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Identification and quantification of antitumor thioproline and methylthioproline in Korean traditional foods by a liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry

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ABSTRACT

A liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometric method (LC–APCI–MS/MS) has been developed for the sensitive determination of antitumor thioproline and methylthioproline from fermented foods. Thioproline and methylthioproline were derivatized in one step with ethyl chloroformate at room temperature. These compounds were identified and quantified in various traditional Korean fermented foods by LC–APCI–MS/MS. The concentration range of thioproline of each food was found for *doenjang* (0.011–0.032 mg/kg), *gochujang* (0.010–0.038 mg/kg), and *ganjang* (0.010–0.038 mg/kg). Those of methylthioproline of each food was found for *doenjang* (0.098–0.632 mg/kg), *gochujang* (0.015–0.112 mg/kg), and *ganjang* (0.023–1.468 mg/kg). A prolonged aging time leads to an increase in both the thioproline and methylthioproline contents, suggesting that the storage time plays a key role in the formation of thioproline and methylthioproline in Korean traditional foods. The results here suggest that thioproline and methylthioproline are related to the biological activities of traditional Korean fermented foods.

1. Introduction

Doenjang, ganjang and *gochujang* are the Korean terms for the traditional fermented soybean paste, soy sauce and red pepper paste of Korea. These foods are traditionally manufactured from *meju*, which is a fermented rectangular block of crushed and cooked soybeans. Attention from both the public and industry has increased due to a number of reports about the biologically functional activities of these foods such as an anticancer effect [1,2], an antioxidative effect [3,4], antihypertensive activity [5], hepatoprotective properties [6] and immune functions [7]. It is well known that biologic activity originates from isoflavones [8,9]. Although these functions of the foods have been extensively studied, little is known about biological active compounds apart from isoflavones. We attempted to investigate new bioactive compounds and found thioproline and methylthioproline in various fermented Korean foods.

Thioproline and methylthioproline (Fig. 1) have been well documented in biological and medicinal chemistry. There are extensive studies of these compounds in many pharmaceutical applications, including detailed investigations of their antioxidant behavior [10], hepatoprotective properties [11], immune functions [10,12], antitumor properties [10,13,14], their detoxifying potential against nitroso compounds and nitrites [15], their elevated glutathione functions [16], and their functions as influenza NA inhibitors [17].

Thioproline has been found in various cooked foods such as cod [18] and Shiitake mushroom [19]. A remarkable amount of thioproline was found in the Djenkol beans eaten in Indonesia and in *Parkia speciosa* seeds and other edible leguminous beans eaten in Thailand and Malaysia [20]. This study is the first paper, in which thioproline and methylthioproline are identified in fermented foods.

Several methods that can be used for the determination of thioproline and methylthioproline have been reported. Examples include ion exchange chromatography [21] and high-performance liquid chromatography (HPLC) [22,23]. An earlier paper by

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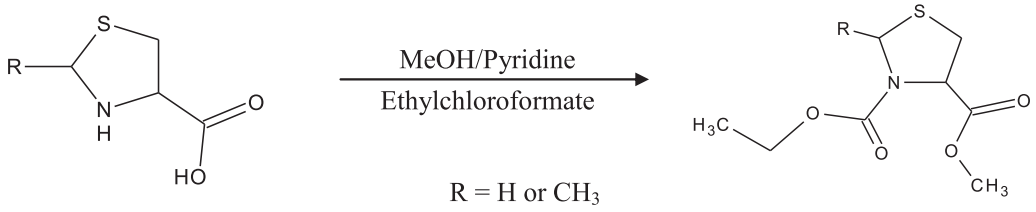

Fig. 1. Derivatization reaction of thioproline and methylthioproline with ethylchloroformate/methanol/pyridine.

the authors described the determination of thioproline and methylthioproline in urine by means of gas chromatography–mass spectrometry (GC–MS) [24].

This study aimed to develop the simple and sensitive analytical method of thioproline and methylthioproline in fermented foods by liquid chromatography–tandem mass spectrometry (LC–MS/MS), and quantify the biologically active compounds of thioproline and methylthioproline in traditional Korean fermented foods.

2. Materials and methods

2.1. Materials

Thioproline (99%) and methylthioproline (97%) were purchased from Sigma (St. Louis, MO, USA), and d_4-acetaldehyde and D-cysteine were obtained from Aldrich (Milwaukee, WI, USA). Analytical grade ethyl chloroformate and pyridine (Sigma–Aldrich, St. Louis, MO, USA) were used as reagents and methanol (E. Merck, Darmstadt, Germany) was used as a solvent.

The synthesis of d_4-methylthioproline as an internal standard was performed as described our previous paper [24]. To a solution of D-cysteine (0.20 mmol) in distilled water (1.0 mL), d4-acetaldehyde (0.20 mmol) was added at 50 °C. The mixture was stirred for 10 min and allowed to stand overnight at −4 °C. The precipitate was collected and washed with water and ethanol continually.

2.2. Food sampling

Traditional Korean fermented foods containing *doenjang* (soybean paste), *gochujang* (pepper soybean paste) and *ganjang* (soybean souse) were home-made products and were procured by a traditional food producer.

2.3. Derivatization

Derivatization and extraction were performed by modifying the method developed by Shin et al. [24]. In a 15 mL test tube, 1.0 mL of the prepared sample solution was placed and approximately 50 mg of carbonate buffer (Na_2CO_3 + $NaHCO_3$, 1:2, w/w) was added. Approximately 150 μL of ethyl chloroformate, 1.5 mL of methanol/pyridine solution (4/1, v/v) and 50 μL of d_4-methylthioproline (ISTD) solution (2.0 mg/L in water) were then added to the solution.

The solution was allowed to stand for 10 min at room temperature, after which a 5 μL sample of the solution was injected in the LC–MS/MS system.

2.4. Liquid chromatography–mass spectrometry

The analytes were separated using a 50.0 × 2.1 mM Eclipse Plus C8 column with a 1.8 μm pore size (Agilent, USA). A binary gradient with a flow rate of 0.2 mL min^{-1} was used. Mobile phase A was 5 mM ammonium acetate in water. Mobile phase B was methanol. The gradient was as follows: B = 0% for the first, increased to 100% by 8 min. All of the compounds were eluted within 8.0 min. Mass spectra were acquired in positive mode under atmospheric pressure chemical ionization (+APCI) or electrospray ionization (+ESI) on an Agilent 1290 LC coupled to a 6490 triple quadruple LC/MS (Agilent, Palo Alto, CA, USA). For each compound, the protonated molecular ion, $[M+H]^+$ was acquired (Table 1). The drying gas was operated at a flow rate of 8 mL min^{-1} at 325 °C. The nebulizer pressure was 45 psi, and the capillary was set at 4000 V. The capillary voltage was set to 3.2 kV. The source temperature was 120 °C and the desolvation temperature was 450 °C. Nitrogen was used as desolvation gas (flow 500 L/h) and argon was used as collision gas at a pressure of 3×10^{-3} mbar. Detection was performed in the multiple-reactions monitoring (MRM) mode. The fragment voltage and the collision energy were optimized for the different analytes (Table 1).

2.5. Calibration and quantification

The soybean sauce or soybean paste (aged within a week), in which analytes were detected in the low concentration, were used a control sample for the validation.

Calibration curves for thioproline and methylthioproline were established after adding 5, 10, 50, 100, 250, 500, 750 and 1500 ng of standard solution (1.0 or 10.0 mg/L in water) and 50 μL of d4-methylthioproline solution (2.0 mg/L) to 1.0 g of control sample. The subsequent procedures were performed by the same methods as used during the derivatization processes. The ions selected for quantification and confirmation are presented in Table 1. The ratios of the peak areas of the standards to that of the internal standard were used during the quantification of the compounds.

The LODs and LOQs were assessed by determining the analytes in seven control samples spiked at concentrations of 0.005 mg/kg. The accuracy and precision were assessed by determining the analytes in five control samples spiked at concentrations of 0.050 and 0.500 mg/kg. The inter-day accuracy and precision were evaluated by determining the analytes in three spiked samples at concentrations of 0.050 and 0.500 mg/kg on five different days.

3. Results and discussion

3.1. Derivatization and detection

Thioproline and methylthioproline were assessed by LC–MS/MS without a derivatization step, but the detection limits were too high to detect less than 0.1 mg/kg of both. The derivatization of the amino and carboxylic acid groups of thioproline and methylthioproline was performed with ethylchloroformate after some modification of the method reported in an earlier paper by the authors [24]. The derivatives were 20-fold more sensitive than thioproline and methylthioproline at the same conditions using LC–MS/MS, and had good chromatographic peak shape. The derivatives are thought to sensitive sufficiently for the injection by LC–MS/MS, therefore, the formed derivatives were designed to be directly injected into the LC–MS/MS without an extraction procedure.