was obtained with the formation of many unwanted side products. Sulfuric acid is a strong acid catalyst; thus has the capability of reacting with both the carboxylic and hydroxyl groups of the HMB. In addition, as molecular sieves were not used in the reaction catalyzed by sulfuric acid, there is a possibility that water molecules formed during the reaction further hydrolyzed the products forming unwanted side products. In an attempt to increase the yield of ethyl HMB esters, sulfuric acid was replaced with *p*-toluensulfonic acid anhydrous as catalyst. In addition, cyclohexane and molecular sieves were also added to the reaction mixture. This reaction proved itself to be more efficient with 80 % of purified ethyl HMB esters obtained following the purification step. The cyclohexane, ethanol, and water mixture formed an azeotrope which resulted in a lower evaporation temperature. As a result, the water molecules formed can be effectively absorbed by the molecular sieves without causing unwanted hydrolysis of the ethyl HMB ester. The structure of the purified ethyl HMB ester was confirmed by using ^{1}H NMR. 1H NMR (CDCl3, 400 MHz) δ_H 4.12 (q, 2H, *J* 7.2 Hz, $OC\underline{H}_2CH_3$), 2.41 (s, 2H, $C\underline{H}_2C(O)$), 1.21 (s, 6H, $C\underline{H}_3$), 1.21 (t, 3H, $OCH_2C\underline{H}_3$).

The ethyl HMB ester obtained was then transesterified with tricaprylin using lipase Novozym 435. Ethyl HMB esters and tricaprylin at different molar ratio [3:1 (3.29:3.53 mg), 2:1 (2.19:3.53 mg), 1:1 (1.10:3.53 mg), 1:2 (1.10:7.06 mg) and 1:3 (1.10:10.59 mg)] were weighed accurately into jacketed glass reactors and stirred at 250 rpm. The jacketed glass reactors were then heated constantly to 65 °C using a circulating water bath. The acidolysis reactions were initiated by the addition of Novozym 435 (10 wt% of substrate). Two different products were formed, namely product 1 with HMB being incorporated at *sn*-1 or *sn*-3 position (1-hydroxy-methylbutyl-2,3-dicaprylin or 3-hydroxy-methylbutyl-1,2-dicaprylin) (MW:429.29) and product 2 with HMB being incorporated at *sn*-1 and *sn*-3 (2-caprylyl-1,3 di-hydroxy-methylbutyrin) (MW:418.26) positions.

In order to develop an efficient synthesis method for a high yield of HMB–MCT complexes, the effects of the substrate molar ratio were examined. As expected, HMB–MCT complexes increased with the amount of ethyl HMB esters (Fig. 2a). After 72 h of reaction, approximately

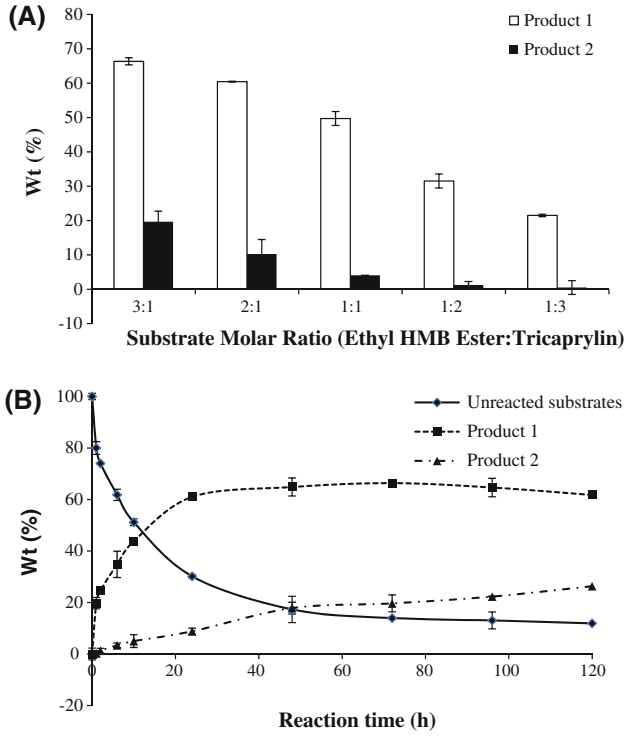

Fig. 2 Transesterification of ethyl HMB ester with tricaprylin. **a** Effects of substrate molar ratio on transesterification **b** Time course of the transesterification

85 wt% of HMB–MCT complexes was synthesized with 66 wt% of product 1 and 19 wt% of product 2 when the molar ratio of ethyl HMB ester to tricaprylin was 3 to 1.

In order to further elucidate the reaction mechanism, the aforementioned reaction was carried out for 120 h. As shown in Fig. 2b, the reaction reached equilibrium at approximately 60 h. The rate and extent for the syntheses of product 1 was higher as compared to those of product 2. Formation of product 1 reached a maximum at equilibrium (60 h). After that, it started to decline and meanwhile product 2 started to increase. This is mainly due to the increasing amount of product 1 in the reaction pool which prompted it to act as a substrate for transesterification with tricaprylin forming product 2.

In summary, a high-yield two-step chemo-enzymatic pathway for the synthesis of HMB–MCT complexes has been developed. Ethyl HMB ester was first synthesized using a chemical catalyst. Subsequently, it was transesterified with MCT by using a biocatalyst. Approximately 85 wt% of HMB–MCT complexes (66 wt% of product 1 and 19 wt% of product 2) can be achieved within 72 h using this two-step synthetic pathway.

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