Original

Methods for Determination of Milt Protein and ϵ -Polylysine in Food Additive Preparations and Processed Foods by Capillary Zone Electrophoresis

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A simple and rapid method using capillary zone electrophoresis (CZE) for the determination of milt protein (MP), which contains mainly protamine, and polylysine (PL) in food additive preparations and processed foods was developed. CZE separation was performed on poly(vinyl alcohol)-coated capillaries at a column temperature of 20°C with 120 mmol/L phosphate buffer (pH 2.5) as the running buffer.

The influence of various components in food additive preparations on CZE analysis of MP and PL was examined. Egg white lysozyme, glycine, sodium acetate, glycerol, fumaric acid, calcium carbonate, dextrin, emulsifiers and sodium polyphosphate and pyrophosphate had no effect. No peak of protamine was detected in preparations containing metaphosphate.

The analysis method for processed foods was composed of extraction with 4% formic acid, precipitation of macromolecular compounds with ethanol, concentration in a water bath and determination by CZE. The average recoveries were 108.4% for protamine sulfate (PS) in red bean sticky rice, and 81.3% for PL in white rice, 118% in egg sandwiches, and 115% in shiraae. The limits of detection of PS in red bean sticky rice and PL in white rice were both 50 ppm.

Key words: natural preservative; milt protein; protamine; polylysine; capillary zone electrophoresis; poly(vinyl alcohol)-coated capillary

Introduction

There is growing interest in preventing deterioration of food, as well as in methods of improving shelf life, along with enforcement of product liability law and the introduction of a consume-by date. Freezing and low temperature preservation of food have markedly improved quality, but the deterioration of food cannot be completely prevented by these methods alone. There is a growing awareness of, and a growing demand for, the use of natural preservatives.

The List of Existing Food Additives (list of natural additives)¹⁾ includes ten kinds of natural preservatives. Of these, milt protein and ϵ -polylysine (polylysine) are often used in processed foods, while the others are hardly used.

The present study was performed to develop an analytical method for milt protein and polylysine. Milt protein from salmon sperm or herring sperm contains basic proteins, the main component of which is protamine. Polylysine is a basic protein produced by *Streptomyces albus*.

Capillary electrophoresis is an important tool for analysis of proteins and peptides²⁾⁻⁶⁾. Coated capillaries

prevent protein adsorption on the capillary walls^{3), 6)-10)}, and analysis of lysozyme (a basic protein) using coated capillaries has been reported⁶⁾⁻¹⁰⁾. In this study, we developed an analytical method for milt protein and polylysine in food additive preparations and processed foods by capillary zone electrophoresis (CZE) using poly(vinyl alcohol)-coated capillaries (PVA capillaries).

Experimental

1. Samples and their solutions

1.1 Food additives

Milt protein from salmon sperm (MP-A), milt protein from herring sperm (MP-B) and commercial polylysine (containing 50% dextrin) (Sa-PL) were obtained from the Japan Association of Food Additives.

Sample solutions of MP-A, MP-B and Sa-PL were prepared at the concentration of 0.01% as milt protein or polylysine in 1% formic acid, and filtered through 0.45 μ m membrane filters for CZE.

1.2 Food additive preparations

(1) Preparations made in our laboratory

The names and contents of preparations made in our laboratory were as follows.

①SA was a mixture of 4% PS, 35% glycine, 31%

dextrin, 25% sodium acetate and 5% polyphosphoric acid.

- ②SB was a mixture of 6.0% PS, 93.5% glycine and 0.5% egg white lysozyme.
- ③LA was a mixture of 8.35% Sa-PL, 83.3% glycine and 8.35% dextrin.
- ④LB was a mixture of 0.6% Sa-PL, 9.54% acetic acid, 0.75% fatty acid sugar ester, 1.5% sodium acetate and 87.61% water.
- ⑤LC was a mixture of 1% Sa-PL, 2% fatty acid glycerol ester, 30% glycerol, 0.75% fatty acid sugar ester, 1% glycine, 1.5% sodium acetate and 63.75% water.

(2) Commercial milt protein preparations

The names and contents of the preparations obtained from the Japan Association of Food Additives were as follows.

- ①SC was a mixture of 2% MP-B, sodium acetate (anhydrous), fumaric acid, sodium metaphosphate, food material and sodium polyphosphate.
- ②SD was a mixture of 2.0% MP-B, sodium acetate (anhydrous), pyrophosphate (acidic), sodium metaphosphate, sodium polyphosphate, fatty acid glycerol ester, calcium carbonate, sodium casein and food material.

Sample solutions of SA, SB, SC, SD, LA, LB, LC were prepared as follows. Samples (1–5 g) were mixed with water, adjusted to pH 2.5 with 1 mol/L hydrochloric acid, made up to 200 mL with water and filtered through 0.45 μ m membrane filters for CZE.

1.3 Processed foods

Sweet potato cakes, cream puffs and red bean sticky rice balls, the ingredient labels of all of which indicated that they contained milt protein, and rice balls containing salmon, egg sandwiches and shiraae (a mixture of vegetables, konnyaku and paste made from a ground mixture of miso, *i.e.* white soybean paste, bean curd and white sesame), the ingredient labels of all of which indicated that they contained polylysine, were purchased from convenience stores and retail stores in Tokyo.

Sample solutions were prepared as follows. Samples (20 g) of rice were mixed with 30 mL of 4% formic acid, homogenized (10,000 strokes, 20 minutes), mixed with about 30 mL of ethanol, made up to 100 mL with 4% formic acid and then centrifuged (4,000 rpm, 10 min). The supernatant (50 mL) was concentrated to about 20 mL in a water bath (less than 70°C), made up to 25 mL with water and filtered through a 0.45 μ m membrane filter for CZE.

For sweet potato cakes, cream puffs, shiraae and egg sandwiches, fat was removed with *n*-hexane from the supernatants obtained in the same way as described above for the rice samples.

2. Reagents

Protamine sulfate (salmon sperm) (PS) purchased from Tokyo Chemical Industry (Tokyo, Japan) and ϵ -poly-L-lysine hydrochloride (PL) of biochemical grade purchased from Wako Pure Chemical Industries

(Tokyo, Japan) were used as authentic standards.

Standard solutions of PS and PL were prepared at 0.02–1.00 mg/mL and 0.04–1.00 mg/mL in 1% formic acid, respectively, then filtered through 0.45 μ m membrane filters for CZE. Calibration curves were prepared by plotting the concentrations and areas (the total areas of peaks S1, S2 and S3 for PS, and the area of peak L for PL).

3. Capillary zone electrophoresis

CZE was carried out using a Hewlett-Packard HP^{3D} CE system. PVA capillaries (Hewlett-Packard) (64.5 cm in length, with an effective length of 56.0 cm, 50 μ m i.d. and 150 μ m optical path length) were used. The capillary temperature was maintained at 20°C. The 120 mmol/L phosphate buffer (pH 2.5) was prepared and filtered through a 0.45 μ m membrane filter for use as the running buffer. Prior to injection of each sample, the capillaries were rinsed with water for 1 min, methanol for 2 min, water for 4 min, and the running buffer for 5 min. The voltage applied was 30 kV (positive), and the electric current was 100 μ A. The samples were injected under a pressure of 50 hPa for 3 sec. Detection was performed by measuring the absorbance at 200 nm and 214 nm.

The electropherograms of PS and PL obtained under these conditions are shown in Fig. 1.

Fig. 1. Electropherograms of protamine sulfate and ϵ -poly-L-lysine hydrochloride (100 μ g/mL)

Instrument: HP^{3D} CE System

Capillary: poly(vinyl alcohol)-coated (with an effective length of 56 cm, 50 μ m i.d., and 150 μ m optical path length)

Running buffer: 120 mmol/L phosphate buffer (pH 2.5)

Preconditioning: flush with water for 1 min, methanol for 2 min, water for 4 min and running buffer for 5 min.

Injection: pressure at 50 hPa for 3.0 sec

Voltage: positive 30 kV

Capillary temperature: 20°C

Detector: diode array

Wavelength of detection: 200 nm.

Results and Discussion

1. Solvent for preparation of standard solutions

When water was used for preparation of the standard solutions, tailing of the PL peak was observed on the electropherogram. The tailing was improved when more than 0.5% formic acid was added. The migration times and sensitivities of PS and PL were good at formic acid concentrations of up to 5%. Therefore, the standard solutions were prepared using 1.0% formic acid.

2. Conditions of CZE

2.1 Capillary columns

Capillary columns coated with various reagents have been used for analysis of the basic protein lysozyme⁶⁾. In this study, PVA capillaries gave good results without tailing of the PS and PL peaks in acidic running buffers. Rinsing of capillaries with methanol prior to injection of each sample gave good reproducibility of peaks and increased the life of capillaries.

2.2 pH of running buffer

The optimal pH of the running buffer for good separation of PS and PL was examined in the range of 1.6 to 7.0 using 120 mmol/L phosphate buffer at a column temperature of 20°C. Mutual separation of the three peaks of PS and PL was improved as the pH was reduced. The manufacturer of the column reported that PVA capillaries are stable within a pH range of 2.5 to 9.5. Therefore, pH 2.5 was used for the running buffer.

2.3 Phosphate concentration of running buffer

The phosphate concentration of running buffer giving good separation was examined in the range of

Fig. 2. The effects of phosphate concentration in running buffer on separation of protamine sulfate and ϵ -poly-L-lysine hydrochloride (100 μ g/mL)
Peaks S1, S2, S3: protamine sulfate
Peak L: ϵ -poly-L-lysine hydrochloride
100 mmol/L, 120 mmol/L, 140 mmol/L: concentrations of phosphate in running buffer
The CZE conditions except the running buffer were as described in the legend to Fig. 1.

Fig. 3. The effects of column temperature on separation of protamine sulfate and ϵ -poly-L-lysine hydrochloride
Peaks S1, S2, S3: protamine sulfate
Peak L : ϵ -poly-L-lysine hydrochloride
Conditions except column temperature were as described in the legend to Fig. 1.

50–140 mmol/L at pH 2.5 at a column temperature of 20°C. The results at phosphate concentrations of 100, 120 and 140 mmol/L are shown in Fig. 2. The mutual separation of the three peaks of PS was improved with increasing phosphate concentration. However, high electrical charge adversely affected the capillary at concentrations of more than 130 mmol/L. A concentration of 120 mmol/L was adopted, since satisfactory separation was obtained at this value.

2.4 Capillary column temperature

The capillary column temperature giving good separation was examined in the range of 16 to 30°C using 120 mmol/L phosphate buffer (pH 2.5). The results are shown in Fig. 3. The S2 and S3 peaks of PS and the peak L of PL were separated best at 20°C. Under these conditions, the peak of lysozyme, which is commonly used to increase the shelf life of foods and is sometimes used together with milt protein, appeared at a migration time (MT) of over 20 min and was fully separated from protamine.

3. Preparation method of sample solution for processed foods

As milt protein and polylysine are water-soluble basic

proteins with comparatively low molecular weights (2–12 kDa) and are resistant to heat¹¹⁾, acids such as formic acid, acetic acid, hydrochloric acid and sulfuric acid were expected to be suitable for extraction solvent and a heating method was adopted for concentration after extraction. The boiling point (100.8°C) of formic acid was the lowest among the available acids, so it was expected to evaporate during concentration of the extraction solution by heating, and might not cause hydrolysis of milt protein and polylysine. Therefore, formic acid was adopted for extraction solvent.

Then addition of 4% formic acid to homogenates of processed foods was examined in the range of 10–50 mL. The main peaks of PS and PL were decreased by 3% of the respective maximum levels with increase in the amount of 4% formic acid added. Thus, it is desirable to minimize the amount of formic acid added, although at least 50 mL is necessary for precipitation of macromolecular compounds in processed foods.

Then heating at 70, 80, and 90°C was examined using standard solution of PS or PL containing 2% formic acid and 30% ethanol. The peaks of PS were not affected, but one peak of PL (MT : 14.3 min) was divided into 2 peaks (MT : 14.3 min, 14.8 min) at temperatures above 70°C, and the late peak increased with increasing temperature. Therefore, a temperature of less than 70°C was used.

4. Comparison of the CZE patterns of protamine and polylysine from different origins

The electropherograms of MP-A and MP-B, commercial milt proteins from salmon sperm and herring sperm, were different (Fig. 4). MP-A had three peaks, similarly to PS, but MP-B had two peaks, and the elec-

tropherogram was different from that of PS. This may be because the pattern of peptides containing arginine in MP-A was different from that of MP-B.

Sa-PL showed only one peak and the electropherogram was the same as that of the PL.

5. Influence of components in food additive preparations on the determination of protamine and polylysine

Various compounds are frequently incorporated in food additive preparations. The influence of components in SA, SB, SC, and SD milt protein preparations, and the LA, LB, and LC polylysine preparations on CZE analysis of protamine and polylysine were examined. Egg white lysozyme, glycine, glycerol, acetate, emulsifiers, sodium polyphosphate and pyrophosphate, carbonate, casein and dextrin showed no effect. No peak of protamine was detected in SC or SD containing metaphosphate, or in the mixture of PS and metaphosphate. Protamine was thought to bind strongly with metaphosphate, resulting in a shift in its MT.

6. Recoveries of protamine and polylysine from processed foods

A standard (2 mg) of PS was added to 20 g of red bean sticky rice, and 2 mg of PL was added to 20 g of white rice, egg sandwich or shiraae. The concentrations of PS and PL in the samples were equivalent to 100 ppm. These processed foods were confirmed in advance to contain neither milt protein nor polylysine. The samples were allowed to stand for one hour prior to analysis in accordance with our method. The average recovery of PS was 108.4%, and the average recoveries of PL were 81.3% for white rice, 118% for egg sandwich and 115% for shiraae. The limit of detection of PS from red bean sticky rice and PL from white rice was 50 ppm. The standard concentration of milt protein or polylysine used individually in processed foods is about 100 ppm, so the detection limit (50 ppm) of PS or PL is thought to be suitable when PS or PL is used individually in processed foods. However, they are sometimes used with glycine and acetate and so on, and the detection limit might be a little high in such cases.

7. Analytical results of protamine and polylysine in commercial processed foods

Our method was applied to analysis of commercial processed foods. Milt protein from herring sperm was detected in cream puffs (Fig. 5). Polylysine was detected in shiraae (Fig. 6). It was necessary to confirm the results by the standard addition method because the MTs of milt protein and polylysine were shifted in some cases. Protamine was not detected in sweet potato cakes or red bean sticky rice balls, and polylysine was not detected in rice balls containing salmon or in egg sandwiches, although the labels indicated the presence of milt protein or polylysine in these foods. This may have been because protamine and polylysine formed complexes with dextran at the high temperatures used in cooking¹¹⁾, they may not actually have been used in

Fig. 4. Comparison of electropherograms of protamine sulfate and commercial milt proteins from two different sources (salmon sperm and herring sperm)

The conditions were as described in the legend to Fig. 1.

Fig. 5. Electropherograms of milt protein extracted from cream puff

- (A) Extract from the cream puff with label indicating the presence of milt protein
(B) Extract A plus 100 ppm of milt protein from herring sperm

The conditions were as described in the legend to Fig. 1.

Fig. 6. Electropherograms of polylysine extracted from shiraae

- (A) Extract from the shiraae with label indicating the presence of polylysine
(B) Extract (A) plus 100 ppm of polylysine

The conditions were as described in the legend to Fig. 1.

processing of these foods, or the amounts used may have been below the limits of detection of our assay system.

Conclusions

CZE using PVA capillaries is a simple and rapid method for the determination of milt protein and polylysine in food additive preparations and processed foods.

There are two kinds of milt proteins from salmon

sperm and herring sperm on the market, and their electropherograms are different. This may be because the patterns of arginine-containing peptide in them are different.

In food additive preparations containing metaphosphate, no protamine peak was detected. Protamine was thought to be strongly bound with metaphosphate, resulting in a shift in its MT. This problem requires further study.

Our method for analyzing processed foods was applied to commercial processed foods. Milt protein from herring sperm and polylysine were detected in cream puffs and in shiraae with labels indicating the presence of milt protein and polylysine, respectively.

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