Solvent effects in TLS determination of beta-lactoglobulin

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Abstract. Accidental presence of small quantities of different allergens such as beta-lactoglobulin in food products is a problem for unaware allergic consumers. Therefore, sensitive methods are needed for detection of trace quantities of allergens. By implementing TLS detection into a FIA system, the sensitivity of beta-lactoglobulin detection was improved. The use of different solvents in the FIA system, however, causes perturbations in the TLS signals. The aim of this research was to reduce the influence of spurious signals and to improve the detection limits of the analytical assay. The optimal performance of the FIA-TLS system for beta-lactoglobulin determination was achieved by using PBS buffer solutions (pH 7.5), which resulted in lowest limits of detection of 2 pM BLG and the total assay time of 10 min.

1. Introduction

Milk is consumed as a basic food product from early infancy. However, in some individuals trace quantities of milk allergens, like beta-lactoglobulin (BLG), can already provoke an allergic reaction [1]. Accidental presence of milk allergen in food products is a problem for unaware allergic consumers. Therefore, there is a need for rapid assays, which provide sufficiently low detection limits for allergens in food products. In recent years a variety of different immunological tests were developed in order to achieve the demanded characteristics of assays [2-6].

Such immunoassays can be implemented in biosensors, where the biological recognition is coupled to a suitable transducer [7, 8]. Moreover, it is possible to gain response to more than one analyte, by simply using multiple immobilized biomolecules, such as enzymes and antibodies [9].

In this research we have coupled a biosensing flow injection (FIA) system for beta-lactoglobulin detection to a thermal lens spectrometer (TLS). However, it was demonstrated that incomplete mixing of eluents in flow system can produced optical perturbation of the probe beam resulting in considerable oscillations of detected signals. Consequently a significant background noise is formed [10]. These effects results in difficult evaluation of the analyte concentration, but can be reduced by applying long chromatographic columns, mixing coils or by matching of FIA eluents [10]. In case of bioanalytical systems there are several limitations for application of such solutions. This is due to technical details of bioanalytical column construction as well as due to sensitivity of bioamolecules to parameters such as pH and ionic strength of the medium. The aim of the presented research was to study the eluent effects on TLS detection in a bioanalytical flow injection detection system. The goal was to reduce or eliminate spurious signals resulting from incomplete solvent mixing in order to facilitate the determination of the allergen.
2. Methods and materials

2.1. Biosensing element
The biosensing element of the detection system consists of a packed column containing polyclonal antibodies raised against BLG (Bethyl laboratories, Texas, USA). The antibodies were bound on controlled pore glass (CPG) beads (particle size 80-120 mesh) by cross-linking. Prior immobilization, the CPG beads were alkylaminated with 3-aminopropylethoxysilane (Sigma-Aldrich, min 98%). The binding of the antibodies was performed on alkylaminated CPG in the presence of the cross-linking agent glutaraldehyde (Merck, 50%). The bioanalytical column was implemented in a flow-injection system.

2.2. FIA-TLS
The FIA system consists of a HPLC pump connected to two injection valves positioned in series and further connected to the biosensing column. The biosensing column is connected to a flow-through cell. The flow-through cell is placed in the TLS spectrophotometer. The first injection valve with an injection loop of 100 µL is used for sample injection, while the second injection valve with a 5 µL injection loop is used for reagent injection. The flow rate of the carrier buffer was 0.4 mL/min.

The TLS spectrophotometer is an in-house built pump/probe dual beam instrument. The excitation laser used is an Ar-ion laser (Coherent CR4, Moorpark, CA, USA) operating at 458.9 nm emission line with a 50 mW power. The probe beam is derived from a Helium-Neon laser (Uniphase 1103P, 2 mW, 632.8 nm).

2.3. Analytical procedure
HRP conjugated secondary antibodies (Bethyl laboratories, Texas, USA) were added to a standard solution of BLG prior to the analysis. Final concentration of secondary antibodies in the injected mixture was 100 ng/mL. 100 µL of the preincubated mixture was injected into the FIA system. Beta-lactoglobulin and the co-bound labelled antibodies were retained in the bioanalytical column by binding to immobilized anti-BLG antibodies. The activity of retained HRP conjugated antibodies was evaluated by injecting 5 µL of o-phenylenediamine (OPD) solution with added H₂O₂. The formation of the enzymatic product was measured with TLS. The assay was performed at room temperature (25±2°C).

3. Results and discussion
According to literature [11], the best working pH for immobilized HRP is closed to pH 5. The commonly used working solution for OPD reagent preparation is an acidic sodium acetate buffer (pH = 4.5), while the most suitable medium for immunological interaction between the preincubated analyte and the immobilized antibodies is a PBS buffer (pH = 7.4). These two buffers are however incompatible for TLS measurements in a FIA system, because their incomplete mixing results in the formation of concentration independent signals which have no optothermal origin and are superimposed to real TLS signal as seen in Fig. 1. After OPD injection into the FIA system, a signal perturbation is formed at the interface of the two solutions, which causes irregular double peak shaped TLS signals and makes it difficult to evaluate the concentration of the analyte in the system (Figure 1).

Different combinations of acetate and PBS buffer solutions were tested in order to achieve unperturbed TLS signals. The combination of acetate buffer as carrier and acetate as solvent for OPD was soon rejected because of irreproducible measurements and high background signals.
More reproducible data were obtained with PBS buffers. The shape of the signals corresponding to the amount of HRP labelled secondary antibodies in the bioanalytical column was symmetrical, which made the evaluation of the BLG concentration more reliable. Although, the HRP was found to work best at pH 6.5, the lowest limits of detection were achieved in a combination of PBS buffers at pH 7.5 (Figure 1). Despite lower activity of HRP under such conditions, the limit of detection was lowered to about 2 pM BLG (S/N=3), which can be attributed to lower background noise. The differences in slopes of calibration lines (Figure 2) can only be related to activity of HRP under different pH. All buffers were prepared in 10 mM concentration, which shall not affect the thermooptical properties of solutions. Earlier reports [12] have confirmed that signal enhancements of 35 – 70% can only be expected at much higher electrolyte concentrations (1 M).

The results showed a ten-fold improvement of LOD compared to the measurements performed in acetate-PBS system, confirming the negative influence of solution mixing in a FIA-TLS detection system. The achieved LOD represents also a significant improvement compared to ELISA detection kits (LOD = 100 pM). This is partly due to the constant rinsing of excess sample matrix in case of FIA-TLS, thus lowering the background signal, which is not possible in case of microtiter plates used in ELISA kits.

In addition to lower LOD the important advantage of the FIA-TLS detection system is its fast response and high sample throughput. On Figure 3, there is a time representation of the analytical procedure. The sequence begins with an injection of the sample on the bioanalytical column (red
arrows). The application of the sample is followed by three consecutive injections of the OPD reagent (blue arrows), in order to detect the analyte retained on the bioanalytical column. This is followed by the regeneration of the column by washing-off of the analyte (black arrows). The concentration of BLG is evaluated from the difference in signals ($\Delta$ signal) resulting from an unknown sample or standard BLG solution and the blank, based on the corresponding calibration curves such as those shown on Figure 2. The entire detection run takes about 10 min for each sample, which is much faster when compared to the overnight incubation required for common ELISA detection.

![Figure 3. FIA-TLS signals obtained for sample without BLG and with BLG (10 pM).](image)

4. Conclusions
The FIA-TLS system for beta-lactoglobulin detection is a highly sensitive analytical tool. To achieve reproducible and reliable results as well as high sample throughput, the use of phosphate buffer is required. The use of PBS buffers results in more reproducible and unperturbed signals. The complete assay takes about 10 min for each sample, which is much faster when compared to the overnight incubation required for common ELISA detection. Moreover, the sensitivity of FIA-TLS system for BLG determination is higher resulting in lower detection limits (LOD = 2 pM), which represents a 50 fold improvement compared to ELISA tests.

References
[1] M. Morisset, D.A. Moneret-Vautrin, G. Kanny, L. Guenard, E. Beaudouin, J. Flabbee, R. Hatahet, Clinical and Experimental Allergy, 33 (2003) 1046.
[2] B.W. Blais, M. Gaudreault, L.M. Phillippe, Food Control, 14 (2003) 43.
[3] V.M. Haarmann, J. Lewitzki, Fleischwirtschaft, 87 (2007) 111.
[4] V. Hohensinner, I. Maier, F. Pittner, Journal of Biotechnology, 130 (2007) 385.
[5] E. Molina, L. Amigo, M. Ramos, Journal of Food Protection, 61 (1998) 1691.
[6] A. Puerta, J.C. Diez-Masa, M. de Frutos, International Dairy Journal, 16 (2006) 406.
[7] H.E. Indyk, International Dairy Journal, 19 (2009) 36.
[8] M. la Farre, D. Barcelo, in: Y. Pico (Ed.), Food Toxicants Analysis Techniques, Strategies and Developments, Elsevier, Amsterdam, 2007, p. 599.
[9] B.E. Eggins, Chemical Sensors and Biosensors, John Wiley & Sons, Kent, 2002.
[10] J.K. Logar, M. Sikovec, A. Malej, M. Franko, Analytical and Bioanalytical Chemistry, 374 (2002) 323.
[11] A. Puerta, J.C. Diez-Masa, M. de Frutos, Analytica Chimica Acta, 537 (2005) 69.
[12] M. Franko, C.D. Tran, Journal of Physical Chemistry 95 (1991) 6688