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Development of an Enhanced Chemiluminescence ELISA for the Rapid Detection of Acrylamide in Food Products

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ABSTRACT: In this work, a polyclonal antibody for acrylamide (AA) was obtained by immunization of rabbits with *N*-acryloxysuccinimide (NAS) and keyhole limpet hemocyanin (KLH) conjugate. A direct enzyme-linked immunosorbent assay (ELISA) based on this antibody was developed with enhanced chemiluminescent (ECL) detection of AA in food samples. Assay conditions, such as concentrations of antibody and enzyme conjugate and competition time, were optimized. The effects of ionic strength and pH value were investigated. The optimized ECL-ELISA system allowed AA determination in a linear working range of 26.3−221.1 ng mL^{-1} with an IC_{50} value of 60.6 ng mL^{-1} and a limit of detection of 18.6 ng mL^{-1}. Good recoveries with spiked food samples were obtained with a recovery range from 74.4 to 98.1%, and these results correlated well with those obtained using an HPLC method. This indicates that ECL-ELISA is applicable to the specific detection and routine monitoring of AA in food samples.

KEYWORDS: acrylamide, polyclonal antibody, enhanced chemiluminescent (ECL), enzyme-linked immunosorbent assay (ELISA)

INTRODUCTION

Acrylamide is a chemical substance that has found widespread application in industry. Polyacrylamide is commonly used in the purification of drinking water and in food packaging. Acrylamide is a suspected human carcinogen, showing reproductive toxicity, genotoxicity, and severe neurotoxicity.[1,2] Following the announcement by the National Food Administration in Sweden that acrylamide was found in many types of food, especially starch-containing foodd with high-temperature treatment, high levels of acrylamide were detected in widely consumed processed foods such as potato chips, biscuits, bread, and a number of other cereal products.

A variety of analytical screening methods for acrylamide and acrylamide metabolites have been developed. Among them, the majority are based on high-performance liquid chromatography (HPLC), liquid chromatography with tandem mass spectrometric detection (LC-MS-MS), gas chromatography−mass spectrometry (GC-MS) and capillary electrophoresis.[3−6] All of these methods require sohpisticated and expensive instruments and extensive sample extraction and purification steps as well as highly qualified personnel and, thus, are not suitable for the rapid screening of large amounts of samples.

Immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) have become a popular and useful screening tool for deleterious compound residues, because they are of low cost, high selectivity, and specificity. Moreover, ELISAs are able to simultaneously analyze a large number of samples with a simple extraction process. So far, only two kinds of immunosorbent assay for acrylamide have been reported, because of the lack of strong epitope groups in the acrylamide molecule and its too low molecular weight (71.09 Da).[7,8] In the work reported by Zhou et al.,[7] *N*-acryloxysuccinimide, a structural analogue of acrylamide, was selected to conjugate to a carrier protein as a complete antigen for the production of antibody. A competitive ELISA with a biotin−abidin system was developed using a polyclonal antibody to detect acrylamide in food samples. The detection limit of the assay was 6 ng mL^{-1}. However, this method needs a long period of about 2 h. Preston et al.[8] achieved a polyclonal antibody by the synthesis of an immunogen comprising acrylamide derivative. The detection limit of the ELISA developed by this antibody was 65.7 ng g^{-1}, but the ectracted acrylamide from food samples required an additional 1 h of derivatization before ELISA analysis.

The sensitivity of an immunoassay strongly depends on the affinity of specific antibodies and the sensitivity of the detection method. The enhanced chemiluminescence (ECL) reaction offers the possibility of improving the sensitivity of immunoassays to at least 2−3 orders of magnitude higher than conventional colorimetric detection. The light intensity of ECL reaches the maximum within 1−2 min after the start of reaction, thus providing a rapid detection of the analytical signal.[9] These advantages of chemiluminescent (CL) techniques make them useful detection systems for acrylamide analysis.[10]

In this study, we adapted the ECL detection method to the development and optimization of a sensitive ELISA (ECL-ELISA) for the detection of acrylamide in food samples. The validation of these assays with spiked samples is fully discussed.

MATERIALS AND METHODS

Materials and Chemicals. Horseradish peroxidase (HRP) was obtained from Roche (Switzerland). Fish skin gelatin (FG), Tween-20, 3,3′,5,5′-tetramethylbenzidine, hydrogen peroxide, keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA) were obtained from Merck (Darmstadt, Germany). Purified water was obtained using a Millipore Milli-Q water system (Millipore, Bedford, MA). Maxisorp

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polystyrene 96-well plates for conventional competition ELISA were purchased from Nunc (Roskilde, Denmark). Acrylamide (AA), *N*-acryloxysuccinimide (NAS), Freund's incomplete and complete adjuvants, and glutaraldehyde (GA) were all purchased from Sigma (St. Louis, MO). Opaque high binding plates for chemiluminescent measurements were bought from Costar (Cambridge, MA). Maxisorp polystyrene 96-well plates for conventional competition ELISA were purchased from Nunc.

Instrumentation. Plates were washed in a 96PW microplate washer from Bio-Rad (Hercules, CA). Immunoassay absorbance was read with a Multiskan Spectrum purchased from Thermo (Finland) in dual wavelength mode (450−650 nm), and chemiluminescent intensity was measured with a Fluoroskan Ascent FL also purchased from Thermo. An HPLC system (Shimadzu LC-10 ATvp pump) with a UV detector and a 4.6 mm × 150 mm i.d., 5 μm, Shim-pack GVP-ODS column (Shimadzu) was used.

Buffers and Solutions. The following buffers and solutions were used: 10 mmol L^{-1} phosphate-buffered saline (PBS, pH 7.4), PBS with 0.05% Tween-20 (PBST), coating buffer (50 mmol L^{-1} sodium carbonate buffer, pH 9.6), TMB substrate solution (1.25 mmol L^{-1} 3,3′,5,5′-tetramethylbenzidine, and 1.6 mM hydrogen peroxide in acetate buffer, pH 5.0), and stopping solution (1.25 mol L^{-1} H_2SO_4).

Preparation of the Complete Antigen. Two methods were used to prepare complete antigen. One is described by Zhou et al.[7] with some modification. A quantity of 20 mg of NAS was dissolved in 1 mL of dimethyl sulfoxide (DMSO), and then 0.1 mL of NAS/DMSO was added dropwise to 1 mL of 1 mg mL^{-1} KLH in PBS at room temperature under gentle stirring for 3 h. The reaction mixture was kept stirring overnight at 4 °C. A Sephadex G-25 with gel filtration was used to remove autocoupling products. The resulting solution was dialyzed against 0.01 mol L^{-1} PBS at 4 °C for 24 h with five changes of PBS. The solution was then lyophilized, and the white powder obtained was stored at −20 °C.

In another method, glutaraldehyde was used as a linker coupling AA and KLH. A quantity of 10 mg of AA was dissolved in 1 mL of PBS, and the solution was added dropwise to 1 mL of 10 mg mL^{-1} KLH in 2% GA at room temperature under gentle stirring. The reaction mixture was kept stirring overnight. The resulting solution was dialyzed against 0.01 mol L^{-1} PBS at 4 °C for 72 h with 10 changes of PBS. The solution was then lyophilized, and the white powder obtained was stored at −20 °C.

The NAS−OVA and NAS−HRP conjugates were prepared in the same way according to the first method, and NAS−OVA was used as a coating conjugate for antibody titer detection.

Antibody Production. Six New Zealand white rabbits (three for each immunogen) were immunized by intradermal and intramuscular injections with the above NAS−KLH and AA−KLH. The procedure of antibody production was the same as that reported by Wang et al.[11] The antisera that exhibited a higher titer after being detected by indirect competitive ELISA were purified using a Protein A Sepharose 4B affinity column. The IgG fraction was adequately dialyzed and then used for the immunoassay as described below.

Antisera Titer Monitoring by Indirect Competitive ELISA. Flat-bottom polystyrene ELISA plates were coated with NAS−OVA conjugates at 1 μg per well in 100 μL of coating buffer and incubated overnight at room temperature. Plates were then washed three times with 10 mmol L^{-1} PBST, and unbound active sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h. After the plate had been washed four times, 100 μL of the appropriate serum dilution in PBS was added per well and incubated for a further 1 h at room temperature. After four washings, plates were incubated for 0.5 h with peroxidase-labeled goat anti-rabbit immunoglobulins diluted 1:10000 in PBS (100 μL per well). After five washings, 150 μL of TMB peroxide-based substrate solution was added to each well. The enzymatic reaction was stopped after 30 min by adding 2.5 mol L^{-1} H_2SO_4 (50 μL per well), and the absorbance was read in dual-wavelength mode (450 nm as test and 650 nm as reference).

ECL-ELISA. The procedure of the ECL-ELISA was the same as that reported by Quan et al.[12] with a few changes. AA standard or diluted sample extract (100 μL) and NAS−HRP conjugate (60 μL, diluted in 1% BSA in PBST) were added to the antibody-coated wells and incubated for 1 h. Following three washings of the plates with PBST washing buffer, 150 μL of substrate solution (1.0 mM luminol, 1.25 mM *p*-iodophenol, and 1.0 mM H_2O_2 in 0.1 mol L^{-1} Tris-HCl buffer, pH 8.6) was added automatically to each well by a Fluoroskan Ascent FL. The intensity of light emission was measured immediately after the addition of the substrate (30 s), and the results were expressed in relative light units (RLU).

Sample Preparation and HPLC Analysis of AA. AA was found in many types of food, especially starch-containing foods with high-temperature treatment. Potato crisps, instant noodles, biscuits, and cakes purchased locally were chosen as typical samples for evaluating the performance of the ECL-ELISA. They were finely ground with a laboratory blender (IKA, Germany). Samples of 10 g were mixed with 30 mL of PBS and then shaken using a rotary shaker (IKA Labortechnik, Germany) at 250 rpm for 60 min. The mixture was kept for 10−20 min, and then another 30 mL of PBS was added to precipitate to repeat the above procedure. The supernatant from double extraction was divided for AA analysis by immunoassay and HPLC. For immunoassay detection, an aliquot of the extract was diluted with PBST or PBS and then directly added to microwells without a cleanup procedure. For detection by HPLC, the extract was further purified by flowing through a SPE column (Waters, Oasis HLB 6 cm^3/500 mg, USA).

The mobile phase was methanol/water (5:95, v/v) at a flow rate of 0.7 mL min^{-1}. The temperature of the column oven was set at 25 °C, and the detection wavelength was 206 nm.

RESULTS AND DISCUSSION

Antibody Production and Screening of Antisera. The antiserum titer was monitored using an indirect ELISA format with NAS−OVA coating antigens after the fourth immunization. Antiserum tirration was optimized to give absorbance values ranging from 0.7 to 1.2 in the absence of analytes. As a very small molecule, AA is a typical hapten and not capable of initiating an immune response unless it is conjugated with a carrier protein to form a complete antigen. Glutaraldehyde was commonly used to couple hapten and carrier protein or enzyme protein. The conjugation reaction happens between the aldehyde group and the amine group. Glutaraldehyde was chosen as a linker, because of the amine group on AA. However, we failed to get an anti-AA antibody from this method because the antiserum titers were very low without any increase from the first bleed to the end (data not shown).

A higher titer was achieved from NAS−KLH as a complete antigen with antiserum titer ranges from 6000, 8000, 13000, and 12000 during the immunization, respectively. After purification on a Protein A Sepharose 4B affinity column, the antibody from the third bleed was used for the immunoassay as described below.

Development and Optimization of the Direct Competitive ECL-ELISA. To develop a sensitive immunoassay for the detection of AA in food samples, several parameters were optimized, including the amount of coating antibody, the concentrations of NAS−HRP and buffers for diluting NAS−HRP, and the competition time of assay. The results showed that conventional colorimetric ELISA was not appropriate for AA dectection because of its low sensitivity, with an IC_{50} (concentration of analyte giving 50% inhibition of color development) and strong matrix effects (especially for potato crisps and biscuits).

For ECL-ELISA, a standard curve was obtained by plotting chemiluminescence intensity against the logarithm of analyte

Table 1. Analytical Characteristics of ECL-ELISA Procedure for Acrylamide Detection

characteristic	results
ELISA parameters	
coating antibody (μg/well)	1.5
competition reaction time (min)	60
diluent buffer of NAS−HRP	0.5% BSA−PBST
NAS−HRP concentration	1:160.00
analytical parameters of standard curve	
IC_{50} (ng mL^{-1})	60.6 ± 1.2
LOD (IC_{15}) (ng mL^{-1})	18.6 ± 0.5
20−80% inhibition (ng mL^{-1})	26.3−221.1

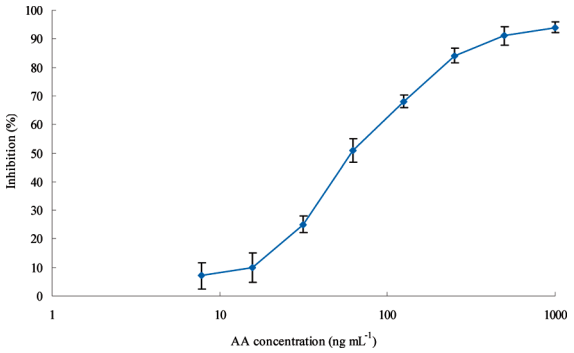

Figure 1. Standard inhibition curve of the ECL-ELISA of AA.

concentration. The optimal reagent concentration was defined as that which gave the maximum intensity of chemiluminescence with minimum reagent expense. The RLU_{max}/IC_{50} ratio was used as a parameter to estimate the effect of factors described above on the ECL-ELISA performance, the highest ratio indicating the highest sensitivity.[13] Final assay conditions are summarized in Table 1. Such conditions as 1.5 μg/well of antibody concentration, 60 min of competition time, and the use of 0.5% BSA−PBST as a NAS−HRP diluent buffer produced a higher RLU_{max}/IC_{50} ratio than any other evaluated conditions.

An eight-point competitive standard curve according to the optimal parameters is shown in Figure 1. The limit of detection (LOD), which was calculated as the concentration that gives a 15% inhibition (IC_{15}) of the maximal chemiluminescence intensity) was 18.6 ± 0.5 ng mL^{-1}. The linear working range determined as the concentrations causing 20−80% inhibition of chemiluminescence intensity was 26.3−221.1 ng mL^{-1}.

As previously reported, organic solvents are commonly considered in many ELISA optimizations because they usually affect the reaction between antigen and antibody by decreasing the bioactivity of antibody hindered the enzyme activity.[14−16] However, AA is water-soluble, and water solvents are commonly used for the extraction of AA in food samples;[17] thus, the influence of ionic strength and the pH value of the PBS on immunoassay performance was studied. The effect of ionic strength was evaluated using equimolar and 2-, 3-, 4-, and 5-fold PBS (10 mmol L^{-1}), at pH 7.4. At the optimum ionic strength, the effect of the pH value was estimated between pH 4.5 9.5. AA standard curves were obtained at several ionic strengths and pH values. The result was evaluated on the basis of the shape and RLU_{max}/IC_{50} ratio of each curve as shown in Figures 2 and 3. Different ionic

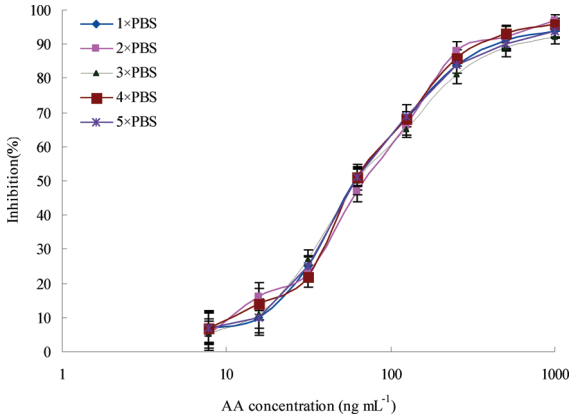

Figure 2. Effect of ionic strength on the ECL-ELISA.

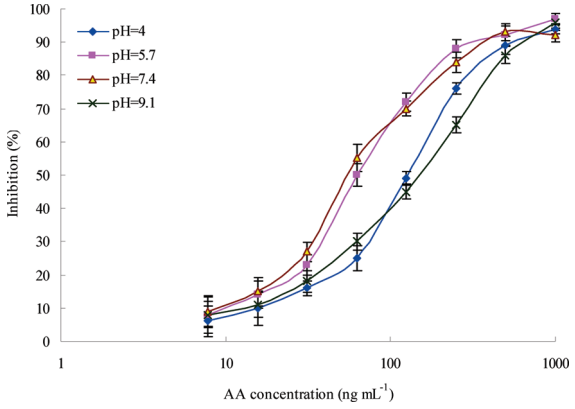

Figure 3. Effect of pH on the ECL-ELISA.

strengths had little influence on the standard curves except that a lower RLU_{max} appeared from 50 mmol L^{-1} PBS as dilution medium. The results from Figure 3 show that the curve slopes appeared to decrease with extreme pH values (4.0, 9.0). The maximal RLU_{max}/IC_{50} ratio was obtained at neural (pH 7.4). The standard curve (pH 7.4) shows a standard sigmoidal shape, which indicates that the system tolerated slightly acidic media better than alkaline media. Taking all of these results into account, the best performance was achieved with 10 mmol L^{-1} PBS at pH 7.4.

Assessment of the Assay Precision. The intra-assay reproducibility and interassay reproducibility were determined to verify the precision of the direct competitive ECL-ELISA assay. The variations of percent inhibition for 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 ng mL^{-1} of AA in PBS, tested 10 times on the same day, were 1.6, 2.4, 3.1, 3.2, 4.8, 4.6, 5.3, and 5.9%, respectively. The assay of the same material run during 10 days gave deviations from the mean values of 2.8, 3.5, 5.3, 4.3, 6.2, 8.7, 11.5, and 12.0% for each of the respective concentrations.

Assessment of the Assay Specificity. Some chemicals (acrylic acid, propanoic acid, propanamide, and asparagine) were selected for investigating the selectivity of the antibody and assessing the specificity of ECL-ELISA for AA determination. Among them, acrylic acid and asparagine are the major precursors during the formation of AA by the Maillard reaction during food processing at high temperature according to the mechanism of formation of AA in food.[18] Their cross-reactivities were

Table 2. Cross-Reactivity of the Antibody with Structural Analogues

compound	IC_{50} (ng mL^{-1})	cross-reactivity (%)
acrylamide	60.6	100
acrylic acid	>100000	<0.01
propanoic acid	841.7	7.2
propanamide	>100000	<0.01
asparagine	>100000	<0.01

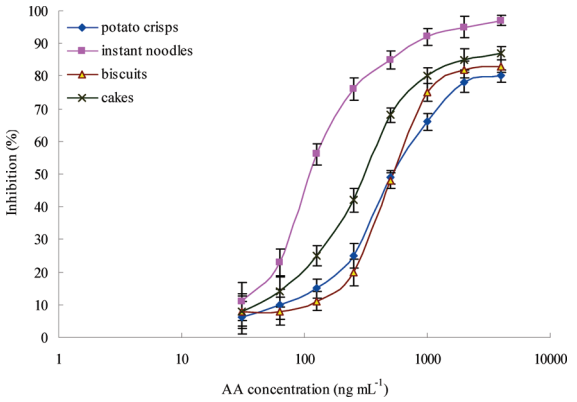

Figure 4. Standard curves of AA in four food samples by ECL-ELISA.

determined using the optimized ECL-ELISA system and were calculated as follows:

$$CR = IC_{50}(AA)/IC_{50}(\text{cross-reacting compounds}) \times 100$$

The cross-reaction results are listed in Table 2. Among the tested compounds, a higher CR value found for propanoic acid is 7.2%, and for another three chemicals, their cross-reactivities are <0.01% and can be considered as negligible. The results indicate that the assays developed from the antibody were very specific to AA.

Matrix Interferences and Their Removal. One of the common challenges of immunoassay for food analysis is matrix interference. These interferences can be reduced in a number of ways. Dilution with some buffers is a commonly used procedure. However, this procedure reduces the sensitivity of the assay to some extent. The effects of the matrix can be neglected when the sample curve and standard curve are superimposable. To study possible matrix interferences from different food samples (potato crisps, instant noodles, biscuits, and cakes), AA standard curves were prepared in these samples (concentration of AA < 5 ng g^{-1} tested by HPLC) and in PBS as a control. It can be seen from Figure 4 that potato crisps and biscuits showed stronger matrix effects than the other two kinds of food. The LODs of these four kinds of food were 126 ± 2.5, 41 ± 1.2, 137 ± 3.1, and 69 ± 1.7 ng mL^{-1}, respectively.

Different dilution ratios with different diluent buffers including PBST (pH 7.4), 0.1−0.5% FG/PBS, and 0.1−0.5% BSA−PBS were tested for reducing matrix effects. AA standard curves were produced in several dilutions of each sample extract (1:5, 1:10, 1:20, 1:40, 1:80, and 1:100 prepared in the above diluent buffers, respectively). By comparison of three diluent buffers under the identical experimental conditions, for potato crisps and biscuits, PBST produced a higher chemiluminescence intensity than any other buffers. A dilution of 1:100 or 1:80 with PBST was adequate to remove the matrix interference, respectively. For instant noodles and cakes, a 1:20 dilution with PBS was preferred.

Table 3. Recovery Studies of Four Food Samples at Three Levels by ECL-ELISA (n = 3)

food sample	spiked level (ng g^{-1})	recovery (%)	CV (%)
potato crisps	500	85.3	13.2
	1000	79.6	9.8
	2000	91.7	7.1
instant noodles	500	93.5	10.0
	1000	92.2	8.6
	2000	80.5	4.6
biscuits	500	74.4	12.8
	1000	76.0	10.1
	2000	83.5	5.2
cakes	500	88.4	8.9
	1000	87.6	3.6
	2000	98.1	6.2

Table 4. Comparison of Results Obtained by ECL-ELISA and HPLC from Spiked Samples (n = 3)

sample	spiked level (ng g^{-1})	ELISA results (ng g^{-1})	HPLC results (ng g^{-1})	ELISA/HPLC (%)
instant noodles	500	480.4	429.3	112
	1000	965.0	911.5	106
	2000	1933.6	1902.6	102
cakes	500	475.2	432.5	110
	1000	946.8	911.1	104
	2000	1960.1	1908.4	103

Recovery Studies of Food Samples. Recovery of spiked food samples with the target analyte was studied to assess the analytical performance of ECL-ELISA, and it was calculated as follows: recovery % = (detected concentration/concentration of added AA) × 100. Food samples were spiked with AA at 500, 1000, and 2000 ng g^{-1} and then analyzed by using the developed immunoassay and validated by HPLC for confirming the applicability of the ECL-ELISA. The LOD for the HPLC method was 32.0 ng mL^{-1}. In this study, PBS was used as the solvent for extraction of AA from food matrices. The condition of extract was also investigated, and the recovery results showed that extracting twice with 30 mL of PBS produced better recoveries than did only one extraction. Each sample was evaluated three times in duplicate to verify the repeatability. The results of spiking recovery and validation are shown in Tables 3 and 4, respectively. Good recoveries were obtained from these spiked samples with a recovery range from 74.4 to 98.1%. The results (Table 4) also showed that the ECL-ELISA gave a good correlation with HPLC results, although there was a slight tendency for ELISA values to be slightly higher than HPLC values. This may be due to the cleanup procedure required prior to HPLC, which causes some loss of target compound and results in lower estimation.

Chemiluminescent detection appears to be an effective analytical technique for use in ecotoxicological monitoring due to its high sensitivity, low cost, and easy handling. In the current study, a fast and reliable method based on a direct competition ELISA format was developed by introducing an ECL reaction as the endpoint detection system. The established ECL-ELISA offers a simple and low-cost alternative to chromatographic methods and is suitable and sensitive for the rapid determination of acrylamide in food samples.

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