

ENZYMATIC SYNTHESIS OF STRUCTURED LIPID BASED ON SILKWORM OIL AND PALM OLEIN

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ABSTRACT

The present study describes the synthesis and characterisation of structured lipid (SL) containing α -linolenic acid (ALA) prepared from palm olein (POo) and silkworm oils (SWO) employing enzymatic interesterification method. POo was chosen as one of the substrate because it does not contain ALA and is oxidatively more stable compared to other vegetable oils. The study was aimed to obtain an ALA content of about 10% in the structured lipid which was achieved with the blend of POo and SWO in the ratio of 80:20 (wt/wt). The reactions were catalysed by a sn-1, 3-regiospecific lipase, Lipozyme RM IM. The effect of reaction time, temperature were studied and were found to be at 4 hr and 65°C-70°C respectively at a lipase concentration 4% (w/w) of substrates. Physico-chemical characteristics of both the substrate oils were determined and compared with those of initial physical blends and the prepared SL. The oxidative stability of SL was also studied and the induction time was found to be at 13.2 hr which was higher with respect to SWO. The SL prepared using refined POo, SWO has potential applications in nutraceutical, food and industrial applications.

Keywords: POo, eri SWO, α -linolenic acid, interesterification, structured lipid, physico-chemical characterisation.

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INTRODUCTION

Structured triacylglycerols with the desired fatty acids are the most effective designer lipids which can be used as nutraceuticals or functional foods to target specific diseases and metabolic conditions. A number of SL are known to have applications as reduced calorie fats, infant nutrition, medium chain triglycerides (MCT), polyunsaturated fatty acids (PUFA) and newer products are being developed with physiologically important fatty acids (Osborn, 2002; Sanjit, 2013; Marek, 2004). According to Kris

et al., PUFA including α -linolenic acid (ALA) are vital for maintaining biofunctions in mammals and have important functions related to heart, circulation and inflammation (Kris, 2004). Studies showed that SL containing medium chain and n-3 fatty acid is highly desirable for the inhibitory effect on tumour growth and also showed good nitrogen balance in Yoshida sarcoma-bearing rats (Akoh, 1995). Among the several PUFA available, recent reports suggest that ALA is gaining importance as important nutraceutical agent in disease conditions associated with brain (Nicolas, 2015). Previous reports on ALA containing fat spreads were obtained from the blends of flaxseed and palm oil (EI-Waseif, 2013). Flaxseed oil was also used for the biosynthesis of structured lipids by interesterification with tricaprylin (Khodadadi, 2013). In one of the study, improved thermal properties were observed for

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the structured lipid prepared from palm stearin and linseed oil (Cecile, 2012). Linseed oil which contains a high amount of ALA was used for the preparation of SL with balanced omega-3 fatty acids with ground nut oil (Sharma, 2009). The SL enriched with n-3 PUFA (ALA) was used for the prevention of cholesterol and heart related diseases (Rupani, 2014). Reports are also available on the use of perilla oil which contains 63% ALA for interesterification with corn oil to produce ALA containing SL (Mitra, 2012). However, the SL prepared from POo or palm stearin blended with flaxseed oil was found to be a unique mixture having n-3 and n-6 fatty acids in considerable amounts (Long, 2003).

Among various oils, palm oil (PO) and its fractions are considered as potential substrates for lipid modification for edible purposes because of their high thermal, oxidative stability and plasticity. Literature studies also suggest that PO and its fractions have been widely employed for the preparation of SL (Kaki, 2012; Long, 2003; Cecile, 2012). As PO does not contain any ALA, it is a better substrate to be studied for incorporation of ALA.

In the present study, we have employed eri silkworm as a source of ALA for the preparation of SL. It was reported that eri silkworm oils contain a high amount of ALA in the range of 45%-55% depending on the feeding source (Kaki, 2006). The oil has been studied for its toxicity and was found that it is equivalent to other vegetable oils and also the oxidative stability of the SWO was shown to be improved when stored with appropriate antioxidants (Thumu, 2015; Longvah, 2012). As part of our research interest on value addition to eri silkworm oil (Ravinder, 2015; Kaki, 2014; Thumu, 2015), we attempted to prepare a SL with eri SWO and PO as substrates.

MATERIALS AND METHODS

Materials

Fatty acid mixtures (C4-24), reference triacylglycerols were purchased from Sigma Chemical Co., USA. Lipozyme TL IM [granulated silica preparation of a microbial lipase from *Thermomyces lanuginosus* with an activity of 75 IUN g⁻¹ (IUN is Interesterification Unit Novo g⁻¹)] was a gift sample from M/s Novozymes South Asia Pvt Ltd, Bangalore, India. High performance liquid chromatography (HPLC) grade solvents were procured from M/s Merck, Mumbai, India. All chemicals and reagents used in the present work were of analytical reagent-grade and were purchased from SD Fine-Chemicals, Mumbai, India.

Refined POo sample was purchased from the local market, manufactured by M/s Ushodaya Enterprises Pvt Ltd (Foods Division),

Kakinada, Andhra Pradesh, India. The silkworm pupae of tapioca leaf fed was collected from Rampachodavaram, E G District, Andhra Pradesh, India.

Methods

Extraction and refining of SWO. SWO was extracted from dried eri silkworm pupae according to a reported method (Kaki, 2006). The crude tapioca leaf fed SWO was refined by degumming, neutralisation, bleaching and deodorisation methods to remove impurities such as free fatty acid, phospholipids, dark colour pigments and odoriferous components (Ravinder, 2015).

Preparation of physical blend. Refined POo and refined SWO blend was prepared by taking in the ratio of 80:20 according to standard specifications and homogenised (PFA, 2005; BIS, 1995). The mixture of blend was heated under stirring for 30 min at 70°C under reduced pressure (10 mmHg) for the preparation of physical blend (Kaki, 2012). The blend was prepared to get the ALA content around 10% in the sample.

Enzymatic interesterification of SWO and PO. Palm and SWO binary blend (100 g) was used as substrate for enzymatic interesterification using Lipozyme TL-IM (4% wt/wt of total substrate) following a reported protocol (Kaki, 2012). The reaction mixture was magnetically stirred for 8 hr at 65°C-70°C. Aliquots were collected at 0, 1, 2, 4, 6 and 8 hr time interval for HPLC analysis. Before sampling, stirring was stopped and the enzyme was allowed to settle at the bottom and sample was withdrawn from the top for further analysis. After 8 hr, the product was separated from the enzyme and was stored in the refrigerator until further use. The product was analysed for fatty acid composition by gas chromatography (GC), molecular species by HPLC, oxidative stability by Rancimat assay and other physico-chemical parameters.

Physico-chemical characterisation. POo, SWO and their blends before, after interesterification were analysed for different physico-chemical characteristics like free fatty acids, iodine value, peroxide value, unsaponifiable matter, saponification value, p-anisidine value, refractive index, cloud point and colour according to AOCS methods (AOCS, 2003). The phosphorous content determined followed by IUPAC method (IUPAC, 1987).

Total oxidation (TOTOX) value. TOTOX value measures the total oxidation, which includes primary and secondary oxidation products. These values reflect the initial and later stages of the oil

oxidation. TOTOX values of the oil samples were determined based on the obtained peroxide value and p-anisidine values. It was calculated according to a reported method (Shahidi, 2008) using Equation (1):

$$\text{Total oxidation (TOTOX) value} = 2 \times \text{PV} + \text{PAV}$$

where PV - peroxide value, and PAV - p-anisidine.

Thiobarbituric acid-reactive substances (TBARS) test. TBARS measures the formation of secondary oxidation products, which are determined according to a reported method (Mariod, 2014). The oil samples (200 mg of each) were weighed into a 25 ml volumetric flask separately. The samples were dissolved in 1-butanol and make up to the mark with butanol. The solution of 5 ml portion was transferred into a dry test tube and a 5 ml quantity of fresh thiobarbituric (TBA) reagent (200 mg TBA in 100 ml 1-butanol) was added to it. Then, the test tube was kept at 95°C in a water bath and heated for about 120 min. After that the tube was taken out and cooled to room temperature. The absorbance of the solution was measured at 532 nm. The values obtained in the test are commonly described as TBARS (TBA reactive substances) and the TBARS value calculated as follows:

$$\text{TBARS value} = \frac{(A \times 0.415)}{m}$$

where A - absorbance at 532 nm, and
m - mass of the sample (g).

Preparation of fatty acid methyl esters. POo, SWO and their blends before, after reaction were converted to fatty acid methyl esters (FAME) according to the method described by Christie (Christie, 1982). The oil (10-20 mg) was taken in 15 ml of 2% H₂SO₄ in methanol reagent and refluxed for about 4 hr. After complete conversion as confirmed by TLC, solvent was partially removed and the remaining mixture was extracted with ethyl acetate (2 x 20 ml), followed by water washings till neutral. Then the ethyl acetate extract was dried over anhydrous sodium sulphate and evaporated under reduced pressure on a rotary evaporator to obtain FAME.

GC analysis. The fatty acid methyl esters analysis of oils were performed on Agilent 6890N Series Gas Chromatograph equipped with a FID and the capillary column DB- 225 (30 m x 0.25 mm i.d. x 0.5 µm film thickness). The injector and detector temperatures were maintained at 230°C and 250°C respectively. The oven temperature was programmed for 2 min at 160°C, further increased to 230°C at 5°C min⁻¹ and finally maintained for 10 min at 230°C. The carrier gas, nitrogen was used at a flow rate of 1.5 ml min⁻¹. The injection volume was 1 µl, with a split ratio of 50:1.

Triacylglycerol (TAG) molecular species determination by HPLC. Triglyceride molecular species were determined by reversed phase HPLC equipped with a quaternary pump and an evaporative light scattering detector (ELSD 2000, Alltech, Deerfield, IL, USA). The drift tube temperature of the ELSD was set at 50°C and the nebulising gas (nitrogen) flow rate was set at 1.5 litre min⁻¹. POo, SWO and structured lipid prepared from their physical blend were injected about 25 µl of 1 mg ml⁻¹ concentration in to the reversed phase silica column (X Bridge TM C18, 5 µm, 4.6 x 250 mm). The molecular species were eluted with mobile phase acetone (100%) at a flow rate of 1 ml min⁻¹. The molecular species of oils and SL were tentatively identified by their equivalent carbon numbers (ECN) and with the elution order (Reena, 2009; Buchgraber, 2004).

Rancimat assay. The oxidative stability of individual oils and the interesterified products was evaluated by the Rancimat method (Trujillo, 2006). The test was performed on a Rancimat apparatus (Metrohm Co., Herisau, Switzerland). The Rancimat apparatus was operated at 110°C with a constant dry air flow (20 litres hr⁻¹), which was blown through the sample (5 g). The volatile oxidation products which are produced during the oxidation of oils are causes to increase the conductivity of the water. The oxidative stability index (OSI) was obtained by induction periods (IP) which were calculated automatically by Rancimat method (Ley, 2003). When the conductivity of the distilled water is recorded continuously an oxidation curve is obtained whose point of inflection is known as the induction time expressed in hours. The high induction time indicates the increased of oxidative stability of the oils. All the analyses were performed in triplicate and the values given are average of the three measurements.

RESULTS AND DISCUSSION

The crude SWO was extracted from eri silkworm pupae and the oil was found to be 20.4% which was refined to remove undesirable components. Refined POo and refined SWO were analysed for different physico-chemical characteristics and the results are given in *Table 1*.

For the preparation of SL, the ratio of PO and SWO blend was restricted to 80:20 (wt/wt) based on the regulations of Prevention of Food Adulteration Act (PFA, 2005) and Bureau of Indian Standards methods (BIS, 1995). The interesterification reaction was initiated by the addition of lipase and the reported conditions were employed for the preparation of SL. The properties of initial oils, physical blend of palm and SWO along with SL were analysed for different physico-chemical characteristics and the results are shown in *Table 1*.

TABLE 1. PHYSICO-CHEMICAL CHARACTERISTICS OF STRUCTURED LIPID AND SUBSTRATES

Characteristic	SWO	PO	SWO:PO Blend	Structured Lipid
FFA (%)	0.17±0.01	0.16±0.01	0.16±0.01	0.25±0.07
Peroxide value	2.81±0.22	0.98±0.12	1.38±0.12	1.51±0.09
Iodine value (g 100 g ⁻¹)	154.80±0.31	58.72±0.11	71.61±0.08	72.81±0.15
Unsaponifiable matter (%)	2.18±0.17	0.31±0.04	0.49±0.09	0.48±0.10
Saponification value (mg of KOH g ⁻¹)	195.32±0.10	199.05±0.14	198.22±0.14	198.51±0.14
Phosphorous (ppm)	0	0	0	0
p-anisidine value	8.38±0.12	4.25±0.07	5.31±0.08	5.40±0.09
TOTOX value	13.99±0.57	6.20±0.31	8.06±0.32	8.74±0.27
TBAR	0.42±0.02	0.39±0.01	0.40±0.01	0.40±0.02
Colour in 1" cell as (Y + 5 R)	28 (18Y, 2R)	14 (7Y, 1.4R)	20 (15Y, 1R)	18 (12Y, 1.2R)
Refractive index (40°C)	1.4697	1.4691	1.4612	1.4613
Cloud point (°C)	3	14.5	14.0	23.0

Note: SWO – silkworm oils.
 PO – palm oil.
 FFA – free fatty acid.
 TOTOX – total oxidation.
 TBAR – tribarbituric acid-refractive.

The results showed that, the iodine value (154.8) was much higher in SWO than the PO (58.72) which is due to the presence of linolenic acid in higher quantity in silkworm oil. Peroxide value was also observed to be slightly higher in SWO (2.81) compared to PO (0.98). Phospholipids were not present in both the oils, which were removed completely during refining. Saponification value of PO (199.05) is slightly higher compared to SWO (195.32). The colour of the SWO was observed to be higher compared to PO. The p-anisidine value, TOTOX and TBAR values were observed to be higher in SWO compared to PO.

In the blend, the peroxide value was observed to be more than 1.38 compared to PO which had a peroxide value of 0.98. The iodine value was increased from 58.72 to 71.61 compared to the initial palm oil. This increase in peroxide value and iodine value is due to the increase in unsaturated fatty acids which are present in SWO. A slight increase in p-anisidine value, TOTOX and TBAR values are observed in the physical blend compared to PO. The results also showed that there was no significant difference in the physico-chemical characteristics of the prepared SL and physical blend of palm and

SWO. However, there was considerable difference in the cloud point of the prepared SL compared to the blend. This shows that the interesterification resulted in increase in the cloud point which could be due to the exchange of acyl groups on the triglyceride back bone. The fatty acid composition of the structured lipid prepared from palm and SWO is given in Table 2 along with the initial oils and the physical blend.

The results showed that, the ALA content in SWO was observed to be 52.23%, which is similar to flaxseed oil composition (Fazary, 2015). However, the content of linoleic acid (18:2, n-6) is higher in PO (10.17%) compared to SWO (4.18%). Similarly oleic and palmitic acids are higher in PO compared to SWO. Over all, saturated fatty acids were observed to be more in PO and unsaturated fatty acids were found to be more in SWO. ALA content was present at 9.23% in the blend of palm and SWO. Linoleic acid (18:2) was reduced from 10.17% to 8.82% in the blend compared to PO. The reduction in palmitic acid was also observed from 41.36% to 38.19% in the physical blend compared to PO. The same trend was observed in case of oleic acid, which was reduced from 44.69% to 38.23% in physical blend. The *trans*

TABLE 2. FATTY ACID (%) COMPOSITION OF STRUCTURED LIPID AND SUBSTRATES

Fatty acid	Silkworm oil (SWO)	Palm oil (PO)	SWO:PO blend	Structured lipid
Myristic acid (14:0)	0.25±0.08	0.69±0.06	0.66±0.15	0.78±0.12
Palmitic acid (16:0)	24.71±0.04	41.36±0.16	38.19±0.58	38.04±0.21
Palmitoleic acid (16:1)	1.14±0.17	-	0.16±0.03	0.24±0.01
Stearic acid (18:0)	3.93±0.03	3.09±0.04	4.69±0.75	4.34±0.17
Oleic acid (18:1)	13.54±0.32	44.69±0.18	38.23±0.46	38.31±0.05
Linoleic acid (18:2)	4.18±0.06	10.17±0.11	8.82±0.09	8.88±0.18
Linolenic acid (18:3)	52.23±0.19	-	9.23±0.09	9.38±0.14

fatty acid content in the SL was also determined and it was found that no *trans* fatty acid content was observed as lipase was used for interesterification reaction.

The molecular species of TAG of both the oils before and after interesterification were determined by using reversed phase HPLC and the results are shown in Table 3. The analysis indicated the formation of newer molecular species in modified lipid which were not present in the physical blend. The data indicated that the molecular species in SWO and SL were in the range of ECN C36-C48, where as in PO it was observed from C42-C48. The molecular species of SL was observed to possess C48 as the major species component, followed by C46 and other species. The formation of new molecular species indicates that the enzymatic reaction was successful in exchange of fatty acids as reported by many studies in the literature (Kaki, 2012).

The prepared SL was further studied for OSI and was compared with substrate oils along with physical blend. The results are shown in Figure 1.

The rancimat assay results showed that the PO has higher oxidative stability (17.67 hr) compared to SWO (3.86 hr). The large difference observed in

oxidative stability for both palm and SWO is due to the variation in fatty acid composition particularly unsaturated fatty acids. The oxidative stability of the blend and interesterified SL is almost similar. After blending the palm and SWO at 80:20 ratios (wt/wt), a better oxidative stability was observed which is imparted by the saturated fatty acids from palm oil. In a reported study, the SL having 32.39% of ALA prepared from perilla and corn oil was also shown to have higher oxidative stability compared to perilla oil due to the balanced saturated and unsaturated fatty acids (Mitra, 2014). The SL obtained after interesterification of palm and SWO at 80:20 ratios (wt/wt) can be claimed as novel SL with better oxidative stability with potential value addition to both substrate oils. Further, the oxidative stability of the SL can be improved if a suitable antioxidant is added (Thumu, 2015). The SL prepared contains ALA in similar amounts to soyabean oil and hence, can be tested for the applications where soyabean oil is used to further expand its application. Further studies for producing additional SL with SWO could be of interest based on preliminary evaluation of the SL as SWO is reported to be safe based on its toxicological evaluation (Longvah, 2012).

TABLE 3. TRIACYGLYCEROL (TAG) MOLECULAR SPECIES OF OILS AND PHYSICAL BLEND

Effective carbon No.	Expected mol. species	Palm olein	Silkworm oil	Structured lipid
C36	LnLnLn	-	16.71±0.20	2.31±0.38
C38	LLnLn	-	5.77±0.15	2.83±0.14
C40	PLnLn/LLLn/OLnLn	-	39.24±0.32	3.11±0.79
C42	LLL/PLLn/SLnLn/LLnO	2.59±0.49	7.96±0.56	1.95±0.21
C44	OOLn/LLO/PLL/PLnP/SLLn	9.63±0.11	22.08±0.61	7.92±0.48
C46	POL/OOL/PLP	11.55±0.35	2.64±0.04	11.71±0.16
C48	OOO/POP/SLnS	76.22±0.53	5.59±0.03	70.16±1.08

Note: P - palmitic acid, S - stearic acid, O - oleic acid, L - linoleic acid, Ln - linolenic acid.

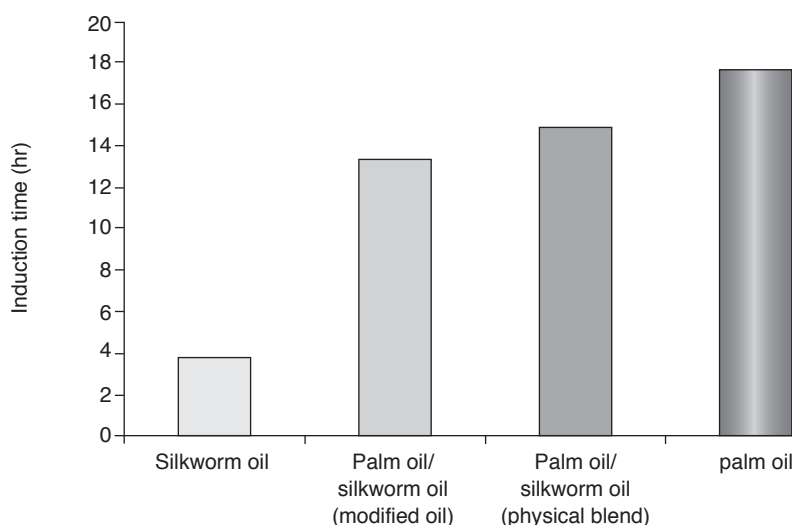


Figure 1. Oxidative stability of oils and modified oils.

CONCLUSION

SL based on palm and SWO was designed and synthesised in this study. Physico-chemical characteristics of both the refined palm olein, silkworm oils were determined and compared with before and after interesterification. Refined PO and refined SWO were subjected to enzymatic interesterification to obtain SL with about 10% of ALA. The SL exhibited good oxidative stability compared to SWO. The SL prepared by the interesterification of refined PO and SWO has potential applications in nutraceutical, food and industrial applications.

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