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Development of a molecularly imprinted polymer for the recovery of lactoferrin

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A B S T R A C T

Lactoferrin (LF) was recovered by molecular imprinting using either vinylpirydin alone (MIP1) or mixed with methacrylic acid (MIP2) as functional monomers. In order to create the specific cavity, pure LF was used as template. Controls were prepared for every MIP. The polymers obtained were tested against a protein mixture containing LF, and the recovery efficiency was calculated determining the diminution of LF from the supernatant by titration of the iron of the LF, as well as native PAGE to determine the protein composition of the supernatants. The only polymer which was able to specifically bind LF was MIP1 (vinylpirydin alone). Measuring the proportions of the different proteins in the mixture, it was demonstrated that before the separation with MIP1, LF represented 81.9% of the total protein content of the mixture, and diminished to 70.5% after the exposure to MIP1, suggesting that LF was specifically sorbed by this polymer. The retention efficiency of the polymer showed that MIP1 retained 34.5% of the total LF content, while its control did not retain any, demonstrating that the retention of the protein is not due to unspecific adsorption in the polymer, but rather to a selective retention in the cavity formed by the template.

Keywords: Lactoferrin; Whey proteins; Molecular imprinting; Solvent-Accessible Surface analysis

1. Introduction

Lactoferrin (LF) is an iron transporter glycoprotein isolated from bovine milk, constituting a minor component of whey proteins. It is a glycoprotein composed by 703 amino acids and with a molecular mass of 80 kDa (Balcão et al., 2013). It has a great capacity to bind Fe^{3+}, containing two molecules of this metal chain. Its potent iron binding properties can locally create iron deficiency, preventing growing and biofilm forming by bacteria (Vogel, 2012).

In recent years, LF has become one of the most valuable proteins of whey due to its nutraceutical properties. In addition to having antibacterial activity (bacteriostasis/bactericidal effect), lactoferrin is now known to have a long list of other beneficial biological properties such as antiviral, antifungal, immunomodulating properties, antinflammatory effect, growth stimulating properties, and even some anticancer activities (Balcão et al., 2013; Brock, 2012; Korhonen and Marnila, 2003; Lönnerdal, 2003; Vogel, 2012). One of the main sources of LF is bovine milk whey; yet, purification of LF from

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whey has proven to be very complicated due to the various whey proteins that range in the same shape and molecular weight as LF. Beyond the scientific interest on lactoferrin due to biological and health promoting properties, the commercial interest on this protein has been greatly increased in recent years leading to the search of proper methodologies for its isolation and purification from whey. However, current techniques are expensive and with low specificity, leading to commercial products that are just lactoferrin enriched mixtures of several whey proteins.

Molecular imprinting is a method for preparing synthetic materials able to mimic the molecular recognition phenomena present in living systems. Molecularly imprinted polymers (MIPs) represent a new class of materials possessing high selectivity and affinity for the target molecule (Ruigrok et al., 2011; Xu et al., 2004). It consists in the selection of a template molecule which is later associated with some functional monomers through non-covalent bonds; then, a polymerization around the template-monomer complex is conducted resulting in a molecularly imprinted polymer (MIP) which after extracting the template molecule has a cavity that recognizes the template molecule. The imprinted polymer material is able to recognize a single target molecule allowing to specifically separate it from a complex mixture (Ruigrok et al., 2011). Up to date, this technique has mainly been used for the separation of small organic molecules based on their shape, size and chemical functionality (Piletsky et al., 2001; Ruigrok et al., 2011). A few recent reports establish the possibility of using molecular imprinting for the separation of peptides and even larger proteins; however, several difficulties involving the use of this technique for the template imprinting of proteins have raised due to their large molecular sizes, and the fragility and complexity of the molecules; in spite of this, there have been several attempts to prepare protein imprinted polymers via different strategies (Bossi et al., 2007, 2012; Haupt, 2010; Whitcombe et al., 2011). The evaluation of the different groups of the template molecule available to interact with the functional monomers could be of use when selecting the kind and proportion of functional monomers to be used in molecular imprinting. The aim of this work was to develop a molecularly imprinted polymer which allows the selective recovery of lactoferrin using Solvent-Accessible Surface Area (ASA) Analysis to determine the available functional groups of LF as a tool for the selection of the functional monomers.

2. Materials and methods

2.1. Protein analysis

Total protein concentration was determined by Bradford's method (Bradford, 1976).

The protein composition of the different samples was assessed by native polyacrylamide gel electrophoresis (PAGE) ($T = 10\%$, $c = 4\%$). Electophoresis was run in a mini-PROTEAN III cell, with a PowerPac-300 power supply using constant voltage of 200 V. Gels were stained with Sypro Orange Protein Stain and protein quantification was done by means of an image analyzer in the UV spectrum (Gel-Doc 1000 with the Molecular Analyst software). The concentration of the different proteins was calculated by comparing the absorption units (AU/mm^2) of each protein band with the absorption units of a known concentration protein band. All reagents and equipment were from Bio Rad Laboratories (Hercules CA, USA).

2.2. Molecular imprinting

2.2.1. Bovine Lactoferrin Solvent-Accessible Surface Area (ASA) analysis

The X-ray diffraction coordinates of the crystal structure of bovine lactoferrin C-lobe in complex with lactose, PDB ID: 2H4I were submitted for bovine lactoferrin ASA calculation to the "Protein Interfaces, Surfaces and Assemblies" service (PISA) at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel and Henrick, 2007). In order to evaluate the convenience of using vinylpirydine or methacrylic acid as a functional monomers, the type of contacts of bovine lactoferrin with the reported pyridine derivative as well as those with the carboxylic derivatives structures (PDB IDs: 3USD, 3O97, 3IAZ, 3TTR, 4GRK, 3UGW, 3U72) were obtained from PDBsum website (Laskowski, 2009).

2.2.2. Functional monomers and polymerization conditions

Vinylpirydin (Sigma–Aldrich, USA) alone prepared to a final concentration of 50 mg/mL (MIP1) or mixed with methacrylic acid (1:1) (Mallinckrodt Organic Reagent, USA) (MIP2) were used as functional monomers. 50 μL ethyleneglycol dimethacrylate (Sigma–Aldrich, USA) were used as crosslinker. For MIP2, 20 μg potassium persulfate (J.T. Baker, Xalostoc, México) were used as catalyst to start the polymerization. 250 mL of functional monomer solutions were prepared separately in acetonitrile, which was used as reaction media. They were mixed with 25 mL of a 50 mg/mL pure LF solution (template) in 0.05 M phosphate buffer pH 7.0 and bubbled with nitrogen for 5 min before mixing them. In order to displace the remaining oxygen, the reaction mixture was bubbled with nitrogen 5 min more at the beginning of the reaction. After the mixture was de-aerated it was allowed to polymerize at 60 °C for 16 h.

The molecular imprinted polymers were washed for 1 h in a solution of methanol and acetic acid (1:1) to remove the template. This procedure was performed 3 times to ensure that the template was completely removed, after which the solution was centrifuged (5000 rpm) for 10 min to separate the MIP. The supernatant was collected and PAGE was performed to verify that LF had been successfully extracted.

2.2.3. Template

In order to create the specific cavity a 50 mg/mL pure LF solution in 0.05 M phosphate buffer pH 7.0 was used was used as template. A control without the template molecule was prepared for each MIP (C1, C2) using the same polymerization conditions and monomers but without the template molecule.

2.2.4. Lactoferrin purification

LF was purified from a commercial preparation (Bioferrin 2000, Glanbia Nutritionals, Monroe, WI, USA) by isoelctrofocusing using Bio-Lyte 3/10 Ampholyte (Bio Rad, Hercules, E.U.A.) (Rotofor, BioRad Laboratories, Hercules, CA, USA).

2.2.5. Desorption of the template

The weak electrostatic interactions that take place between the template and the polymer are easily broken by vigorously shaking the MIP in a 50:50 solution of methanol–acetic acid.