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Original Article

The Effect of Different Stabilizers on Stability of Horseradish Peroxidase-Bovine Serum Albumin-Aflatoxin B1, a Conjugated Tracer for Detection of Aflatoxin B1 in Immunoassay-Based Methods

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Abstract

Aflatoxins are a group of fungal toxic metabolites, which are contaminated certain food commodities. ELISA is one of the sensitive methods for detection of aflatoxins. Preparation and stabilizing of a proper conjugated tracer for detection of aflatoxins is probably the main step for designing an ELISA method. In current study, different stabilizers were applied to stabilize a newly prepared conjugated molecule, Horseradish peroxidase-Bovine serum albumin- Aflatoxin B1 (HRP-BSA-AFB1). Stabilizing effects of six different stabilizers were compared during 10 months verification at room temperature and 6 weeks verification at 37 °C. Based on the results, it was concluded that trehalose-containing stabilizers especially those contain casein in their compositions show the best stabilizing effects on HRP-BSA-AFB1 conjugated tracer.

Keywords: Aflatoxin; BSA; Conjugate; HRP, Immunoassay; Stabilizer.

Introduction

Aflatoxins are a group of toxic agents belonging to the category of mycotoxins. These fungal metabolites are produced by at least three species of *Aspergillus*, *A. flavus*, *A. parasiticus* and *A. nomius*, which may grow on a number of corps. Aflatoxin B1, B2, G1, G2 and their metabolites M1 and M2 are the most common and of these, and B1 and G1 are observed most frequently in food commodities (1-3).

A. flavus, main producer of aflatoxins, is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as drought (4).

Aflatoxins can produce dangerous illnesses including acute liver damage, cirrhosis, tumor induction and teratogenicity (5). Hence, the long-term chronic exposure to extremely low levels of aflatoxins in the diet is an important consideration for human health (3). Aflatoxin B1 is the most toxic aflatoxin and is considered as one of the most potent carcinogens especially hepato-carcinogens (6, 7).

To avoid human exposure to aflatoxins, rigorous programs have been established by different government agencies to control aflatoxin levels in food (8, 9). As a consequence, a number of sensitive methods for the detection, determination and analysis of aflatoxins have been developed. Chemical methods such as thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are most commonly used (10-12). Although chromatography analysis especially HPLC

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are accepted as official methods for aflatoxin determination, these methods are expensive, time-consuming, and unsuitable for large amount of samples and need precise and expensive instruments. Investigations in the last two decades have further led to the development of several immunochemical methods for such purpose, even in Iran (13-17). Among several techniques, enzyme-linked immunosorbent assay (ELISA) is the most useful technique due to its quickness, sensitivity and application (18, 19).

For designing an immunoassay-based method, one of the main functions is conjugation of analyte to an enzyme to make a suitable tracer for detection of desired analyte. After preparation of a conjugate, second point of importance is stability of the prepared conjugate. Stability of a conjugate shows that different components keep their functional structures as well as biological activities. This will be more important when one of these components is an enzyme (19). The enzyme tracer is commonly used at high dilutions in ELISA procedures and is not often stable (18). HRP-conjugated analytes are often unstable and can lose a significant level of activity within a short period of time after dilution unless properly stored. In this study, a conjugated tracer, based on a three-component molecule, HRP-BSA-AFB1 was prepared. This conjugated tracer was designed for detection and determination of aflatoxin B1 based on ELISA. Different combinations of stabilizers were applied to this conjugated molecule and the stabilizing effects of these combinations were verified and discussed.

Experimental

Materials

Horseradish peroxidase (HRP), bovine serum albumin (BSA) and aflatoxin B1 (AFB1) were purchased from Sigma (St Louis, USA). The AgraQuant ® aflatoxin B1 (1-20 ppb) test kit was obtained from Romer Labs (MO, USA). 3, 3′, 5, 5′-tetra methyl benzydine (TMB) one shot Plus (ready-to-use substrate solution) obtained from Kem-En-Tec Diagnostic Co (Taastrup, Denmark). All other chemicals and reagents were prepared from Merck (Darmstadt, Germany).

Methods

Preparation of HRP-BSA-AFB1

To prepare HRP-BSA-AFB1, one mg of HRP was dissolved in 1 ml of 0.1 M sodium acetate buffer. 4 µl of sodium m-periodate 0.1 M was added and the solution was incubated in dark place for 20 minutes. Then, desalting was done using Sephadex G-25 column equilibrated with 1 M carbonate-hydrocarbonate buffer (pH 9.5). One mg of AFB1-BSA conjugate was added to collected fractions of this column and the mixture was incubated for 180 minutes at 2-8 °C. 4 µl of sodium cyanoborohydride was added and the mixture was incubated at 2-8 °C for 15 minutes. Then, 1 M sodium dihydrogen phosphate was added to raise pH and to quench excess reaction of sodium cyanoborohydride. This solution was eluted through G-75 Sephadex column and fractions were collected. Collected fractions were checked by UV absorbance as well as reaction with TMB and intensity of color developed. Best fractions according to these verifications were chosen for further use.

To verify formation of HRP-BSA-AFB1, it was used as alternative conjugate of Agraquant AFB1 test kit and compared with original conjugate of the kit. According to the observed ODs, formation of conjugate was confirmed (2.43 for new conjugate versus 2.78 for original)

Preparation of stabilizers

Six different stabilizers were prepared and applied for stability verification of the conjugated tracer. Ingredients of these stabilizers were as follows:

Stabilizer 1. BSA 0.02% in 0.05 M Tris-HCl buffer, Kathon CG 0.1% and 0.05 M CaCl2

Stabilizer 2. BSA 0.1% and Kathon CG 0.2% in phosphate buffer saline (PBS).

Stabilizer 3. Casein 1% and Kathon CG 0.2% in PBS.

Stabilizer 4. Glycerol 50% and Kathon CG 0.2%.

Stabilizer 5. Trehalose 5% and Kathon CG 0.2%.

Stabilizer 6. Casein 1%, Trehalose 5% and Kathon CG 0.2%.

Tests on conjugates with different stabilizers

HRP-BSA-AFB1 conjugate was diluted

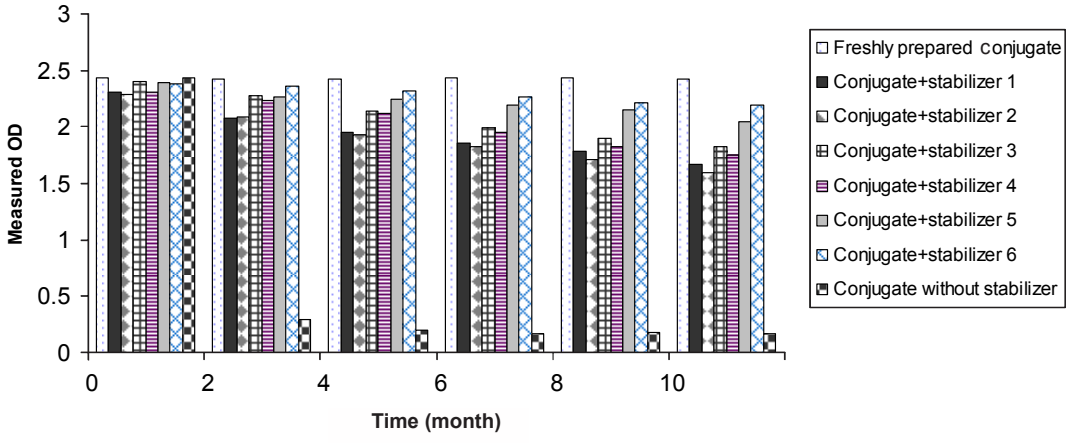

Figure 1. Stability verification of different conjugate-stabilizer mixtures kept at room temperature during 10 months in comparison with conjugate without stabilizer as well as freshly prepared conjugate as controls. ODs were measured at 450 nm.

in phosphate buffer saline (PBS) and kept in thoroughly closed brown glass vials at room temperature together with different stabilizers.

The ELISA was performed in two different methods, normal and accelerated. In normal method, the conjugate-stabilizer mixture was maintained at room temperature and checked every other month. Two different assays were used for verification of conjugate stability. First assay was done with a direct ELISA which was previously designed for such purpose. In this method, prepared conjugates mixed with different stabilizers were added to microplates coated with Anti-AFB1 antibodies. After 1 h incubation at 37 °C, microplate was washed 5 times with 0.05% PBS and then TMB was added. After 10 minutes incubation in a dark place, the reaction was stopped by 2 M sulfuric acid and ODs were measured at 450 nm. All six conjugate-stabilizer mixtures together with conjugate without stabilizer and freshly prepared conjugate were verified by this method. As an alternative method, prepared conjugate-stabilizer mixtures were used as alternative conjugate in AgraQuant ® aflatoxin B1 test kit according to its instruction manual. The samples were incubated for 15 minutes at room temperature and the plate was washed and then TMB was added. After 10 minutes incubation in a dark place, the reaction was stopped by 2 M sulfuric acid and ODs were measured at 450 nm.

In the accelerated method, the conjugate-stabilizer mixtures as well as conjugate without stabilizer were maintained at 37 °C and checked every week with the same ELISA methods.

The performance of stabilized conjugated tracers in ELISA tests was determined and compared with freshly prepared HRP-BSA-AFB1 conjugate.

Results and Discussion

Different stabilizers were checked during 10 months verification in normal and 6 weeks in accelerated procedures. In the normal procedure, all six mixtures were checked by in-house ELISA method (Figure 1). Only stabilizer 6 together with freshly prepared conjugate as well as conjugate without stabilizer were verified by alternative method using AgraQuant ® aflatoxin B1 test kit for 6 months (Figure 2).

In the accelerated procedure, all six mixtures were checked by in-house ELISA method (Figure 3). Only stabilizer 6 together with freshly prepared conjugate as well as conjugate without stabilizer were verified by alternative method using AgraQuant ® aflatoxin B1 test kit for the same period of 6 weeks (Figure 4).

According to the results, best stabilizing effect was shown when stabilizer 6 containing casein1%, trehalose 5% and Kathon CG 0.2 % was used. The stabilizer 6 maintained the activity of HRP-BSA-AFB1 at room temperature yielding 90% of initial activity for 10 months in normal and for 6 weeks in accelerated verification procedures.

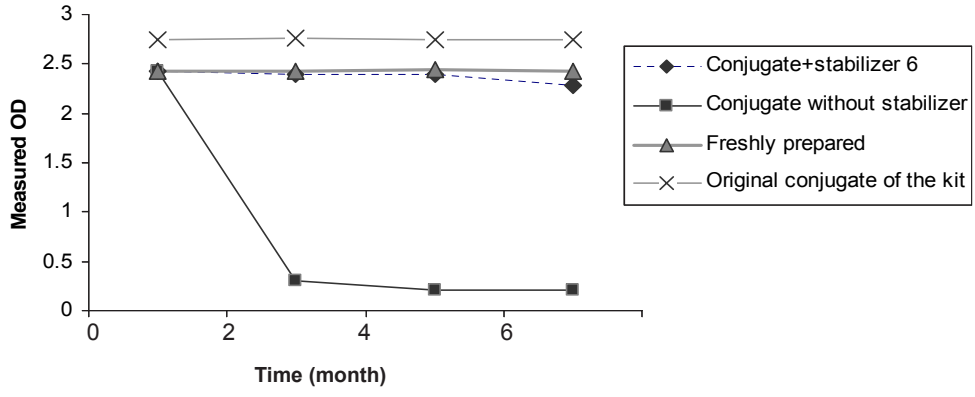

Figure 2. Stability verification of conjugate-stabilizer 6 mixture kept at room temperature using AgraQuant ® aflatoxin B_1 test kit. Freshly prepared conjugate, original conjugate of the kit stored according to kit instruction (2-8 °C) and conjugate without stabilizer with same storage condition of stabilized one were tested as controls. Each sample was tested as duplicate and mean of measured ODs at 450 nm were shown.

Compositions of different stabilizers used in this study were essentially similar to those cited in another published experiment (18); however, concentrations of the ingredients were slightly modified.

BSA was shown to have a strong stabilizing effect on HRP in buffer solution without any additives (21). The stabilizing effect of BSA relies on an increase in soluble protein concentration, a general principle of protein stabilization, and may be achieved as a result of protein-protein interactions after aggregation of BSA and the conjugates (18). In this study, the conjugate, however, had BSA in its own molecule. It seems that an interaction between BSA inside the molecule and BSA used in stabilizer is not favorable. So, unexpectedly, it was observed that stabilizers with BSA in their compositions did not show a suitable stabilizing effect in comparison with other stabilizers. It could be due to a new conformation induced by BSA. It seems that for the design and development of a stabilizer, similarity between conjugate and stabilizer components must be taken into consideration.

Sugars and polyols are also widely used to stabilize proteins (22). In all cases, the protein was preferentially hydrated, i.e. addition of sugars to an aqueous protein solution resulted in free-energy change. This effect was shown to increase with increasing of protein surface area, explaining the protein stabilizing effect of these sugars and their enhancing effect of protein associations. Correlation of the preferential interaction parameter with the effect of the sugars on the surface tension of water, i.e. their positive surface increment has led to this conclusion that the surface free energy perturbation by sugars plays a predominant role in their preferential

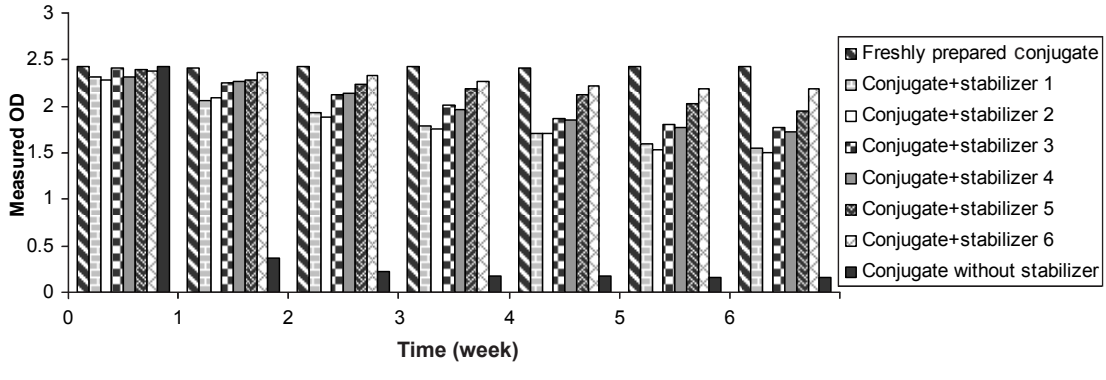

Figure 3. Accelerated stability verification of different conjugate-stabilizer mixtures kept at 37 °C during 6 weeks in comparison with conjugate without stabilizer and freshly prepared conjugate as controls. ODs were measured at 450 nm.

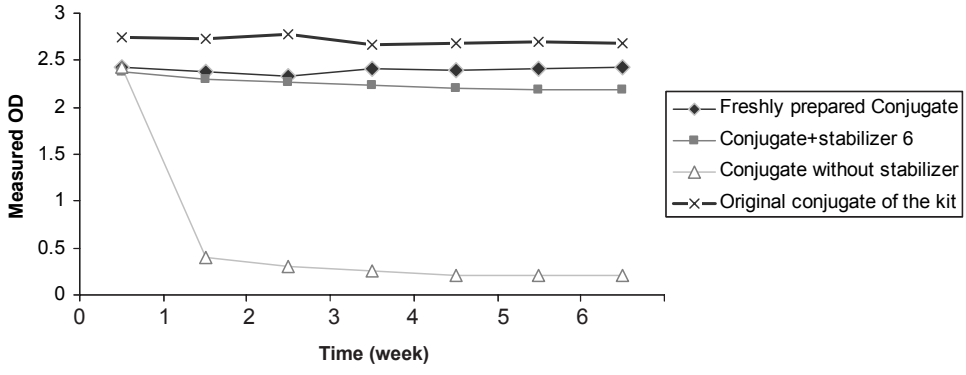

Figure 4. Accelerated stability verification of conjugate-stabilizer 6 mixture which was kept at 37 °C using AgraQuant ® aflatoxin B_1 test kit. Freshly prepared conjugate, original conjugate of the kit which was stored according to kit instruction (2-8 °C) and conjugate without stabilizer with same storage condition of stabilized conjugate were tested as controls. Each sample was tested as duplicate and mean of measured ODs at 450 nm were shown.

interaction with proteins (23, 24). In this study, trehalose was used as a stabilizing agent. Trehalose is a disaccharide composed of two glucose molecules linked together. It has a very important property that helps it to stabilize proteins and thus can be used as a biologic preservative (25).

Trehalose can be expected to work as a universal stabilizer of protein conformation due to its exceptional effect on the structure and properties of solvent water compared to other sugars and polyols (26). Our findings also approved of trehalose superiority to other stabilizers.

We noted when casein together with trehalose was used to stabilize formulated conjugate a more stable structure was achieved. Probably, besides the discussed effect of trehalose, a protein-protein interaction might account for stabilizing the conjugate. Presence of a protein like casein could make a stereo-conformation in which the conjugate molecule could be more stable.

Glycerol is also act as other sugars and polyols but its effect on different conjugated molecules may be varied. In this study, its effect as a stabilizer was better than BSA-containing stabilizers but weaker than those containing trehalose.

In general, it was concluded that trehalose-containing stabilizers especially those which have casein in their compositions show the best result as stabilizing agents for HRP-BSA-AFB1 conjugated tracer.

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References

(1) FAO/WHO. *News Archive: Codex Committee in Food Additives and Contaminats,* Rothschild, Geneva, Switzerland (1998) 1992

(2) Richard JL and Payne GA. (eds.) *Mycotoxins: Risks in Plant, Animal and Humans.* Council of Agricultural Science and Technology (CAST), Task Force Report No. 139, Ames, Iowa (2003) 195

(3) Chu FS and Bhatnagar D. Fungal biotechnology in agricultural, food and environmental application. In: Arora DK. (ed.) *Mycotoxins*. Marcel Decker, New York (2004) 325-342

(4) United States Department of Agriculture (USDA). *Aflatoxin Handbook.* The Institute, Washington DC (2002) 1-13

(5) Deshpande SS. (ed). *Handbook of Food Toxicology.* Marcel Decker, New York (2002) 387- 456

(6) Bakirci I. A study on the occurrence of aflatoxin M1 in milk products produced in Van province of Turkey. *Food Control* (2001) 12: 47-51

(7) Alborzi S, Pourabbas B, Rashidi M and Astaneh B. Aflatoxin M_1 contamination in pasteurized milk in Shiraz. *Food Control* (2006) 17: 582-584

(8) Chu FS. Mycotoxins. In: Chiver DO. (ed.) *Foodborne Diseases*. 2nd ed. Academic Press, New York (2002) 271-303

(9) Xiulan S, Xiaolian Z, Jian T, Zhou J and Chu FS. Preparation of gold-labeled antibody probe and its use in immunochromatography assay for detection of aflatoxin B1. *Int. J. Food Microbiol.* (2005) 99:

185-94

(10) Stroka J, van Otterdijk R and Anklam E. Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices. *J. Chromatogr. A* (2000) 904: 251-256

(11) Jaimez J, Fente CA, Vazquez BI, Franco CM, Cepeda A, Mahuzier G and Prognon P. Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chromatogr. A* (2000) 882: 1-10

(12) Papp E, Otta KH and Bagocsi B. Determination of aflatoxins in food by overpressured-layer chromatography. *J. Chromatogr. A* (2000) 882: 11-6

(13) Escobar A and Regueiro OS. Determination of aflatoxin B_1 in food and feedstuffs in Cuba (1990 through 1996) using an immunoenzymatic reagent kit (Aflacen). *J. Food Prot.* (2002) 65: 219-221

(14) Paknejad M and Rassaee MJ. *Development and Characterization of Enzyme-Linked Immumosorbent Assay for Aflatoxin B_1 Measurement in Urine Samples Using Penicillinase as Label* [dissertation]. Tehran, Tarbiat Modares University (1996)

(15) Lee NA, Wang S, Allan RD and Kennedy IR. A rapid aflatoxin B_1 ELISA: development and validation with reduced matrix effects for peanuts, corn, pistachio and Soybeans. *J. Agric. Food Chem.* (2004) 52: 2746-2755

(16) Xiulan S, Xiaolian Z, Jian T, Xiaohong G, Zhou J and Chu FS. Development of an immunochromatographic assay for detection of aflatoxin B_1 in foods. *Food Control* (2006) 17: 256-262

(17) Chu FS. Immunochemical methods for mycotoxin analysis: from radioimmunoassay to biosensors. *Mycotoxins* (2004) 54: 1-14

(18) Kolosova AY, Shim WB, Yang ZY, Eremin SA and Chung DH. Direct Competitive ELISA based on a monoclonal antibody for detection of Aflatoxin B_1. Stabilization of ELISA kit components and application to grain samples. *Anal. Bioanal. Chem.* (2006) 384: 286-94

(19) Boyd S and Yamazaki H. Use of polyvinyl alcohol as a stabilizer of proxidase-antibody conjugate for enzyme immunoassay. *Biotechnology Techniques* (1994) 8: 123-8

(20) Burkin AA, Kononenko GP and Soboleva NA. Products of spontaneous conjugation of aflatoxins with bovine serum albumin: immunochemical properties. *Prikl. Biokhim. Mikrobiol.* (2003) 39: 228-36

(21) Eremin AN, Budnikova LP, Sviridov OV and Metelitsa DI. Stabilization of diluted aqueous solutions of horseradish peroxidase. *Appl. Biochem. Microbiol.* (2002) 38: 151-158

(22) Taylor LS, York P and Williams AC. Sucrose reduce the efficiency of protein denturation; by a chaotropic agent. *Biochim. Biophys. Acta* (1995) 1253: 39-46

(23) Arakawa T and Timashell SN. Stabilization of protein structure by sugars.*Biochemistry* (1982) 21: 6536-44

(24) Wimmer R, Olssen M and Peterson MT. Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. *J. Biotechnology* (1997) 55: 85-100

(25) Draber P, Draberova E and Novakova M. Stability of monoclonal IgM antibodies freeze-dried in the presence of trehalose. *J. Immunol. Methods* (1995) 181: 37-43

(26) Kaushik JK and Bhat R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolytre trehalose. *J. Biol. Chem.* (2003) 278: 26458-65

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