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Preparation and characterization of liprotides prepared from protein extracts of mung beans (*Vigna radiata* (L.))

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ABSTRACT

Liprotides are protein-fatty acid complexes that have been shown to exhibit promising drug carrier and anti-cancer activities. Most published studies have focused thus far on animal serum albumin proteins, and there is scarce data on liprotides prepared from other protein molecules, particularly those derived from plants. Therefore, to explore new liprotide preparations, mung bean albumins and globulins were extracted, and their complexation with oleic acid at 40 °C and 80 °C was investigated. The prepared complexes were then subjected to ATR-FTIR spectroscopy, particle size, and zeta potential analyses. The FTIR spectra of the liprotides showed that the proteins' characteristic amide I and II bands shifted to higher wavenumbers, indicating changes in secondary structure in the presence of oleic acid. This was also accompanied by changes in the characteristic bands for C=O and C-H stretching of oleic acid in the presence of the protein. Particle size analysis showed that the prepared liprotides were highly polydispersed (PdI > 0.4), ranging from 658.7 nm and 1946 nm in diameter, that was also larger than those reported previously. Finally, zeta-potential measurement revealed that the liprotides had values ranging from -24.19 mV and -34.88 mV, which generally suggested an incipient to moderate stability of the colloidal suspension against aggregation. These data suggest that the preparation temperature employed in the study resulted in liprotides prepared from the crude albumin and globulin extracts having comparable characteristics.

Keywords: *albumin, globulin, liprotide, mung beans, oleic acid*

INTRODUCTION

Liprotides are complexes composed of a micellar oleic acid core and a flexible, partially unfolded protein shell. This type of core-shell structure was first reported by Håkansson et al. (1995) via the synthesis of the cytotoxic human alpha-lactalbumin made lethal to tumor cells, which they called HAMLET (Svensson et al., 2000), a complex consisting of cis-monounsaturated oleic acid surrounded by alpha-lactalbumin. Alpha-lactalbumin, comprising of 123 amino acids, is one of the most abundant components of whey protein that was previously identified as an apoptosis-inducing agent in human milk with broad, but selective cytotoxic action contributing to the possible role of milk in stimulating mucosal immunity (Håkansson et al. ,1995). A remarkable feature of this molecule is its ability to undergo changes in tertiary structure, under mild denaturing conditions, to a molten globule-like state that induces apoptosis; in contrast, native alpha lactalbumin is unable to cause a similar effect (Svensson et al., 1999). It was initially thought that this type of transition, and the corresponding biological effect, was unique to human alpha lactalbumin, however, a similar observation was seen with bovine alpha lactalbumin leading to the development of an analogous cytotoxic complex named BAMLET (Brinkmann et al., 2011). Over the years, various unrelated proteins such as lysozyme, parvalbumin, and beta-lactoglobulin, and different kinds of fatty acids have been used to prepare these complexes having a core-shell structure or even larger multi-layered-structure, i.e., an extra layer of fatty acids and proteins enveloping the inner core-shell body, when a higher fatty acid-protein molar ratio is used (Frislev et al., 2016). Liprotides are known for their cancer cell cytotoxicity, which has been proposed to arise from the transfer of the fatty acid, e.g., oleic acid, to the cell membrane, increasing its fluidity and compromising its integrity (Frislev et al., 2017). Furthermore, liprotides have also been shown to accommodate small molecules cargoes, provided that they have hydrophobic terminal moieties, demonstrating their potential as drug delivery carriers (Pedersen et al., 2015).

Previous studies have often only incorporated alpha-lactalbumin and other animal-based proteins into liprotides, while plant-derived proteins, to the best of our knowledge, have not been subjected to possible liprotide use. However, given the variety of proteins already shown to be compatible to liprotide formation, it is highly likely that proteins derived from plants would be a suitable component as well. In this regard, beans are primarily known to be abundant in protein content, including mung beans (Vigna radiata (L.)) which have protein content ranging from 20.97 - 31.32%, significantly higher than other commonly utilized beans such as soybeans (18-22%) and kidney beans (20-39%) (Anwar et al., 2007; Shevkani et al., 2015; Xu et al., 2015). The high percentage of proteins in these beans provides a feasible starting material for forming the liprotide's protein shell. Therefore, as an alternative to the established use of alpha-lactalbumin and other animal-derived proteins, this study investigated using mung bean protein extracts with oleic acid in the preparation of liprotides to expand the current knowledge on suitable protein components of this promising bioactive complex.

METHODS

Protein Extraction. Cracked mung beans were purchased from a local supermarket in Las Piñas City, Philippines. Cracked mung beans were chosen instead of the shelled type mainly to reduce cellular wall debris contamination during extraction. The procedure for the acid-base extraction of mung bean proteins—primarily albumins and globulins—was based on the methodology of Yang et al. (2022) with some modifications. Mung beans were first ground using an electric grinder and sifted using a cheesecloth. Thirty (30) g of the powder was then dispersed in 300 ml of 0.01 M PBS buffer to achieve a 10% (w/w) dispersion. To increase protein solubility, the resulting mixture was stirred for 2 hours with a magnetic stirrer with a constant pH adjustment to 8.0 with 1 M NaOH. Afterwards, the mixture was centrifuged at 10,000 rpm for 15 minutes

(4°C) in a Hermle® Z36-HK Refrigerated Centrifuge (Hermle AG, Gosheim, Baden-Württemberg, Germany). The supernatant was collected and stirred for 1 hour, with a constant pH adjustment to 4.5 with 1 M HCl. This precipitated the globulins, which were then centrifuged into a pellet at 10,000 rpm for 15 min (4°C) and, after reconstituting in an ample amount of PBS buffer, was further stirred for 2 hours with constant pH adjustment to 7.0. Meanwhile, the supernatant containing the albumins was set aside. The globulin and albumin solutions were then freeze-dried using a Telstar® LyoQuest Benchtop Freeze-dryer (Azbil Telstar Technologies, S.L.U., Terrassa, Barcelona, Spain) for approximately two days. Subsequently, 0.8 g was taken from each of the lyophilized albumin and globulin samples and dissolved in 8 ml of PBS buffer to create a 100 mg/ml crude protein solution. The obtained solutions were dialyzed overnight using 6-kDa MWCO dialysis tubes (3 ml) in PBS buffer at 4°C. Biuret and Bradford assays were used as qualitative and quantitative tests for the proteins, respectively.

Liprotide Preparation. The preparation of liporotides was adapted from Frislev et al. (2017). In this study, four types of liprotides were synthesized based on different proteins and temperatures: albumin-oleic acid liprotide synthesized at 40 °C, albumin-oleic liprotide synthesized at 80 °C, globulin-oleic liprotide synthesized at 40 °C, and globulin-oleic liprotide synthesized at 80 °C. Liprotides were formed by mixing each protein solution with 125 mM oleic acid (in 20% ethanol) to an approximate molar ratio of 1:15 (crude protein:oleic acid) in PBS buffer, calculated using the molecular mass of albumin and globulin and without considering the crude nature of the extracts. The resulting mixtures were incubated at either 40 °C or 80 °C for one hour. Albumin, globulin, and oleic acid controls—that is, each component alone dissolved in PBS buffer—were also prepared at the respective temperatures for one hour using the identical amounts to those used in the liprotides. The as-prepared liprotides and control samples were then lyophilized and stored at four °C until use.

ATR-FTIR Spectroscopy. Analysis was carried out using a Shimadzu® IRSpirit Compact FTIR Spectrometer accessorized with a QATR™-S Single-Reflection ATR (Shimadzu Scientific Instruments Inc, Columbia, Maryland, U.S.A). Sixteen scans at a 4 cm^{-1} resolution in the range of 4,000–400 cm^{-1} were used for all samples in the solid, lyophilized form, obtained from a liprotide preparation having a 1:15 (crude protein:oleic acid) molar ratio. After each run, a new reference air background spectrum was obtained, followed by ATR plate cleansing. ATR correction, smoothing, and multiple baseline correction were applied to each individual IR spectrum.

Particle Size and Zeta Potential Measurements. Particle size and zeta-potential analyses were conducted using a Malvern® Zetasizer Pro (Malvern Panalytical Ltd., Worcestershire, U.K.). The samples were each dispersed in 5 ml PBS buffer and lightly vortexed before transferring to dip cells. The Z-average (d.nm) and polydispersity indices (PdI) of the liprotide samples were measured at 25 °C. A total run time of 6 minutes comprised of three measurements with 1-minute intervals was performed for each sample. Zeta-potential measurement was conducted in an automatic mode, and the average zeta-potential was obtained through three trials at 25 °C.

RESULTS AND DISCUSSION

The protein content of the extracts was estimated at 7.84 mg/mL for albumins and 23.78 mg/mL for globulins based on a standard Bradford assay. These values agree with the higher percentage of globulins compared with albumins previously reported in the literature, at 60% and 25% respectively of the total protein content of mung beans, with the other 15% being reported to include trypsin inhibitors, non-specific lipid transfer peptides, and thiamine-binding proteins (Yi-shen et al., 2018). A more recent report, whose extraction protocol was adapted in this study, reported that globulin accounted and albumin accounted for 66.6% and 10.5%, respectively, of the total protein content of mung beans (Yang et al., 2022). As liprotides prepared from plant-derived proteins have not yet been reported, it is worth mentioning that previous studies showed

that total protein isolates from mung beans demonstrated a denaturation temperature of ~158 °C, determined using differential scanning calorimetry (Brishti et al., 2017; Brishti et al., 2021). Thus, the preparation temperature employed in this study can be safely assumed to have resulted only in partial protein denaturation required for liprotide formation. Additionally, literature data indicated that heat-induced liprotide preparation have been carried out using a temperature range of between 45 – 90 °C, encompassing the chosen conditions in this study (Pedersen et al., 2015).

Encapsulation of oleic acid within a protein shell is expected to result in changes in the IR spectrum of both molecules, and the presence or absence of peaks and/or peak shifts of these characteristic absorption bands served as critical indicators for liprotide formation. Among these, emphasis was given to the peak at ~1711 cm-1 attributed to the C=O stretching, ~1550 cm-1 attributed to the COO asymmetric stretching, and between ~2800 – 3000 cm-1 for C–H stretching of the fatty acyl chains as the distinguishing features of oleic acid, while the amide I and II bands at around ~1650 cm-1 and ~1550 cm-1, respectively, were monitored with respect to the protein shell (De Meutter & Goormaghtigh, 2021; Usoltsev et al., 2019; Guillén & Cabo, 1999; Kachel et al., 2018; Nishikawa et al., 1993). Figure 1 shows the FTIR spectra of the prepared liprotides, overlayed with the respective protein and oleic acid controls. In all cases, the intensity of the peaks attributed to C=O, COO, and C–H stretching of oleic acid (black traces) was significantly reduced in the presence of the proteins (blue traces). Peak shifts also occurred between the albumin-oleic acid complexes and the oleic acid controls. Interestingly, previous works attributed frequency shifts to potential complexation, which in the current study is between oleic acid and the protein (Bagchi et al., 2012; Sharma & Lahiri, 2008; Yan et al., 2020). Such instances of peak shifting in FTIR analysis have two common perspectives: either the change in peak position is caused by the actual frequency shift of a distinct absorption band or by the relative intensity contributions of coinciding bands, such as the amide I band. The first case is the classical view wherein peak shifts have been correlated with changes in the degree of molecular interactions, most especially dipole-dipole and hydrogen bonding interactions. For instance, Nie et al. (2005) demonstrated that C=O stretching frequency can be strongly correlated with the hydrogen bond number of a protein's polar side chain COOH group as well as the hydrogen bond strength. The oleic acid peak shifts observed in this work could indicate the disruption of intermolecular hydrogen bonds between oleic acid molecules upon protein interaction, followed by complexation. For protein secondary structure changes assessment, amide I and II are the most insightful spectral bands reflecting C=O stretching in the peptide backbone (De Meutter & Goormaghtigh, 2021). The usefulness of the amide I band in FTIR analysis arises from the fact that it is the most sensitive region of the protein where each type of secondary structure corresponds to a different C=O stretching frequency due to their distinctive molecular geometry and hydrogen bond network. The amide II band, on the other hand, probes mostly in-plane N–H bending and C–H stretching, providing a lesser specificity and sensitivity for detecting conformational changes (Sadat & Joye, 2020). In this work, shifts of amide I and II bands to higher wavenumbers were observed in all prepared liprotides, as opposed to the corresponding protein controls (Figure 1, blue and red traces), implying changes in the secondary structure of the proteins upon complexation with oleic acid. These results are summarized in Table 1 and support the notion of a successful complexation of oleic acid with the extracted proteins.

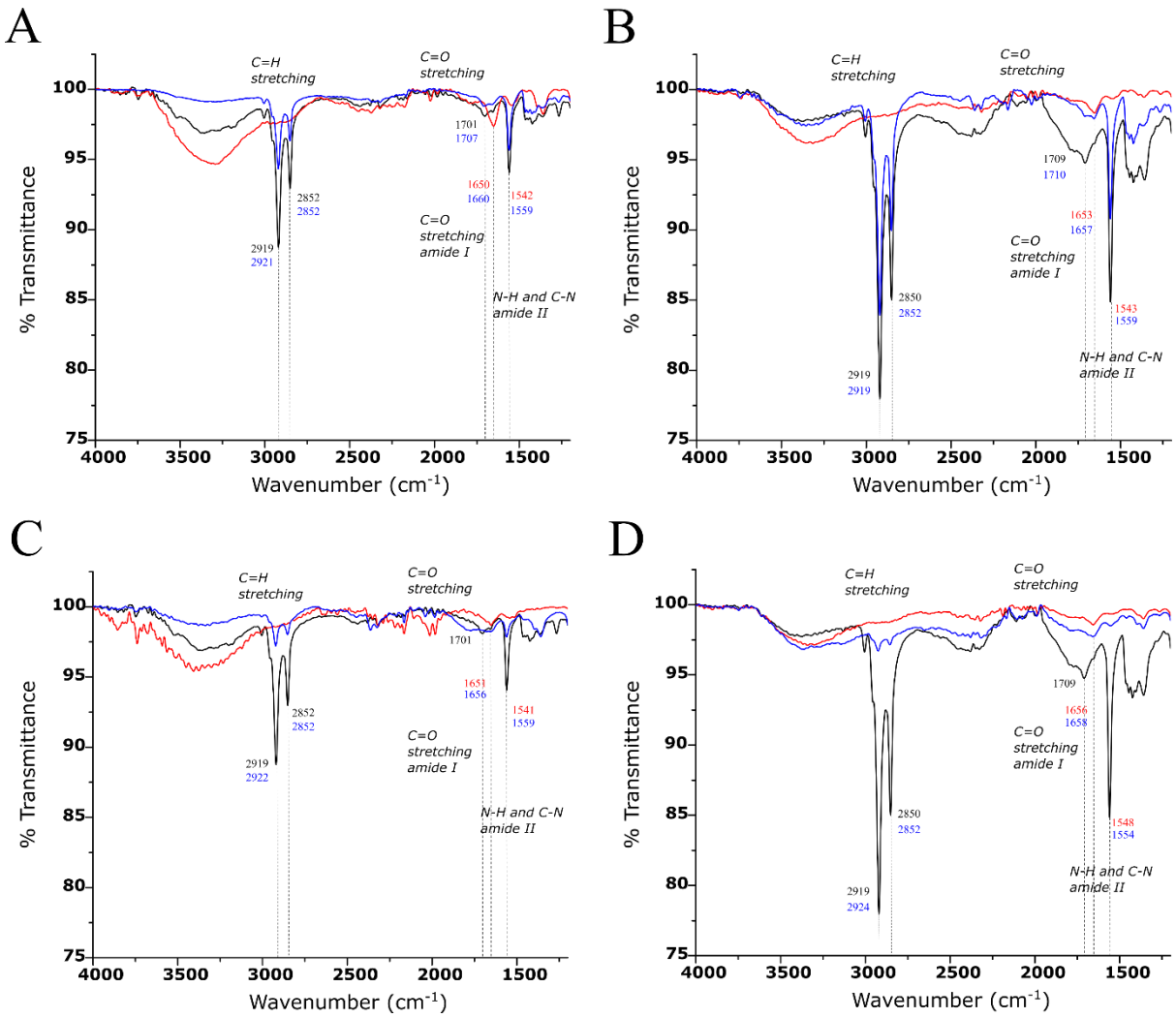

Figure 1. ATR-FTIR spectra of albumin – oleic acid liprotides (A and B, blue) and globulin – oleic liprotides (C and D, blue) prepared at 40 °C (A and C)and 80 °C (B and D) with the corresponding uncomplexed albumin (A and B, red) and globulin (C and D, red) (red) and pure oleic acid (A-D, black) at the same temperature serving as controls.

Table 1. Summary of the most notable peaks in the FTIR spectrum of the liprotides and control samples.

Sample	Peak position (cm^{-1}) & Band assignment			
	C – H stretching (oleic acid)	C = O stretching (oleic acid)	C = O stretching (protein amide I band)	N – H and C – N (protein amide II band)
Oleic acid 40°C	2919, 2852	1701	-	-
Oleic acid 80°C	2919, 2850	1709	-	-
Albumin 40 °C	-	-	1650	1542
Albumin 80°C	-	-	1653	1543
Globulin 40°C	-	-	1651	1541
Globulin 80°C	-	-	1656	1548
Albumin-oleic acid 40°C	2921, 2852	1707	1660	1559
Albumin-oleic acid 80°C	2919, 2852	1710	1657	1559
Globulin-oleic acid 40°C	2922, 2852	-	1656	1559
Globulin-oleic acid 80°C	2924, 2852	-	1658	1554

The prepared liprotides were further characterized for their size distribution and zeta potential, and results are summarized in Table 2. The reported diameter of a generic core-shell liprotide is ~100 Å or around 10 nm (Frislev et al., 2017), while multilayered liprotides are reported to be between 20 – 30 nm (Pedersen et al., 2020) based on small angle x-ray scattering (SAXS) measurements. The measured diameter of the liprotides prepared in this work was significantly larger (658.7 – 1946 nm), which may be primarily attributed to the crude nature of the proteins used as evidenced by the already large sizes for the uncomplexed protein controls, in contrast with the highly purified proteins utilized in previous studies. Furthermore, high polydispersity indices (PDI), generally considered with values above 0.4 (Costa et al., 2021), were also recorded for the crude protein controls and the prepared liprotides suggesting varied size distribution for all cases in an aqueous suspension. Finally, the zeta potential was measured, whose magnitude provide a good index for the stability of most colloidal suspension systems analogous to the liprotides in solution. A higher absolute value, more than 60 mV, of zeta-potentials is preferred due to their stronger repulsive forces that prevent flocculation and further coagulation of the particles. All the prepared liprotides in this work generated zeta potential values from -24.19 to -34.88 mV, which are generally classified as having incipient or moderate stability. This observed negative potential arises from a negative surface charge, which in the context of this study, could be explained by the partial unraveling of the protein to expose acidic amino acid residues which would be deprotonated at the solution pH (7.4) used during measurement. Alternatively, it may also arise from exposed basic amino acid residues resulting in the accumulation of anions present in the solution, e.g., Cl-, on the protein surface contributing to a net negative surface charge. Nonetheless, the albumin-oleic acid liprotides prepared at 40 °C showed the most significant change in measured zeta potential relative to their corresponding control, an increase of approximately 27% and 32%, respectively, suggesting that complexation of oleic acid with these proteins having a lesser extent of unfolding results to better stabilization. The extent of protein unfolding is known to affect liprotide formation, as previously demonstrated where α-lactalbumin formed liprotides at room temperature, while ovalbumin and bovine serum albumin required a temperature of at least 60 °C (Sørensen et al., 2017). Notwithstanding these differences, it is still apparent from the results that the preparation temperature of 40 °C and 80 °C employed in this work resulted, overall, in liprotides from crude mung bean albumin and globulin extracts with comparable characteristics. Finally, due to the different liprotide preparation methods reported so far, in addition to the crude protein extracts used in this study, a direct comparison between these different liprotides is challenging (Pedersen et al., 2015).

Table 2. Z-average, polydispersity index, and zeta potential values of the prepared liprotide and control samples. Data is presented as mean ± standard deviation of three measurements of the same sample.

Sample	Z-average (d.nm)	Polydispersity (PdI) index	Zeta potential (mV)
Albumin 40°C	1497 ± 272	0.8454 ± 0.14	-23.22 ± 4.59
Albumin 80°C	1158 ±233	0.8216 ± 0.12	-33.71 ± 2.70
Globulin 40°C	793 ± 259	0.6885 ± 0.13	-23.64 ± 7.14
Globulin 80°C	748 ± 157	0.6276 ±0.04	-26.01 ± 9.10
Albumin-oleic acid 40°C	1946 ± 801	0.8109 ± 0.18	-29.39 ± 1.86
Albumin-oleic acid 80°C	838 ± 52	0.8820 ± 0.08	-34.88 ± 4.29
Globulin-oleic acid 40°C	658 ± 11	0.6408 ± 0.03	-31.23 ± 5.92
Globulin-oleic acidv80°C	950 ± 188	0.6829 ± 0.19	-24.19 ± 4.12

CONCLUSIONS

This study extracted albumins and globulins from mung beans and served as protein shells for liprotide formation with oleic acid at two different preparation temperatures. ATR-FTIR spectroscopy, particle size analysis, and zeta-potential measurements were conducted on the prepared complexes, and the results of the analyses suggested the formation of the liprotides exhibiting acceptable zeta-potential values, albeit with larger particle size and high polydispersity. Despite this, further studies will be beneficial to fully characterize the liprotides generated including morphological investigations using tools such as transmission electron microscopy. Furthermore, additional purification of the crude protein extracts and identification of the protein components will also be ideal. Finally, as liprotides exhibit promising anticancer and drug delivery applications, *in vitro* work on these new complexes will be crucial to determine their possible value in the clinical setting.

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