Heat Deterioration of Phospholipids. III. Thermally Deteriorated Products from Phosphatidylethanolamine and Hexose

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Abstract: Novel four 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum derivatives were obtained with increase of UV absorption at 350 nm and browning of the solution by heating paste lecithin from soybean (SL) in octane. These four derivatives were characterized the factor based on UV absorption at 350 nm. The SL was separated into the hexane (DSSL fr.) and 60% ethanol in water frs. by liquid- liquid partition method. Even when the DSSL fr. was heated in octane more than 9 h, the four derivatives were not formed and increase of any UV absorption was also not observed but the solution color was slightly yellowing. On the other hand, the formation of four derivatives and increase of UV absorption at 350 nm were observed when DSSL and 60% ethanol in water frs. were combined and, then, heated in octane. From the 60% ethanol in water fr, sucrose, raffinose and stachyose were isolated and identified as compounds which involved the formation of the derivatives with the DSSL fr. When DSPE (1,2-di-*O*-stearoyl-*sn*-glycero-3-phosphatidylethanolamine) and any hexose were combined and then heated, two 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum derivatives generated with increase of UV absorption at 350 nm and browning. From these results, it is concluded that the formation of derivatives, with increase of the UV absorption at 350 nm and browning, are based on the reaction of sugars and phosphatidylethanolamine (PE) in SL.

Key words: soybean lecithin, phospholipid, heat decomposition, phosphatidylethanolamine, 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum derivatives

1 Introduction

Recently, lecithin is not only used as an emulsifier, but also in various fields of application. Lecithin is improved into various forms to fit to use and application. However, these improvement processes sometimes cause its quality to lower, especially the improvement process *via* heating is known to make lecithin brown lowering its quality.

There are many reports for the browning and change of UV absorption of lecithin by heating. For example, Tomioka *et al.* (1-3) investigated that the browning of lecithin by heating involved polymerization of phosphatidylcholine (PC) and conjugated dienone compounds. Reaction of carbonyl compounds and PE browns lecithin, and forms pyridinium compounds with UV absorption at 280 nm (4, 5). Increase of UV absorption at 240 nm in lecithin by heating is based on

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the carbonyl compounds (6). However, there is no report about browning and change of UV absorption of lecithin by heating in detail and the browning mechanism of lecithin by heating has been not known yet.

We recently discovered the absorption bands appeared at 350 nm besides those of 240 nm and 280 nm during the heating process of paste lecithin from soybean (SL) (7, 8). Novel four 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum derivatives, namely compound **A**: 1-[2'-(2'',3'')-dilinoleoyl-*sn*-glycero-1''-phosphoethyl)]-6-methyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, compound **B**: 1-[2'-(2'')-linoleoyl*-3'')-palmitoyl*-*sn*-glycero-1''-phosphoethyl)]-6-methyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, compound **C**: 1-[2'-(2'',3'')-dilinoleoyl-*sn*-glycero-1''-phosphoethyl)]-6-hydroxymethyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, compound **D**: 1-[2'-(2'')-linoleoyl*-3'')-palmitoyl*-*sn*-glycero-1''-phosphoethyl)]-6-hydroxymethyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, were isolated and identified as the compounds involving the increase of the UV absorption at 350 nm of SL by heating in octane (* means interchangeable, shown in Fig. 5). Each of four derivatives has two molar of PE moiety in its structure. Compounds **A** and **B** have a methyl group at 6 position of the 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum ring. Compounds **C** and **D** have a hydroxymethyl group at 6 position of the ring. Compounds **A** and **C** have two molar of linoleoyl moiety in PE part and compounds **B** and **D** have one molar of linoleoyl moiety and one molar of palmitoyl moiety in PE part. The structures of these four derivatives are very similar. It is, therefore, very useful to consider the formation mechanism of these four compounds for understanding thermal deterioration of lecithin. In this paper, we report about compounds involving the formation of the derivatives and the mechanism in SL.

2 Experimental

2.1 Instruments

GC data were measured with a HP 6890 Series Gas Chromatograph instrument fitted with a fused silica column {HP-5 (crosslinked 5% PH ME Siloxane, 0.25 μ m, thickness, 25m $\times$ 0.2 mm i.d.)} and programmed 170°C with FID mode. GC-MS data were measured by a JEOL JMS600 instrument with EI mode. ¹H- and ¹³C-NMR data were recorded by a JEOL JNM-AL400

(400MHz) spectrometer in CDCl₃ or D₂O with TMS as an internal standard. Letters (br)-s, (br)-d, t, q, and m represent (broad)singlet, (broad)doublet, triplet, quartet, and multiplet, respectively, and coupling constants are given in Hz. IR data were measured by a SHIMADZU FT-IR 4300 in the liquid membrane method. HPLC were carried out with a Shimadzu LC-10AD pump equipped with Shimadzu RID-10A and Shimadzu SPD-M10A diode array detectors. LC-MS data were recorded with a Shimadzu LC-MS 2010 mass spectrometer when using a column (Inertsil ODS-3 150 mm $\times$ 4.6 mm i.d., GL Sciences Inc., Japan, flow rate: 1.0 mL/min) eluted with 50% methanol in water in the APCI-positive mode. UV data were measured by a Hitachi 200-20 Spectrophotometer. In a two dimensional TLC analysis, chloroform/methanol/7 molar of aqueous ammonia (130/60/8) and chloroform/methanol/acetic acid/water (170/25/25/6) were used as a developer, respectively (9).

2.2 Material

Paste soybean lecithin (SL) including PC(29.3%), PE(25.7%), PI(15.2%) and PA(11.2%) was manufactured by Tsuji Oil Mill Co., Ltd. (Mie, Japan). 1,2-Di-*O*-stearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE) was purchased from SIGMA (Missouri, USA). D-Galactose, D-glucose, D-fructose, sucrose, D-raffinose pentahydrate, stachyose tetrahydrate, Wakogel C-100 type and 5% hydrochloric acid in methanol solution were purchased from Wako (Osaka, Japan). Sep-Pak C18 cartridge was purchased from Waters (Massachusetts, USA).

2.3 Extraction and Isolation

2.3.1 SL separation

SL (20.0 g) was separated into hexane (DSSL, 250 mL) and 60% ethanol in water fr. (250 mL $\times$ 4) by liquid-liquid partition, respectively. After drying the 60% ethanol fr. under reduced pressure, the fraction was applied on an ODS open column and successively eluted with water, 40% methanol in water and methanol.

2.3.2 SL, DSSL, DSPE heating method

SL (100 g) and DSSL (100 g of equivalent to SL) was dissolved in flasks with octane (10 L) and heated for 9 h, respectively. After adding same equivalent of separated fractions or authentic sugars (2 mmol) in small amount of aqueous methanol solution to the DSSL (20 g of equivalent to SL) in octane solution, the solvent was removed from octane. The DSSL octane

solution (250 mL) was then heated for 9 h. A mixture of DSPE (480 mg, 642 μ mol) and galactose (115 mg, 642 μ mol) was also dissolved into octane (100 mL) and heated for 9 h.

2·3·3 Isolation of compounds **1** and **2**

After heating for 9 h, the reactant of DSPE and galactose was chromatographed on a silica gel column (Wakogel C-100, 150 mm $\times$ 10 mm i.d.), eluting in sequence with an increasing concentration of methanol in chloroform to obtain the chloroform, 25% methanol in chloroform, and 50% methanol in chloroform frs. The two compounds (**1** and **2** *Rt* = 26.9 min and 36.0 min, respectively) were isolated from the 50% methanol in chloroform fr. by using HPLC (column: Senshu Pak. AQUASIL SS-4251(60) 250 mm $\times$ 10 mm i.d, Senshu, Japan, flow rate: 3.0 mL/min, solvent: chloroform: methanol: water = 70: 30: 1, Detector: UV at 350 nm). The yields of compounds **1** and **2** were 16.45 mg/g and 6.46 mg/g based on a weight of DSPE and galactose, respectively.

2·3·4 Methanolysis of compounds **1** and **2**

Compounds **1** (7.9mg) or **2** (3.1mg) was mixed with 1 mL of 5% hydrochloric acid in methanol, and heated at 85°C for 3 h. After vacuum concentration, the reaction mixture was dissolved in 5 mL of ether and extracted four times with 4 mL of water. From the ether fr., methyl stearate was identified by using GC instrument and GC-MS {(programmed from 170°C (3 min hold) to 250°C at a rate of 10°C /min, measured at 70eV)}.

On the other hand, the water fr. was passed through a Sep-Pak C18 cartridge and successively eluted in sequence with an increasing concentration of methanol in water to obtain the water and 20% methanol frs. Compounds **AM** and **CM** were recovered from the water eluate. Compounds **AM** and **CM** were isolated by HPLC (column: Senshu Pak. AQUASIL SS-4251(60) 250 mm $\times$ 10 mm i.d, Senshu, Japan, flow rate: 3.0 mL/min, solvent: chloroform: methanol: water = 70: 30: 1, Detector: UV at 350 nm). The spectral data of compounds **1**, **2**, **AM** and **CM** were given as follows.

Compound **1**

¹H-NMR (CDCl₃) δ : 0.88 (6H, *m*), 1.25 (about 50H, *m*), 1.56 (4H, *m*), 2.26 (4H, *m*), 2.38 (3H, *s*), 4.10 (*H, *m*), 4.12 (*H, *m*), 4.23 (*H, *m*), 4.38 (*H, *m*), 5.16 (1H, *br-s*), 6.26 (1H, *br-s*), 7.27 (1H, *br-s*), 8.65 (1H, *br-s*).

* means not clear.

IR ν_{MAX} (liquid film) cm^{-1} : 1737 (C = O), 1236 (P = O),

1060 (P-O).

Compound **2**

¹H-NMR (CDCl₃) δ : 0.89 (6H, *m*), 1.29 (about 50H, *m*), 1.56 (4H, *m*), 2.26 (4H, *m*), 3.93 (*H, *m*), 4.11 (*H, *m*), 4.34 (*H, *m*), 4.51 (*H, *m*), 5.18 (1H, *br-s*), 6.40 (1H, *br-s*), 7.00 (1H, *br-s*), 8.60 (1H, *br-s*).

* means not clear.

Compound **AM**

Rt = 18.2 min. λ_{max} 351 nm

LC-MS: *m/z* (%): 179M⁺

¹H-NMR (D₂O) δ : 2.23 (3H, *s*, methyl), 3.77 (4H, *m*, H-1' and H-2'), 3.89 (2H, *t*, *J* = 6.5, H-3), 4.16 (2H, *t*, *J* = 6.5, H-2), 6.26 (1H, *d*, *J* = 4.3, H-8), 7.10 (1H, *d*, *J* = 4.3, H-7), 8.14 (1H, *s*, H-5).

¹³C-NMR (D₂O) δ : 11.90 (*q*, methyl), 40.94 (*t*, C-2), 47.61(*t*, C-3), 58.74 (*t*, C-1'), 60.53 (*t*, C-2'), 116.44 (*d*, C-8), 123.12 (*s*, C-6), 127.88 (*d*, C-7), 147.82 (*s*, C-9), 150.68 (*d*, C-5).

Compound **CM**

Rt = 10.6 min. λ_{max} 346 nm

LC-MS: *m/z* (%): 195M⁺

¹H-NMR (D₂O) δ : 3.84 (4H, *s*, H-1' and H-2'), 3.99 (2H, *br-t*, H-2), 4.30 (2H, *t*, *J* = 6.6, hydroxymethyl), 4.62 (2H, *s*, H-3), 6.45 (1H, *d*, *J* = 4.4, H-8), 7.16 (1H, *br-d*, H-7), 8.32 (1H, *s*, H-5)

¹³C-NMR (D₂O) δ : 41.30 (*t*, hydroxymethyl), 47.94(*t*, C-2), 55.40 (*t*, C-3), 58.73 (*t*, C-1'), 60.99 (*t*, C-2'), 115.52 (*d*, C-8), 123.96 (*s*, C-6), 127.07 (*d*, C-7), 146.62 (*s*, C-9), 152.74 (*d*, C-5).

3 Results and Discussion

3·1 UV Absorption Changes of SL and DSSL by Heating

Heating SL in octane results in a change of its color from yellow to brown and gives an especially UV absorption at 350 nm with disappearance of PE in the solution on a two dimensional TLC analysis (7, 9). The color, was, however, slightly yellowing and any UV absorptions did not increase at all even when DSSL was heated in octane for more than 9 h in spite of this DSSL fr. contains the all lecithins from SL on the TLC analysis. Slight decrease of PE in the DSSL fr. was only observed on the TLC analysis. On the other hand, when the 60% ethanol in water fr., in which the lecithins

Fig. 1 UV Absorption Changes of SL and DSSL by Heating.

Fig. 2 UV Absorption Changes of DSSL by Heating with ODS Separated Frs.

could not be detected on the TLC analysis, was added to DSSL and heated in octane, the color of reactant turned brown with increasing the UV absorption especially at 350 nm similar to that of SL (**Fig. 1**) and disappearance of PE in the solution was also observed on the TLC analysis. These results clearly show that the 60% ethanol in water fr. contains some compound(s) which are responsible for changing color and increasing especially UV absorption at 350 nm of the octane solution in cooperation with the DSSL. Of three fractions separated from the 60% ethanol in water fr. by using ODS open column (ODS-water, ODS-40% methanol in water and ODS-MeOH frs), only the ODS-water fr. caused the resultant mixture to change color and to increase the UV absorption at 350 nm along with the DSSL (**Fig. 2**). The four 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-yl cation derivatives (compounds **A~D**) with UV absorption at 350 nm were also formed in the ODS-water fr (8). The other fractions (ODS-40% methanol in water and ODS-methanol frs.) could not change the color, increase the UV absorption of the octane solution and form the four derivatives by heating. These results indicate that ODS-water fr. only contains some compound(s) which are responsible for changing color, increasing especially UV absorption at 350 nm of the octane solution and formation of the four compounds in cooperation with the DSSL.

3·2 Cooperation Function of Sugars with DSSL

The components of the ODS-water fr. were further analyzed by using HPLC (column: Nakarai tesuque COSMOSIL 5NH₂-MS, 250 mm × 10 mm i.d., solvent: 75% acetonitrile in water, flow rate: 4.0 mL/min, detector: RI). Based on HPLC analysis, that the ODS-water fr. mainly composed of three compounds.

The structures of these three compounds were determined to be sucrose (12.69 mg/g of SL), raffinose (1.64 mg/g of SL) and stachyose (2.66 mg/g of SL) by comparing their HPLC chromatograms with those of authentic samples, respectively. These sugars are reported as typical sugars in soybean lecithin (10).

When the mixture of these sugars was added to the DSSL maintaining their ratio in the water fr. and heated under the same condition previously described, the resultant octane solution changed the color and increased the UV absorption at 350 nm like when SL was heated. The four 2,3-dihydro-1*H*-imidazo[1,2-

Fig. 3 HPLC Profiles of Compounds A~D and Compounds 1 and 2.

Fig. 4 UV Spectra of the Reactant of DSSL with Several Sugar.

R =	2'',3''-diacyl- <i>sn</i> -glycero-1''-phospho moiety			H
Acyl groups	stearoyl	linoleoyl	linoleoyl and palmitoyl	
①	Compound 1	Compound A	Compound B	Compound AM*
②	Compound 2	Compound C	Compound D	Compound CM**

* 6-methyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum

** 6-hydroxymethyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum

Fig. 5 The New Compounds with $\lambda_{\max}$ at 350 nm Isolated from Heated SL, DSPE with Hexose.

a]pyridine-4-ylum derivatives (compounds **A~D**) with UV absorption at 350 nm were also formed (Fig. 3).

From these results, we concluded that sugars in cooperation with the DSSL causes the typical UV absorption increase at 350 nm and color change from yellow to brown, due to formation of the compounds during heating.

3.3 UV Spectra of the Reactants of DSSL with Several Sugars

To confirm the ability of cooperation function of sugars, several sugars, for example, galactose (Gal), glucose (Glu), fructose (Fru), sucrose (Suc), raffinose (Raf) and stachyose (Sta) were combined with DSSL and then heated, respectively. Even when any sugar were heated with DSSL, resultant octane solutions changed the color and increased UV absorption like when SL was heated as shown in Fig. 4 and four compounds (compounds **A~D**) with UV absorption at 350 nm were also formed (Fig. 5). From these results, we concluded that hexoses or oligosaccharides including a hexose moiety are based on making the color of DSSL solution brown and increasing the UV absorption at 350 nm by heating in octane.

Regardless a kind of hexoses including an aldose and a ketose, same results were obtained. These results might indicate that an oxygen atom on 2 position of sugar is concerned in the resultant reaction by heating and thus, it might be necessary to test 2-deoxysugars. A

pentose, or heptose might be also investigated because only hexoses or oligosaccharides including a hexose moiety were used in this experiment.

3.4 Heating Galactose with DSPE

The compounds **A~D** have two PE (phosphatidylethanolamine) moieties in the structure, respectively. Amount of PE in SL was decreased during heating in octane (9). These things may suggest that the decomposition of PE and formation of the four compounds occur simultaneously. To confirm the compound(s) being in cooperation with sugars in DSSL, DSPE (1,2-di-*O*-stearyl-*sn*-glycero-3-phosphatidylethanolamine) and galactose were, therefore, heated in octane solution. When DSPE and galactose were heated in octane, increasing UV absorption at 350 nm and change of the color to brownish were observed in the reactant solution (Fig. 6). The four compounds **A~D** were not formed in the reactant solution but two compounds (compounds **1** and **2**) with UV absorption at 350 nm, which have similar retention times to those of compounds **A~D** (Fig. 3), were formed. Even when various hexoses were used instead of galactose, the same compounds **1** and **2** were formed. These results clearly show that turning the color and increasing the UV absorption are based on the reaction a sugar with phosphatidylethanolamine.

Of these sugars, galactose made the UV absorption of the reactant highly increase. It is considered that this result is reasonable because open-chain form of galactose is most stable among hexoses tested, that is, aldehyde group in open-chain form of galactose could be attacked easier by nucleophilic PE than that of the other hexoses.

3.5 Structural Determination of Compounds **1** and **2**

¹H- and ¹³C-NMR spectra of compounds **1** and **2** were very similar to those of compounds **A~D** (8). All compounds contained a 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum moiety in their structures, respectively. Structural difference between the compounds **1** and **2** are only substitute group on 6 position in the 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum moiety, that is, 6 position in the ring of compound **1** is a methyl group, that of compound **2** is a hydroxymethyl group. There are not any other differences between the ¹H-NMR data of compounds **1** and **2** except those of aro-

Fig. 6 UV Spectra of the Reactant of DSPE with Sugar.

matic ring. After conducting the methanolysis of compound **1**, methyl stearate was only recovered from ether fr., while 1-(2'-hydroxyethyl)-6-methyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, that is, compound **AM** which we previously reported, was obtained from water fr (8). On the other hand, 1-(2'-hydroxyethyl)-6-hydroxymethyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, that is compound **CM**, was obtained by methanolysis of compound **2** from water fr (8).

According to these results, compounds **1** and **2** were determined as a 1-[2'-(2'',3'')-distearoyl-*sn*-glycero-1''-phosphoethyl)]-6-methyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum and 1-[2'-(2'',3'')-distearoyl-*sn*-glycero-1''-phosphoethyl)]-6-hydroxymethyl-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum, respectively (**Fig. 5**). The difference of retention times on HPLC between the compounds **A~D** and compounds **1** and **2** is based on the difference of fatty acid moieties in their structures.

Change of the color from colorless to brown, increase of the UV absorption at 350 nm, and formation of the compounds are caused at the same time even when only the DSPE and galactose were heated in octane. Formation mechanism of all six compounds is, therefore, same and each of six compounds is generated by reaction of two molar of PE and one molar of sugar during heating. It is concluded that reaction of PE with sugars is based on the deterioration in quality of SL by heating and it is very useful to remove the PE from SL to prevent SL from deteriorating in quality.

PE reacts with any hexose, regardless an aldose or a ketose, in octane solution by heating to give the same 2,3-dihydro-1*H*-imidazo[1,2-*a*]pyridine-4-ylum derivatives. It will be, therefore, very important to investigate this reaction mechanism and identify of origin of car-

bons in the derivatives.

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