Research Article

Emulsifier and antioxidant properties of by-products obtained by enzymatic degumming of soybean oil†

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Running title: By-products obtained by enzymatic degumming

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Keywords: enzymatic oil degumming, phospholipids, fractionation process, $^{31}$P NMR

Abbreviation: DSL deoiled sunflower lecithin; RG recovered gum obtained by enzymatic degumming of crude soybean oil; RG deoiled deoiled gum; RG soluble ethanol soluble fraction of recovered gum, RG insoluble ethanol insoluble fraction of recovered gum

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/ejlt.201200333].

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Received: September 20, 2012 / Revised: February 1, 2013 / Accepted: March 1, 2013
Summary

The enzymes used in degumming, called phospholipases, specifically act on phospholipids without degrading the oil itself. Degumming using a phospholipase C enzyme allows to meet all market specifications while it increases the oil yield. The aim of this study was to evaluate antioxidant and emulsifier properties of the recovered gum (RG) obtained by enzymatic oil degumming process of crude soybean oil subjected to modifications as deoiling (RG deoiled) or ethanol fractionation (RG soluble and insoluble). RG soluble allowed obtaining more stable O/W emulsions (30:70 wt/wt) in comparison with those by-products assayed at different concentrations (0.1-1.0%). Also, Deoiled Soybean Lecithin (DSL) and RG deoiled had a similar behavior in relation to the kinetic destabilization (%BS profiles), despite the different degumming processes used to obtain these samples. The study on induction times (Metrohm Rancimat) showed a significant antioxidant effect ($p < 0.05$) against a refined sunflower oil associated with all the by-products analyzed. However, RG soluble and DSL showed a strong effect on the oil stability at high concentrations (1000-2000 ppm). These results showed that the deoiled recovered gum and its derivates obtained by ethanol fractionation are a potential alternative for industrial application as additive.

Practical applications

The economic benefits of enzymatic degumming process have also been quantified by several oilseed processors. This process allows obtaining a by-product with a high concentration of different phospholipids. This study intends to increase the commercial value of this recovered gum contributing to the food industry with useful information about their functional properties.
1. Introduction

Remotion of undesirable impurities (nontriglycerides) from the crude vegetable oil that affect its quality (taste, smell, appearance) and storage, with the least possible damage to the triglycerides and the minimal loss of desirable parts are the main objectives of the refining process. The quantities of these nontriglycerides -free fatty acids, phosphatides, color pigments, metal ions, odours, moisture, etc- vary with the oil source, extraction process, season, and geographical area [1, 2].

Untreated vegetable oils contain different proportions of phospholipids (PLs) such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA). These phosphatides can degrade and cause dark colors when the oil is heated as in a later deodorization step or cause dangerous foaming if these compounds are present in frying oils [3]. Thus, degumming is an important processing step during the refining of vegetable oils to remove phosphatides and other impurities. This process consists of the treatment of crude oils with water, salt solutions, enzymes, caustic soda, or dilute acids to convert phosphatides to hydrated gums, which are insoluble in oil and readily separated as a sludge by settling, filtering, or centrifugal action [4].

The main types of phospholipase are A1, A2, C, and D with their target sites can be shown in Scheme 1. The use of this type of enzymes for degumming vegetable oils was firstly reported in the 1990’s by Roehm and Lurgi concerning the commercial “Enzy-Max process” with phospholipase A2 from porcine pancreas (Lecitase® 10L). Later, Novozymes substituted Lecitase® 10L with Le citase® Novo, a phospholipase A1 from Aspergillus oryzae, owing to limited enzyme source, high optimal pH and non-compliance of kosher and halal specifications [5]. Phospholipase C also has been considered for oil degumming because the phosphate moiety generated by its action on phospholipids exhibit high solubility in water and it is easy to remove, and the diglyceride would stay with the oil and reduce losses [6]. Use of enzymes diminishes the emulsification properties of the phospholipids in the oil system, allowing the reduction of oil loss. In this way, the application of this new technology contributes to yield gains up to 2% [7].

Enzymatic degumming process allows recovering a gum with a high concentration of different phospholipids (RG). Although some authors have made contributions to the study of the enzymatic degumming process, most of them did not analyze the feasibility of applying this by-product as industrial additive [8].

Phospholipids with hydrophilic and lipophilic portions in their molecular structure are concentrated at the interface between oil and water. This behaviour facilitates the formation of an emulsion during the homogenization process and prevents destabilizing
processes such as creaming, coalescence, thus increasing the life of the product [9-11]. On the other hand, PLs can contribute to the improvement of the oxidative stability of fats and oils. Various antioxidative mechanisms have been proposed for the phospholipid actions [12]. For example, the amino functions of PC, PS, or PE, or the sugar moiety of PI have been shown to have metal-chelating properties and PC and PE presented a synergistic effect, with phenolic antioxidants such as tocopherols and flavonoids [13,14].

The objective of this work was to evaluate the potential applications of the by-products of the enzymatic degumming process of crude soybean oil as emulsifier or antioxidant agent.

2. Materials and Methods

2.1. Materials

Native soybean lecithin and by-products of the enzymatic degumming process used as starting material (RG) were provided by a local oil industry (Vicentin S.A.I.C.). The enzymatic degumming process used 50 ppm of Phospholipase C (PLC, Purifine, Verenium) for 2 h at 55°C.

Native soybean lecithin and the recovered gum were deoiled with acetone according to AOCS Official Method Ja 4-46, procedures 1–5 [15, 16]. This process allowed obtaining the deoiled soybean lecithin (DSL) and the deoiled gum (RG deoiled), respectively (Fig. 1). Both samples were stored at 0 °C. Deoiling procedure was performed in duplicate.

All solvents used were of analytical grade.

2.2. Recovered gum fractionation

Fractionation process was performed on 30 g of by-products obtained by enzymatic degumming with the addition of absolute ethanol (absolute ethanol/lecithin ratio 3:1). This sample was incubated in a water bath at 65 °C for 90 min with moderate agitation (60 rpm), and then centrifuged at 1880 g, 10 °C for 10 min. Afterwards, the corresponding ethanolic extracts and residues were obtained and ethanol was eliminated by evaporation under vacuum [17].

Ethanol soluble and insoluble phases were further deoiled with acetone, obtaining the soluble (RG soluble) and insoluble (RG insoluble) fractions, respectively (Fig. 1). Then, both fractions were stored at 0 °C. Fractionation procedure was performed in duplicate.
2.3. Phospholipid composition

Phospholipid composition of samples obtained after different modification processes was determined by $^{31}$P NMR analysis in a Bruker Avance 600 MHz automatic spectrometer, using triphenyl phosphate as internal standard (Spectral Service GmbH, Köln, Germany) [18]. For this purpose, 100 mg of each sample were diluted in 1 ml of deuterated chloroform, 1 ml of methanol and 1 ml of 0.2 M Cs-EDTA (pH 8.0). The organic layer was separated after 15 min shaking, and analyzed using the described spectroscopic technique. Phospholipid composition was expressed in terms of molar concentration (mol / 100 mol lecithin) (Table 1).

2.4. Coarse Oil-in-Water (O/W) emulsions preparation

Refined sunflower oil was utilized to prepare oil-in-water (O/W) emulsions with a formulation of 30:70 wt/wt. Coarse emulsions were prepared at room temperature in an Ultra-Turrax T25 homogenizer using S 25 N–10 G dispersing tool (7.5 mm rotor diameter) at 25,000 rpm for 1 min, with the addition of different emulsifying agents (DSL deoiled, RG deoiled, RG soluble, RG insoluble) in a range of 0.1–1.0% (wt/wt) according to Cabezas et al. [19]. The behavior of these emulsions as a function of the storage time was analyzed for 90 min in a QuickScan Vertical Scan Analyzer (Coulter Corp., Miami, FL).

2.5. Fine Oil-in-Water (O/W) emulsions preparation

Coarse emulsions previously obtained in Ultra-Turrax homogenizer, with 1% of different emulsifying agents, were homogenized in an ultrasound homogenizer (SONICS Vibra Cell VCX750) at a power level of 70%, with the standard tip immersed 1/3 in a glass of 28 mm diameter for 1 min. In addition, these fine O/W emulsions were subjected to different temperatures (0 and 24ºC) and their behavior was analyzed in a QuickScan Vertical Scan Analyzer (Coulter Corp., Miami, FL) in storage for 21 days.

2.6. Optical characterization of O/W emulsions

The backscattering of light was measured using a QuickScan Vertical Scan Analyzer. The backscattering of monochromatic light ($\lambda = 850$ nm) from the emulsions was determined as a function of the height of the sample tube (ca. 65 mm) in order to quantify the rate of different destabilization processes at different stages of the analysis. This methodology allowed us to discriminate between particle migration (sedimentation, creaming) and particle size variation (flocculation, coalescence) processes [19, 20]
2.7. Particle size

Particle size distribution, and De Brouker (D[4,3]) and Sauter (D[3,2]) mean diameters of particles of the emulsions were determined with a particle size analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd., Worcestershire, U.K.). Samples were diluted in the water bath of the dispersion system (Hydro 2000MU), which is a laser diffraction based particle size analyzer [9, 21]. This determination was carried out in triplicate for each sample.

2.8. Antioxidant properties

The antioxidant properties of the different samples were evaluated using the Rancimat (Mod 679, Metrohm) method. 5 g of sunflower oil (α-tocopherol 512.84 μg/g; β-tocopherol 4.55 μg/g) were added at different concentrations of the analyzed samples (250 to 2000 ppm), heated at 98 °C, air flow 20 L/h. Stability was expressed as the induction time according to Gutiérrez [22,23].

2.9. Statistical analysis

Data were evaluated using analysis of variance (ANOVA) with the software Systat® 12.0 [24]. For this purpose, differences were considered significant at \( p < 0.05 \).

3. Results and Discussion

3.1. Phospholipid composition

Native soybean lecithin (10.0% PI, 15.5% PC, 8.5% PE, 2.4% PA and 6.1% minor phospholipids) and the by-product obtained after an enzymatic degumming process (15.0% PI, 4.8% PC, 3.2% PE, 4.6% PA, and 9.6% minor phospholipids) were used as starting materials.

The phospholipid composition of the different phospholipidic-products obtained in this research work is shown in Table 1. A marked difference was recorded regarding the phospholipid composition between the deoiled soybean lecithin and the samples obtained from the recovered gum (RG deoiled, soluble, and insoluble). PLC (Purifine, Verenium) only affects PC and PE, but it does not catalyse the hydrolysis of PI or PA and their nonhydratable salts [25]. In correlation with this feature, RG deoiled presented a lower concentration of PC and PE and a higher concentration of PI in comparison with the deoiled soybean lecithin. On the other hand, the ethanol soluble and insoluble fractions presented a high concentration of PC (19.3% mol PC/100 mol lecithin) and PI (22.3% mol...
PI/100 mol lecithin), respectively. These results are in agreement with those obtained by applying different fractionation processes with ethanol on different vegetable lecithins [17, 26, 27].

3.2. Coarse O/W emulsions

O/W emulsions prepared in Ultra-Turrax homogenizer (30:70 wt/wt) were studied recording the backscattering (%BS) profiles as a function of cell length and time using a vertical scan analyzer (QuickScan). The analysis of the %BS values on the tube Zone I (10-20 mm) and Zone II (55-60 mm) allowed the characterization of the emulsion stability in relation to the destabilization processes of creaming and coalescence, respectively [19]. Figure 2 shows %BS values during 90 min of the different O/W emulsions prepared in Ultra-Turrax homogenizer.

The QuickScan profiles corresponding to the zone I (10-20 mm) showed an increase in the emulsion stability against the creaming process as a function of an increasing concentration of different phospholipidic-products (Fig. 2). In particular, the addition of RG soluble reached a higher stability in O/W emulsions than the other products analysed at the bottom zone of the tube over the range of concentration studied. On the other hand, O/W emulsions with 0.1-0.5% of deoiled and insoluble recovered gum showed a sharp decrease of %BS in the Zone I because of the migration of the oil particles to the upper portion of the tube.

Emulsions are highly dynamic systems, therefore if droplets are not protected by a sufficiently strong interfacial film, they tend to coalesce with one another during the frequent collisions [9]. The destabilization process of the cream phase by coalescence was analyzed in the upper zone of the tube (55-60 mm) (Zone II). A sharp decrease in the %BS values was observed with 0.1% of RG deoiled, RG insoluble and DSL, suggesting the occurrence of a rapid cream phase destabilization such as coalescence. A similar observation was also recorded by adding 1% of RG soluble.

Figure 3 depicts the evolution of De Brouker (D [4,3]) and Sauter (D [3,2]) mean diameters as a function of type and concentration of the emulsifying agents. A high concentration of large particles produces a fast creaming process according to the Stokes' law [28]. On the other hand, an increase in the mean diameters of the oil droplet as a function of time could be correlated with destabilization by coalescence. Thus, these results are in agreement with the previous analyses of the %BS values by the corresponding QuickScan profiles. Most unstable emulsions presented a high initial particle size and/or a significant increase of these values (p < 0.05) after 90 min.
Particularly, emulsion with 0.1% of the ethanol insoluble fraction presented a fast destabilization, where creaming and coalescence processes took place simultaneously without enabling the formation of a stable cream phase.

PC forms a lamellar phase at the interface between oil and water with well-ordered mono- and bi- layers. These structures are of importance for the stabilisation of O/W emulsions [19]. However, PE and occasionally PA give a reversed hexagonal phase, which is more difficult to arrange at the interface [29]. These behaviours are in relation to the previous analysis of the QuickScan profiles and the low mean diameters recorded in emulsions using 0.1-0.5% of RG soluble, instead of the poor characteristics as emulsifying agent of the other samples studied at low concentration. On the other hand, the increase of RG soluble in the formulation of O/W emulsions also increases the PE and PA content. The formation of the unstable cream phase in relation to the coalescence process at concentration of 1% of this emulsifier could be generated (Figs. 2 and 3).

3.3. Fine O/W emulsions

Fine O/W emulsions prepared in an ultrasound homogenizer (30:70 wt/wt) by adding 1% of each emulsifier were studied recording the backscattering (%BS) profiles for 21 days (Fig. 4). The analysis of the %BS values was measured in the Zone I (10-20 mm) of the tube.

Emulsions formulated with the ethanol soluble fraction presented higher %BS values than those obtained using the other phospholipidic-products for both temperatures studied. In this sense, it should be noted that QuickScan profiles obtained with this emulsifier did not show significant variations of %BS values ($p > 0.05$), especially during the entire storage time at 0°C or the first 15 days at 24°C. In addition, emulsions after the addition of RG soluble presented the smallest particle size (D [4,3], D [3,2]) in all conditions assayed (Fig. 5). These results coupled with those previously analyzed showed the best condition as emulsifying agent of this soluble fraction of the recovered gum compared to other samples assayed.

DSL and RG deoiled samples had a similar behavior in relation to the %BS profiles in the lowest zone of the tube (creaming) and the mean particle sizes (Figs. 4 and 5), despite the different degumming processes used to obtain these samples. On the other hand, emulsions formulated with the ethanol insoluble fraction showed the lowest stability against the creaming process, especially during the first week.

O/W emulsions obtained in an ultrasound homogenizer were not affected by the coalescence process. These emulsions did not show significant changes ($p > 0.05$) either
in the % BS profiles on the upper region of the tube (data not shown) nor in the values of De Brouker and Sauter mean diameters as a function of time (Fig. 5). This fact could be associated with the formation of a stable cream phase, with a high density of particles, hence with a lower proportion of continuous phase [28].

3.4. Antioxidant properties

Antioxidant properties of the different phospholipidic-products were evaluated by analyzing the respective induction times (t_i) recorded on a Rancimat equipment (Mod 679, Metrohm). This methodology can be used to evaluate the capacity of synthetic or natural antioxidants to stabilize different fats and oils against the oxidation process through an oxidation accelerated test [30].

The ability of phospholipids to inhibit lipid oxidation in bulk oils has been known for several decades, but the mechanism of stabilization still remains controversial [31]. However, many research works have proposed different antioxidative mechanisms for these compounds. Particularly, PC and PE have been shown to have metal-chelating and scavenging properties. Also, this type of phospholipids presents a synergistic effect with the different tocopherols (α-, γ-, δ-) regenerating the oxidized tocopherol molecule by donation of a hydrogen atom of their amino function [32]. This fact and the high PC and PE concentrations are in correlation with the best antioxidant characteristics observed for the RG soluble and DSL assayed in this research work (Fig. 6).

The analysis of the induction times values (t_i) showed a significant antioxidant effect (p < 0.05) associated with the different samples analysed, over the concentration range tested with respect to the control system (refined sunflower oil) (Fig. 6). At low concentration (250-500ppm), samples obtained from modification of the by-products obtained by enzymatic degumming of soybean oil did not show a significant difference (p > 0.05) on the respective t_i values. However, RG soluble and DSL showed a strong effect on the oxidative stability of oil at high concentrations (1000-2000 ppm), increasing this parameter by 87% with respect to the control oil. At concentration of 2000 ppm, these samples doubled the t_i value obtained by using refined sunflower oil. The behaviour of this variable as a function of the concentration of the emulsifier agent is in accordance with the results of Pokorný et al. (1990) on soybean and rapeseed lecithins which present a pronounced autooxidation inhibition activity toward seed oils at high concentration (0.5-2.0%), but only a moderate one at low levels (0.02-0.10%) [33]. This feature could be associated with the need for a minimum concentration of amino alcohol phospholipids (PC
and PE) to produce the mentioned synergistic effect with the different tocopherols presents in the oil control.

4. Conclusion

The emulsifying and antioxidant properties of the modified recovered gum obtained by deoiling or ethanol fractionation in comparison with the deoiled soybean lecithin suggest that their application by the local oil industry could revalue the by-product of the enzymatic degumming process of soybean oil. These products, specially RG soluble, show a potential industrial application.

Acknowledgments

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina (PICT 2007-1085), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, PIP 1735 (CONICET); and Universidad Nacional de La Plata (UNLP), Argentina, 11/X502 (UNLP).

D. M. Cabezas and M. C. Tomás are members of the Career of Scientific and Technological Researcher of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. B. W. K. Diehl is Director of Spectral Service GmbH, Cologne, Germany.

Soybean lecithin and lyso-gum were provided by Néstor Buseghin (Vicentin S.A.I.C., Argentina). Thorsten Buchen and Rute Azevedo (Spectral Service, Germany), Jorge Wagner and Paula Sceni (Universidad Nacional de Quilmes, Argentina) are acknowledged for technical assistance.

The authors have declared no conflict of interest

References


**Table 1.** Phospholipid (PL) composition (mol PL/100 mol lecithin) of different phospholipidic-products obtained from a by-product of the enzymatic degumming process (RG deoiled, RG soluble, RG insoluble)\textsuperscript{a)} and a deoiled soybean lecithin (DSL)\textsuperscript{a)} by \textsuperscript{31}PNMR \textsuperscript{b)}

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<th>RG insoluble</th>
<th>DSL</th>
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\textsuperscript{ns}= no signal assignment
\textsuperscript{a)} See Figure 1
\textsuperscript{b)} Average values are shown ($n = 2$). The coefficient of variation was lower than 4%
Figure captions

**Scheme 1.** General representation of a PL indicating the position of cleavage of different phospholipases

**Fig. 1.** Flow diagram of the process used for producing different phospholipidic-products obtained from a by-product of the enzymatic degumming process (RG deoiled, RG soluble, RG insoluble) and deoiled soybean lecithin (DSL)

**Fig. 2.** Backscattering (%BS) profiles of coarse O/W emulsions (30:70 wt/wt) obtained in an Ultra-Turrax homogenizer with the addition of different phospholipidic-products (RG deoiled, RG soluble, RG insoluble, DSL) in Zone I (10-20 mm) and Zone II (50-55 mm) of the measuring tube. Mean values ($n = 3$) ± sd

**Fig. 3.** Mean diameters for coarse O/W emulsions (30:70 wt/wt) obtained in an Ultra-Turrax homogenizer with the addition of different phospholipidic-products (RG deoiled, RG soluble, RG insoluble, DSL): (A) $D[4,3]$; (B) $D[3,2]$. Mean values ($n = 3$) ± sd

**Fig. 4.** Backscattering (%BS) profiles of fine O/W emulsions (30:70 wt/wt) obtained in an ultrasound homogenizer with the addition of different phospholipidic-products (RG deoiled, RG soluble, RG insoluble, DSL) in Zone I (10-20 mm) of the measuring tube analyzed at different store temperatures. Mean values ($n = 3$) ± sd

**Fig. 5.** De Brouker ($D[4,3]$) and Sauter ($D[3,2]$) mean diameters for fine O/W emulsions (30:70 wt/wt) obtained in an ultrasound homogenizer with the addition of different phospholipidic-products (RG deoiled, RG soluble, RG insoluble, DSL). Mean values ($n = 3$) ± sd

**Fig. 6.** Percentage induction time increase of sunflower oil added with different concentrations of phospholipidic-products in relation to the induction time of the control sample (refined sunflower oil) obtained in Rancimat equipment (Mod 679, Metrohm). Mean values ($n = 3$) ± sd
By-product of Enzymatic Degumming (RG)  

Ethanol Fractionation  
65°C, 90 min  
Absolute ethanol / Lecithin 3:1v/wt  

Ethanol Extract  
Residue  

Soybean Lecithin (SL)  

Deoiling  
Acetone / Lecithin 4:1v/wt  

RG soluble  
%Yield  
RG soluble/RG 7.1%  

RG insoluble  
%Yield  
RG insoluble/RG 66.0%  

RG deoiled  
%Yield  
RG deoiled/RG 73.1%  

DSL  
%Yield  
DSL/SL 68.3%  

Figure 1, Cabezas et al.
Figure 2, Cabezas et al.
Figure 3, Cabezas et al.
Figure 4, Cabezas et al.
Figure 5, Cabezas et al.
Figure 6, Cabezas et al.
Phospholipase A$_1$

Phospholipase A$_2$

R$_2$\[\text{O}\]\text{O}^{-}\text{CH}

\[\text{CH}_2\text{O}\text{P}^{-}\text{O}^{-}\text{X}\]

Phospholipase C

Phospholipase D

X = H, choline, ethanolamine, inositol, etc.

Scheme 1, Cabezas et al.