A Sandwich ELISA for the Determination of Beef Meat Content in Processed Foods

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Two kinds of polyclonal antibodies against beef myoglobin were produced by immunizing rabbits with denatured myoglobin or with a peptide having the amino acid sequence unique to beef myoglobin. The latter peptide antibody reacted specifically with beef myoglobin without cross-reacting with other food proteins or with pork and chicken myoglobin. A sandwich ELISA, in which this peptide antibody was used as a capture antibody and the anti-denatured myoglobin antibody as an enzyme-labeled antibody, was also specific against beef myoglobin. When the beef content was determined in pork- or chicken-based model foods by this sandwich ELISA, the recoveries of beef protein agreed well with the actual mixing ratio of beef. Moreover, it could be confirmed by this method that the labeling of beef usage in several commercially processed foods was reasonable. Thus, a sandwich ELISA suitable for the evaluation of beef content in various foods was established.

Keywords: beef myoglobin, peptide antibody, sandwich ELISA, food allergy, meat deception

Introduction

Together with the increasing number of patients with food allergy, the health hazards triggered by unfortunate ingestion of foods containing allergenic substances have been reported in the world (Imai and Iikura, 2003; Sicherer et al., 2003; Buhl et al., 2008). For the prevention of such incidents, proper labeling is necessary to correctly inform consumers of the presence of allergenic substances in foods. Subsequently, the Ministry of Health, Labour and Welfare in Japan made labeling mandatory for five major allergenic foods (eggs, milk, wheat, buckwheat, and peanuts) and recommendatory for 19 other foods (abalone, squid, salmon roe, shrimp/prawn, oranges, crab, kiwi fruit, beef, tree nuts, salmon, mackerel, soybeans, chicken, pork, mushrooms, peaches, yams, apples and gelatin) in 2002. In accordance to the labeling directive, two types of sandwich enzyme-linked immunosorbent assay (ELISA), for the detection of the 5 major allergenic foods, were established and notified as official methods in Japan, the first in the world (Mamegoshi et al.; 2002; Takahata et al., 2002). Thereafter, in 2004, banana was added to the recommendatory foods. Labeling of shrimp/prawn and crab was made mandatory in 2008 and their detection methods, in addition to those of recommendatory foods, are currently being developed in Japan (Seiki et al., 2007, Sakai et al., 2008).

Three kinds of meat, beef, pork and chicken, are included in the recommendatory foods, since the number of patients with meat allergy has been increasing recently (Tanabe and Nishimura, 2005). Takahata et al. (2000) reported that beef is more allergenic than pork, chicken, rabbit and turkey meat. This prompted us to develop the assay system for beef. Bovine serum albumin (Fiocchi et al., 2000; Tanabe et al., 2002) and immunoglobulin (Ayuso et al., 2000; Han et al., 2000) are reported to be the major allergens in beef. These allergenic proteins, or myofibril abundant proteins such as myosin and actin, may normally be selected as a target antigen for the detection of beef by sandwich ELISA. However, ELISA suitable for the current Japanese allergen labeling of beef should be, at the minimum, capable of distinguishing beef from pork, chicken and cow’s milk. Therefore, we selected myoglobin (Mb) as a target antigen.

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Mb is a sarcoplasmic protein and is relatively rich in beef (4–10 mg/g) as an oxygen acceptor. Moreover, Fuentes et al. (2004) reported that beef Mb is a heat-stable allergenic protein. Though amino acid sequences of major myofibril proteins are highly conserved with over 95% homology, that of Mb can vary among animals (Rousseaux and Dautrevaux, 1976). Mb homology between beef (Han et al., 1970) and pork (Rousseaux and Dautrevaux, 1976) is about 88%, and that between beef and chicken (Deconinck et al., 1975) is 74%. Even if antibodies are produced using beef Mb as an antigen, it is still possible that the obtained antibodies cross-react with pork or chicken Mb. In establishing sandwich ELISA specific to beef, at least one antibody is required that is able to recognize an amino acid sequence unique to beef Mb and differing from pork and chicken. Such sandwich ELISA will be helpful in proactive measures with respect to allergen labeling, beef adulteration, and bovine spongiform encephalopathy.

Materials and Methods
Preparation of Mbs and protein standards from beef, pork and chicken
Beef Mb was purified from fresh leg meat, and those of pork and chicken were from fresh heart meat. Minced meat (100 g each) was homogenized with two-fold (w/v) of distilled water and mixed for 30 min at 4°C. Minced meat (100 g each) was homogenized with meat, and those of pork and chicken were from fresh heart meat. Beef Mb was purified from fresh leg meat was brought to 90% ammonium sulfate saturation and those from pork and chicken heart meat were brought to 70% ammonium sulfate saturation. After centrifugation at 4,500 × g for 15 min at 4°C, the supernatant was filtered through No. 1 filter paper (Whatman Japan Inc., Tokyo, Japan). The filtrate from beef leg meat was brought to 90% ammonium sulfate saturation and those from pork and chicken heart meat were brought to 70% ammonium sulfate saturation. After centrifugation at 4°C, each solution was centrifuged at 20,000 × g for 15 min at 4°C. After filtration, the supernatant was adjusted to pH 6.85 and brought to 95% ammonium sulfate saturation. After stirring for 1 h at 4°C, it was centrifuged at 20,000 × g for 15 min at 4°C. The precipitate was dissolved in distilled water and dialyzed against 20 mM Tris–HCl (pH 8.3). The solution was applied to an ion exchanged column (Toyoscreen DEAE 650M; Tosoh, Inc., Tokyo, Japan) equilibrated with 20 mM Tris–HCl (pH 8.3). The column was eluted at a flow rate of 1.0 mL/min by a linear gradient of 0.5 M NaCl in 20 mM Tris–HCl (pH 8.3). The purified Mbs were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% gel) under a reduced condition according to the method of Laemmli (1970). Determination of their N-terminal amino acid sequences was entrusted to Takara Bio Inc. (Shiga, Japan).

Minced fresh leg meat (250 g each) of beef, pork or chicken was homogenized with an equal volume of distilled water and freeze-dried. Freeze-dried powder (20 mg each) was added to 20 mL of phosphate buffered saline (PBS, pH 7.2) containing 0.5% sodium dodecyl sulfate and 2% 2-mercaptoethanol and shaken for 12 h at room temperature for extraction. After centrifugation at 20,000 × g for 15 min at room temperature, the supernatant was filtered to obtain each protein standard. Protein content was determined by a 2-D Quant Protein Assay Kit (GE Healthcare UK Ltd., Buckinghamshire, England).

Preparation of polyclonal antibodies
The purified beef Mb was used as an immunogen after denaturation with 1% SDS and heating at 95°C for 10 min (denatured Mb: D-Mb). Peptide A was synthesized by Toray Research Center Inc. (Tokyo, Japan). Peptide A (Cys – Ala$^{84}$ – Glu$^{85}$ – Val$^{86}$ – Lys$^{87}$ – His$^{88}$ – Leu$^{89}$ – Ala$^{90}$ – Glu$^{91}$ – Ser$^{92}$ – His$^{93}$ – Ala$^{94}$ – Asn$^{95}$ of beef Mb) was conjugated with mariculture keyhole limpet hemocyanin by Inject Maleimide Activated Immunoconjugation Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) for immunization of rabbits. D-Mb and the conjugate were sent to Operon Biotechnologies Inc. (Tokyo, Japan) for production of rabbit antisera. Polyclonal antibody against D-Mb or Peptide A was purified using a Hitrap NHS-activated HP column (GE Healthcare UK Ltd.) coupled with D-Mb. horseradish peroxidase (HRP)-conjugated polyclonal antibody against D-Mb was made with a Peroxidase Labeling Kit-SH (Dojindo Laboratories, Inc., Kumamoto, Japan).

Western blotting
After SDS-PAGE on a 15% gel under reduced condition, the resolved protein bands were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., CA, USA) at 1 mA/cm². After electroblotting, the membrane was blocked by PBST (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$·12H$_2$O, 1.5 mM KH$_2$PO$_4$, 0.1% Tween20) for 12 h at 4°C. After washing three times with PBST, the membrane was incubated with diluted polyclonal antibody against D-Mb or polyclonal antibody against Peptide A for 1 h at room temperature. The excess antibodies were removed by washing three times with PBST, and the membrane was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Zymed Inc., Vienna, Austria), diluted with PBST, for 1 h at room temperature. After washing three times with PBST, the membrane was equilibrated with 0.1 M Tris buffer (pH 9.5), and then incubated with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride in 0.1 M Tris buffer (pH 9.5). Color development was stopped by washing the membrane in distilled water.

Competitive ELISA
A polystyrene 96-well microtiter plate (Corning Inc., Washington D.C., USA) was coated with 100 µL of D-Mb (1 µg/mL) in the coating buffer (0.1
M phosphate buffer, pH 7.2) for 12 h at 4°C. After washing four times with the washing buffer (10 mM phosphate buffer, 0.15 M NaCl, 0.1% Tween20, pH 7.4), the plate was blocked with the 10% blocking buffer (Blocking One; Nacalai Tesque Inc., Kyoto, Japan) for 2 h at 37°C. To 80 µL each of diluted antibody against D-Mb or Peptide A, 80 µL of various concentration of Peptide A, D-Mb or native Mb was added as a competitor, and then the mixture was incubated for 1 h at room temperature. To each of the wells of the above D-Mb coated plate, 100 µL of the mixture was transferred, and then incubated for 1 h at room temperature. After removal of unbound antibodies by washing, 100 µL of HRP-conjugated goat anti-rabbit IgG (Zymed) was added to each well, and then incubated for 30 min at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was incubated for 10 min at room temperature in the dark. The reaction was stopped by the addition of 1.0 M sulfuric acid (50 µL/well), and the absorbance was then measured at 450 nm with a microplate reader (Spectra Fluor; Tecan, Salzburg, Austria).

**Sandwich ELISA** A polystyrene 96-well microtiter plate (Corning Inc.) was coated with 100 µL of antibody against D-Mb or Peptide A (5 µg/mL) in the coating buffer for 2 h at 4°C. After washing four times with the washing buffer, the plate was blocked with 10% Sea Block Blocking buffer (Thermo Fisher Scientific Inc.) for 2 h at 37°C. The dilution buffer (the washing buffer containing 1% Sea Block Blocking buffer, 0.05% SDS, and 0.2% 2-mercaptoethanol) was used for dilution of D-Mb and each sample. Diluted D-Mb or sample was added to the antibody coated plate in triplicate, and the plate was then incubated for 1 h at room temperature. After washing six times with the washing buffer, 100 µL of HRP-conjugated antibody against D-Mb was added to each well, and the plate was then incubated for 10 min at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was then incubated for 1 h at room temperature. After removal of unbound antibodies by washing, 100 µL of HRP-conjugated antibody against D-Mb was added to each well, and then incubated for 30 min at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was then incubated for 1 h at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was then incubated for 1 h at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was then incubated for 1 h at room temperature. After washing, 100 µL of the substrate solution (Sure Blue; TMB Microwell Peroxidase Substrate, KPL Inc., MD, USA) was added to each well, and the plate was then incubated for 1 h at room temperature.
a haptenic antigen. The N-terminal Cys in Peptide A was added to simplify the conjugation with a carrier protein, because Glu and Lys were present in the sequence of Peptide A.

**Polyclonal antibody** Rabbits were immunized with D-Mb or Peptide A conjugated to mariculture keyhole limpet hemocyanin by disulfide bond. Polyclonal antibodies against D-Mb were affinity-purified with a Hitrap Protein G HP column coupled with D-Mb. First, their cross-reactivity was analyzed by western blot analysis (Fig. 2B and C). The antibody against D-Mb reacted with beef and pork Mb, but not with chicken Mb or other proteins. In contrast, the antibody against Peptide A reacted only with beef Mb, with no cross-reactivity. Next, their reactivity was further analyzed using competitive ELISA. The antibody against D-Mb reacted with D-Mb and native Mb, but not with Peptide A (Fig. 3A). In contrast, the antibody against Peptide A reacted not only with D-Mb and native Mb but also with Peptide A (Fig. 3B), indicating that it reacted with beef Mb by recognizing the sequence Ala<sup>84</sup> to Asn<sup>95</sup>. From these results, it was shown that the epitopes of the two antibodies differed and it was, therefore, possible to develop sandwich ELISA with them.

**Sandwich ELISA** The sensitivity and specificity of sandwich ELISA constructed for beef Mb is shown in Fig. 4 with the antibody against D-Mb as the enzyme-labeled antibody. When the antibody against D-Mb was used as the capture antibody, relatively high sensitivity, but weak cross-reactivity, against pork was observed. In contrast, when the antibody against Peptide A was used as the capture antibody, cross-reactivity against pork and chicken was not observed as expected from the data shown in Fig. 2. Thus, the latter sandwich ELISA was used for evaluating specificity rather than sensitivity. This sandwich ELISA exhibited no cross-reactivity against egg, milk, wheat, buckwheat, peanuts, shrimp and crab (data not shown). According to Akiyama et al. (2003), its detection limit and quantification limit were calculated to be 29.4 and 36.4 ng/mL, respectively, from the linear beef protein standard curve (Fig. 4B).

**Determination of beef protein content in processed foods** To evaluate the practical applicability of sandwich ELISA specific against beef Mb, beef protein content was determined in model foods produced with varying amounts of beef in pork or chicken and at different temperatures. When 2 g of model foods prepared with 100% beef was extracted with 18 mL of PBS containing 0.5% SDS and 2% 2-mercaptoethanol, beef protein concentrations in the extracts (determined by 2-D Quant kit) were 8.0, 3.0 and 2.2 mg/mL, respectively, for the non-heated sample and the heated samples at 80°C and 120°C. The decrease in recovery was shown using SDS-PAGE (Fig. 5). It was probably due to the aggregation and the resulting insolubilization of beef proteins by heating. Although Mb is a myoplasmic protein and extraction was performed under the denatured condition, its remarkable decrease was observed unexpectedly at 120°C (Fig. 5, lane 4). Corresponding results were obtained by sandwich ELISA of 10.83, 3.88, and 0.61 mg/mL, respectively, for the non-heated sample and the heated samples at 80°C and 120°C. Thus, because the decrease in extraction efficiency was a serious problem, even if SDS and 2-mercaptoethanol were used for extraction, further studies are needed.

Although the extraction efficiency was low in heated
samples, good agreement was obtained between 0.1% ~ 10% of the actual mixing ratio of beef and the recoveries determined with sandwich ELISA, regardless of differences in basic meats and heating conditions (Table 1). Beef is generally added to processed foods from 1 to 50%. Therefore, this sandwich ELISA is thought to be applicable for the detection of beef proteins in commercial processed foods. The high performance in specificity is probably accomplished through the use of the antibody against Peptide A as the capture antibody.

As shown in Table 2, this method could be used to qualitatively confirm that the labeling of beef usage in several commercial foods was reasonable. Based on the protein content of beef (20%) and the proportion of beef (30%) in the sausage listed in Table 2, the beef protein content of the sausage was expected to be about 60 mg/g. The recovery of about 70% suggested the quantitative applicability of our method to commercial foods. However, improvements to the sensitivity of this method is necessary, because labeling of the allergenic material is mandatory in Japan, when greater than 10 µg of protein of the corresponding allergenic material/g of food weight is contained in processed foods (Matsuda...
et al., 2006; Sakai et al., 2008). Taking into account the 400-fold dilution according to the official extraction method (i), the detection limit required for beef labeling is 25 ng/mL in the standard curve (Fig. 4). The slightly higher detection limit (29.4 ng/mL) of our method should be improved by the optimization of the system. Currently, we are attempting to introduce monoclonal antibodies against beef Mb and its peptide.

Thus, in this study, we developed sandwich ELISA specific against beef Mb for evaluation of beef protein content in processed foods.

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References


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