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A Reliable Enzyme Linked Immunosorbent Assay for the Determination of Bovine and Porcine Gelatin in Processed Foods

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Since gelatin-containing foods pose a risk for eliciting allergic reactions in sensitized individuals, a novel sandwich enzyme linked-immunosorbent assay (ELISA) for the detection and quantification of bovine and porcine gelatin in processed foods was developed. Rabbits and goats were immunized with bovine gelatin, and three antisera (pAb1 and pAb2 from rabbits, and pAb3 from goats) were obtained. We established a sandwich ELISA method based on a combination of these antibodies. In this study, two sandwich ELISA methods, rabbit pAb2-pAb1 and goat pAb3-pAb3, were evaluated for sensitivity, specificity, cross-reactivity, and applicability. Both ELISA methods were highly specific for bovine and porcine gelatin but had little reactivity with fish gelatin. The detection and quantification limits for porcine gelatin were found to be 0.78 ng/mL and 1.56 ng/mL, respectively. The established sandwich ELISA methods produced no false-positives, except for heated meat products or false negatives when various commercial foods were analyzed for their gelatin content. The rabbit pAb2-pAb1 ELISA cross-reacted with boiled squid, while the goat pAb3-pAb3 ELISA did not. Thus, the proposed goat pAb3-pAb3 ELISA method is a reliable tool for the detection of gelatin contaminants present in processed foods.

KEYWORDS: Gelatin; enzyme linked immunosorbent assay (ELISA); food allergy

INTRODUCTION

It is estimated that up to 8% of children and 2% of adults are affected by food allergies in industrialized countries (*1*−*3*). The clinical manifestations of food allergies vary from mild symptoms, such as oral allergy syndrome or mild urticaria, to severe anaphylactic reactions with fatal consequences.

Gelatin is widely distributed in all multicellular animals and has long been considered not to cause immune reactions in humans. Therefore, gelatin has been generally used as a stabilizer in vaccines (*4*). However, it was reported that vaccines containing gelatin sometimes cause severe anaphylactic reactions (*5, 6*). Beginning in 1993, anaphylactic reactions were increasingly reported after administration with gelatin-containing vaccines in Japan (*7*−*9*). Furthermore, it was reported that gelatin-containing foods also triggered allergic reactions (*10, 11*). Gelatin has been mainly used as a food ingredient for processed foods such as jelly, yogurt, and soft candy. There are five types available for industrial uses including bovine gelatin treated with alkali (BA gelatin), bovine gelatin treated with hydrochloric acid (BHA gelatin), porcine gelatin treated with alkali (PA gelatin), porcine gelatin treated with hydrochloric acid (PHA gelatin), and fish gelatin (F gelatin). Among these five types, BA gelatin from bovine bone and PHA gelatin from porcine skin are predominantly used. It was reported that most gelatin-allergic patients develop allergic reactions to bovine and porcine gelatin, but do not react to fish gelatin (*12, 13*). Thus, in order to avoid the onset of allergic reactions from bovine and porcine gelatin, the ministry of ordinance in Japan has recommended that bovine and porcine gelatin are appropriately labeled. Despite the labeling precautions, bovine and porcine gelatin present a high degree of risk for sensitized patients because they are often present in commercial foods as a nondisclosed allergen, due to cross-contamination during food processing in factories in which many different products are manufactured with various ingredients on the same production line.

Even small amounts of bovine and porcine gelatin can cause severe reactions. Thus, strict management is required to minimize the potential harm. In order to practically and effectively manage bovine and porcine gelatin in food and food ingredients, especially concealed traces of bovine and porcine gelatin, a simple and reliable detection method for bovine and porcine gelatin contaminants is necessary.

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In the present study, we developed reliable, highly specific and sensitive sandwich enzyme-linked immunosorbent assay (ELISA) methods to detect bovine and porcine gelatin in processed foods. We showed that these detection methods could be applicable to food-processed products and that trace amounts of bovine and porcine gelatin contained in commercial food products can be detected by these methods.

MATERIALS AND METHODS

Food Materials. Bovine gelatin treated with alkali (BA gelatin), bovine gelatin treated with hydrochloric acid (BHA gelatin), porcine gelatin treated with alkali (PA gelatin), porcine gelatin treated with hydrochloric acid (PHA gelatin), and fish gelatin (F gelatin) from tilapia were kindly provided by Nitta Gelatin Inc. (Osaka, Japan). Samples of commercial processed foods and food ingredients were purchased at supermarkets (Yokohama, Japan). For the cross-reactivity evaluation, the portions of meat and seafood samples were boiled at 95 °C for 10 min.

Chemicals and Reagent. Polyoxyethylene (20) sorbitan monolaurate (Tween 20) was supplied by Nacalai Tesque, Ibc. (Kyoto, Japan). Bovine serum albumin (BSA), horseradish peroxidase (HRP), and 3,3′5,5′-tetramethylbenzidine (TMB, TMB Super Sensitive One Component HRP Microwell Substrate) were provided by Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), Toyobo Co., Ltd. (Osaka, Japan), and Biofix (Maryland, USA), respectively.

Compositions of Buffer Solutions. The following buffers were prepared for the experiment. Buffer A consisted of 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. Buffer B consisted of 0.1 M glycine-HCl buffer (pH 2.3). Buffers A and B were used for the purification of specific antibodies. Buffer C was used for sample extraction and dilution and consisted of 150 mM Tris-HCl (pH 7.4) containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20. Buffer D was used for the preparation of gelatin standard solution and consisted of 750 mM Tris-HCl (pH 7.4) containing 0.5% (w/v) BSA and 0.25% (v/v) Tween 20. Buffer E was used for ELISA and consisted of 150 mM Tris-HCl (pH 7.4) containing 1% (w/v) BSA and 0.05% (v/v) Tween 20. The coating buffer consisted of 50 mM sodium carbonate, pH 9.6. The blocking buffer consisted of buffer C with 150 mM NaCl.

Preparation and Purification of Specific Antibodies. *Polyclonal Antibody 1 (pAb1).* For immunization, a complete Freund's adjuvant containing BA gelatin (initially 10 mg and subsequently 1 mg) was hypodermically injected into five rabbits as an antigen 7 times at 2-week intervals. Whole blood was collected, and the serum was separated for antibody purification. Total rabbit IgG was purified, using a HiTrap Protein G column (GE Healthcare UK Ltd., Buckinghamshire, England), by applying the serum to the column, equilibrated with buffer A, and eluted with buffer B. Next, a HiTrap NHS-activated HP column (GE Healthcare UK Ltd., Buckinghamshire, England) was conjugated with PHA gelatin and used to purify the PHA gelatin-specific antibody. Briefly, total rabbit IgG was applied to the column and equilibrated with buffer A, and the specific anti-BA and -PHA gelatin antibody was eluted with buffer B.

Polyclonal Antibody 2 (pAb2). The epitope peptide of gelatin-allergic patients, IPGEFGLPGP (*14*), was synthesized and conjugated C-terminal to keyhole limpet hemocyanin (KLH) using the maleimide method by Invitrogen (Carlsbad, CA). For immunization, a complete Freund's adjuvant containing KLH conjugated synthetic peptide (initially 1 mg and subsequently 0.5 mg) was hypodermically injected into five rabbits as an antigen 6 times at 2-week intervals. Whole blood was collected and the serum separated for antibody purification using the same method as that described above (HiTrap Protein G and the synthetic peptide-conjugated HiTrap NHS-activated HP column). Additionally, a HiTrap NHS-activated HP column was conjugated with boiled squid extract, prepared according to the method described below, and was used to remove the antibody with cross-reactivity to boiled squid extracts.

Polyclonal Antibody 3 (pAb3). Immunization was performed using complete Freund's adjuvant containing BA gelatin (initially 0.5 mg and subsequently 1.0 mg) as an antigen by hypodermic injection in three goats 5 times at 2-week intervals. Whole blood was collected and the serum separated for antibody purification using HiTrap Protein G and the PHA gelatin-conjugated HiTrap NHS-activated HP column by the same method as that described above.

Sample Extraction from the Food Samples. A Millser IFN-700G homogenizer (Iwatani International Corp., Osaka, Japan) was used to homogenize food samples. Thirty-eight milliliters of buffer C was added to 2 g of a homogenized sample and homogenized again (three times for 30 s) for extraction. After confirming that the pH was around 6.0−8.0 with pH paper, the sample was centrifuged at 3000 × *g* for 20 min, and the supernatant was filtered through 5A filter paper (Advantec Toyo, Tokyo, Japan) to obtain the extract.

Preparation of Gelatin Standard Solution. PHA gelatin was dissolved in water at a final concentration of 10 mg/mL and was warmed to 60 °C for 15 min. This was used as a standard solution and adjusted to a concentration of 1 μg/mL using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd., Buckinghamshire, England). The gelatin solution was diluted to 250 ng/mL with buffer D and stocked in 200-μL aliquots at 4 °C after freeze-drying. The standard solution was resuspended to a concentration of 50 ng/mL with distilled water, and serially diluted to make up a concentration range of 25, 12.5, 6.25, 3.125, 1.56, 0.78 ng/mL, and a blank (0 ng/mL). Sample extracts were diluted 20-fold with buffer C. A 100 μL volume of the standard and sample solutions were applied to the ELISA plate wells.

ELISA. A microtiter plate (F8 Maxisorp Nunc-Immuno module, Thermo Fisher Scientific Inc., Waltham, USA) was coated with pAb2 (8 μg/mL) or pAb3 (4.5 μg/mL), diluted with coating buffer. The plate was then blocked with blocking buffer (*15*). The blocking buffer was removed, and the plate was subsequently dried. The diluted food samples and PHA gelatin standard solutions were added to the plate (100 μL/well) and incubated for 1 h at 25 °C. The wells were then washed six times and pAb1 (0.35 μg/mL) or pAb3 (0.5 μg/mL) (labeled with horseradish peroxidase (HRP) (*16*) diluted with buffer E) was added to the ELISA plate at 100 μL/well and allowed to stand for 30 min for the secondary reaction. After washing six times, 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μL/well) was added, and the enzyme reaction was allowed to run at 25 °C for exactly 10 min. The reaction was stopped by the addition of 100 μL/well of 0.5 M H_2SO_4. The absorbance was measured at 450/620 nm, the dominant/subdominant wavelength. The amount of gelatin was calculated from the absorbance of the sample using the standard curve. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

Since adding gelatin is common practice in the production of processed foods, manufacturers are obligated to label raw materials properly. The verification of food labeling is of great importance in decreasing the unexpected occurrence of allergic reactions to foods. Hence, it is necessary to establish a rapid method for detecting gelatin in processed foods in order to verify the labeling. Currently, a number of methods are available for detecting food contaminants that are based on the detection of species-specific proteins and DNA by ELISA and polymerase chain reaction (PCR). Unfortunately, as for the determination of gelatin, the PCR method is not suitable for DNA that is degraded by the gelatin manufacturing process. In addition, it was reported that most gelatin-allergic patients react to bovine and porcine gelatin, yet remain unreactive to fish gelatin. Thus, the ability to distinguish bovine and porcine gelatin from fish gelatin is a societal need that is met by the ELISA method described here.

Establishment of Sandwich ELISA. First, we selected BA gelatin and the epitope peptide from gelatin-allergic patients, IPGEFGLPGP (*14*), as antigens to obtain two rabbit polyclonal antibodies (pAb1 and pAb2) for the detection of bovine and porcine gelatin. Next, we tried to establish the rabbit pAb1-pAb1 ELISA method using pAb1 for both the first and the second reactions, but this approach failed due to a high level

Table 1. Reactivity to Various Gelatins Using the Developed ELISA[a]

(A) Rabbit pAb2-pAb1 ELISA

sample	the origin	production process	ELISA mean (ng/mL)	protein assay (ng/mL)	ELISA/Protein assay (%)	CV (%)
PHA	porcine	acid treatment	15.5	12.5	124	8.9
PA	porcine	alkalitreatment	36.7	12.5	294	6.6
BHA	bovine	acid treatment	8.7	12.5	69.6	11.8
BA	bovine	alkalitreatment	18	12.5	144	2.4
F	fish		43.7	25,000	0.2	0.3

(B) Goat pAb3-pAb3 ELISA

sample	the origin	production process	ELISA mean (ng/mL)	protein assay (ng/mL)	ELISA/Protein assay (%)	CV (%)
PHA	porcine	acid treatment	14.2	12.5	114	3.7
PA	porcine	alkalitreatment	27.7	12.5	222	2.3
BHA	bovine	acid treatment	7.8	12.5	62.4	8.5
BA	bovine	alkalitreatment	29.4	12.5	235	6.4
F	fish		34.3	25,000	0.1	6.9

[a] The reactivity of the developed ELISA for five kinds of gelatin is listed. The gelatin was dissolved in water at 10 mg/mL and was warmed at 60 °C for 15 min. This solution was adjusted to a concentration of 12.5 ng/mL (25,000 ng/mL for F) using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd., Buckinghamshire, England). The ELISA mean value denotes the calculated mean concentration (gelatin protein weight/gelatin solution weight) of the gelatin ((A) $n = 2$; (B) $n = 3$). Reactivity was calculated as ELISA mean/protein assay × 100 (%).

Table 2. Cross-Reactivity to Different Meats Using the Developed ELISA[a]

(A) Rabbit pAb2-pAb1 ELISA

samples	mean (μg/g)	samples	mean (μg/g)
pork	<0.31	boiled pork	1,200
beef	<0.31	boiled beef	77
chicken	<0.31	boiled chicken	45
spareribs (pork)	<0.31	boiled spareribs (pork)	290
gristle (beef)	0.7	boiled gristle (beef)	220
wing chip (chicken)	1.5	boiled wing chip (chicken)	410
gristle (chicken)	<0.31	boiled gristle (chicken)	150

(B) Goat pAb3-pAb3 ELISA

samples	mean (μg/g)	samples	mean (μg/g)
pork	<0.31	boiled pork	300
beef	0.6	boiled beef	67
chicken	<0.31	boiled chicken	0.5
spareribs (pork)	<0.31	boiled spareribs (pork)	290
gristle (beef)	2.1	boiled gristle (beef)	330
wing chip (chicken)	<0.31	boiled wing chip (chicken)	4.7
gristle (chicken)	<0.31	boiled gristle (chicken)	3.8

[a] The cross-reactivity of the developed ELISA for raw or boiled meats is listed. The sample extracts were prepared according to the procedure described in the Materials and Methods section. Listed values are the calculated mean concentrations (PHA gelatin weight/sample weight) of the gelatin ($n = 2$).

of cross-reactivity to fish gelatin (data not shown). We also found that pAb2 was not suitable for the second reaction due to low reactivity (data not found). We then tried to establish sandwich ELISA by the combinations of these two antibodies. We established the rabbit pAb2-pAb1 ELISA method; pAb2 and pAb1 were used for the first (coating) and the second (capture) reactions, respectively. We found that it showed only a slight cross-reactivity to fish gelatin (as shown in **Table 2**), and as described later, it was unexpectedly found that the rabbit pAb2-pAb1 strongly (440 μg/g) cross-reacted with boiled squid (**Table 3**). In addition, this cross-reactivity was not resolved even after we tried to remove the antibody, which cross-reacted to boiled squid extracts by affinity chromatography on a HiTrap NHS-activated HP column conjugated with boiled squid extract prior to use. It is unclear what kind of protein in squid is causing this phenomenon. Because the binding between the antisera and squid extract may be nonspecific, it was assumed that the squid extract would bind with the Fc region of the rabbit antibody. Thus, we prepared another polyclonal antibody (pAb3) derived

Table 3. Cross-Reactivity to Various Seafood Using the Developed ELISA[a]

(A) Rabbit pAb2-pAb1 ELISA

samples	mean (μg/g)	samples	mean (μg/g)
salmon	1.0	boiled salmon	<0.31
mackerel	<0.31	boiled mackerel	<0.31
bonito	<0.31	boiled bonito	4.9
tuna	<0.31	boiled tuna	2.6
squid	<0.31	boiled squid	440
octopus	<0.31	boiled octopus	1.1
prawn	<0.31	boiled prawn	0.7
short-neck clam	<0.31	boiled short-neck clam	<0.31
capelin roe	<0.31	boiled capelin roe	<0.31
cod roe	<0.31	boiled cod roe	<0.31
scallop	<0.31	boiled scallop	0.5
snow crab	<0.31	boiled snow crab	<0.31

(B) Goat pAb3-pAb3 ELISA

samples	mean (μg/g)	samples	mean (μg/g)
salmon	0.6	boiled salmon	<0.31
mackerel	<0.31	boiled mackerel	<0.31
bonito	<0.31	boiled bonito	2.4
tuna	<0.31	boiled tuna	3.2
squid	<0.31	boiled squid	4.8
octopus	<0.31	boiled octopus	2.1
prawn	<0.31	boiled prawn	<0.31
short-neck clam	<0.31	boiled short-neck clam	<0.31
capelin roe	<0.31	boiled capelin roe	<0.31
cod roe	<0.31	boiled cod roe	<0.31
scallop	<0.31	boiled scallop	0.8
snow crab	<0.31	boiled snow crab	<0.31

[a] The cross-reactivity of the developed ELISA for 12 kinds of raw or boiled seafood is listed. The sample extracts were prepared according to the procedure described in the Materials and Methods section. Listed values are the calculated mean concentrations (gelatin weight/sample weight) of the gelatin ($n = 2$).

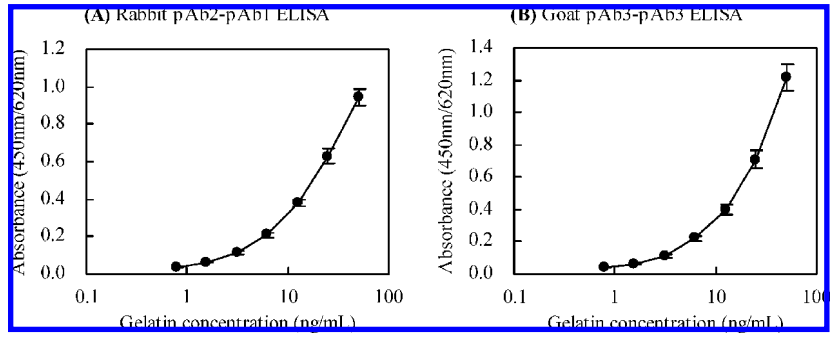

Figure 1. Calibration curves for the developed ELISA. The calibration curves were obtained from an average of eight experiments. Concentrations of standard solution were 0 ng/mL (0 μg/g), 0.78 ng/mL (0.31 μg/g), 1.56 ng/mL (0.62 μg/g), 3.13 ng/mL (1.25 μg/g), 6.25 ng/mL (2.5 μg/g), 12.5 ng/mL (5 μg/g), 25 ng/mL (10 μg/g), and 50 ng/mL (20 μg/g). The value in parentheses was converted into the concentration in foods using the dilution factor. (**A**) Rabbit pAb2-pAb1 ELISA; (**B**) goat pAb3-pAb3 ELISA.

from goats and established an alternative ELISA system (goat pAb3-pAb3 ELISA) in which pAb3 was used for both the first and the second reactions because the Fc regions of rabbits and goats are distinct. As a result, the goat pAb3-pAb3 ELISA we developed successfully avoided cross-reactivity with boiled squid, which is one of the most important advances of this experiment.

Figure 1 shows representative nonlinear calibration curves for the two sandwich ELISAs developed for the gelatin determination using PHA gelatin as a calibration standard. Calibration data were measured using eight replicates. The model that best describes the relationship between the absorbance and antigen concentration is a four-parameter logistic curve, ranging from 0.78 ng/mL (0.31 μg/g) to 50 ng/mL (20 μg/g). The signal-to-noise ratio for the pAb2-pAb1 and pAb3-pAb3 methods was shown to be 68.5 and 67.6, respectively, by serial titrations. For both methods, the limits of detection (LOD) and quantification (LOQ) were 0.78 ng/mL and 1.56 ng/mL, respectively, when PA gelatin is used as the standard. The LOD and LOQ values correspond to food samples contaminated with 0.31 μg/g and 0.62 μg/g PHA gelatin, respectively.

Reactivity to Various Gelatins. Five kinds of gelatin (BA, BHA, F, PA, and PHA gelatins) were tested for reactivity and specificity using the established ELISA methods. As shown in **Table 1** section A, the reactivity of rabbit pAb2-pAb1 ELISA for bovine and porcine gelatin was found to be 69.6−294%. The rabbit pAb2-pAb1 reacted most strongly with PA gelatin (294%) but less with fish gelatin (only 0.2%).

Also, the reactivity of the goat pAb3-pAb3 ELISA for bovine and porcine gelatin was found to be 62.4−235% (**Table 1** section B). The goat pAb3-pAb3 ELISA reacted with bovine and porcine gelatins treated with alkali more strongly than with those treated with hydrochloric acid. BA gelatin was detected more efficiently by the goat pAb3-pAb3 (235%) than the rabbit pAb2-pAb1 (144%) ELISA. The goat pAb3-pAb3 ELISA had little reactivity with fish gelatin (only 0.1%), which is similar to the rabbit pAb2-pAb1 ELISA.

To obtain antibodies, rabbits and goats were immunized with bovine gelatin in this study. However, as shown in **Table 1**, the rabbit pAb2-pAb1 ELISA and the goat pAb3-pAb3 ELISA both reacted with porcine gelatin to an extent equal to or greater than that with bovine gelatin. Therefore, it is assumed that the structures of bovine and porcine gelatin are relatively similar to each other or at least that the epitope regions of bovine and porcine gelatin are highly similar. In addition, the present method showed that bovine and porcine gelatins treated with alkali were well recognized than those treated with hydrochloric acid, probably because alkali-treated gelatin was immunized to rabbits and goats. Since gelatin structures are different depending on the manufacturing method, it was considered that the avidity of antibodies to react epitopes of antigen was changeable. We assumed that this is the probable reason why the antibody detected upward of 3 times the amount of antigen than was put into the assay. It is still unclear as to which gelatin (alkali-treated or hydrochloric acid-treated) is more allergenic to sensitized patients, and further clinical evaluations are necessary to clarify this point.

In this study, the standard gelatin sample was prepared by freeze-drying because of the stable preservation at 4 °C. By this freeze-drying process, the reactivity of PHA gelatin solution was decreased to about 80% (data not shown). This was, in turn, the reason why the value of the ELISA/protein assay displayed over 100% (124% and 114% for the rabbit and goat ELISA, respectively).

Assay precision was determined by calculating the coefficients of variation (CVs), which were found to be below 10% except for BHA gelatin in the rabbit pAb2-pAb1 ELISA (11.8%). Therefore, the methods are shown to have an acceptable precision.

Cross-Reactivity to Meat. As shown in **Table 2**, cross-reactivity was evaluated with seven kinds of raw and boiled meat. The rabbit pAb2-pAb1 ELISA showed slight (0.7−1.5 μg/g) cross-reactivity to raw gristle (beef) and wing chip (chicken), and showed no cross-reactivity to the other five kinds of raw meats. However, the rabbit pAb2-pAb1 cross-reacted with all seven kinds of heated meat (over 45 μg/g). The goat pAb3-pAb3 ELISA showed slight (0.6−2.1 μg/g) cross-reactivity to raw beef and showed no cross-reactivity to the other five kinds of raw meats. However, the goat pAb3-pAb3 cross-reacted with heated meat (over 67 μg/g) except chicken. The goat pAb3-pAb3 ELISA also cross-reacted with heated goat meat, venison, boar, and rabbit meats, although it did not reacted with these raw meats (data not shown). The probable reason for the high cross-reactivity with heated meats was that collagen in meats was denatured by heating and subsequently recognized by the antibodies used in this study. Nevertheless, because the cross-reactivity of the goat pAb3-pAb3 with heated meats was generally lower than that of the rabbit pAb2-pAb1, it was judged that the goat ELISA performed better for pork, beef, and chicken.

The developed ELISA methods showed a high cross-reactivity to heated meats and meat products (**Tables 2** and **5**). It is obvious that, upon heating, the binding between collagen chains is dissociated and gelatinization occurs. Thus, if heated meat is gelatinized, it is recognized by antisera against gelatin. We are

Table 4. Cross-Reactivity to Various Foods Using the Developed ELISA[a]

foods	Rabbit pAb2-pAb1 ELISA (μg/g)	Goat pAb3-pAb3 ELISA (μg/g)
rice	<0.31	<0.31
buckwheat	<0.31	<0.31
wheat	<0.31	<0.31
rye	<0.31	<0.31
barley	<0.31	<0.31
oatmeal	<0.31	<0.31
corn	<0.31	<0.31
sago	<0.31	<0.31
potato	<0.31	<0.31
tomato	<0.31	<0.31
onion	<0.31	<0.31
spinach	<0.31	<0.31
brawn mushroom	<0.31	<0.31
orange	<0.31	<0.31
kiwi fruit	<0.31	<0.31
apple	<0.31	<0.31
peanut	<0.31	<0.31
almond	<0.31	<0.31
pecan nut	<0.31	<0.31
cashew	<0.31	<0.31
macadamia	<0.31	<0.31
pistachio	<0.31	<0.31
hazelnut	<0.31	<0.31
walnut	<0.31	<0.31
sesame	<0.31	<0.31
pine nut	<0.31	<0.31
cumin	<0.31	<0.31
black pepper	<0.31	<0.31
red pepper	<0.31	<0.31
ginger	<0.31	<0.31
cinnamon	<0.31	<0.31
coriander	<0.31	<0.31
soybean	<0.31	<0.31
adzukil bean	<0.31	<0.31
green peas	<0.31	<0.31
tiger bean	<0.31	0.9
miso	0.4	<0.31
ketchup	<0.31	<0.31
chili XO sauce 1	15.7	0.8
chili XO sauce 2	1.5	<0.31
egg	<0.31	<0.31
boiled egg	<0.31	<0.31
milk	<0.31	<0.31
butter	<0.31	<0.31
skim milk	<0.31	<0.31

[a] The cross-reactivity of the developed ELISA for 45 kinds of foods is listed. The sample extracts were prepared according to the procedure described in the Materials and Methods section. Listed values are the calculated mean concentrations (gelatin weight/sample weight) of the gelatin ($n = 2$).

now pursuing the establishment of methods that can distinguish gelatin used as a food ingredient from gelatinized heated meat. The methods will exploit the ELISA system using monoclonal antibodies that are specific to the epitope structures occurring only in the gelatin production process or a PCR system that targets a DNA region remaining only in heated meat, but not in gelatin.

Cross-Reactivity to Seafood (Fish, Squid, Octopus, Prawn, Crab, Shellfish, and Roe). As shown in **Table 3**, cross-reactivity was evaluated with 12 kinds of raw and boiled seafood (fish, squid, octopus, prawn, crab, shellfish, and roe). The rabbit pAb2-pAb1 ELISA showed slight (1.0 μg/g) cross-reactivity to raw salmon and showed no cross-reactivity to the other raw seafood, but the rabbit pAb2-pAb1 cross-reacted strongly with boiled squid (440 μg/g), which led us prepare another polyclonal antibody (pAb3) by changing the immunization animal. The alternative goat pAb3-pAb3 ELISA showed slight (0.6 μg/g) cross-reactivity to raw salmon and showed no cross-reactivity to other raw seafood. In addition, the goat pAb3-pAb3 ELISA showed no cross-reactivity to boiled salmon, boiled mackerel, boiled prawn, boiled short-neck clam, boiled capelin roe, boiled cod roe, and boiled snow crab, and slight (0.8−4.8 μg/g) cross-reactivity to boiled bonito, boiled tuna, boiled squid, boiled octopus, and boiled scallop. Because the cross-reactivity of goat pAb3-pAb3 with heated squid was substantially lower than that of rabbit pAb2-pAb1, it was decided that the goat pAb3-pAb3 ELISA performed better for seafood (fish, squid, octopus, prawn, crab, shellfish, and roe).

Table 5. Analysis of Gelatin Content in Commercial Foods Using Sandwich ELISA[a]

samples	declaration on label	rabbit pAb2-pAb1 ELISA mean (μg/g)	goat pAb3-pAb3 ELISA mean (μg/g)
coffee jelly	none	<0.31	<0.31
almond jelly	gelatin	2,200	4,500
custard pudding	gelatin	580	820
soft candy	gelatin	990	630
yogurt	gelatin	390	500
cheese cake	gelatin	5,000	10,000
marshmallow	gelatin	140	230
home-cooked jelly	gelatin	83,000	76,000
gelatin powder	gelatin	370,000	510,000
bacon	none	510	590
vienna sousage	none	890	820
ham	none	1,800	1,300
boiled beef	none	670	700
pork (foot)	none	95,000	55,000
gristle (beef)	none	90,000	32,000

[a] Gelatin in 15 kinds of commercial foods was assayed. The sample extracts were prepared according to procedure described in the Materials and Methods section. Listed values are the calculated mean concentrations (gelatin weight/sample weight) of the gelatin ($n = 2$).

Cross-Reactivity to Various Foods. One hundred and twenty foods (17 grains, 10 legumes, 4 fruits, 6 vegetables, 20 nuts and seeds, 19 spices, 9 seasonings, 7 seaweeds, and 28 others) excluding meat and seafood and such grains as wheat, eggs, soybeans, milk, sesames, were evaluated for gelatin contamination using the two ELISA methods developed in this study. **Table 4** shows representative results for 45 food materials among the 120 items. The rabbit pAb2-pAb1 ELISA showed cross-reactivity to miso (0.4 μg/g) and two kinds of chili XO sauces (15.7 and 1.5 μg/g), and no cross-reactivity to the other 117 foods. The goat pAb3-pAb3 ELISA showed slight cross-reactivity to tiger bean (0.9 μg/g) and chili XO sauce 1 (0.8 μg/g), and no cross-reactivity to the other 118 foods. These results indicated that the rabbit pAb2-pAb1 and the goat pAb3-pAb3 ELISA developed in this study had high specificity.

Application to Commercial Foods. A variety of 15 commercial foods were analyzed for their gelatin content by the two ELISA methods. Each commercial food and food ingredient was homogenized, and extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, the existence of gelatin in eight commercial foods with gelatin listed in the ingredients was clearly detected by the rabbit pAb2-pAb1 and the goat pAb3-pAb3 ELISA. In addition, both ELISA methods failed to detect gelatin (less than 0.31 μg/g) in coffee jelly, which does not have gelatin on its ingredients list. However, six cooked meats (without declared gelatin content) showed very high reactivity (510−95,000 μg/g for the rabbit pAb2-pAb1 ELISA; 590−55,000 μg/g for the goat pAb3-pAb3 ELISA), similar to what was observed with boiled meat (**Table 2**). This indicates that it was difficult to distinguish between gelatin and cooked meat by our ELISA. Except for this, there were no false positives from nondeclared samples, nor false negatives from declared samples

and food ingredients analyzed in this study. These results show that the established ELISA methods could appropriately determine bovine and porcine gelatin in commercial processed foods excluding those containing cooked meat.

Conclusion. We established two sandwich ELISAs based on the use of polyclonal antibodies. First, the rabbit pAb2-pAb1 ELISA employed rabbit polyclonal antibodies derived against a pentapeptide as the capture antibody and rabbit polyclonal antibodies derived against BA gelatin as the detector antibody. Second, the goat pAb3-pAb3 ELISA employed goat polyclonal antibodies derived against BA gelatin as both the capture and detector antibodies. Both ELISAs displayed comparable limits of detection and quantification for porcine gelatin of 0.78 ng/mL and 1.56 ng/mL, respectively. The two ELISAs differed in their cross-reactivity profiles. Both ELISAs also generated positive responses with boiled meat, though the goat pAb3-pAb3 ELISA did not generate positive responses with boiled chicken. The goat pAb3-pAb3 ELISA is expected to be useful for verifying food labeling because it displayed less cross-reactivity with boiled squid and weaker cross-reactivity to cooked meat, and produced no false positives (except for gelatinized heated meat) or false negatives.

ABBREVIATIONS USED

BA gelatin, bovine gelatin treated with alkali; BHA gelatin, bovine gelatin treated with hydrochloric acid; F gelatin, fish gelatin; PA gelatin, porcine gelatin treated with alkali, PHA gelatin, porcine gelatin treated with hydrochloric acid.

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