Journal of Chromatography A, 1220 (2012) 108–114

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Purification of L-alpha glycerylphosphorylcholine by column chromatography

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ARTICLE INFO

Article history:
Received 19 August 2011
Received in revised form
16 November 2011
Accepted 1 December 2011
Available online 11 December 2011

Keywords:
Glycerylphosphorylcholine
Phospholipase A_1
Purification
Resin column chromatography
Silica gel column chromatography

ABSTRACT

Colorless L-alpha glycerylphosphorylcholine (L-α-GPC) was obtained at 99.8% purity, 69.8% recovery, and with a specific rotation of −2.5° via a five-step procedure. L-α-GPC was first produced by phospholipase A_1 hydrolysis of soy lecithin powder. Ca^{2+} and Cl^- were then effectively removed using two successive 001 × 7 cation and D311 anion exchange resin column chromatography procedures. Silica gel column chromatography and decoloration with active carbon were then applied to remove remaining impurities and colorant. Characterization of the L-α-GPC product was well in agreement with the standard. The resin and silica gel showed remarkable ability for L-α-GPC isolation after 10 uses. Thus, this study presents a simple and cost-effective method for preparing L-α-GPC with high yield and purity, low cost, and environmental friendliness, and encourages future investigation into its adaptation for industrial applications.

1. Introduction

L-Alpha glycerylphosphorylcholine (L-α-GPC), comprised of choline, glycerol, and phosphate, is well known as the precursor for producing acetylcholine and phosphatidylcholine (PC) in the body [1,2], and for having important medical applications in neurological and psychiatric disorders of the human brain, such as Alzheimer's disease, cerebellar ataxia, schizophrenia, and bipolar affective disorder [3,4]. These potential, valuable uses for L-α-GPC have prompted exploration into its possible role in the brain. Unfortunately, L-α-GPC is scarce in natural sources, and thus research into the production of high-purity L-α-GPC would have important medicinal, social, economic, and research value.

To date, major methods in domestic and international research for preparing and purifying L-α-GPC include isolation from natural organ extracts [5], chemical synthesis and alcoholysis from egg or soy lecithin [6–8], solvent extraction [6], precipitation [9], recrystallization [10], and resin column chromatography [5]. However, these methods possess various difficulties in preparing and purifying L-α-GPC with suitable yield, purity, and environmental effects. For instance, solvent extraction is limited by raw material availability, which thus limits the scale of manufacturing, while chemical hydrolysis and alcoholysis present environmental challenges, and calcium precipitation and recrystallization have poor separation efficiency. Therefore, there is a great demand for a technologically useful and economical method for producing pure, high quality L-α-GPC.

An enzymatic preparation of L-α-GPC would be a very attractive alternative strategy for high efficiency, economic, and environmental friendliness. Phospholipase A_1 (Lecitase® Ultra enzyme) as one of the industry scale enzyme, with the lower cost, has been widely utilized in the food and pharmaceutical industries [11], and has potential application for enzymatic preparation of L-α-GPC on scale-up. Soy lecithin powder is a by-product of soy oil refining [12], which has been extensively used in food, nutrition compounding and emulsifiers [13], but application of developing high value-added pharmaceutical grade L-α-GPC is still minor.

However, the purity of enzymatic reaction product is not high and requires further purification. Owing to high adsorption specificities, facile desorption, mechanical strength, its low cost, and successfully application in the industrial refining and purifying of bioactive substances from natural resources [14–16], resin column chromatography is now used to remove ion residue. Moreover, silica gel column chromatography has major advantages such as its simple operation, high yield, reusability, and ability to separate chemicals with similar character and structure, such as isomers [17–19]. But to the best of our knowledge, no data has been reported regarding the performance of resin and silica gel column chromatography in purifying L-α-GPC.

The present work reports a detailed method for preparing and purifying L-α-GPC via five steps. L-α-GPC was first produced by phospholipase A_1 hydrolysis of soy lecithin powder, the calcium and chloride ions then removed using resin column

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doi:10.1016/j.chroma.2011.12.003

chromatography, and the eluted product purified using silica gel column chromatography and activated carbon.

2. Experimental

2.1. Materials

Food-grade soy lecithin powder was kindly provided by East Ocean Oils & Grains Industries Co., Ltd. (Zhangjiagang, China). $CaCl_2$ (ultrahigh purity, certified >99.99%), L-α-GPC standards (specific rotation ($[\alpha]_D^{20}$) of −2.0° to 3.5°) and PC from soybeans and HPLC-grade chloroform and methanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phospholipase A_1 (Lecitase® Ultra, 9500 U mL^{-1}) was a generous gift from Novozymes A/S (Bagsvaerd, Denmark). Silica gel for column chromatography (100–200 mesh, 3% moisture content) was provided by Qingdao Haiyang Chemical Co., Ltd. (Qingdao, PR China). Resin 001 × 7 and D311 were provided by Suqing Group (Jiangyin, PR China). All other reagents were analytical grade.

2.2. Enzymatic procedure of L-α-GPC

A 50 g weight of soy lecithin powder was placed in a three-necked flask with 500 mL deionized water and stirred to homogeneity at 300 rpm. Then, 2 g of $CaCl_2$ and 9500 U of phospholipase A_1 were added to the solution, incubated at 35 °C for 360 min, and the insoluble material removed from the mixture by centrifugation and vacuum filtration. The PC conversion and L-α-GPC yield were calculated by high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) analysis.

2.3. L-α-GPC purification procedures

2.3.1. Resin column chromatography

Pretreatment of ion exchange resins was conducted as previously reported [20]. In resin column chromatography, pretreated resins were suspended in water and packed individually into columns. The cation exchange resin was used to remove Ca^{2+}, and the anion exchange resin was used to remove Cl^-. Calcium ions and chloride ions were quantified as previously reported [21,22].

2.3.1.1. Static adsorption assay. Static adsorption tests were performed by placing 2 g (dry wt) of pretreated resin in a flask, adding 100 mL of enzyme reaction solution, and capping and shaking the flask in a 25 °C water bath at 120 rpm for 120 min until equilibrium was attained.

2.3.1.2. Dynamic adsorption. Based on the results of preceding tests, dynamic adsorption experiments were carried out in glass columns (1.6 cm × 50 cm) wet-packed with 4 g (dry wt) of pretreated resin and equipped with a constant-flux pump to control the eluent flow rate. Reactions solutions were applied to the column in 100 mL lots and pushed through at a flow rate of 1.5 mL min^{-1}.

2.3.1.3. Calculation of adsorption capacity and adsorption ratio. The following equations were used to quantify the adsorption capacity and adsorption ratio [16,23]. Adsorption capacity (Eq. (1)),

$$q_e = \frac{V_1(C_0 - C_e)}{W} \quad (1)$$

where C_0 and C_e are the initial and equilibrium ion concentrations in the reaction solution (μg mL^{-1}), respectively; V_1 the solution volume (mL), and W the resin weight (g). Adsorption ratio (Eq. (2)),

$$X(\%) = \frac{C_0 - C_e}{C_0} \times 100 \quad (2)$$

where X is the adsorption ratio, defined as the percentage of quantity adsorbed at equilibrium relative to the initial, and C_0 and C_e the same parameters as in Eq. (1).

For the next adsorption, the resin columns needed to be regenerated by removing adsorbed Ca^{2+} and Cl^- using HCl and NaOH, respectively.

2.3.2. Silica gel column chromatography

In silica gel chromatography, 100 g silica gel was activated in a drying oven at 120 °C for 60 min, slurried in methanol, and packed into a column (3.0 cm × 50 cm).

L-α-GPC purification experiments were carried out in silica gel columns equipped with a constant-flux pump. The chromatography conditions were methanol as the eluent, loading of 20 mg g^{-1} silica gel, loading concentration of 30 mg mL^{-1}, and flow rate at 2 mL min^{-1}. Eluate fractions were collected every 30 mL until no response for L-α-GPC was detected by HPLC-ELSD analysis, and fractions of the same peak combined. The combined L-α-GPC fractions were reduced to dryness in a rotary evaporator at 60 °C under vacuum.

The final L-α-GPC fraction was decolorized at 60 °C for 90 min to remove colorant and minor impurities. A weighed sample of the residue was taken and dissolved in methanol for HPLC-ELSD quantitative analysis. The purity and recovery of L-α-GPC were calculated according to the following equations:

$$P = \frac{n \times v}{w} \times 100\% \quad (3)$$

$$R = \frac{W \times P}{m_{GPC}} \times 100\% \quad (4)$$

where P and R represent L-α-GPC purity and recovery, respectively; n the concentration determined by quantitative HPLC-ELSD analyses, v the flask volume, w the sample dry weight, W the residue total weight, and m_{GPC} the theoretical L-α-GPC yield (mg).

After each purification, silica gel was regenerated by heating at 150 °C for 3 h and reused.

2.4. HPLC-ELSD analysis

Samples were analyzed by HPLC-ELSD, using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a LiChrospher Si column (25 cm × 0.46 cm I.D., 5 μm particle size, Sigma–Aldrich Corp. K.K., Tokyo, Japan) at 35 °C, and eluted with a binary gradient of solvent A (methanol) and solvent B (methanol/water, 8/1, v/v) at 0.97 mL min^{-1}. Samples were applied as 5 μL injections and eluted with a linear gradient from 40 to 60% B (v/v) over 10 min, 60% B for 5 min, followed by a linear decrease to 40% B over 3 min. Resolved sample components were identified and quantified by comparison with peak retention times and calibration curves of standard compounds.

3. Results and discussion

3.1. HPLC-ELSD analysis

HPLC-ELSD profiles (Fig. 1A) of the chemical composition of initial enzymatic reaction solutions showed significant PC peaks and weak lysophosphatidylcholine (LPC) peaks. After 360 min of reaction, new peaks for L-α-GPC and glycerylphosphorylethanolamine (GPE) became clearly visible, while the PC peak intensity became very weak and the LPC peak undetectable (Fig. 1B), resulting overall in a 98% PC conversion and 94.5% L-α-GPC yield, which indicated that phospholipase A_1 was a very efficient catalyst for converting PC to L-α-GPC. The main reasons are that the PC *sn*-1