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On-chip single column transient isotachophoresis with free zone electrophoresis for preconcentration and separation of α-lactalbumin and β-lactoglobulin

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ABSTRACT

Isotachophoresis (ITP) coupled to zone electrophoresis (ZE), either free zone electrophoresis (FZE) or gel electrophoresis (GE), carried out mainly in capillaries and, although less frequently, also in microchips, is a powerful preconcentration and separation technique which has been successfully used for the study of many low molecular-weight analytes. However, this analytical technique has been scarcely applied for proteins separation. In this work, an on-chip transient ITP coupled to free zone electrophoresis (t-ITP-MFZE) mode with LIF detection is developed for the preconcentration and separation of the proteins α-lactalbumin and β-lactoglobulin.

Firstly, for LIF detection, the proteins were off-chip fluorescently labeled with the fluorogenic reagent Chromeo P503. Then, several separation parameters in t-ITP-MFZE mode such as leading electrolyte, terminating electrolyte, separation voltage, and injection time were optimized to achieve the maximum sensitivity while maintaining an adequate resolution between α-lactalbumin and β-lactoglobulin in a single column configuration t-ITP. Using the optimized electrolytes (50 mM imidazole/HCl pH = 8 as leading electrolyte and 100 mM imidazole/12 mM HEPES pH = 8 as terminating electrolyte) separation of both proteins was achieved in less than 4 min with peak resolution of 1.5. The LODs were 55 nM and 380 nM, for α-lactalbumin and β-lactoglobulin, respectively, which are adequate for some food allergenicity studies.

Finally, comparison of the optimized t-ITP-MFZE method to the equivalent MFZE method, carried out also in microchips but without the isotachophoretic preconcentration step, provided preconcentration indexes for both proteins around 10.

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1. Introduction

Capillary electrophoresis (CE) is a powerful separation technique that offers high efficiency, high resolution capability, and high analytical throughput [1]. However, using the almost universal UV detector, the sensitivity in terms of concentration is limited in comparison with other separation techniques, such as HPLC [2]. The appearance and development of microchip based electrophoresis (ME) with its inherent and promising properties (parallelization, low reagent and sample consumption, low waste generation, integration of multiple analytical steps, and portability) has not overcome these sensitivity limitations. For that reason, the majority of applications in ME employ LIF detection instead of UV detection [3].

However, taking advantage of the different electromigration phenomena, a certain number of preconcentration techniques, such as field-amplified sample injection (FASI), field-amplified sample stacking (FASS), sweeping, and isotachophoresis (ITP), among others [2,4], have been developed which allow to overcome in part the sensitivity limitation of CE. Among them, ITP coupled to zone electrophoresis (ZE) —also called transient-ITP (t-ITP)— has achieved the highest concentration index (CI) reported [5].

The mechanism of transition between ITP and ZE is complex and has been widely studied [6–10]. In this context, Bahga and Santiago [10] proposed an enlightening classification for t-ITP methods based on the principles used for disrupting the band of the ITP preconcentrated analyte and triggering CE separation. According to them, t-ITP methods can be divided in two main groups: (1) those *"disrupting the zone order"*, so

Abbreviations: CE, capillary electrophoresis; CI, concentration index; CZE, capillary zone electrophoresis; FZE, free zone electrophoresis; GE, gel electrophoresis; ITP, isotachophoresis; LA, α-lactalbumin; LE, leading electrolyte; LG, β-lactoglobulin; LIF, laser induced fluorescence; ME, microchip based electrophoresis; MFZE, microchip free zone electrophoresis; TE, terminating electrolyte; t-ITP, transient ITP, this is ITP with zone electrophoresis (either FZE and or GE); t-ITP-FZE, t-ITP with free zone electrophoresis; t-ITP-MFZE, t-ITP with microchip free zone electrophoresis; ZE, zone electrophoresis.

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that leading electrolyte (LE) and terminating electrolyte (TE) zones no longer sandwich in space the analyte zones; and (2) those *"changing the mobility order of species"*, so that the mobility value of the ionic analytes at one point become independent of that of LE and TE ions, by reaching a region with a sieving matrix for gel electrophoresis or where a pH gradient exists. Additionally, this group of techniques can be categorized according to their instrumental set-up, in single column and column-coupled configurations. Single column configuration yields lower separation resolution but it is easier to implement than column-coupled one.

t-ITP has been successfully applied to a variety of low-molecular weight analytes [11,12]. However, ITP preconcentration of proteins has received relatively little attention, both in capillary and in microchip formats [13,14] although in the last few years there has been an increase in the number of publications using t-ITP techniques for proteins in microchips [15,16]. The majority of on-chip t-ITP applications for proteins have been carried out combining ITP with gel electrophoresis (GE) [17–21] while ITP combined with free zone electrophoresis (t-ITP-FZE) has been scarcely used. There are two main reasons for this low applicability of t-ITP-FZE to protein analysis. First, it is complicated to perform a t-ITP-FZE of proteins because of the low intrinsic mobility of these compounds, which in turn, makes difficult to find ions to be used as TE with lower mobility than proteins. This limitation is easily overcome in denaturing GE where the presence of the tensioactive sodium dodecyl sulfate in the sample and in the separation media increases the effective charge of proteins and, consequently, their electrophoretic mobility. The second reason is that the disruption of ITP step for CE initiation happens faster and the mechanism is simpler in GE (is the *"changing the mobility order of species"* group of t-ITP techniques mentioned above) than in FZE (the *"disrupting the zone order"* group) [10] due to the presence of gel. However, in spite of the difficulties, FZE would be required for those studies in which proteins need to be in native state, such as isoform separation [22], affinity studies [23], or protein-ligand interaction [24].

Notwithstanding these difficulties, there are two interesting publications about t-ITP-FZE on microchip format (t-ITP-MFZE) [25,26]. Olvecka et al. [25] used a poly(methyl methacrylate) microchip with a column-coupled configuration and conductivity detection. In this work, six proteins were concentrated and separated by cationic on-chip t-ITP-MFZE. Wu et al. [26] developed a multidimensional microchip coupled with a capillary for determination of functional proteins in infant milk formula. The isoelectric focusing step was performed for fractionation of functional proteins (lactoferrin, IgG, α-lactalbumin, β-lactoglobulin A and β-lactoglobulin B) which were concentrated afterwards by on-chip ITP. However, the separation step was carried out in capillary format (CZE-UV).

In the present work, we developed an anionic t-ITP-MFZE method for the determination of α-lactalbumin (LA) and β-lactoglobulin (LG). LA and LG are the main allergenic whey proteins [27] and the development of new methods for their quick and sensitive detection is of interest in food analysis control [28–31]. The need of these analytical methods has increased due to the new European Union Regulation Nº 1169/2011 on the provision of food information to consumers which entered into application on December 2014. The last changes introduced in this Regulation refer mainly to the obligation of including information about allergens in different types of food.

The method developed in this work uses a simple-cross glass microchip and LIF detection. The t-ITP was of type *disrupting the zone order* [10] in a single channel configuration. This combination is easy to implement in a simple-cross microchip and avoids the need of using sophisticated multichannel chips or replacing the liquids in the chip reservoirs. Selection of the terminating electrolyte was critical and several analytical parameters such as separation length, voltage, and injection time were optimized to achieve the maximum preconcentration with adequate resolution between LA and LG in the separation step. Prior to the load in the sample reservoir, both proteins were fluorescently labeled off-chip using the fluorogenic reagent Chromeo P-503 to be detected using LIF.

2. Materials and methods

2.1. Reagents

Sodium tetraborate, boric acid, rhodamine B, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), α-lactalbumin (LA, M_w = 14 kDa, pI = 4.2–4.5) and β-lactoglobulin B (LG, M_w = 18 kDa, pI = 4.7–5.2) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) was from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was obtained from Panreac (Barcelona, Spain). Imidazole was purchased from Scharlau (Barcelona, Spain). Chromeo P503™ was obtained from Active Motif (Tegernheim, Germany). EOTrol™ LN (3% w/v polymer) was purchased from Target Discovery (Palo Alto, CA, USA). All aqueous solutions were prepared in Milli-Q water (Millipore, Molsheim, France).

2.2. Instrumentation

Simple-cross glass microchips from Micronit (Ref. X8050 CE CHIP, Enschede, The Netherlands) were used. The channel section was 50 μm width and 20 μm depth. The length of the short channels was 5 mm. The length of the long channel (separation channel) was 80 mm. The glass microchips were used in a home-made microchip holder [32]. This holder consisted of two poly(methyl methacrylate) plates, which sandwiched the microchip by using plastic screws. The top plate has four platinum electrodes for voltage applying and four threaded holes that allow the connection of the plastic tubing from the syringe pump. The bottom plate had an elongated hole aligned with the separation channel, so that the laser light impinges directly on the microchip (Fig. S1).

A high voltage sequencer (model HVS448 from LabSmith, Livermore, CA, USA) with eight channels and double polarity was used to apply the electric field in the microchannels through the platinum electrodes in the top plate of the microchip holder.

Fluorescence detection was performed with an epifluorescence inverted microscope (model DM IL LED, Leica, Wetzlar, Germany) provided with a DPSS laser (model SDL-473-030T, Shanghai Dream Laser, Shanghai, China) with emission at 473 nm and a nominal power of 50 mW (output at the objective 12 mW) and a long working distance plan fluor 20× objective together with a filter cube (model L5 from Leica) (excitation filter BP 480/40 nm, emission filter BP 527/30 nm, and a dicroic mirror 505 nm). An Orca R2 CCD camera (Hamamatsu, Hamamatsu City, Japan) and the HCImage Live software also from Hamamatsu were used for detection and image processing.

An Aladdin 1000 syringe pump (WPI, Sarasota, FL, USA) was employed for fluid manipulation connected to the microchip-holder with Valco plastic connections and Teflon tubing (Symta, Madrid, Spain). A forward (pushing) or backward (aspirating) flow-rate of 1600 μL/h was used in all experiments.

For CZE-UV studies, a P/ACE 2050 equipment (Beckman, Brea, CA, USA) was used. The capillary employed was of 375 μm O.D., 25 μm I.D. and 27 cm length (Polymicro Technologies, Phoenix, AZ, USA). The control and data acquisition software was System Gold 8.0 from Beckman.

2.3. Procedures

2.3.1. Sample preparation and labeling reaction

Standard solutions of LA and LG were made at concentration of 5×10^{-5} M in an aqueous buffer 50 mM imidazole adjusted with HCl to pH = 8. These protein solutions were fluorescently labeled with Chromeo P503, which react with primary amino groups of the protein, in non-denaturing conditions.

The fluorogenic reagent Chromeo P503 was first aliquoted in MeOH as received, obtaining aliquots of 80 μg (2×10^{-7} mol) which were vacuum-dried and stored at 4 °C in the dark in 1.5 mL Eppendorf tubes. For labeling reaction, 200 μL of standard protein solution (1×10^{-8} mol)

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