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Development of an immunochromatographic method to determine β-lactoglobulin at trace levels

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Abstract

β-Lactoglobulin (βLG) is one of the main components of whey proteins. Among other reasons, its allergenic character makes necessary its determination in hypoallergenic foods and biopharmaceutical products at very low concentration making use of very sensitive methods. In this paper, the development of an enzyme linked immunoaffinity chromatographic (ELIAC) method in sandwich format is described. The optimized method includes the use of a minicolumn (4.9 mm × 1 mm) packed with Nucleosil® 4000-7OH derivatized with high density of antibody coverage, the employment of rabbit serum and Zwittergent® 3–12 as mobile phase additives, and the incubation of the sample containing βLG with anti βLG labeled with horseradish peroxidase prior to their injection into the immunochromatographic system. The method allows determination of βLG with a practical detection limit of 20.7 pM and a sensitivity of 1.05×10^9 AU mol^{-1} L. The application of this ELIAC method to determine βLG in a commercial hypoallergenic formula based on caseins is shown. The amount of allergen, 0.32 pmol βLG per gram of sample, is about 5 million times lower than that naturally occurring in cow's milk.

Keywords: β-Lactoglobulin; Enzyme linked immunoaffinity chromatography; ELIAC; Minicolumn; Hypoallergenic formula

1. Introduction

β-Lactoglobulin (βLG), the most abundant protein in the whey fraction, is the main allergen in cow's milk [1], being able of promoting allergic reactions even when present at very low concentrations. Cow's milk protein allergy, the most common allergy in early childhood, is estimated to affect to 2–3% of infants in their first years [2]. Symptoms such as gastrointestinal alterations, urticaria, angioedema, atopic dermatitis, allergic rhinitis, asthma, or chronic cough have been described. In order to diminish the incidence of cow's milk allergy, hypoallergenic formulas intended to be βLG free have been commercialized as substitutes of milk for allergic people. These formulas are usually prepared employing cow's milk proteins, which are hydrolyzed by different methods in order to avoid their allergenic character [3,4]. Unfortunately, in spite of the very low residual amounts of βLG expected to be present in these hypoallergenic formulas, allergic reactions have been described in some instances in infants fed on these formulas [5,6]. Therefore, a method capable to determine the amount of residual βLG present in these formulas in order to assess their safety to be consumed is necessary.

Quantitation of βLG in hypoallergenic formulas requires very sensitive and very selective methods. Enzyme immunoassays (EIA) fulfill both requirements. Due to the specificity of antigen–antibody interactions, methods based on immunorecognition are very selective, and due to the enzymatic reaction employed in the detection step, the analytical signal can be enhanced, leading to very sensitive methods. The few instances in which βLG has been detected in hypoallergenic formulas, it has been mainly made by classic enzyme linked immunosorbent assays (ELISA) performed in microwells plates [7]. Competitive [8] and non-competitive [9] ELISA including different enzymes, such as horseradish peroxidase [10] or alkaline phosphatase [7], have been em-

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ployed. Using these methods, βLG has been detected in hypoallergenic formulas in the pM–μM range.

Although classic ELISA methods are sensitive, selective, and allow the analysis of several samples simultaneously, they have several disadvantages. The range of concentrations at which the method works is usually narrow and the sample volume is limited by the well volume. Besides, they are tedious, time consuming, and sometimes these methods are only semiquantitative assays due to their low reproducibility. In order to solve these limitations, flow systems have been applied to perform ELISA. They allow higher control on reaction times, on addition of reactives, and on kinetics than classic systems. Also, the assays performed on flow systems are more repetitive and easy to automate than ELISA methods. Besides, the work range of concentrations is larger and sample volumes even lower than 1 μL can be employed [11].

Immunoaffinity chromatography is one of the flow system methods employed to perform immunoassays in different formats (see for example Refs. [12–19]). One of the formats of classical immunoassays (IA) performed in immunoaffinity chromatography is the sandwich IA [20,21]. The sandwich format, a type of non-competitive assay, consists on the formation of a complex between the antibody (Ab) linked to the stationary phase of the immunocolumn (Ab_1), the antigen (Ag) of the sample and a second Ab labeled with a tag (Ab_2-L). Ag and Ab_2-L can be introduced in the column sequentially or simultaneously [21,22]. The term sandwich has also been applied to the procedure to quantitate Ab capturing a complex Ab–Ag-label [23] or a complex Ab–(anti Ab)-label [24] onto a protein G column. Sandwich immunoassays are usually more specific than other formats of immunoassays.

In spite of the advantages of the immunoaffinity chromatography (IAC) methods, to our knowledge there are not IAC methods developed to determine βLG. The determination of any Ag by IAC demands the development of its own method due to the specificity of the conditions of the antibody–antigen reactions in which these kinds of methods are based. The aim of this work was to develop an enzyme linked immunoaffinity chromatography method (ELIAC) that allows determining βLG in hypoallergenic formulas, even if it exists at the pM level.

2. Experimental

2.1. Chemicals, samples, and buffers

Trizma base, trizma hydrochloride, potassium chloride, glycine, *o*-phenylenediamine (OPD), *p*-nitrophenyl phosphate (PNPP), ovoalbumin (OVA), bovine seroalbumin (BSA), bovine β-lactoglobulin A + B (L-0130) (βLG), bovine α-lactalbumin (αLA), gelatine from pig skin (type A), pig immunoglobulin G, horse serum, rabbit (rb) serum, zein, Triton® X-100, polyetileneglycol 8000 (PEG_{8000}) and Pluronic F68, were purchased from Sigma (St. Louis, MO, USA).

Acetic acid, magnesium chloride hexahydrate, sodium borohydride, sodium chloride, sodium dihydrogenphosphate, disodium hydrogenphosphate dihydrate, potassium dihydrogenphosphate, Brij® 35, Tween® 20, Tween® 80 and Zwittergent® 3–12 were from Merck (Darmstadt, Germany).

Hydrochloric acid and sulphuric acid were purchased from Panreac (Barcelona, Spain). Sodium *m*-periodate was from Carlo Erba (Milan, Italy). Glycerol was obtained from Foret (Barcelona, Spain). Sodium azide was from J.T. Baker (Deventer, The Netherlands). Ethanolamine was purchased from Scharlau (Barcelona, Spain) and sodium cyanoborohydride and Gohsenol were from Aldrich (Milwaukee, WI, USA). Polyacrylamide 700,000–1,000,000 ($PAA_{700,000–1,000,000}$) was obtained from Polysciences (Warrington, PA, USA). Concentrated buffer with stabilized hydrogen peroxide for enzymatic reaction with horseradish peroxidase was purchased form Pierce (Rockford, IL, USA).

Affinity-purified anti bovine βLG A + B raised in rabbit (anti βLG (rb)) unconjugated and conjugated with either horseradish peroxidase (anti βLG (rb)–HRP) or alkaline phosphatase (anti βLG (rb)–ALP) were purchased from Bethyl Labs (Montgomery, TX, USA). Concentration of these anti βLG (rb) in commercial solutions was 1, 1 and 0.1 mg/mL, respectively.

Immunoglobulin (Ig) fraction with anti bovine βLG B raised in sheep labeled with ALP (anti βLG (sh)–ALP) was obtained from Bio Trend (Köln, Germany) in a commercial solution of 11 mg/mL.

A hypoallergenic formula based on extensively hydrolyzed caseins was purchased in a local store.

All the buffers and solutions were prepared in ultrapure water obtained from a Milli-Q unit (Millipore, Bedford, MA, USA).

Application, detection, and elution buffer compositions depended of the enzyme (E) used as label of the second antibody during the assay. When ALP was used, the application buffer was Tris–buffer saline (TBS = 0.01 M Tris, 0.137 M NaCl, 0.0027 M KCl, pH 7.4), the detection buffer consisted on 0.1 M Tris–buffer, 5 mM $MgCl_2$, pH 9, and the elution buffer was 4 M $MgCl_2$ in 20 mM Tris–buffer pH 5.9. For HRP as labeling enzyme, phosphate buffer saline (PBS = 0.01 M phosphate buffer, 0.137 M NaCl, 0.0027 M KCl, pH 7.4) was used as application and detection buffers, while 0.1 M Gly/HCl pH 2.5 was the elution buffer.

2.2. Preparation of samples and reagents

βLG aqueous standard solutions were prepared from a 1 mg/mL βLG solution filtered through a low protein binding filter of 0.22 μm pore size (Acrodisc®, Pall Corporation, Ann Arbor, MI, USA). The concentration of this stock solution was determined by its absorbance at 278 nm (ε = 17,280 L mol^{-1} cm^{-1}). The stock solution was aliquoted and stored at −4 °C. Every working day, an aliquot was thawed, stored at 4 °C during use to prepare the standard solutions, and discarded at the end of the day. Concentrations of