Indirect competitive ELISA based on monoclonal antibody for the detection of 5-hydroxymethyl-2-furfural in milk, compared with HPLC

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ABSTRACT

In this study, a method for rapid detection of 5-hydroxymethyl-2-furfural (HMF) was investigated. Monoclonal antibody (anti-HMF) was prepared and evaluated by an indirect competitive ELISA (ic-ELISA) format. The optimized standard curve was $y = -0.2097x + 1.0432$ [where x is the logarithm (base 10) of the values of the HMF concentration and y is the absorbance of ic-ELISA results tested at 490 nm] and the linear detection range was 0.008 to 32.768 mg/L. The percentage of cross-reactivity of HMF with 5 major furfural derivatives was less than 2.92%. Finally, the established ic-ELISA format was used to test HMF in milk, and compared with the result obtained by HPLC, which produced an error of about 0.3%. Based on the data in this experiment, we concluded that the established ic-ELISA format was reliable with a high specificity.

Key words: 5-hydroxymethyl-2-furfural, monoclonal antibody, indirect competitive ELISA, HPLC

INTRODUCTION

5-Hydroxymethyl-2-furfural (HMF) is a well-known heterocyclic compound produced by nonenzymatic browning reactions such as ascorbic acid degradation, caramelization, and the Maillard reaction (Hodge, 1953; Rufián-Henares et al., 2004). The content of HMF in food has been tested by several research groups. For example, 3.2 to 220 mg of HMF/kg was estimated in bread (Bachmann et al., 1997). Early studies showed that HMF was a potential toxin, mutagen, and carcinogen (Ulbricht et al., 1984; Nässberger, 1990). For example, research found that HMF had significant adverse effects on human blood cells (Rufián-Henares and de la Cueva, 2008). Generally, many countries require that the content of HMF in food must be less than 20

mg/kg (Fang et al., 2011). Therefore, developing a fast method to detect HMF is necessary.

5-Hydroxymethyl-2-furfural has always been measured using HPLC, gas chromatography, and GC-MS (Jun et al., 2003; Gaspar and Lopes, 2009; Guan et al., 2012). Although these methods are reliable, some disadvantages such as expensive test cost might restrict their wide use. Indirect competitive ELISA (**ic-ELISA**) has been used to detect pesticides and veterinary drugs in food and the environment (Lee et al., 2001; Wang et al., 2011), which has proven to be fast and inexpensive (Lee et al., 2001; Wang et al., 2011). Moreover, ic-ELISA based on monoclonal antibody has proven to be one of the best means to test low-molecular weight compounds with a high specificity (Wang et al., 2011). Therefore, developing an ic-ELISA method based on monoclonal antibody to detect HMF is necessary.

In this research, an ic-ELISA method based on monoclonal antibody to detect HMF was established. Moreover, we used the established ic-ELISA method to detect HMF in milk and compared the result with results using HPLC.

MATERIALS AND METHODS

Chemicals

Methanol and pyridine were chromatographic grade and purchased from Merck KGaA (Darmstadt, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl); *N*-hydroxysuccinimide; HMF; glutaric anhydride; polyoxyethylene sorbitan monolaurate (Tween-20); hypoxanthine, aminopterin, and thymidine; hypoxanthine and thymidine; BSA, ovalbumin, dimethyl sulfoxide; and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, MO). The RPMI Medium 1640 was purchased from AppliChem GmbH (Darmstadt, Germany). The horseradish peroxidase-labeled goat anti-mouse IgG was purchased from Vector Laboratories Inc. (Burlingame, CA). Polyethylene glycol (PEG 1500) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Color reagents A (0.004% urea

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hydrogen peroxide acetate solution, pH 5.4–5.6) and B (3,3',5,5'-tetramethylbenzidine, TMB; 0.2 mg/mL ethanol-water solution) were purchased from Tai Tian He Biological Ltd. Co. (Jinan, Shandong, China).

Synthesis, Separation, and Structure Identification of the Hapten

5-Hydroxymethyl-2-furfural (2 mmol, 252.22 mg) was mixed with glutaric anhydride (3 mmol, 342.30 mg), and dissolved in 5 mL of pyridine. After full reaction at 75°C for 1 h, thin-layer chromatography was used to monitor whether the hapten was formed. For further isolation, the hapten was separated by a preparative liquid chromatography using a C18 (10- μ m) column (25-mm i.d. $\times$ 600-mm length; lisui II) with a UV detector (Lisui, Suzhou Lisui Technology Co. Ltd., Suzhou, China). The gradient used was 100% water to 100% methanol for 120 min, with a flow rate of 15 mL/min. The monitor was set at 254 nm. The purified hapten was placed in vacuum at 45°C for 24 h to remove the solvent. Electrospray ionization-mass spectrometry (**ESI-MS**) and nuclear magnetic resonance (**NMR**) analysis are necessary for further identification of the hapten structure. The ESI-MS was carried out in a LCQ-Fleet mass spectrometer (Thermo Fisher Scientific, Waltham, MA), with an electrospray ionization source using a negative mode (m/z : 50–800). The NMR was operated at 400 MHz for ^1H NMR and 125 MHz for ^{13}C NMR in a Bruker Avance 400 instrument (Bruker Co., Switzerland). The data were processed by the MestReNova program (Mestrelab Research, Santiago de Compostela, Spain). Data of the hapten were obtained in deuterated chloroform (**CDCl₃**).

The hapten structure data were as follows: ESI-MS m/z 239.03 $[\text{M-H}]^-$, 478.91 $[2\text{M-H}]^-$; ^1H NMR data (400 MHz, **CDCl₃**): δ : 9.60 (s, 1H, CHO); δ : 7.22 (d, 1H, H2); δ : 6.60 (d, 1H, H3); δ : 5.15 (s, 2H, H6); δ : 2.49 (t, 2H, H9); δ : 1.98 (m, 2H, H10); δ : 2.44 (t, 2H, H11), and ^{13}C NMR data (125 MHz, **CDCl₃**): C1: (155.39 ppm); C2: (121.88 ppm); C3: (112.66 ppm); C4: (152.82 ppm); C6: (57.85 ppm); C8: (172.27 ppm); C9: (32.80 ppm); C10: (19.63 ppm); C11: (32.86 ppm); C13: (177.97 ppm); C15: (178.79 ppm), where M-H = mass of molecular ion minus a proton; δ = chemical shift; CHO = formyl group; s = singlet; d = doublet; t = triplet; m = multiplet; C1 to C15 = carbon atom no. 1 to 15.

Synthesis of the Conjugates of the Hapten and Protein

Hapten-BSA and hapten-ovalbumin conjugates were synthesized as immunogen and coating antigen, respectively; 0.01 mmol of the hapten was prepared

containing 0.15 mmol of EDC·HCl and 0.15 mmol of *N*-hydroxysuccinimide, and then dissolved in 0.2 mL of dimethylformamide. After stirring at room temperature in the dark for 12 h, 2 mL of protein (5 mg/mL) PBS (pH 6.0, 0.05 mol/L) was gently added in an ice bath. The mixture was reacted for 12 h with a magnetic stirrer. After reaction, each sample was added into a wet cellulose dialysis tube [33-mm flat width, 21-mm diameter, 12.4-kDa molecular weight cutoff (MWCO)]. Dialysis was performed in 3 L of 0.015 mol/L PBS (pH 7.4) for 2 d at 4°C. The PBS was replaced every 6 h. After dialysis, samples were freeze dried and stored at 4°C.

Immunization of Mice

Eight-week-old BALB/c female mice were immunized with a 1:1 mixture (vol/vol) of 100 μ g of hapten-BSA conjugate dissolved in 100 μ L of 0.9% NaCl solution and 100 μ L of Freund's complete adjuvant. Booster injections were given at 15-d intervals with the same amount of antigen emulsified with incomplete Freund's adjuvant. Tail blood of mice was obtained and the serum titer was determined by ic-ELISA after 7 d of each booster injection. Mice that produced a high titer and significant competition with HMF after 10 d of the third booster injection were put to death for further experiment.

Cell Fusion, Hybridoma Selection, and Subclone for Monoclonal Antibody Preparation

The splenocytes of mice were fused with SP2/0 myeloma cells at a 5:1 ratio in the presence of PEG 1500 (0.7 mL) at 40°C for 4 min. The RPMI Medium 1640 (20 mL) was added to end the cell fusion. The fused cells were centrifuged ($68 \times g$ for 5 min) before adding 10 mL of fetal bovine serum; 800 μ L of 50-fold diluted hypoxanthine, aminopterin, and thymidine selection medium; and 25 mL of methyl cellulose medium. The fused cells were incubated at 37°C with 5% CO_2 . After 7 d of incubation, colony-forming unit-monocyte was transferred to 96-well culture plates, and 150 μ L of RPMI Medium 1640, including 15% fetal bovine serum and 1% hypoxanthine and thymidine medium, was added to each well. The selected colony transferred to the 96-well culture plates was incubated at 37°C with 5% CO_2 . Subsequently, half of the media in the wells was replaced by fresh RPMI Medium 1640, including 15% fetal bovine serum media, every 2 d. After 6 d of incubation, subcloning was done by a limited dilution technique. Stable antibody-producing clones were injected into the enterocoelia of a first filial generation (F1) mouse. After 8 d of incubation, the ascites was

Figure 1. Chemical structure of the hapten.

collected and purified by salting out (with caprylic acid-ammonium sulfate) to prepare the monoclonal antibody. The purified monoclonal antibody was stored at -20°C in the presence of fetal bovine serum with 10% dimethyl sulfoxide.

ic-ELISA Based on Monoclonal Antibody

The ic-ELISA was carried out on 96-well polystyrene plates. Plate wells were coated with coating antigen (1 $\mu\text{g/mL}$ in 0.05 mol/L carbonate buffer at pH 9.6; 100 $\mu\text{L/well}$) for 2 h at 37°C . The plate was washed 3 times with 0.015 mol/L PBS-with-Tween-20 (PBST) solution (1,000 mL of phosphate buffer with 0.5 mL of Tween-20) and blocked by adding 3% BSA phosphate buffer solution (0.015 mol/L; 200 $\mu\text{L/well}$). After incubation for 2 h at 37°C , the plate was washed 3 times with 0.015 mol/L PBST solution, subsequently, HMF with different concentrations (50 $\mu\text{L/well}$) was added, followed by the addition of the excretive liquid of cells (diluted 1,000 times for the excretive liquid from cells; 50 $\mu\text{L/well}$). The plate was shaken for 10 s and incubated for 1 h at 37°C . After another washing step, the secondary antibody (the goat anti-mouse IgG-horseradish peroxidase, 1:20,000 in PBST; 100 $\mu\text{L/well}$) was added. The mixture was allowed to incubate for 40 min, and after another washing step, an equal volume of color reagents A and B was added (total volume: 100 $\mu\text{L/well}$). The reaction was stopped with sulfuric acid (2 mol/L; 50 $\mu\text{L/well}$) after 20 min of incubation at 37°C , and subsequently measured at 490 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific).

The optimization assay was carried out as follows: (1) the concentration of the coating antigen was optimized (0.25, 0.5, 1.0, and 2.0 $\mu\text{g/mL}$), (2) the dilution of antibody was optimized (1:2,000, 1:4,000, 1:8,000, and 1:16,000), (3) the effect of ionic strength of running buffer was evaluated with different concentrations (20, 50, 110, and 230 mmol/L PBST, which became 15, 30, 60, and 120 mmol/L, respectively, after combining with an equal volume of monoclonal antibody diluted with 10 mmol/L PBST), and (4) the influence of pH (pH value was 5.5, 6.5, 7.4, and 8.5) of 30 mmol/L PBST was also evaluated. Working standard curves

were obtained by plotting absorbance against the logarithm of HMF concentration, from which half-maximal inhibitory concentration (IC_{50}) values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

Determination of Cross-Reactivities

The cross-reactivity (CR) ratio reflected the specificity of the antigen. The CR was determined by the IC_{50} of HMF assigned to be 100% compared with the IC_{50} of other analogs. The CR ratio was calculated according to the following formula: $\text{CR} (\%) = (\text{IC}_{50} \text{ of HMF}) / (\text{IC}_{50} \text{ of other analogs}) \times 100$.

HPLC Assay

The HPLC analysis of HMF was carried out as follows: the sample was filtered through a Millex-HN nylon clarification kit of 0.45- μm pore size (Millipore Corp., Billerica, MA) for analysis with an HPLC-photodiode array detector (PAD) system [chromatogram controller: Waters 600; pump: Waters 600E; injector: Rheodyne 7725i Manual Injector; detector: Waters 2998 PAD; and column: 5- μm Waters Atlantis T3 150 $\times$ 4.6-mm column (all from Waters Corp., Milford, MA)]. The mobile phase was 100% water (0 to 10 min), and then a linear gradient up from 100% water to 100% methanol (from 10 to 55 min), and last, 100% methanol (from 55 to 70 min) delivered at a flow rate of 0.5 mL/min. The column temperature was set at 25°C .

Figure 2. Standard curves for the 5-hydroxymethyl-2-furfural (HMF) indirect competitive ELISA (ic-ELISA) assay based on monoclonal antibody, where x is the logarithm (base 10) of the values of the HMF concentration and y is the absorbance of ic-ELISA results tested at 490 nm. A_{max} = the ic-ELISA absorbance at an HMF concentration of 0%; IC_{50} = half-maximal inhibitory concentration (the concentration of the HMF inhibiting the absorbance of the control by 50%).

Table 1. Effects of different factors on indirect competitive ELISA (ic-ELISA)¹

Item	IC ₅₀ (mg/L)	Amax/IC ₅₀
Coating antigen concentration (μg/mL)		
0.25	1.40	0.30
0.5	1.49	0.43
1.0	1.09	0.83
2.0	1.58	0.82
Dilution of antibody		
1:2,000	1.63	1.00
1:4,000	0.98	1.07
1:8,000	1.21	0.56
1:16,000	1.51	0.25
Ionic strength of PBST (mmol/L)		
15	1.66	0.78
30	0.96	0.88
60	1.47	0.48
120	1.52	0.37
pH of PBST (30 mmol/L)		
5.5	2.23	0.36
6.5	1.90	0.55
7.4	1.29	0.93
8.5	1.11	0.72

¹IC₅₀ = half-maximal inhibitory concentration (the concentration of the HMF inhibiting the absorbance of the control by 50%); Amax = the ic-ELISA absorbance at an HMF concentration of 0%; PBST = PBS with Tween-20.

Analogue	Major furfural derivative structure	Mouse monoclonal antibody	
		IC ₅₀ (mg/L, mg/kg)	CR (%)
HMF		0.9123	100.0
Furfural		31.2873	2.92
5-Ethyl-2-furfaldehyde		47.2941	1.93
5-Methylfurfural		65.8923	1.38
5-Nitrofurfural		>100	<0.1
4,5-Dimethyl-2-furfaldehyde		>100	<0.1

Figure 3. Recognizability of 5-hydroxymethyl-2-furfural (HMF) and its major furfural derivative structure. IC₅₀ = half-maximal inhibitory concentration (the concentration of the HMF inhibiting the absorbance of the control by 50%); CR = cross-reactivity, reflecting the specificity of the antigen [calculated as (IC₅₀ of HMF)/(IC₅₀ of other analogs) × 100].

Figure 4. 5-Hydroxymethyl-2-furfural (HMF) analysis in milk using (ic-ELISA; $n = 3$). Color version available in the online PDF.

As in our earlier study (Guan et al., 2012), the regression equation for the HMF standard was $y^* = 10^8 C - 80,361$ ($R^2 = 0.999$), where y^* = peak area of HMF and C = HMF concentration (mg/mL).

Statistical Analysis

All experiments were carried out in triplicate. Means and standard deviations of the data were calculated for each treatment. Analysis of variance was carried out to determine any significant differences ($P < 0.05$) among the applied treatments using the SPSS package (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Synthesis of the Hapten

The chemical structure of the hapten is shown in Figure 1. Synthesis of the hapten was performed by introducing a straight-chain spacer arm (i.e., glutaric anhydride) using acylation reaction in a Lewis alkali medium, which can form stable HMF-glutaric anhydride addition reaction product (i.e., hapten of HMF).

Optimization of the ic-ELISA

The sensitivity of the ic-ELISA was evaluated by the ratio value of A_{max} (the ic-ELISA absorbance at an HMF concentration of 0%) to IC_{50} (the concentration of the HMF inhibiting the absorbance of the control by 50%; Lee et al., 2001). A higher $A_{max}:IC_{50}$ ratio indicated higher sensitivity. To qualitatively and quantitatively determine HMF, it is necessary to optimize the ic-ELISA sensitivity. The optimized ic-ELISA results are shown in Table 1. Summarizing the results of the ic-ELISA optimization, it was found that a coating antigen concentration of 1.0 $\mu\text{g/mL}$, dilution of anti-

body at a ratio of 1:4,000, ionic strength of PBST at 30 mmol/L, and pH value of PBST solution of 7.4 were most suitable for ic-ELISA detection of HMF.

In the optimum conditions, the standard curve for HMF detection using ic-ELISA was obtained, and is shown in Figure 2. The standard curve was $y = -0.2097x + 1.0432$ [where x is the logarithm (base 10) of the values of the HMF concentration and y is the absorbance of ic-ELISA results tested at 490 nm] and the linear detection range was 0.008 to 32.768 mg/L. Although a relatively narrow linearity detection range was obtained, significant sensitivity was gained.

CR Analysis

It was a common phenomenon that antibodies recognized compounds structurally similar to the immunizing hapten. In this experiment, 5 major furfural derivatives were used to evaluate the CR of the monoclonal antibody (Figure 3). Obviously, the percentage of CR of HMF with the other 5 major furfural derivatives was less than 2.92%. This result indicated that the established ic-ELISA had the specificity to detect HMF.

Application of ic-ELISA to Detect HMF in Milk Compared with the HPLC Assay

The established ic-ELISA based on monoclonal antibody was applied to detect HMF in milk. As shown in Figure 4 and from the comparative results (HPLC: 0.617 ± 0.03 mg of HMF/kg; ic-ELISA: 0.619 ± 0.08 mg of HMF/kg), detection of HMF using established ic-ELISA showed a very low error [i.e., 0.3%; $n = 3$; the error was calculated as follows: (ic-ELISA result – HPLC result)/HPLC result $\times 100\%$] compared with the result obtained by HPLC. Thus, it can be seen that the established ic-ELISA is suitable for HMF detection in milk, and may be appropriate for being applied to detect HMF in other food samples.

CONCLUSIONS

The ic-ELISA based on monoclonal antibody with a high specificity to detect HMF was established. The established ic-ELISA method is sensitive for quantification of HMF in milk, which produced a very low error (about 0.3%) compared with the HPLC result. In this case, correction is not needed and the established ic-ELISA method can be used commercially in HMF detection in milk samples.

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