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One-step chromatographic method to purify α-lactalbumin from whey for nanotube synthesis purposes

Clara Fuciños[a,b,*], Pablo Fuciños[c], Natalia Estévez[a], Lorenzo M. Pastrana[c], Antonio A. Vicente[b], María Luisa Rúa[a]

[a] Biotechnology Group, Department of Analytical Chemistry and Food Science, University of Vigo, As Lagoas s/n, 32004 Ourense, Spain
[b] Centre of Biological Engineering, University of Minho, Campus de Gualtar s/n, 4710-057 Braga, Portugal
[c] The International Iberian Nanotechnology Laboratory (INL), Avenida Mestre José Veiga s/n, 4715-330 Braga, Portugal

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ABSTRACT

A one-step anion-exchange chromatography method (NaCl gradient elution on a DEAE Sepharose™ Fast Flow gel column) was developed to purify α-lactalbumin (α-LA) from whey protein isolate. α-LA nearly 100% pure (based on the total protein content) was obtained with a yield of about 39%. Besides pure α-LA, which was the main objective of this work, highly pure β-lactoglobulin was also obtained with a yield of about 59%. The high purity of the obtained α-LA samples allowed its use to synthesise protein nanotubes with excellent gelation properties for their use as food thickeners and bioactive carriers. The samples' purity degree obtained (based on the total protein content) was critical in the formation of proper nanotubes instead of random aggregates, which produced opaque and weak gels, less useful for food applications.

1. Introduction

Food nanotechnology has gained enormous interest during the last few years. Industries are particularly interested in developing smart and active delivery devices for a broad range of applications, from food packaging and safety to health and biomedical uses (Brody, Bugusu, Han, Sand & McHugh, 2008). In particular, nanotube-based materials are now starting to have an impact on the food industry (Weiss, Takhistov & McClements, 2006). Carbon nanotubes are popular as low-resistance conductors and catalytic reaction vessels, and as containers to release or protect valuable molecules. Although carbon nanotubes are not food-grade substances due to their potential toxicity for humans, some of their properties would find a place for food and health applications if they had characteristics such as biocompatibility, biodegradability, low cost and absence of toxicity.

Milk whey is a by-product of cheese manufacturing that is growing all over the world. In 2010, the global whey production was around 180 million tons (estimated as 9-fold the cheese production) (FAO, 2013; Guimarães, Teixeira & Domingues, 2010). Whey removal and treatment is expensive. Nevertheless, whey content in high-value proteins (Tarhan & Harsa, 2014) makes it not only a dairy by-product but also a valuable raw material.

β-Lactoglobulin (β-LG, 18.4 kDa) and α-lactalbumin (α-LA, 14.2 kDa) are the two major proteins in whey. They are present in an 80:20 (β-LG:α-LA) ratio and together represent almost 70% of the total whey protein content (Walstra, Wouters & Geurts, 2006). Both proteins are a valuable source of essential amino acids, although α-LA has some properties that make it especially interesting for the industry. α-LA contains high levels of tryptophan, which causes positive effects on consumers' well-being. Also purified fractions of α-LA are used in infant formula to avoid the allergenic potential of β-LG (Toro-Sierra, Tolkach & Kulozik, 2013). In addition to these properties, under appropriate conditions, α-LA can self-assemble into nanotube structures (Graveland-Bikker, Fritz, Glatter & De Kruif, 2006). These nanotubes have many potential applications (Ipsen & Otte, 2007; Ramos et al., 2017). Within the food industry, encapsulation for controlled release of bioactive compounds or the protection of functional ingredients against degradation during food processing, storage, and usage are among the most promising applications. Basic applications for α-LA nanotubes as thickening agents are also possible since linear structures are very efficient in increasing viscosity. Combining both applications makes these structures useful to be incorporated as fat substitutes into low-fat products, obtaining functional products with rheological properties similar to those of the full-fat products (mainly creaminess).

Despite the potential use of these materials, reports on their application are scarce (Fuciños et al., 2017). The production process of α-LA

* Corresponding author at: Biotechnology Group, Department of Analytical Chemistry and Food Science, University of Vigo, As Lagoas s/n, 32004 Ourense, Spain.
E-mail address: clarafg@gmail.com (C. Fuciños).

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nanotubes is simple, and they can be easily produced in large quantities. However, the synthesis of nanotubes requires highly pure α-LA, which is costly to produce, therefore limiting its use to only high value-added applications.

The development of cheaper purification methods will boost the incomes of dairy industries, allowing not only to reduce the generation of wastes but also to reuse them obtaining a new high added value product. However, a major challenge is developing an easily scalable, economical process capable of providing high purity and yield enough for subsequent applications.

Several methods have been developed during the last decades to purify whey proteins. In the literature, we can find a wide variety of methods with common pre-treatments to obtain a whey protein isolate (WPI) or a whey protein concentrate (WPC), that includes different steps for removing fat, precipitate the major whey proteins to separate the target proteins from other proteinic contaminants at the end. However, in general, commercial WPI or WPC was used to optimise the methods. New methods were developed based on successive precipitations and separations by microfiltration and/or ultrafiltration (Alomirah & Alli, 2004; Arunkumar & Etzel, 2014; Toro-Sierra et al., 2013), selective enzymatic hydrolysis (Konrad & Kleinschmidt, 2008; Lisak, Toro-Sierra, Kulozik, Bozanic & Cheison, 2013), selective adsorption on hydroxyapatite microbeads (Cetinkaya & Akkaya, 2016), processes applying supercritical carbon dioxide (Bonnaillie & Tomasula, 2012; Yver, Bonnaillie, Yee, Mcaloon & Tomasula, 2012), or methods based on two-phase systems (Sivakumar & Iyyaswami, 2015). These methods involve multiple steps to achieve high purity fractions (Table 1), which could hinder their implementation. The simplest and most common methods are those that involve chromatographic approaches. However, the reported chromatographic methods to obtain highly pure α-LA always consist of at least two steps (El-Sayed & Chase, 2010; Neyestani, Djalali & Pezeshki, 2003; Tarhan & Harsa, 2014), or even three steps (Pilbrow, Bekhit & Carne, 2016). In alternating order, these steps are usually gel filtration chromatography (also known as size exclusion chromatography), where proteins are separated by their molecular size, and ion exchange chromatography, where proteins are separated as a function of their overall charge. In the last chromatographic method, proteins are selectively eluted using a salt gradient (Neyestani et al., 2003) or a pH gradient (Tarhan & Harsa, 2014). Only a few works describing the application of a single chromatographic method are found in the literature (Geng, Tolkach, Otte, & Ipsen, 2015; Liang, Chen, Chen, & Chen, 2006; Mao et al., 2017), whose results (Table 1) we pretend to improve.

In this paper, we propose a one-step purification method based on ion-exchange chromatography to obtain highly pure α-LA fractions. Subsequently, we demonstrate that these α-LA fractions are suitable for the synthesis of protein nanotubes for food applications.

2. Material and methods

2.1. Standards and chemicals

WPI and α-LA, were kindly supplied by Davisco Foods International, Inc. (Eden Prairie, MN, USA). β-LG from bovine milk (> 90%), bovine serum albumin (BSA, ≥98.0%), trifluoroacetic acid CHROMASOLV®, for HPLC (TFA, ≥99.0%), acetonitrile CHROMASOLV® Plus, for HPLC (ACN, ≥99.9%), Coomassie Brilliant Blue R-250 (technical grade) and 2-mercaptoethanol for electrophoresis (> 98%) were purchased from Sigma-Aldrich (Munich, Germany). TRIS buffer (tris-(hydroxymethyl)-aminomethane, extra pure) was from Scharlau. Hydrochloric acid (HCl, 37%), sodium chloride (NaCl), sodium dihydrogen phosphate anhydrous (NaH_2PO_4), disodium hydrogen phosphate anhydrous (Na_2HPO_4), sodium azide (99%), sodium dodecyl sulfate (SDS), glycerol and bromophenol blue were from Panreac (Barcelona, Spain). Diethylaminoethanol Sepharose™ Fast Flow gel (DEAE-S FF, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). BCA Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). Acrylamide/Bis 30% (29:1) and broad range SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Watford, UK). Serine protease from Bacillus licheniformis (BLP) was gently supplied by Novozymes A/S (Bagsværd, Denmark). Manganese (II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$) was from Merck (Darmstadt, Germany). Sodium cacodylate EM and glutaraldehyde EM 25% were purchased from TAAB (Reading, UK).

2.2. Preparation of the whey protein samples

Samples for the purification processes were prepared by dissolving WPI at different concentrations, depending on the experiment, in 25 mM Tris-HCl buffer (pH 7.5 at 25 °C). The solutions were then filtered through a 0.22 μm filter. The obtained purified samples were prepared in the same way for their further characterisation.

2.3. Whey proteins purification by anion-exchange chromatography

In a first part of our research (Fig. 1), to purify α-LA from WPI, the anion-exchange chromatography (AEC) conditions were optimised by using a HiPrep™ 16/10 DEAE FF column (volume: 20 mL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) coupled to an ÄKTA Purifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the instructions provided by the manufacturer. The column was equilibrated at 5 mL min^{-1} with 5 column volumes (CV) of binding buffer (Tris-HCl 25 mM buffer, pH 7.5 at 25 °C). Then, an appropriate amount of protein was loaded onto the column at a flow rate of 1 mL min^{-1}. The column was washed at 4 mL min^{-1} with 5 CV of binding buffer, and then eluted in four steps: (1) 3 CV of 5% elution buffer (Tris-HCl 25 mM buffer, pH 7.5 at 25 °C, with NaCl 1 M), (2) 20 CV increasing the elution buffer from 5 to 11%, (3) 10 CV increasing the elution buffer from 11 to 35%, and (4) 3 CV of 100% elution buffer. Protein elution was monitored by absorbance at 280 nm. The collected fractions were concentrated (3.75x) and analysed by reverse-phase high-performance liquid chromatography (RP-HPLC).

In a subsequent part of the research the one-step purification process was scaled-up (Fig. 1). A chromatography was conducted, loading an appropriate amount of protein at a flow rate of 2.5 mL min^{-1} onto a DEAE-S FF gel packed in a column with 5 cm of length and 5 cm of internal diameter (volume: 98 mL). Equilibration, elution and detection conditions were kept the same as described above for the optimisation with the HiPrep DEAE FF 16/10 column. The collected fractions were desalted by diafiltration with MilliQ water using Ultracel® 10 kDa molecular weight cut-off (MWCO) membranes (Millipore, MA, USA) mounted on an Amicon® model 8200 stirred cell (model 8200; Millipore, CA, USA) under 40-psi pressure of nitrogen gas at room temperature, until the conductivity reached ~0.2 mS. The retentate was then freeze-dried for further analysis.

2.3.1. Optimisation of the protein loading capacity

WPI samples with different protein concentration were prepared in binding buffer. The same volume of WPI samples and DEAE-S FF gel, also equilibrated in binding buffer, were placed in microtubes. After that, the microtubes were gently mixed in a rotatory shaker for 15 min and then centrifuged at 300 × *g* for 10 min. After that, the supernatant was removed from samples and analysed by BCA Protein Assay Kit to obtain the not binding protein onto the DEAE-S FF gel (*NBP*, mg mL^{-1}). The percentage of not bound protein (*%NBP*) was calculated as follows:

$$\%NBP = \frac{NBP}{LP_i} \times 100 \quad (1)$$

where LP_i is the initial amount of loaded protein in mg per mL of DEAE-S FF gel. *%NBP* is plotted against LP_i (Fig. 2) and these experimental data were fitted with a logistic equation, adapted from Vázquez, Docasal, Prieto, González, and Murado (2008), to calculate the protein