Evaluation of Musk by Enzyme-Linked Immunosorbent Assay

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We report the development of enzyme-linked immunosorbent assay (ELISA) for the quantitative analysis of a unique musk protein (MP-1) in musk samples. Musk defatted with ethyl acetate/methanol (9:1, v/v) was dipped in cold water and ammonium sulfate was added to the supernatant up to 85% saturation. The resulting precipitate was applied to a Bio-Gel P-100 chromatography. The fraction eluted at the void region was collected and it was consecutively purified by affinity chromatography on a DEAE Affi-Gel Blue and on anion-exchange columns containing DEAE-Sepharose CL-6-B. This protein was determined to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions with an apparent molecular weight of 35000 Da and was called as musk protein-1 (MP-1). Polyclonal antibodies of MP-1 were produced by injecting it into a rabbit. These antibodies were reactive to the aqueous extract of musk and the pure antigen. The ELISA could be applied to detect nano gram quantities of the antigen in musk samples. This method made it possible to distinguish musk samples from different origins.

Key words musk; evaluation; ELISA; purification; musk protein-1

Musk is the dried secretion from the male pocket of the musk deer (*Moschus moschiferus* LINNE). It is one of the most famous traditional Chinese medicines, and is used as a cardiac and general stimulant, an aphrodisiac, and an anti-spasmodic. Although its active principles are unknown, muscone (3-methylcyclopentadecanone) is a unique substance with a very distinctive aroma. In addition to muscone, musk also contains muscopyridine, cardiotonic musclide A and B, and some steroid hormones, such as androstanediols and cholesterols. An anti-inflammatory peptide was also isolated from the aqueous extract of musk. As musk is considered a precious drug in Eastern Asia, artificial materials are fraudulently offered or the real product is mixed with chemicals. The quality control of musk is generally conducted by gas-chromatographic (GC) or high-performance liquid chromatographic (HPLC) determination. As a synthetic muscone is now available, it seems highly likely that this compound will be offered as musk. For this reason we undertook to develop a new analytical method to identify and evaluate this precious drug.

The enzyme-linked immunosorbent assay (ELISA) is one of the analytical techniques that is generally more sensitive and specific than chromatographic assays. It offers a quick, easy, and reliable means of measuring protein in test samples. In the present study, we purified a unique protein (MP-1) from musk and produced polyclonal antibodies in rabbits. We then developed a direct ELISA for quantitative analysis using anti-MP-1 polyclonal antibodies. The quantity of MP-1 in the formula containing musk and in musk samples from different locations was then determined by ELISA.

MATERIALS AND METHODS

Materials Musk used for the isolation of components was purchased at Kyung-dong herb drug market, Seoul, Korea in 1999 (ME-Ko). The origin was presumed to be a mixture of *Moschus moschiferus* and *M. berezowskii*. Nine other musk samples (MC-Ko, M01-Ka1, M01-Ka2, M01-Ka3, M02-C1, M02-C2, M02-C3, M02-C4, and M03-M1) were collected from the Korean, Kazakhstan, Chinese and Mongolian markets in 1999. Voucher specimens were deposited in the Korea Food and Drug Administration. Bio-Gel P-100 (medium) and DEAE Affi-Gel Blue Gel were purchased from Bio-Rad (Hercules, CA, U.S.A.). DEAE Sepharose CL-6-B was obtained from Sigma (St. Louis, MO, U.S.A.), and UV readings were taken on a Jasco UV-550 spectrophotometer (Tokyo, Japan).

Purification of Musk Protein 1 The dried musk of ME-Ko (412 g) was repeatedly suspended in 1000 ml ethyl acetate/methanol (9:1, v/v) three times at room temperature and the supernatant was removed. The residue thus obtained was air-dried and extracted with 5 volumes of water. The aqueous layer was collected by centrifugation and ammonium sulfate was added up to 85% by weight. The resulting precipitate was collected and dissolved in 50 mM Tris–HCl (pH 7.4). The solution was syringe-filtered and applied to a Bio-Gel P-100 column (2.5 × 110 cm) equilibrated with 50 mM Tris–HCl buffer containing 0.1 M NaCl (pH 7.4). The column was then eluted with the same buffer at a flow rate of 19 ml/h. The fraction showing 35 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was eluted at the void region and it was collected, dialyzed against 50 mM Tris–HCl (pH 7.4), and concentrated by Molecular/Por® C-10K from Spectrum (Houston, TX, U.S.A.). The concentrate was then loaded onto a DEAE Affi-Gel Blue affinity chromatography column (2.5 × 24 cm) equilibrated with 50 mM Tris-HCl (pH 7.4). It was eluted with a linear NaCl gradient (0–0.5 M) in 50 mM Tris–HCl (pH 7.4) at a flow rate of 52 ml/h. The fraction containing 35 kDa on SDS–PAGE (MP-1) was pooled, dialyzed against 20 mM Tris–HCl (pH 6.8) and concentrated, as described above. For further purification, the fraction was loaded on a DEAE-Sepharose CL-6-B anion-exchange chromatography column

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(1.5 × 8 cm) equilibrated with 0.2 M NaCl in 20 mM Tris–HCl (pH 6.8). The bound protein was eluted with a linear salt gradient (0.2–0.8 M) in 20 mM Tris–HCl (pH 6.8) at a flow rate of 7.5 ml/h and the fractions containing MP-1 were pooled, dialyzed against distilled water and then concentrated.

**Determination of Molecular Weight**  The molecular weight of the protein was determined according to the method of Laemmli using a 4% stacking and a 10% resolving polyacrylamide gel. β-Lactoalbumin (14200 Da), soybean trypsin inhibitor (20100 Da), trypsinogen (24000 Da), carbonic anhydrase (29000 Da), glyceraldehyde-3-phosphate dehydrogenase (36000 Da), ovalbumin (45000 Da), and bovine serum albumin (66000 Da) were used as protein standards. The protein assay was performed using a Bio-Rad Protein Assay kit from Bio-Rad (Hercules, CA, U.S.A.).

**Determination of the N-Terminal Amino Acid Sequence**  The N-terminal amino acid sequence of the purified musk protein I was determined using an Applied Biosystems Procise 491 amino acid sequencer at the Korea Basic Science Center (Seoul).

**Production of Anti-MP-1 Antibodies**  The purified MP-1 was emulsified with an equal volume of Freund’s complete adjuvant and the mixture was injected intramuscularly to two male New Zealand white rabbits. A series of 3 booster injections were given at 3 week intervals in a similar way as the first injection, except that Freund’s incomplete adjuvant was used. Blood samples were taken from the marginal ear vein one week after each immunization, and the antibody titers of the plasma were determined by ELISA as described below. Each animal was bled from the carotid artery and the sera obtained were stored at −80°C.

**Detection of Anti-MP-1 Antibody**  A solution (10 μl) containing the antigen, MP-1 diluted in 50 mM Tris–HCl buffer (pH 7.4) was spotted directly onto a dry nylon membrane. The membrane was then blocked, washed and incubated with antibodies according to conventional procedures, using secondary anti-rabbit IgG conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BICP/NBT) as the chromogenic substrate for color development.

**Western Blot Analysis of MP-1 Antigen**  Crude or purified antigens were separated by SDS–PAGE, as described above, and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc.). Musk extracts were separated by 10% SDS–PAGE and then electrophoretically transferred to nitrocellulose paper at the constant voltage of 30 V at 4°C overnight. The electroblotting buffer was composed of 0.025 M Tris–HCl, 192 mM glycine, 20% methanol and 0.1% SDS. The antigen was detected as described above. The paper was blocked to prevent nonspecific antibody binding and probed with polyclonal antibodies of MP-1. The antigen was detected by alkaline phosphatase-IgG conjugate and visualized by incubating the membrane in the presence of the precipitable chromogenic substrate BICP/NBT, as described above.

**Titer Determination of Anti-MP-1 Antibody**  In brief, 50 μl of purified MP-1 (10 μg/ml), which was decided to be the optimal antigen concentration, was coated on a 96-well ELISA plate. The plate was incubated for 2 h at 37°C and each well was washed with PBS three times. Twofold serial dilutions from 1 : 1000 dilution of polyclonal antiserum in PBS buffer were made and 45 μl of them were plated on each well. Following the same treatment, the secondary antibody conjugated with alkaline phosphatase (1 : 1000 dilution) was added to each well. After washing, 45 μl of p-nitrophenyl phosphate (1 mg/ml) was added and the plate was incubated for 25 min. The absorbance was read by a microplate reader at 405 nm. Bovine serum albumin was used as control. MP-1 concentration in the samples was determined based on the calibration curve prepared using optimal dilutions of primary and secondary antibodies against 10 to 90 ng of MP-1 antigen coated on the 96 well plate as described above.

**RESULTS**

**Purification of MP-1 from Crude Musk**  The isolation of MP-1 was achieved by a combination of ammonium sulfate fractionation, gel filtration, and affinity chromatography. Gel-filtration chromatography of the 85% ammonium sulfate fraction on Bio-Gel P-100 afforded a single fraction eluting at the void region of the column (Fig. 1A). The collected fraction I was separated by DEAE Affi-Gel Blue chromatography with a linear sodium chloride gradient from 0 to 0.5 M NaCl in the same buffer. The fraction II, representing the 35 kDa band on SDS–PAGE, was collected and concentrated (Fig. 1B). Finally, this fraction was further separated into two other fractions, fractions III and IV by DEAE-Sepharose ion-exchange chromatography (Fig. 1C). The fraction III (Fr III) was found to be homogeneous by SDS-PAGE (inset of Fig. 1C) showing a single band at 35000 Da. But under native conditions its molecular weight was estimated to be about 200000 Da (data not shown). The N-terminal amino acid sequence of the Fr III was determined to be Ala–Phe–Arg–Arg–Ser–Phe–Arg–Thr–Gly–Arg–Ser–Tyr. When the Fr III was compared with protein sequences in the Genbank, no matching proteins/peptides showed homology. This strongly suggested that MP-1 is a unique protein of musk and that it was highly possible to prepare an antibody to this antigen. Polyclonal anti-Fr III antibodies were raised by injecting into rabbits and then used as primary antibodies for ELISA.

**Detection of Anti-MP-1 by Dot Blotting Assay**  Primary antibodies were diluted at two concentrations (1 : 100, 1 : 500). A purified protein, MP-1 (Fr III in Fig. 1) was blotted onto a nitrocellulose membrane in the range of 0.1 to 1 μg, and solid-phase enzyme-linked immunospot assay performed. The minimal concentration for the detection of the antigen was 0.25 μg (data not shown).

**Western Blot Analysis of the MP-1 Antigen**  The detection of the MP-1 antigen in the mixture is shown in Fig. 2. Panel A shows Coomassie brilliant blue staining of the membrane, and panel B shows antigen detection after probing with polyclonal antibodies. The locations of the two bands were precisely consistent, which indicated that polyclonal antibodies were raised to the MP-1 antigen.

**Determination of MP-1 in the Musk Samples**  The titer of the primary antibody was determined by reading the absorbance after twofold serial dilution of the antibody. The highest absorbance of the sample was 2.1 and the lowest value was 0.6 and typically the background reading was 0.21. As the convection point was observed to be in the range of 4000 to 8000-fold dilutions, the primary antibody was fixed.
at 4000-fold dilutions. Quantitative analysis indicated that the minimal concentration of MP-1 in the solution was 5 ng/ml and that the maximum concentration for measurement was 100 ng/ml (Fig. 3). In other words, the binding capacity of MP-1 to the plate ranged to 100 ng/ml in considering a nonspecific binding of MP-1 to the plate.

**Quantitative Analysis of MP-1 in Musk Samples Collected from Different Sources**

Nine musk samples were collected from different sources and worked-up as described above, and protein concentrations were determined for calculation of the specific amount of MP-1 in the samples. Figure 4 represents the relative levels of MP-1 in the samples; it was clearly detected in four samples, namely, M02-C2, M02-C3, M03-M1 and MC-Ko. The concentration of MP-1 that could be detected in four samples ranged to a couple of nanograms in 1 g of protein. This method could be applied to formulas with musk. MP-1 was detected in four of the twelve samples in the range 0.2 to 2 ng (data not shown). The results were sufficient to demonstrate that the developed method is capable of evaluating the existence of real musk in a prescription.

**DISCUSSION**

Musk is a traditional medicine in Asia, and has been widely used as a raw material for the manufacture of perfume in Europe. However, because of excessive collection, the
number of musk deer has been significantly reduced and the animals have almost disappeared. Accordingly, the musk deer has been listed in Appendix II of the Convention of International Trade in Endangered Species (CITES). Muscone is an important substance for the quality control of musk samples by chemical analysis. A chromatographic analysis of muscone usually has a sensitivity of $10^{-6} - 10^{-3}$ g/ml, depending on the detection method used. Even though this sensitivity is very high, chromatography cannot determine whether muscone, for example, has been intentionally added. According to our recent observation, muscon content varies significantly depending on the species of musk deer. Musks obtained from Moschus moschiferus have less than 1% muscone content (data not shown). This is one reason why it is difficult to compare muscone content as a criterion. However, the ELISA system is based on the presence of unique proteins or peptides in the biomaterials, and thus it can overcome such difficulties.

The approach used for this study was intended to characterize the unique proteins or peptides in musk and then to produce the appropriate polyclonal antibodies. The N-terminal sequence of a protein, MP-1, strongly indicated that it is probably unique. Under native conditions, the molecular weight of this protein is 200 kDa, but under denaturing conditions, it was determined to be 35 kDa, suggesting that the native form is a hexamer. The antibody produced to MP-1 in rabbits was strongly responsive to the protein, and has a sensitivity in the nanogram range.

In summary, this study is the first designed to discriminate between real musk and a fake musk using ELISA. The musk protein MP-1 is highly antigenic as well as being unique. The ELISA system offers sensitivity at the nanogram level for the detection of MP-1 and is useful for evaluating the authenticity of musk. Further research is required to determine the biological roles and activities of MP-1.

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