Food Chemistry 201 (2016) 185–189

Contents lists available at ScienceDirect

Food Chemistry

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

Short communication

Identification of natural lactoylcholine in lactic acid bacteria-fermented food

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ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 5 December 2015
Accepted 13 January 2016
Available online 14 January 2016

Keywords:
Acetylcholine
Lactoylcholine
Isolated purification
Lactic-fermentation food
Blood pressure-lowering food

ABSTRACT

Acetylcholine (AcCh) is a major neurotransmitter and an agonist of nicotinic and muscarinic receptors in non-neuronal systems. Artificially synthesized lactoylcholine (LaCh) has potent nicotinic activity equal to that of AcCh. In this study, we report the isolation and purification of natural AcCh and LaCh from a lactic-fermented food known to reduce blood pressure. To our knowledge, we are the first to isolate natural LaCh. The choline esters were isolated using a novel purification procedure combining a weak cation-exchange cartridge with ODS and pentafluorophenyl HPLC columns, and the structure of LaCh was identified via various analyses. Assessment of D- and L-LaCh showed that the isolated LaCh was an enantiomer mixture with a D/L ratio of 1.6. D-LaCh induced vasorelaxation of thoracic aortas from spontaneously hypertensive rats ($EC_{50} = 3.83 \times 10^{-7}$ M), while L-LaCh did not. Our results suggest that choline esters could be new functional ingredients in lactic-fermented foods.

1. Introduction

The cholinergic system is a major neurotransmission pathway responsible for motor, cognitive, sensory, and autonomic functions (Wess, 1996). Within this system, the neurotransmitter acetylcholine (AcCh) has several important physiological actions, which are initiated by its binding to nicotinic and muscarinic AcCh receptors (nAChRs and mAChRs, respectively). Both receptors are expressed in both the vertebrate nervous system and the non-neuronal system (Wessler & Kirkpatrick, 2008). In the digestive system, epithelial and mesothelial cells of the esophagus, stomach, and intestines express both nAChRs and mAChRs (Wess, 2004). Endothelial cells of the aorta and pulmonary vessels also express both receptors, and agonist action at M3 mAChRs causes vasodilation and reduces blood pressure (Khurana et al., 2004).

We developed a lactic-fermented food from buckwheat sprouts (hereafter denoted as "neo-FBS," which is an abbreviation for neo-fermented buckwheat sprouts) that, compared with other foods, has a superior blood pressure-lowering (BPL) effect in spontaneously hypertensive rats (SHRs). This effect is thought to be caused by inhibition of tissue angiotensin-converting enzyme and vasodilation (Nakamura, Naramoto, & Koyama, 2013). We previously reported six types of angiotensin-converting enzyme inhibitory peptides in neo-FBS (Koyama et al., 2013); herein, we identify AcCh and lactoylcholine (LaCh) as candidate vasodilatory compounds.

AcCh is ubiquitously expressed in non-neuronal animal tissues, plants, fungi, and bacteria (Horiuchi et al., 2003; Kawashima et al., 2007). Production of AcCh by *Lactobacillus plantarum* isolated from fermented sauerkraut was reported by Stephenson, Rowatt, and Harrison (1947). AcCh has also been identified in milk (Whittaker, 1958) and in edible plants and fungi (Horiuchi et al., 2003); however, its functionality in food, i.e., its biological action, has not been reported to our knowledge. LaCh, 2-(2-hydroxy-1-oxopropoxy)-*N,N,N*-trimethylethanaminium, is an ester of choline with lactic acid. Korey, De Braganza, and Nachmansohn (1951) prepared LaCh from choline, lactic acid, and acetone-dried squid ganglia powder, whereas Rama Sastry, Lasslo, and Pfeiffer (1958) chemically synthesized D-, L-, and DL-LaCh. D-LaCh has nicotinic activity similar to that of AcCh, but the activity of L-LaCh is 20% that of AcCh, and the muscarinic activities of D- and L-LaCh are much weaker than those of AcCh in anesthetized dogs (Rama Sastry,

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http://dx.doi.org/10.1016/j.foodchem.2016.01.055
0308-8146/

Pfeiffer, & Lasslo, 1960). When investigating the bioactivity of LaCh, Lasslo, Meyer, and Rama Sastry (1959) hypothesized that an AcCh-like neuroactive substance with an asymmetric center at the alpha position to carbonyl carbon might occur in nature because LaCh derived from naturally occurring lactic acid and choline is a substrate of cholinesterase. However, natural LaCh has yet to be reported.

Here, we identified AcCh and natural LaCh in neo-FBS. We also report the isolation, purification, and structural determination of these choline esters and chemical synthesis of LaCh. To investigate the formation of LaCh in nature, the D/L ratios of LaCh and lactic acid isolated from neo-FBS were determined. The vasodilatory activities of D- and L-LaCh were also assessed.

2. Materials and methods

Choline esters are usually highly hydrophilic, unstable even in weakly basic conditions, and have weak UV absorbability; therefore, their purification is difficult. Only their charged trimethyl quaternary amine group can be exploited for purification. Therefore, AcCh and LaCh were purified using a novel combination of weak cation exchange treatment and HPLC separations on octadecylsilyl (ODS) and pentafluorophenyl (PFP) columns. The structures of the isolated natural AcCh and LaCh were determined by using NMR, FT-IR, LC/MS, and MALDI-TOF MS analyses, and the optical purity of LaCh was established by using a chiral column for HPLC analysis. Chemically synthesized D- and L-LaCh were used to confirm the structure of isolated LaCh, and their vasodilatory effects on the isolated thoracic aortas of SHRs were examined. For full details of all materials and methods used in this study, see the Supplementary information. Experimental and surgical procedures were performed with the approval of the Animal Care Committee of the Faculty of Agriculture of Shinshu University (approval number: 240083).

3. Results

3.1. Isolation and purification of LaCh

First, lyophilized neo-FBS (11.5 g) was treated with a weak cation-exchange cartridge, and choline compounds were selectively adsorbed to the weak cation-exchange resin at pH 7.0. After unadsorbed compounds were washed out with purified water, the absorbed choline compounds were eluted with 1 M HCl. Using an AcCh standard reagent, the mean recovery rate from three experiments was 98%. The lyophilized product of the HCl eluent (540 mg) was obtained from neo-FBS with a 4.7% yield. Choline compounds in the HCl eluent were separated by ion-pair chromatography using sodium 1-octanesulfonate (SOS) (Kozik & Rapala-Kozik, 1993); separation was performed isocratically on an ODS column using acetate–citrate buffer/CH_3CN (4/1, v/v) containing 7.0 mM SOS as the mobile phase. The 1-octanesulfonic acid strongly interacts with the trimethyl quaternary ammonium moiety of choline compounds, and the conjugates can be separated on an ODS column using UV detection (Fig. 1). Peaks eluted at 11.2–12.5 and 13.5–14.5 min were collected and lyophilized to obtain ODS fraction 1 and fraction 2 at yields of 5.6% and 1.2% of neo-FBS, respectively. SOS was contained in ODS fractions 1 and 2; therefore, the yields on a weight basis were higher than the theoretically expected values. A choline compound in each ODS fraction was purified through a PFP column using 33% aqueous MeOH containing 0.01% HCOOH as the mobile phase. Peaks eluted at 17–18 and 19–20 min were collected from ODS fraction 1 and fraction 2, respectively, and lyophilized to obtain PFP fraction 1 (4.7 mg; 0.044% yield) and fraction 2 (2.3 mg; 0.020% yield), respectively, as the final products.

3.2. Structural determination and identification of isolated AcCh and LaCh

From the results of NMR, FT-IR, LC/MS, and MALDI-TOF MS analyses, AcCh was identified in PFP fraction 1 by comparison with commercially available AcCh. In LC/MS analysis of PFP fraction 2, molecular ion peaks at *m/z* 176.2, 117.0, and 104.1 corresponded to $[M]^+$ of LaCh, $[M\text{-}(NCH_3)_3]^+$ with the loss of the trimethylamine moiety, and $[M\text{-}C_3H_4O_2]^+$ with the loss of the lactoyl moiety, respectively. The PFP fraction was also analyzed by ^1H and ^{13}C NMR in D_2O to identify its molecular structure. The proton signals at 4.51–4.53 (CH_2), 3.65–3.67 (CH_2), and 3.10 ($CH_3 \times 3$) ppm were assigned as the protons of the choline moiety, whereas the coupled quartet and doublet signals resonating at 4.35 (CH) and 1.31 (CH_3) ppm corresponded to the protons of the lactoyl moiety. In the ^{13}C NMR spectrum, the resonance signal at 175.1 ppm indicated the ester carbonyl carbon; CH at 64.4 ppm and CH_3 at 18.9 ppm showed the lactoyl moiety. Signals at 66.4, 58.8, and 53.7 ppm were assigned as a trimethyl and two methylene groups of the choline moiety, respectively. Thus, the compound in PFP fraction 2 was determined as LaCh. FT-IR and MALDI-TOF MS analyses corroborated this determination. FT-IR absorbance values at 3393, 1748, 1196, and 1133 cm^{-1} indicated the existence of a hydroxyl group, an ester, and C–N bonds, respectively. A mass-spectral peak at *m/z* 176.1285 corresponded to a molecular ion $[M]^+$ of LaCh with a mass of 176.1281 ($C_8H_{18}NO_3$). In addition, the small optical rotation value of 5.2 ($[\alpha_D^{20}]$, *c* 0.2, H_2O) indicated low optical purity.

D- and L-LaCh were chemically synthesized to confirm the structure of isolated LaCh (synthesis of D-LaCh is shown in Fig. 2). First, the hydroxyl group on D-ethyl lactate was protected with a *tert*-butyldimethylsilyl (TBDMS) group, and the ethyl ester was cleaved with 4 M lithium hydroxide. The protected lactic acid was condensed with choline using *N,N*-dicyclohexylcarbodiimide/*N,N*-dimethyl-4-aminopyridine, and then the protecting TBDMS was removed with trifluoroacetic acid to give D-LaCh. After purification, D-LaCh was obtained with 64% yield and >98% HPLC purity. Using similar methods with L-ethyl lactate as a starting material, L-LaCh was synthesized with 62% yield and >98% HPLC purity. All spectral data from the synthesized D- and L-LaCh were consistent with those of isolated LaCh, except for the optical rotation value (Table 1).

3.3. Determination of optical purity of isolated LaCh and lactic acid in neo-FBS

Comparison of the optical rotation values from isolated and synthesized LaCh suggested that LaCh in neo-FBS was an enantiomer mixture. To determine the enantiomer ratio of isolated LaCh, we used chiral column HPLC analysis of lactic acids liberated from LaCh. The content of D- and L-LaCh in isolated LaCh, as calculated from the liberated lactic acid analyses, was 61.7% and 38.3%, respectively, and the enantiomeric ratio (D/L) was 1.6. The enantiomeric ratio of D- and L-lactic acid in neo-FBS (1.7) was similar to that in isolated LaCh.

3.4. Vasodilatory activity of D- and L-LaCh

Following previously described methods (Nakamura et al., 2013), the thoracic aortas of SHRs were used to examine the vasodilatory activities of D- and L-LaCh. Briefly, phenylephrine-contracted thoracic aorta rings were obtained from 18-week-old male SHRs, and AcCh was employed as a positive control. D-LaCh