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Competitive Enzyme-Linked Immunoassay for Sialoglycoprotein of Edible Bird's Nest in Food and Cosmetics

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ABSTRACT: The proliferation of fake and inferior edible bird's nest (EBN) products has recently become an increasingly serious concern. To identify and classify EBN products, a competitive enzyme-linked immunoassay (ELISA) was developed to quantitate sialoglycoprotein in EBN used in food and cosmetic applications. The characteristic sialoglycoprotein in EBN was found, extracted, purified, and analyzed. Sialoglycoprotein, considered the main carrier of sialic acid in EBN, consisted of 106 and 128 kDa proteins. A monoclonal antibody that could recognize both proteins was prepared. The heat-treated process did not change the affinity of sialoglycoprotein with the antibody. An optimized ELISA method was established with a cross-reactivity of less than 0.1% and an IC_{50} of 3.3 μg/mL. On the basis of different food and cosmetic samples, the limits of detection (LOD) were 10−18 μg/g. Recoveries of fortified samples at levels of 20 and 80 μg/g ranged from 81.5 to 96.5%, respectively. The coefficients of variation were less than 8.0%.

KEYWORDS: *Enzyme-linked immunoassay, sialoglycoprotein, edible bird's nest, monoclonal antibody*

INTRODUCTION

Edible bird's nest (EBN) is the nest of swifts constructed using salivary glue. EBN is believed to nourish and boost the health of the sick and aging.[1] Studies have shown that the EBN extract has a potent inhibitory activity against infection of host cells with human, avian, and porcine influenza viruses.[2−4] EBN administration stimulates hormone mitosis and epidermal growth factor to help in cell repair and immune system stimulation. Matsukawa et al.[5] found that oral administration of EBN extract improves bone strength and calcium concentration in the femur of ovariectomized rates. Dermal thickness is also increased by EBN supplementation.[6] With the help of modern commercialization and technology, EBN has recently been developed into end products, including mixed congee, can, mask, face cream, etc., because of its various advantages to human health.

In swiftlet habitats in southeast Asia and Pacific islands, the collection of nests on cliffs is potentially hazardous, which makes EBN expensive. From our investigation, nests cost about $6000/kg in China depending upon their grade. Thus, illegal use and sale of fake EBN have become widespread. Various fake materials are added to increase the net weight of the nest prior to sale, including *Tremella* fungus, karaya gum, pork skin, and egg white.[7] Distinguishing real EBN from its fake counterpart by sensory evaluation alone is difficult. The amounts of sialic acid (SA) and other carbohydrates are used to determine EBN content in food products. This method is not a specific detection method for EBN and can cause confusion when SA and other carbohydrates are artificially added. EBN-specific fiber array is determined through X-ray microanalysis.[8] Wu et al.[7] reported that SYBR green polymerase chain reaction (PCR) and dimensional electrophoresis methods could authenticate EBN in food. However, their methods are complicated and time-consuming. More importantly, these methods are not suitable in quantitating EBN in food products because there is a huge difference in the content of EBN in food products. The EBN content in some products is even less than 0.01%. Thus, a detection method that is sensitive, specific, and simple and can eliminate interferences is required. The characteristic protein of EBN is a definite indicator that is specific and closely related to the EBN content.

The enzyme-linked immunosorbent assay (ELISA) has numerous advantages in protein analysis.[9−12] Its accuracy, stability, and sensitivity depend upon the quality of antibody used. However, developing high-quality antibodies requires high-quality immunogens. In this study, the characteristic protein of EBN was obtained to prepare polyclonal and monoclonal antibodies. Two ELISA methods using highly specific antibodies were developed for EBN detection, and these methods were compared in EBN detection in food and cosmetic products.

MATERIALS AND METHODS

Materials and Apparatus. Analytical-grade bovine serum albumin (BSA) and other related proteins, complete and incomplete Freund's adjuvant, and salts were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized pH gradient (IPG) strips, protein marker, and other related chemicals for electrophoresis were purchased from Biorad (Hercules, CA). Double-distilled H_2O (ddH_2O) was used throughout the experiment. Uibracell VCX750 (Sonics and Materials, Inc., Newtown, CT) was used for sample treatment. PowerPac HV System and protean IEF (Biorad, Hercules, CA) were used for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and two-dimensional (2D) PAGE analysis. Rotofor cells were used for liquid-phase isoelectric focusing (LIEF) (Biorad, Hercules, CA). A 422 Electro-Eluter was used for protein electroelution (Biorad, Hercules,

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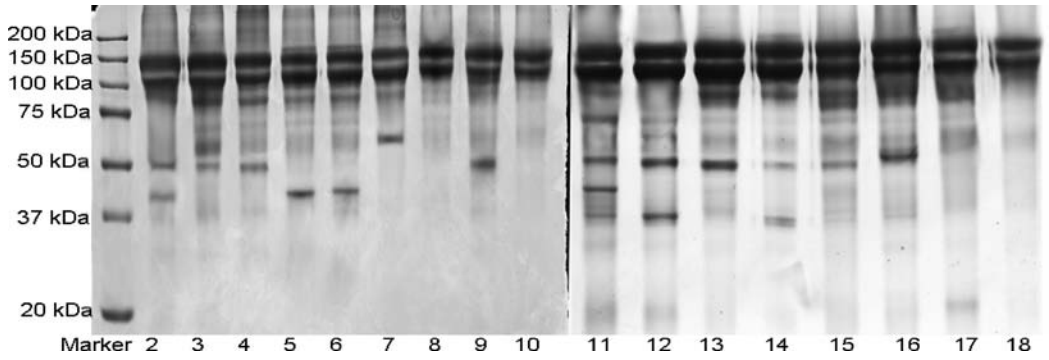

Figure 1. SDS−PAGE of 17 EBNs. Lane 1, marker; lanes 2−4 and 18, EBNs from Vietnam; lanes 5, 6, 14, and 15, EBNs from Malaysia; lanes 7, 8, 16, and 17, EBNs from Vietnam; and lanes 9−13, EBNs from Indonesia.

CA). Capillary electrophoresis analysis was performed with P/ACE MDQ equipped with a diode array detector (Beckman Instruments, Fullerton, CA). Biotek ELX808 (Biotek, Winooski, VT) was used to measure the optical density (OD) of ELISA.

SDS−PAGE. Ground EBN samples (20 mg) and 7 M urea (1 mL) were successively added to the ultrasonic treatment (2 min) and centrifuged (10 000 rpm for 1 min). The upper layer was quantitated for protein via the Lowry method. Electrophoretic separation of the protein samples was carried out using 8% SDS−PAGE, in accordance to the instructions of the manufacturer. Equal amounts (20 μg) of protein samples were used. Separation was performed under reducing conditions for 2 h at 100 V in a SDS running buffer.

Extraction and Purification of Characteristic EBN Protein. The extraction of urea in EBN was performed in a LIEF Rotofor cell according to the instructions of the manufacturer. The machine was operated at a constant power of 1 W at 4 °C for 1 h. The electrolytes in the anode and cathode chambers were 0.1 M H_3PO_4 and 0.1 M NaOH, respectively. The IPG buffer had a pH range from 3 to 10. The characteristic protein of EBN was precipitated at the anode. The protein was collected and washed 3 times by ddH_2O. The crude protein was used as an immunogen and further purified by cutting the protein bands via SDS−PAGE and electroelution. Cutting protein bands were loaded into an electroelution instrument and ran at 10 mA for 6 h. The eluent was 0.01 M Tris-HCl with pH 7.4. The recovered proteins were dialyzed against 1000 mL of ddH_2O for 3 days at 4 °C with 3 changes/day to obtain the analytical standards. Colorimetric analysis of the protein was performed via the phenol−sulfuric acid assay for total saccharide (calculated by glucose),[13] Lowry method for protein quantitation (calculated by BSA),[14] and thiobarbituric acid assay for SA (calculated by *N*-acetylneuraminic acid).[15]

Two-Dimensional PAGE. About 5 mg of lyophilized crude protein extracted using the method described previously was diluted to 34 mL with rehydration buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), and 0.5% ampholyte} and applied to the IPG strips with pH between 3 and 10. The IEF procedure was as follows: (i) 100 V, 20 °C, step-and-hold, 1 h; (ii) 300 V, 20 °C, step-and-hold, 1 h; (iii) 500 V, 20 °C, step-and-hold, 1 h; (iv) 4000 V, 20 °C, gradient, 4 h; and (v) 4000 V, 20 °C step-and-hold, until 24 000 V h was reached. The condition for the secondary electrophoresis was the same as that above.

Capillary Electrophoresis (CE). CE analysis was performed with a built-in diode array detector set at 210 nm for detection of eluted peaks. Separation and analysis were carried out on a 60.2 cm × 50 μm inner diameter uncoated fused silica capillary tube at 25 °C, using 30 mM borate buffer at pH 10 and 20 kV. Samples were introduced using the pressure mode (0.5 psi × 10 s) at the anode (normal polarity). Initially, preconditioning was performed with 1 M NaOH for 2 min, ddH_2O for 1 min, and the operating buffer for 1 min.

Rabbit Polyclonal Antibody Production. For booster immunization, 1 mg of immunogen was dissolved in 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.4, 0.5 mL) and emulsified with Freund's incomplete adjuvant (0.5 mL). The emulsion was then injected subcutaneously. The booster immunizations were repeated every 3 weeks. A New Zealand white rabbit was bled through the ear vein 1 week after each injection. Blood samples were left to coagulate for 1 h at about 25 °C and overnight at 4 °C, followed by centrifugation for 10 min at 10 000 rpm to obtain the antiserum. The clear supernatant phase was carefully collected. The polyclonal antibody was purified from the antiserum by the ammonium sulfate precipitation method, which was then divided into aliquots and stored at −20 °C until use.[16]

Monoclonal Antibody Production. Four BALB/c mice (4−6 week old) were immunized 3 times with 100 μg of immunogen in Freund's adjuvant and injected intraperitoneally at 2 week intervals. The elapsed times between the third to fourth and fourth to fifth immunizations were 4 and 11 weeks, respectively. Monoclonal antibody was prepared following a previously described method.[17]

Competitive ELISA for Sialoglycoprotein Quantitation. Standard sialoglycoprotein was diluted with PBS before the ELISA assay. The checkerboard procedure was used for the sialoglycoprotein as a coating antigen and for the monoclonal antibody. Each well of the microtiter plate was coated with 100 μL of coating antigen at 1 μg/mL, incubated, and blocked. After the sialoglycoprotein was removed from the blocking solution, 50 μL of optimal antibody dilution solution (10 ng/mL) and 50 μL of standard or sample solution were added to the wells and incubated at 37 °C for 30 min. About 100 μL of horseradish-peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) solution (1 μg/mL) was added to each well, and the plate was incubated at 37 °C for 20 min. A total of 100 μL of 1% (w/v) tetramethylbenzidine was then added. Finally, the plate was incubated at 37 °C for 10 min before 50 μL of 2 mol/L H_2SO_4 was added to stop the reaction. After each step, the plate was washed with PBS−0.05% Tween 20 (PBST). The plate was read at 450 nm using an ELISA plate reader. Sigmoidal curves were fitted to a four-parameter logistic equation. The standard deviations (n = 6) were calculated using Origin Program 8.5 software packages.

Sandwich ELISA for Sialoglycoprotein Quantitation. The 96-well microtiter plates were coated with 100 μL of rabbit anti-characteristic EBN Protein IgG (100 ng/mL) and incubated in a wet chamber at 4 °C overnight. The wells were blocked with 5% nonfat dry milk in PBS. After two washes with PBST, 100 μL of the standard or sample was added in PBS and incubated at 37 °C for 30 min. About 100 μL of the monoclonal antibodies (10 ng/mL) were added to the solution and incubated in a wet chamber at 37 °C for 30 min. Succeeding steps were the same as described previously. After each step, the plate was washed with PBST.

Preparation and Analysis of Sample. *EBN.* Ground EBN samples (20 mg) and 7 M urea (1 mL) were successively added to the ultrasonic treatment (2 min) and centrifuged (10 000 rpm for 1 min). The upper layer was diluted to a suitable concentration with PBS for ELISA.

EBN Product. The homogenized sample (1 g) was placed in a 20 mL volumetric flask. The flask was then filled with PBS, ultrasonicated for 2 min, and centrifuged at 10 000 rpm for 1 min. The aqueous phase was diluted to a suitable concentration with PBS for ELISA.

RESULTS AND DISCUSSION

Choice and Verification of Antigen. EBN was constructed using the saliva of the swift. Thus, the composition of protein varied from nest to nest. In choosing the common protein that will be used as the antigen, 17 EBNs obtained from four main producing areas of EBN (Indonesia, Malaysia, Thailand, and Vietnam) were analyzed by SDS−PAGE (Figure 1). Two common abundant protein bands, which were more than 80% of the EBN total protein, were found at 106 and 128 kDa. Thus, these proteins were selected as the antigen to produce the antibody.

Before verification, the proteins should be purified. A simple protein purification method using LIEF was carried out using the isoelectric point. On the basis of the differences in isoelectric points, the compound was separated into 10 parts. Colloidal protein was precipitated at the anode, which was analyzed by 2D PAGE. In 2D PAGE, the proteins are separated by their net charge and mass. As shown in Figure 2, two protein spots were found at the top of pH 3.0. The result indicated that the isoelectric points of the 106 and 128 kDa protein were at a pH ≤ 3.0. Several previous reports have shown that EBN contained about 8% SA, but these studies did not indicate the main modified protein of SA.[18−21] From our analysis, the 106 and 128 kDa proteins correspond to sialoglycoprotein, which was modified by excess SA (17% in the 106 kDa protein and 20% in the 128 kDa protein). The SA content of the other parts of EBN proteins was only 1.2%. SA has many health benefits, such as improving immunity, increasing memory, etc.[22−24] The price of EBNs depends upon their grade. However, there are no definite standard in classifying EBN. The 106 and 128 kDa proteins are the main carriers of SA in EBN, which perform specific nutritional functions. They can therefore be a safe and valid natural source. Thus, quantitative detection for it has great significances in quality characterization of EBN.

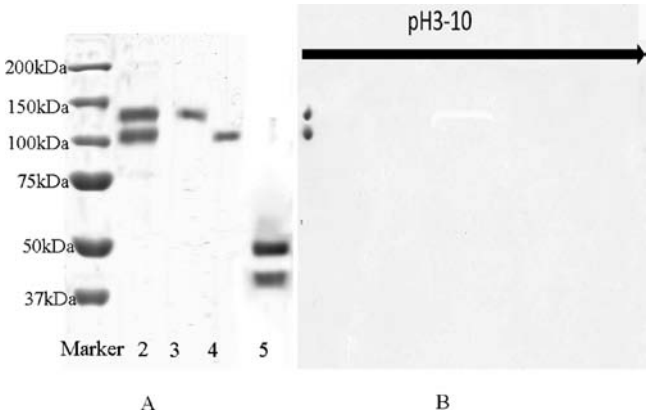

Figure 2. SDS−PAGE and 2D PAGE profiles of EBN proteins. (A) SDS−PAGE. The sample was from Vietnam, as shown in lane 2 of Figure 1. Lane 1, marker; lane 2, total sialoglycoprotein; lane 3, 128 kDa sialoglycoprotein; lane 4, 106 kDa sialoglycoprotein; and lane 5, other proteins in EBN (40 and 50 kDa proteins). (B) 2D PAGE of sialoglycoprotein in EBN.

Analysis of Monoclonal Antibody. Western blot and ELISA are typically used to determine the affinity of monoclonal antibodies. Western blot uses gel electrophoresis to separate proteins. The proteins are then transferred to a membrane, where they are probed using antibodies specific to the target protein. The qualitative analysis method cannot provide specific data. ELISA is an effective quantitative analysis method to investigate the cross-reactivity between 106 and 128 kDa proteins. The two proteins were separated and recovered using SDS−PAGE (Figure 2). ELISA showed that the IC_{50} values were 3.3 μg/mL (31.1 pmol/mL) (106 kDa sialoglycoprotein) and 4.0 μg/mL (31.2 pmol/mL) (128 kDa sialoglycoprotein), respectively. The result indicated that the monoclonal antibody could combine with the two sialoglycoproteins equally, which means that the two sialoglycoproteins had the same potential antigenicity domains. The composition of the sialoglycoproteins, such as protein and total saccharide, was investigated to determine the reason behind the difference in molecular weight between the two sialoglycoproteins. The 106 kDa sialoglycoprotein consisted of 66% protein and 19% total saccharide. The 128 kDa sialoglycoprotein consisted of 60% protein and 24% total saccharide. It indicated that the two sialoglycoproteins may have similar protein structures but are modified by different saccharides. We tried to identify the two protein spots by N-terminal sequence determination (Edman degradation), matrix-assisted laser desorption ionization−tandem time of flight (MALDI−TOF/TOF), and liquid chromatography−tandem mass spectrometry (LC−MS/MS), but no useful result was obtained. The glycosylation of the proteins may influence the identification result. Thus, more research has to be conducted in this field. The subsequent ELISA data were calculated using the 106 kDa protein. The purity of the isolated glycoprotein was further examined by CE. As shown in Figure 3, the isolated 106 kDa protein has migrated as a simple peak at 8.8 min, suggesting a pure

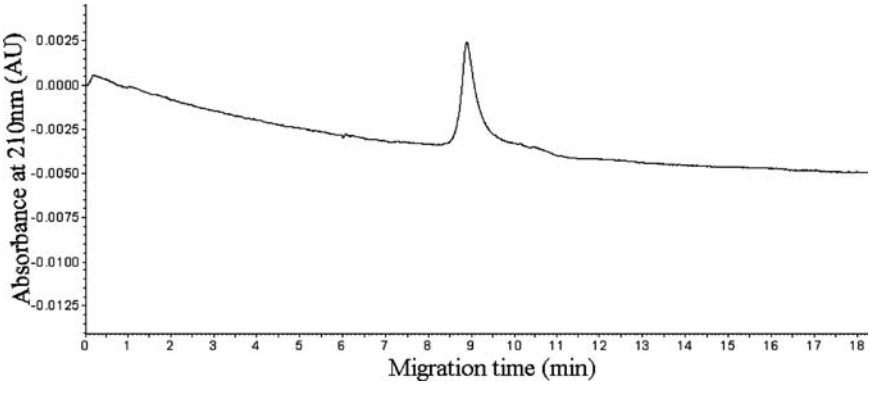

Figure 3. Electropherogram of the isolated 106 kDa sialoglycoprotein.

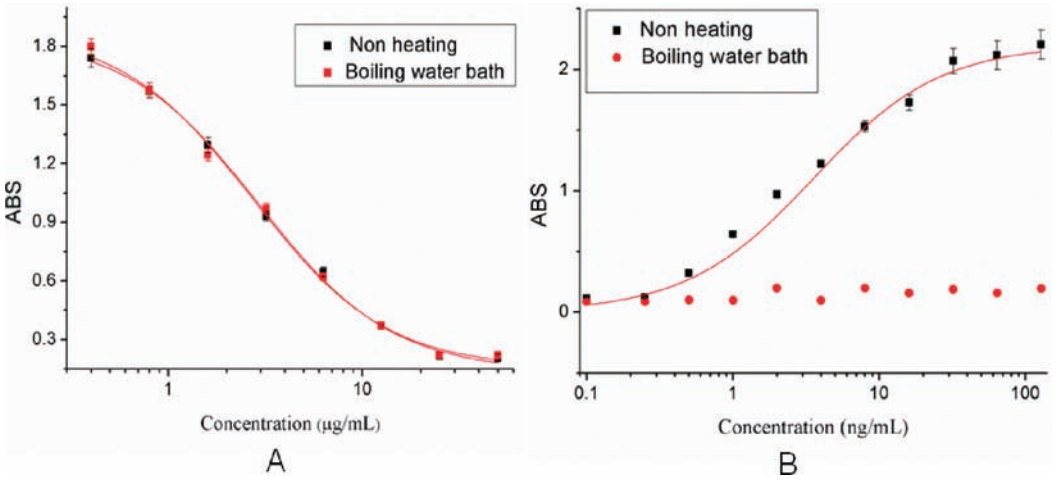

Figure 4. Representative detection standard curves for EBN sialoglycoprotein in ELISA (n = 6). (A) Competitive ELISA. (B) Sandwich ELISA.

preparation. According to SDS−PAGE and CE analysis, the 106 kDa protein was pure enough to be a standard.

Comparison of Competitive and Sandwich ELISA. The competitive ELISA uses a single monoclonal anti-sialoglycoprotein-specific antibody to recognize the sialoglycoprotein in the sample. The sandwich ELISA uses two different anti-sialoglycoprotein antibodies, namely, the rabbit polyclonal antibody coated on the surface of the microtiter plate to capture the sialoglycoprotein in the sample and an antibody conjugated to HRP to bind and quantitate the captured sialoglycoprotein in the plate well. The sialoglycoprotein in the sample is "sandwiched" between two sialoglycoprotein-specific antibodies and, therefore, requires that two sites on a single sialoglycoprotein molecule be recognized and bound. The competitive assay generally has a faster analysis speed but a lower overall sensitivity and specificity than the sandwich assay. Sandwich ELISA is more specific than the competitive assay because it involves two antibody recognition steps.

On the basis of their respective advantages, the immunoquantitation curve of EBN sialoglycoprotein was obtained from the two methods (Figure 4). A control group used the sialoglycoprotein standard, which was treated in boiling water for 1 h, because the EBN product needs be processed at high temperatures. The IC_{50} values were 3.8 ng/mL (sandwich ELISA) and 3.3 μg/mL (competitive ELISA) for non-heat-treated sialoglycoprotein analysis. Sandwich ELISA, which recognizes two antigenic determinants, had higher sensitivity than competitive ELISA but did not react with heat-treated sialoglycoprotein. The degradation or denaturation of the protein may damage the antigenic determinants. Thus, heat-treated food cannot be detected by the sandwich method. Competitive ELISAs for the two types of the sialoglycoprotein had no significant difference (IC_{50} of 3.3 and 3.5 μg/mL). The result indicated that the affinity area of the sialoglycoprotein connected to the monoclonal antibody was not denatured. The competitive ELISA was used for EBN and its product analysis. The linear detection range of the competitive ELISA was 0.6−20 μg/mL (R^2 = 0.993).

The specificity of the developed assay was evaluated by determining the cross-reactivity toward selected various fake materials related to this research. The cross-reactivity studies were carried out using ELISA by adding various free adulterants at different concentrations. Under optimized ELISA conditions, the specificity of the method was estimated (Table 1). The

Table 1. Cross-Reactivity of Antibody with Various Adulterants

	sandwich ELISA		competitive ELISA	
chemicals	IC_{50} (ng/mL)	CR (%)	IC_{50} (μg/mL)	CR (%)
characteristic EBN glycoprotein	3.8	100	3.3	100
N-acetylneuraminic acid	>4000	<0.1	>4000	<0.1
BSA	>4000	<0.1	>4000	<0.1
ovalbumin (OVA)	>4000	<0.1	>4000	<0.1
lysozyme	>4000	<0.1	>4000	<0.1
collagen	>4000	<0.1	>4000	<0.1
sodium alginate	>4000	<0.1	>4000	<0.1
Tremella fuciformis polysaccharides	>4000	<0.1	>4000	<0.1
carrageenan	>4000	<0.1	>4000	<0.1
agar	>4000	<0.1	>4000	<0.1

results from the two ELISA methods showed that the cross-reactivity rates for the eight materials with adulterants were all less than 0.1%, which suggested that the antibody possessed a high specificity for sialoglycoprotein detection in EBN.

Average Content of Sialoglycoprotein in EBN. The sialoglycoprotein content varies among EBNs based on their place of origin. With that being the case, 17 EBNs collected in Indonesia, Malaysia, Thailand, and Vietnam were used as references to investigate the average content of sialoglycoprotein in EBN. The competitive ELISA was used for EBN analysis because all EBNs could be recognized by this method. The results are shown in Table 2. The sialoglycoprotein contents of most samples were between 30 and 50%, which may be influenced by individual difference and geographic factors. The average content was 38.7% (Table 2). This value will be used for calculating the EBN content in food or cosmetic samples.

Addition Recovery of the Method. ELISA is a rapid method with high ability for disturbance resistance. The sample preparation only requires one step of protein extraction, which could be completed within 5 min per sample. A total of 96 samples could be simultaneously screened within 75 min after simple preparation.

Food and cosmetic samples were spiked with known amounts of sialoglycoprotein to determine the assay accuracy and to evaluate the matrix effect. The validation of the ELISA method could be characterized by limits of detection (LOD),

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Table 2. Contents of Characteristic EBN Sialoglycoprotein in Dry EBN

	content (%)					
sample source	number 1	number 2	number 3	number 4	number 5	average
Indonesia	32.2	34.8	36.9	31.1	39.4	34.9
Malaysia	26.3	33.6	51.8	51.0		40.7
Thailand	19.0	22.4	45.4	39.9		31.7
Vietnam	44.6	51.3	49.3	45.1		47.6
average						38.7

recovery (percent) of the fortified sample, and coefficient of variations (CVs). LOD was defined as the mean observed concentration plus three standard derivations (mean + 3 SDs) based on six blank samples. No false-positive rates were obtained. In this study, the LODs in soup, noncarbonated drinks, liquid cosmetics, and cosmetics with milk were 10, 10, 10, and 18 μg/g, respectively. In positive sample detection, the recoveries of sialoglycoprotein ranged from 81.5 to 96.5% at 20.0 and 80.0 μg/g with acceptable interassay variability (<10%) (Table 3). These results indicated that the ELISA method was sensitive and stable in detecting sialoglycoprotein in commercial samples.

Table 3. Analytical Results and Recoveries in Food and Cosmetic Samples (n = 6)

sample	fortification level (μg/g)	mean ± SD (μg/g)	recovery (%)	CV (%)
soup	80.0	71.0 ± 1.9	88.8	2.4
	20.0	18.5 ± 1.0	92.5	5.0
noncarbonated drinks	80.0	72.3 ± 1.8	90.4	2.3
	20.0	17.6 ± 1.5	88.0	7.5
liquid cosmetic	80.0	68.2 ± 3.0	85.3	3.8
	20.0	19.3 ± 1.4	96.5	7.0
cosmetics with milk	80.0	65.2 ± 2.1	81.5	2.6
	20.0	16.4 ± 1.6	82.0	8.0
average			88.1	4.8

Investigation of Commercial Samples from the Market. Because of the highly evaluated function of EBNs in nutrition and medicine, they are added to several food and cosmetic products. Many products claim they contain EBN. However, the EBN content cannot be proven and quantitated. Six food samples and six cosmetic samples were chosen at random in the market while noting the EBN ingredient in the tag. The results are shown in Table 4. The EBN content of each food sample is indicative of the value of the sample. The certified and noncertified products had varying EBN contents (from 0.08 to 20.1 mg/g). Thus, fake EBN products were identified and classified using the ELISA method. However, we did not find cosmetic products positive for EBN using ELISA. This result may be ascribed to two reasons: the concentration of EBN was very low, or the non-protein extraction of EBN was used. This hypothesis should be investigated more thoroughly in future studies.

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Notes

The authors declare no competing financial interest.

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Table 4. Content Determination in Various EBN Products[a]

food sample	sialoglycoprotein content (mg/g)	EBN content (mg/g)	cosmetic sample	sialoglycoprotein content (mg/g)	EBN content (mg/g)
EBN soup A	7.787	20.1	facial mask A	undetected	
EBN soup B	3.576	9.2	facial mask B	undetected	
EBN soup C	8.567	22.1	eye cream	undetected	
EBN soup D	0.437	1.1	face cream	undetected	
EBN oral liquid A	0.764	2.0	whitening serum	undetected	
EBN oral liquid B	0.033	0.08	essence	undetected	

[a]The EBN contents were evaluated using the average content of sialoglycoprotein in 17 EBNs (38.7% of EBN) in Table 2.

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