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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-dimethyllaminopropyl)carbodiie hydrochlide (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-μm) column (25-mm i.d. × 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 ^{1}H 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 (**$CDCl_3$**).

The hapten structure data were as follows: ESI-MS m/z 239.03 $[M–H]^-$, 478.91 $[2M-H]^-$; ^{1}H NMR data (400 MHz, $CDCl_3$): 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, $CDCl_3$): 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 μg of hapten-BSA conjugate dissolved in 100 μL of 0.9% NaCl solution and 100 μL 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 × g for 5 min) before adding 10 mL of fetal bovine serum; 800 μL 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 μL 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

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