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Satoshi Kotoura ^a, Yukie Murakami-Yamaguchi ^b, Kumiko Kizu ^c, Miyuki Nakamura ^a, Hidetaka Fuchu ^a, Kiyotaka Miake ^a, Masaaki Sugiyama ^a & Hiroshi Narita ^c

^a Central Research Institute, Marudai Food Co. Ltd., 21-3 Midori-cho, Takatsuki, Osaka, 569-8577, Japan

^b Kyoto College of Nutritional & Medical Science, 18 Setogawa-cho, Saga Tenryuji, Ukyo-ku, Kyoto, 616-8376, Japan

^c Department of Food and Nutrition, Kyoto Women's University, 35 Imakumano, Kitahiyoshi-cho, Higashiyama-ku, Kyoto, 605-8501, Japan

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Establishment of a sandwich ELISA for the determination of beef content in processed foods by using monoclonal antibodies to myoglobin

Satoshi Kotoura^a, Yukie Murakami-Yamaguchi^b, Kumiko Kizu^c, Miyuki Nakamura^a, Hidetaka Fuchu^a, Kiyotaka Miake^a, Masaaki Sugiyama^a and Hiroshi Narita^{c*}

^aCentral Research Institute, Marudai Food Co. Ltd., 21–3 Midori-cho, Takatsuki, Osaka 569-8577, Japan; ^bKyoto College of Nutritional & Medical Science, 18 Setogawa-cho, Saga Tenryuji, Ukyo-ku, Kyoto 616-8376, Japan; ^cDepartment of Food and Nutrition, Kyoto Women's University, 35 Imakumano, Kitahiyoshi-cho, Higashiyama-ku, Kyoto 605-8501, Japan

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A sandwich enzyme-linked immunosorbent assay specific to beef myoglobin was developed using monoclonal antibodies to sodium dodecyl sulphate-denatured beef myoglobin and a peptide with an amino acid sequence unique to beef myoglobin. This method was very specific for beef myoglobin, and showed no cross-reactivity with not only pork and chicken myoglobins, but also with other food proteins such as egg, cow's milk, wheat and peanut. The limit of detection and that of quantification for the beef protein was 9.5 and 12.8 ng/ml, respectively. When the content of beef protein in pork- and chicken-based model processed foods containing varying amounts of beef were determined by this method, the recovery of beef protein agreed well with the actual ratio of added beef regardless of the processing conditions. Also, the amount of beef protein in several commercially processed foods could be reasonably determined and confirmed by this method.

Keywords: monoclonal antibody; sandwich ELISA; myoglobin; beef protein content; allergen labelling

Introduction

Beef is eaten around the world as a delicious and important source of protein, and various beef products, such as sausages, meat sauce and hamburger steak, are produced and consumed. However, there are cases in which the avoidance of beef ingestion is required due to religious beliefs, personal principles such as vegetarianism, and beef-specific diseases such as allergies and bovine spongiform encephalopathy (Hsieh, Ofori, Rao, & Bridgeman, 2007; Restani, Ballabio, Tripodi, & Fiocchi, 2009). Proper labelling is necessary to ensure the quality, safety and fair trade of beef-containing foods for consumers, and a simple and specific analytical method should be established for the detection of beef in food products and feeds. Enzyme-linked immunosorbent assay (ELISA) is well known (Taylor, Nordlee, Niemann, & Lambrecht, 2009) and is widely utilised as a method for determining the presence of specific proteins in processed foods (Hefle, Jeanniton, & Taylor, 2001; Kiening et al.,

*Corresponding author. Email: narita@kyoto-wu.ac.jp

2005; Matsuda et al., 2006; Sakai et al., 2008). A few ELISA-based methods have been developed for the detection of meat proteins in food products (Andrews, Berger, Mageau, Schwab, & Johnston, 1992; Chen & Hsieh, 2000). In this study, we attempted to develop a sensitive sandwich ELISA using monoclonal antibody (mAb) to a peptide with an amino acid sequence unique to beef myoglobin (Mb).

Food proteins are usually denatured during processing, especially by heating, and this denaturation contributes to the shape and texture of the product. In these cases, potential target proteins for the ELISA are often irreversibly aggregated, insoluble and difficult to extract (Taylor et al., 2009). Watanabe et al. (2005) reported that a solution containing sodium dodecyl sulphate (SDS) and 2-mercaptoethanol could efficiently extract food proteins, and this method was adopted as the official Japanese method for the detection of major allergenic ingredients in food products (Akiyama, Imai, & Ebisawa, 2011). Therefore, we also used SDS and 2-mercaptoethanol for the extraction and dilution of samples to be assayed. Consequently, the target proteins should be able to react with the mAbs under denatured condition.

In this study, we produced mAbs to SDS-denatured beef Mb and a synthetic peptide unique to beef Mb, and developed a sandwich ELISA specific and sensitive to beef Mb. This sandwich ELISA enabled us to accurately determine the beef content in model processed foods and some commercial foods.

Materials and methods

Food materials

Beef leg meat, pork heart and leg meat and chicken heart and breast meat were prepared separately in our meat department unit to prevent cross-contamination. Samples of commercially processed foods were purchased at supermarkets in Osaka.

Chemicals

The following materials were obtained from the indicated sources: Freund's complete and incomplete adjuvants (Difco Laboratories Inc., Detroit, MI, USA); bovine serum albumin (BSA), polyethylene glycol and pristine (Sigma, St. Louis, MO, USA); 3,3',5,5'-tetramethylbenzidine (TMB; Kirkegaard & Perry Laboratories Inc., Baltimore, MD, USA); horseradish peroxidase-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed Inc., Vienna, Austria); Inject Maleimide Activated Immunogen Conjugation Kit and EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA); Peroxidase Labeling Kit-SH (Dojindo Laboratories Inc., Kumamoto, Japan); 2-D Quant Protein Assay Kit and streptavidin-horseradish peroxidase (GE Healthcare UK Ltd., Buckinghamshire, UK); 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) Alkaline Phosphatase Substrate Kit V (Vector Laboratories Inc., Burlingame, CA, USA). All other chemicals used were of analytical grade.

Preparation of myoglobins and standard solutions from beef, pork and chicken

Beef Mb was purified from fresh leg meat, and pork and chicken Mbs were purified from fresh heart meat. Minced meat (100 g each) was homogenised with

two-fold (w/v) of distilled water and mixed for 30 min at 4°C. After centrifugation at $4500 \times g$ for 15 min at 4°C, the supernatant was filtered through filter paper No. 1 (Whatman Japan Inc., Tokyo, Japan). The filtrate from beef leg meat was brought to 90% ammonium sulphate saturation and those from pork and chicken heart meat were brought to 70% ammonium sulphate saturation. After stirring for 1 h at 4°C, each solution was centrifuged at $20,000 \times g$ for 15 min at 4°C. After filtration, the supernatant was adjusted to pH 6.85 and brought to 95% ammonium sulphate saturation. After stirring for 1 h at 4°C, it was centrifuged at $20,000 \times g$ for 15 min at 4°C. The precipitate was dissolved in distilled water and dialysed against 20 mM Tris-HCl (pH 8.3). The solution was applied to an ion exchange column (Toyoscreen DEAE 650 M; 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 0–0.5 M NaCl in 20 mM Tris-HCl (pH 8.3). The purity of each Mb was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions according to the method of Laemmli (1970), and a sequence of 10 amino acids at the N-terminus was determined by Takara Bio Inc. (Shiga, Japan).

Minced meat (250 g each) of beef leg, pork leg or chicken breast meat was homogenised with an equal volume of distilled water and freeze-dried. The resulting lyophilised powder (20 mg each) was added to 20 ml of phosphate buffer saline (PBS: 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2) containing 0.5% SDS and 2% 2-mercaptoethanol and shaken for 12 h at room temperature for extraction (Watanabe et al., 2005). After centrifugation at $20,000 \times g$ for 15 min at room temperature, the supernatant was filtered to obtain a standard solution of which the protein content was determined by a 2-D Quant Protein Assay Kit.

Preparation of monoclonal antibodies

Because the beef Mb would be denatured during extraction, the purified beef Mb was denatured with 1% SDS and heated at 95°C for 10 min (denatured beef Mb: D-Mb) before being used as an immunogen for inducing the production of mAbs to D-Mb. 2-Mercaptoethanol was not used at this step because there are no disulphide bonds in beef Mb (Fuentes, Palacios, Garces, Caballero, & Moneo, 2004). Synthesis of Peptide A and Peptide B was entrusted to Toray Research Center Inc. (Tokyo, Japan). Peptide A (Cys-Ala⁸⁴-Glu⁸⁵-Val⁸⁶-Lys⁸⁷-His⁸⁸-Leu⁸⁹-Ala⁹⁰-Glu⁹¹-Ser⁹²-His⁹³-Ala⁹⁴-Asn⁹⁵ of beef Mb) and Peptide B (Met¹⁴²-Cys-Ala¹⁴⁴-Gln¹⁴⁵-Tyr¹⁴⁶-Lys¹⁴⁷-Val¹⁴⁸-Leu¹⁴⁹-Gly¹⁵⁰-Phe¹⁵¹-His¹⁵²-Gly¹⁵³ of beef Mb) were conjugated with mariculture keyhole limpet haemocyanin from the Inject Maleimide Activated Immunogen Conjugation Kit for immunisation of mice. Five BALB/c mice (female, 8 weeks old) were immunised intraperitoneally with emulsion containing 50 µg of D-Mb or each peptide-conjugate per mouse in Freund's complete adjuvant. After two weeks, mice were boosted once with the same volume of immunogen emulsified in Freund's incomplete adjuvant. Ten days later, sera were collected from the tail vein and tested for specific antibody production using a direct ELISA with D-Mb, or Peptide A or B conjugated with ovalbumin of the Inject Maleimide Activated Immunogen Conjugation Kit on the solid phase of a 96-well microtiter plate (Corning Inc., Washington, DC, USA). The antibodies bound to D-Mb or Peptide A or B were detected with diluted horseradish peroxidase-conjugated goat anti-mouse

IgG and TMB substrate solution. The reaction was stopped by the addition of 1.0 M sulphuric acid (50 µl/well), and then the absorbance was measured at 450 nm with a microplate reader (Spectra Fluor, Tecan, Salzburg, Austria). The mice showing higher titer of antibody were injected with 50 µg D-Mb or each peptide conjugate in PBS into the tail vein. Three days later, myeloma cells (P3X63. AG80. 1; DS Pharma Biomedical Co., Ltd, Osaka, Japan) were fused with spleen cells from immunised mice according to the method of Köhler and Milstein (1975). Ten days after fusion, the cell culture supernatants were screened for specific antibodies by a direct ELISA as described above. The positive cells were cloned twice by limiting dilution and then injected intraperitoneally into mice previously primed with pristine. Ascitic fluids were precipitated by saturated ammonium sulphate solution and dissolved in PBS. The mAbs were named after the code numbers of cells cloned and were purified with a Protein G column (GE Healthcare UK Ltd.). Peroxidase-conjugated and biotin-conjugated mAbs to D-Mb were prepared with a Peroxidase Labeling Kit-SH and EZ-Link Sulfo-NHS Biotinylation Kit, respectively. Mouse experiments in this study were carried out under the guidelines of the Animal Experiment Committee of the university following the bulletin (No.71, 2006) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Competitive ELISA

Polystyrene 96-well microtiter plates were coated with 100 µl of D-Mb (1 µg/ml) in coating buffer (0.1 M phosphate buffer, pH 7.2) for 12 h at 4°C. After washing four times with a washing buffer (10 mM phosphate buffer, 0.15 M NaCl, 0.1% Tween20, pH 7.4), the plates were blocked with 1% BSA for 2 h at 37°C. Diluted mAbs to D-Mb, Peptide A or Peptide B (80 µl each) were added to an equal volume of various concentrations of Peptide A, Peptide B, D-Mb or native beef Mb (N-Mb) as a competitor, then the mixtures were incubated for 1 h at room temperature. D-Mb was diluted in washing buffer containing 0.1% BSA and 0.3% SDS, and Peptide A, Peptide B and N-Mb were diluted in washing buffer containing 0.1% BSA without SDS. Each mixture (100 µl) was transferred to the wells of the D-Mb-coated plate, and incubated for 1 h at room temperature. After removal of unbound materials by washing, 100 µl of diluted peroxidase-conjugated goat anti-mouse IgG was added to each well, and then incubated for 30 min at room temperature. Finally, the enzyme reaction was performed in the plate as mentioned above for the direct ELISA.

Sandwich ELISA

Polystyrene 96-well microtiter plates were coated with 100 µl of mAbs to Peptide B (5 µg/ml) in coating buffer for 12 h at 4°C. After washing four times with washing buffer, the plates were blocked with 1% BSA for 2 h at 37°C. D-Mb and the food sample solution were diluted with dilution buffer (the washing buffer with 0.1% BSA, 0.05% SDS and 0.2% 2-mercaptoethanol). Diluted D-Mb or food sample solution was added to an antibody-coated plate in triplicate, and incubated for 1 h at room temperature. After washing six times with the washing buffer, 100 µl of diluted peroxidase-conjugated mAb to D-Mb was added to each well, and the plate was then incubated for 30 min at room temperature. One hundred microlitres of the diluted biotin-conjugated mAb to D-Mb was then added to each well, and the plate was

incubated for 30 min at room temperature. After washing, 100 μ l of diluted streptavidin-horseradish peroxidase was added to each well, and the plate was incubated for 30 min at room temperature. Finally, the plate was applied to the enzyme reaction as described for the direct ELISA. Limit of detection (LOD) or limit of quantification (LOQ) for the sandwich ELISA were calculated by adding 3 times or 10 times the standard deviation of the buffer blank to the mean of the buffer blank, respectively (Seiki et al., 2007).

Western blotting

Western blotting was carried out according to the method of Towbin, Staehelin, and Gordon (1979) with slight modifications. After SDS-PAGE on a 15% gel under reduced conditions, the resolved protein bands were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., Alfred Nobel Drive Hercules, CA, USA) at 1 mA/cm². After electroblotting, the membrane was blocked with phosphate buffer saline with Tween 20 (PBST: 0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄·12H₂O, 1.5 mM KH₂PO₄, 0.1% Tween20) for 12 h at 4°C. After washing three times with PBST, the membrane was incubated with diluted mAb to D-Mb or Peptide B for 1 h at room temperature. The excess antibodies were removed by washing three times with PBST, then the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG 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 detected with BCIP/NBT Alkaline Phosphatase Substrate Kit V. The colour development was stopped by shaking the membrane in distilled water.

Preparation of model processed foods and sample solutions

Minced beef leg meat was mixed with minced pork leg meat or chicken breast meat to be 100%, 10%, 1%, 0.1%, 0.01% and 0% (w/w). Then, potato starch, salt and water were added at the weight ratio of 0.2:0.2:0.6 to 9 of the mixed meat. The mixtures were incubated at room temperature, 80 or 120°C for 30 min, cooled in running water and then preserved in a freezer at -30°C until use. Eighteen millilitres of PBS (pH 7.2) containing 0.5% SDS and 2% 2-mercaptoethanol was added to 2 g of the mashed sample. After shaking for 12 h at room temperature for extraction, the mixture was centrifuged at 20,000 $\times g$ for 15 min and the supernatant was filtered to obtain a solution for each sample to be assayed.

Results and discussion

Selection and preparation of antigens

To establish a mAbs ELISA specific to beef, the selection of an appropriate target protein is important. BSA (Fiocchi, Rastani, & Riva, 2000; Tanabe et al., 2002) and immunoglobulin (Ayuso et al, 2000; Han, Matsuno, Ito, Ikeuchi, & Suzuki, 2000) are reported to be the major allergen in beef. These allergenic proteins or myofibrillar abundant proteins may usually be selected as a target antigen for the detection of beef by a sandwich ELISA. However, the ELISA specific to beef should be capable of

distinguishing beef at least from cow's milk and also from pork and chicken. Serum albumin and immunoglobulin are constitutive components in cow's milk and amino acid sequences of major myofibrillar proteins such as myosin (Chikuni, Tanabe, Muroya, & Nakajima, 2002) are highly conservative with over 95% homology among these animals. We selected beef myoglobin (Mb) as a target antigen, because it is a constitutive and relatively rich component in meat (4–10 mg/g) but not in milk, and its amino acid sequence is somewhat different among animals: 88% homology between beef Mb (Han, Dautrevaux, Chailax, & Biserte, 1970) and pork Mb (Rousseaux, Dautrevaux, & Han, 1976) and 74% between beef Mb and chicken Mb (Deconinck et al., 1975).

Beef Mb was purified from fresh leg meat (See Figure 3A, lane 4) and was used as an immunogen after denaturation by heating with SDS because the actual samples are also denatured during extraction (Watanabe et al., 2005). If whole beef Mb is used as an immunogen, the obtained antibodies might cross-react with pork or chicken Mb (Levieux & Levieux, 1996). Therefore, we tried to obtain mAbs to peptides with amino acid sequences specific to beef Mb. Two sequences, Ala⁸⁴-Asn⁹⁵ and Met¹⁴²-Gly¹⁵³, which are relatively characteristic of beef Mb, were selected (Figure 1). Amide bonding of the peptides to the carrier protein was avoided to simplify the structure of the conjugates because Glu and Lys were present in the sequences of both peptides. Then, two peptides, Peptide A (Cys-Ala⁸⁴-Asn⁹⁵) and Peptide B (Met¹⁴²-Cys-Ala¹⁴⁴-Gly¹⁵³), were chemically synthesised. The N-terminal Cys in Peptide A was supplemented to Ala⁸⁴-Asn⁹⁵ and the second Cys in Peptide B was substituted for Ala¹⁴³ in Met¹⁴²-Gly¹⁵³. They were conjugated to a carrier haemocyanin through

Beef	1	glsdgewqlv	lnawgkvead	vaghgqevli	rlftghpetl	ekfdkfkhlk
Pork	1	glsdgewqlv	lnvwgkvead	vaghgqevli	rlfkghpetl	ekfdkfkhlk
Chicken	1	glsdqewqv	ltiwigkvead	iaghghevln	rlfhghpetl	drfdkfkglk
Lamb	1	glsdgewqlv	lnawgkvead	vaghgqevli	rlftghpetl	ekfdkfkhlk
Horse	1	glsdgewqv	lnvwgkvead	iaghgqevli	rlftghpetl	ekfdkfkhlk
Beef	51	teaemkased	lkkhgntvlt	alggilkkkg	<u>hheaevkhla</u>	<u>eshankhkip</u>
Pork	51	sedemkased	lkkhgntvlt	alggilkkkg	hheaeltpla	qshatkkip
Chicken	51	tpdomkgsed	lkkhgatvlt	qlgkilkqkg	nheselkpla	qthatkkip
Lamb	51	teaemkased	lkkhgntvlt	alggilkkkg	<u>hheaevkhla</u>	<u>eshankhkip</u>
Horse	51	teaemkased	lkkhgtvlt	alggilkkkg	hheaekpla	qshatkkip
Beef	101	<u>vkylefisa</u>	<u>iihvlhakhp</u>	sdfgadaqaa	mskalelfrn	<u>dmaaykvlg fhg</u>
Pork	101	vkylefisea	iiqvlqskhp	gdfgadaqaa	mskalelfrn	dmaakykelg fgg
Chicken	101	vkylefisev	iiqvlaekha	adfgadsqaa	mkalelfrn	dmaskykefg fgg
Lamb	101	<u>vkylefisa</u>	<u>iihvlhakhp</u>	snfgadaqaa	mskalelfrn	<u>dmaaykvlg fgg</u>
Horse	101	ikylefisa	iihvlhskhp	gdfgadaqaa	mtkalelfrn	diaakykelg fgg

Figure 1. Amino acid sequence of myoglobin (Mb). Selected amino acid sequences relatively unique to beef Mb (Ala⁸⁴-Asn⁹⁵ and Met¹⁴²-Gly¹⁵³) are underlined. Shaded letters indicate the amino acid sequences that are identical with beef Mb.

disulphide bonds to enhance their immunogenicity, and also to ovalbumin to enable screening for cells producing antibodies reactive to the peptides.

Monoclonal antibodies

First, three mAbs reactive to Peptide A conjugated to ovalbumin were obtained. They reacted with D-Mb coated on an ELISA plate. The binding was inhibited at higher concentrations of free Peptide A (IC_{50} was about 10–100 $\mu\text{g/ml}$) but not with free N-Mb nor with free D-Mb on a competitive ELISA (data not shown). Next, mAbs 11H, 1D and 10B reactive to Peptide B conjugated to ovalbumin were obtained. They also reacted with D-Mb coated on an ELISA plate, but the binding was inhibited on a competitive ELISA at lower concentrations of free peptide B (IC_{50} was about 0.01 $\mu\text{g/ml}$) and with free N-Mb or D-Mb. The typical data obtained with mAb 11H, shown in Figure 2A, suggest that these mAbs recognise beef Mb exactly at

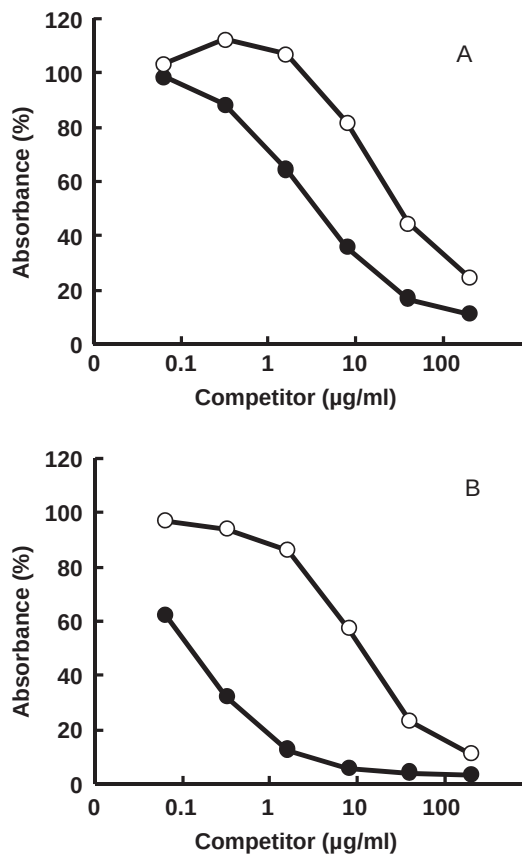


Figure 2. Reactivity of antibodies against beef myoglobin (Mb) on a competitive ELISA. A competitive ELISA for beef Mb (see *Materials and methods*) in the presence of various amounts of D-Mb (●) or N-Mb (○) as competitors. Absorbance obtained with no competitor was set as 100%. Antibody against Peptide B (mAb 11H) was used in panel (A) and that against D-Mb (mAb 11E) in panel (B).

the Met¹⁴²-Gly¹⁵³ sequence close to the C-terminus. The C-terminus of beef Mb seems to be pushed to the outsides of the molecule and is available for antibody binding in its native state. This is consistent with a report by Abaza and Atassi (1992) which showed that the region of 145–151 in any animal Mb is an antigenic site that is exposed on the outside. On western blotting analysis, however, mAbs 1D and 10B weakly cross-reacted with pork Mb but not with chicken Mb, and only mAb 11H was specific to beef Mb (Figure 3B). The difference in the specificity among obtained mAbs to a particular peptide is most likely due to the differences in the amino acid sequence of their antigen-binding sites. To construct a sandwich ELISA with this beef-specific mAb 11H, five mAbs were obtained from mice immunised with D-Mb. Among the selected mAb, mAb 11E reacted with D-Mb more strongly than with N-Mb, and the reaction was stronger than the mAb 11H reaction in the competitive ELISA (Figure 2). The mAb 11E reacted with beef and pork Mb, but not with chicken Mb or other beef proteins on western blotting analysis (Figure 3C).

Sandwich ELISA

As expected from the results above suggesting that epitopes of mAbs 11E to D-Mb and 11H to peptide B are different, a sandwich ELISA was constructed using both mAbs. Monoclonal Ab 11H was selected as the capture antibody because of its strict specificity to beef Mb (Figure 3B) and mAb 11E was used as the peroxidase-conjugated antibody. The ELISA thus constructed was specific to D-Mb without cross-reactivity to pork and chicken D-Mbs even at high concentrations (Figure 4).

For the practical usage of this ELISA, it is best to show the data in terms of beef protein content and not as beef Mb content. Thus, the beef standard solution (see *Materials and methods*) was introduced as the standard material instead of D-Mb. In addition, the biotin–avidin system was incorporated to improve assay sensitivity. As shown in Figure 5, a linear standard curve was obtained at concentrations below 500 ng/ml of beef standard solution. The LOD and LOQ of the beef protein were calculated to be 9.5 and 12.8 ng/ml, respectively (See *Materials and methods*). This

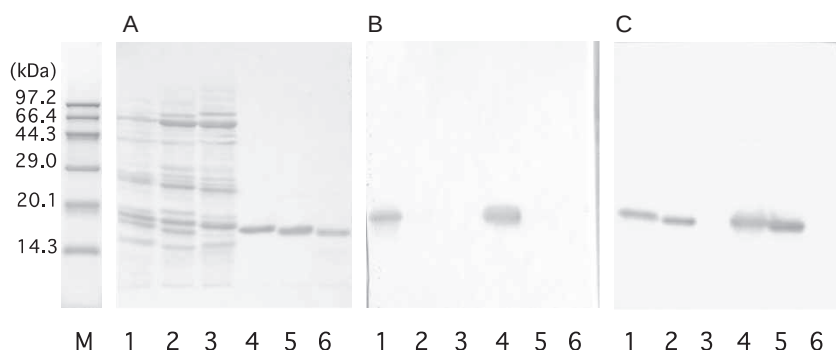


Figure 3. Specificity of mAbs against beef myoglobin (Mb). (A) Protein staining with coomassie brilliant blue; (B) immunostaining with antibody against Peptide B (mAb 11H); (C) immunostaining with antibody against denatured Mb (mAb 11E). M: molecular weight markers. Ten micrograms each of protein extracted from beef (lane 1), pork (lane 2) and chicken (lane 3), and 1 µg each of Mbs from beef (lane 4), pork (lane 5) and chicken (lane 6) were applied to 15% agarose gels.

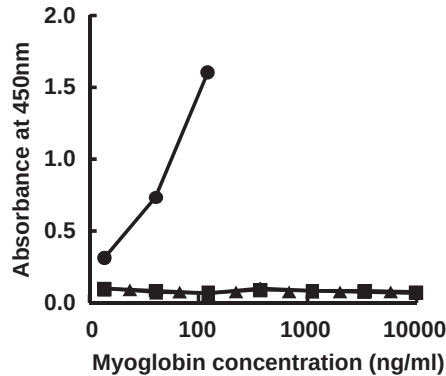


Figure 4. Specificity of the sandwich ELISA. The antibody against Peptide B (mAb 11H) was used as the capture antibody, and the antibody against denatured Mb (D-Mb) (mAb 11E) was used as the peroxidase-conjugated antibody. (●): beef D-Mb; (■): pork D-Mb and (▲): chicken D-Mb.

improved system did not cross-react with pork, chicken and horse standard solutions, but it did react with lamb at approximately 10% of the reactivity to beef (data not shown). This difference is consistent with the differences in amino acid sequence of the Peptide B region between Val¹⁴⁷ in beef and lamb Mb and Glu¹⁴⁷ in pork, chicken and horse Mb (Figure 1). Except for lamb, this system exhibited no cross-reactivity to other food protein, including egg, milk, wheat, buckwheat, peanut, shrimp and crab (data not shown).

Determination of beef protein content in processed foods

To evaluate the applicability of this beef-specific sandwich ELISA, beef protein content was determined in model processed foods produced with varying amount of beef mixed with pork or chicken under different processing temperatures. When 2 g

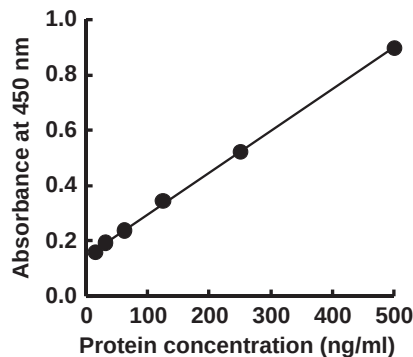


Figure 5. Standard curve of the sandwich ELISA for the beef protein standard. Protein standard from beef was applied to the sandwich ELISA, the sensitivity of which was enhanced with the biotin–avidin system. A linear regression line was obtained for the beef standard at 0–500 ng/ml. The equation was statistically expressed as $Y = 0.0015 X + 0.1456$, $R^2 = 0.9994$, where Y is the absorbance and X is concentration of the beef protein standard.

of model processed foods prepared with 100% beef were extracted with 18 ml of PBS containing 0.5% SDS and 2% 2-mercaptoethanol, this sandwich ELISA determined the beef protein concentrations in the extracts of non-heated samples, samples heated at 80°C and samples heated at 120°C to be 8.4, 7.8 and 6.9 mg/ml, respectively (Table 1). Its coefficient of variation, calculated from nine replicates of the same sample solution, was below 5%. On the other hand, those determined by 2-D Quant Kit were 8.0, 3.0 and 2.2 mg/ml for the non-heated samples, samples heated at 80°C and samples heated at 120°C, respectively. This discrepancy of the values in heated samples might be explained by that the extraction efficiency of whole proteins was decreased by heating but that of beef Mb was not decrease of its high extractability (Fuentes et al., 2004). Consequently, a good agreement was obtained between 0.01% and 10% of the actual mixing ratio of beef and the recoveries determined with this sandwich ELISA, regardless of difference of basic meats and heating conditions (Table 2). This high specificity was accomplished by using mAb 11H, which is specific to beef Mb, as the capture antibody (Figure 2B). An ELISA constructed with mAbs 11H and 11E reversely showed inferior specificity and sensitivity (data not shown). Beef is generally added into processed foods at concentrations of 1% to 50%; therefore, this sandwich ELISA is suitable for use in the detection of beef in commercially processed foods.

As shown in Table 3, this method could qualitatively confirm that the labelled beef content in several commercial foods was reasonable. It is notable that beef Mb was detectable by western analysis using mAb 11H in even demi-glace sauce, which contained beef extracts (data not shown). Beef extract is generally prepared from the liquid obtained from boiling beef meats, tendons or bones in water, and sometimes the liquid is treated with proteinases to add taste and flavour. Shimakura, Tonomura, Hamada, Nagashima, and Shiomi (2005) reported that tropomyosin, the major crustacean allergen, was degraded to peptide fragments during manufacturing of crustacean extractives. However, beef Mb seems to tolerate heating and proteinase treatment during manufacturing. Thus, beef Mb is an excellent target for the detection of beef protein because of its low homology in amino acid sequence with other Mbs, easy-extractability and tolerance to heating and proteinase treatment.

A detection kit utilising methods developed by the United States Department of Agriculture is commercially available for the detection of cooked beef meat in foods (Andrews et al., 1992). However, the kit is qualitative and can only detect the presence of cooked beef meat at concentrations of 1% or more. Previously, we reported the establishment of a sandwich ELISA using polyclonal Abs specific to beef Mb (Kotoura et al., 2009); however, its sensitivity was low (LOD: 29.4 ng/ml,

Table 1. The precision of the sandwich ELISA using beef-based model processed foods.^a

Model processed food	Mean (mg/ml)	SD (mg/ml)	CV (%)
Non-heated	8.4	0.3	3.3
Heated at 80°C	7.5	0.1	1.8
Heated at 120°C	6.4	0.2	2.8

^aThe precision of the sandwich ELISA was evaluated by using model beef-containing processed foods, which were prepared with minced beef meat, potato starch, salt and water, and incubated at room temperature (non-heated), 80°C or 120°C for 30 min. The means were calculated from nine replicates of the same sample solution. SD, standard deviation; CV, coefficient of variation.

Table 2. Recovery of beef protein in pork- or chicken-based model processed foods in relation to those in beef-based model processed foods.^a

Basic meat	Mixing ratio of beef to basic meat (%)	Recovery of beef protein (%)		
		Non-heated	Heated at 80°C	Heated at 120°C
Pork	10	9.2	5.7	10.3
	1	1.0	0.4	0.7
	0.1	0.2	0.1	0.1
	0.01	0.01	0.01	0.01
	0	n.d.	n.d.	n.d.
Chicken	10	13.5	4.8	6.0
	1	0.8	0.6	0.4
	0.1	0.1	0.1	0.1
	0.01	0.01	n.d.	0.01
	0	n.d.	n.d.	n.d.

^aBeef-containing model processed foods were prepared with minced meat (beef, pork or chicken), potato starch, salt and water, and incubated at room temperature (non-heated), 80°C or 120°C for 30 min. Recovery of beef protein (%) was calculated by dividing the beef protein concentration determined by ELISA in pork- and chicken-based model processed foods with those obtained for the beef-based model processed foods shown in Table 1. n.d., not detected.

LOQ: 36.4 ng/ml), the determination of beef protein content in foods was affected by heating, and it cross-reacted with lamb at the same reactivity as with beef (data not shown). We have largely succeeded in improving these problems in the current sandwich ELISA: (1) the sensitivity was higher with a LOD and LOQ of 9.5 and 12.8 ng/ml, respectively; (2) the determination of beef protein content was hardly affected by heating and (3) cross-reactivity with lamb was reduced, at about 10% of the reactivity to beef. Moreover, the new ELISA was composed of mAbs, which have better reliability in terms of quality and quantity than polyclonal antibodies.

In conclusion, we have developed a sandwich ELISA specific to beef protein by using two mAbs. This method is useful in evaluating the accuracy of the food labelling of beef products, and is suitable for use in the determination of beef protein

Table 3. Determination of beef protein content in selected commercial foods.

Sample	Labelling ^a	Substance labelled	Beef protein content (mg/g)
Beef jerky	+	Beef	113.0
Meat sauce	+	Beef	12.4
Hamburger steak	+	Beef	2.8
Demi-glaze sauce	+	Beef extract	0.1
Salami	—	—	n.d.
Pasta sauce	—	—	n.d.
Hamburger steak	—	—	n.d.
Demi-glaze sauce	—	—	n.d.

^aLabelling: a description of beef or beef extract usage was labelled (+) or not (—) on the food packaging. n.d., not detected.

content in processed foods and feeds, regardless of processing conditions such as temperature and enzyme treatment.

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