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Fractionation of sheep cheese whey by a scalable method to sequentially isolate bioactive proteins

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ABSTRACT

This study reports a procedure for the simultaneous purification of glyco(caseino)macropeptide, immunoglobulin, lactoperoxidase, lactoferrin, α-lactalbumin and β-lactoglobulin from sheep cheese sweet whey, an under-utilized by-product of cheese manufacture generated by an emerging sheep dairy industry in New Zealand. These proteins have recognized value in the nutrition, biomedical and health-promoting supplements industries. A sequential fractionation procedure using economical anion and cation exchange chromatography on HiTrap resins was evaluated. The whey protein fractionation is performed under mild conditions, requires only the adjustment of pH between ion exchange chromatography steps, does not require buffer exchange and uses minimal amounts of chemicals. The purity of the whey protein fractions generated were analyzed by reversed phase-high performance liquid chromatography and the identity of the proteins was confirmed by mass spectrometry. This scalable procedure demonstrates that several proteins of recognized value can be fractionated in reasonable yield and purity from sheep cheese whey in one streamlined process.

1. Introduction

Although dairy production in New Zealand is dominated by cow milk, increasing quantities of milk are being produced by the more recently established sheep dairy industry. Sheep dairy has considerable potential for future expansion (Ardern et al., 2013). This has in part been driven by increasing interest in small ruminant milks that are reported to have various benefits including being more digestible (El-Agamy, 2007) and may be better tolerated by individuals with allergic reaction to dairy products, providing an alternative to consumption of cow milk (Pandya & Ghodke, 2007). Sheep milk produces about twice the cheese yield compared to that from cow, due to higher solids content (Hernandez-Ledesma, Ramos, & Gomez-Ruiz, 2011). A major proportion of sheep milk is used in production of special cheeses such as pecorino, resulting in generation of considerable quantities of sheep cheese whey by-product, which currently is under-utilized. In several other countries such as those of the Mediterranean, and the Near and Far East, small ruminant milk production predominates in part due to climate and geographical conditions (Pandya & Ghodke, 2007; Selvaggi, Laudadio, Dario, & Tufarelli, 2014).

Dairy cow milk protein is composed of caseins (~80%), the majority of which are retained in cheese products, and whey (~20%) obtained as a by-product. Beta-lactoglobulin (β-Lg) constitutes a major component of the total whey protein (Tariq, Sameen, Khan, Huma, & Yasmin, 2013). Other major whey proteins include α-lactalbumin (α-La), albumin (Alb), immunoglobulin (Ig), lactoferrin (Lf) and lactoperoxidase (Lp). Glyco(caseino)macropeptide (Gmp) comprising residues 106–169 (cow) and 106–171 (sheep and goat) of κ-casein, is derived by chymosin rennet hydrolysis during cheese making (Manso & Lopez-Fandino, 2004). In addition there is proteose peptone(derived from β-casein by serum plasmin hydrolysis), lysozyme (Lz) and osteopontin.

Whey proteins are well recognized for their health-promoting properties (Krissansen, 2007; Pihlanto, 2011; Tariq et al., 2013), in addition to the bioactivities associated with derived peptide fragments (Mills, Ross, Hill, Fitzgerald, & Stanton, 2011). Lz, Lf and Lp are effective as antibacterial agents and Lf sequesters iron. Uses of these proteins include food fortification and inhibition of bacterial growth in food preparations (Adlerova, Bartoskova, & Faldyna,

Abbreviations: ABC, ammonium bicarbonate; AIEX, anion-exchange; α-La, alpha-lactalbumin; β-Lg, beta-lactoglobulin; CFTII, ceramic fluoroapatite type II; CIEX, cation-exchange; Gmp, glyco(caseino)macropeptide; Ig, immunoglobulin; Lf, lactoferrin; Lp, lactoperoxidase; Lz, lysozyme; MWCO, molecular weight cut off; RP-HPLC, reversed-phase high performance liquid chromatography.

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2008), and they are also potentially useful for application in food antibacterial packaging technology. Gmp being devoid of phenylalanine has application in nutrition for phenylketonuriacs, and several other health-promoting effects have been reviewed, including protection against toxins, bacteria and viruses, and promotion of bifidobacterial growth (Brody, 2000; Taylor & Woonton, 2009; Thoma-Worringer, Sorensen, & Lopez-Fandino, 2006). Igs provide immune support. The major whey protein constituents, α-La and β-Lg, are valued as food additives, primarily due to their gelling and foaming capabilities, and more recently are finding application in production of nanotubes and micro-emulsion encapsulation systems (O'Neill, Egan, Jacquier, O'Sullivan & O'Riordan, 2014; Sullivan, Tang, Kennedy, Talwar, & Khan, 2014).

An established awareness of the value of whey proteins for use in the food, pharmaceutical and chemical industries (Pihlanto, 2011) has resulted in a considerable literature on fractionation of mainly cow whey proteins (Bonnaillie, Qi, Wickham, & Tomasula, 2014; Du, Lin, Zhang, & Yao, 2014; Etzel, 2004; Liang, Wang, Wu, & Zhu, 2011; Perumalsamy & Murugesan, 2012; Rojas & Torres, 2013; Santos, Teixeira, & Rodrigues, 2012; Wu & Xu, 2009). However, many of the fractionation procedures reported have involved the isolation of only one or two of the whey proteins and often only partial purification of the protein has been achieved. In many cases the fractionation procedure has involved one or more of the following: protein precipitation, heat treatment, considerable use of chemicals, use of expensive materials, use of a complex process, all of which are not particularly amenable to industrial scale-up. Whey proteins from different species vary in their physical properties due to differences in amino acid sequence and also in their relative proportions (Ha, Bekhit, McConnell, Mason, & Carne, 2014). Although whey is finding uses in a range of food applications due to gelling, foaming and emulsifying characteristics, it is recognized that the functional properties of un-fractioned whey can be unpredictable due to variations in the composition of whey, as illustrated by the variable effect reported of Gmp on the gelling of whey proteins (Croguennec et al., 2014). Hence, the use of whey proteins in some applications could benefit from sub-fractionation and enrichment of individual whey proteins, while for other applications protein fractionation to high purity may be necessary. A challenge has been to balance level of purification against cost of the processing.

The main whey proteins can be grouped based on those with basic pIs (particularly Lf and Lp) and those with acidic pIs. The acidic pI whey proteins have similar pIs and hence have been more challenging to separate. Ion exchange chromatography provides opportunities to achieve reasonably efficient protein fractionation under mild non-denaturing conditions, can be cost effective and is scalable. Although the use of ion-exchange membrane chromatography technology has shown great promise (Goodall, Grandison, Jauregi, & Price, 2008; Saufi & Fee, 2011), ion exchange column chromatography is still reported to have advantages in terms of binding capacity, cost, and packed column regeneration/cleaning (Santos et al., 2012).

The aim of this study was to develop a simplified procedure for the sequential fractionation of the major sheep cheese whey proteins, under mild conditions that would contribute to the economy of a scaled up process. We therefore evaluated the fractionation of sheep cheese whey proteins that could be achieved with column chromatography using low-cost SP- and Q-Sepharose fast flow resins.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma Aldrich, Auckland, New Zealand, unless otherwise stated. 1D-PAGE electrophoresis materials were from Life Technologies, Auckland, New Zealand. Ion exchange chromatography materials were from GE Healthcare, Auckland, New Zealand.

2.2. Sheep sweet cheese whey sample processing

Sheep sweet cheese whey was obtained as a by-product of cheese manufacture, in which rennet is added to sheep milk (Blue River Dairy Ltd., Invercargill, New Zealand) using pooled milk from pasture fed healthy East Friesian sheep in mid-lactation season. The sweet whey samples were centrifuged (16,000*g*, 30 min, 4 °C) to remove residual lipid (cream) fraction by flotation and to pellet residual casein protein. The clarified whey supernatant was filtered through 6 layers of Calbiochem® Miracloth (Merck-Millipore, USA) and then stored at −20 °C prior to protein fractionation.

2.3. 1D-PAGE

Protein samples were analyzed by 1D-PAGE using a Novex BOLT mini-gel electrophoresis system (Life Technologies, Auckland, New Zealand). Aliquots of protein samples were added to Novex BOLT LDS sample buffer (4×) and Novex BOLT sample reducing agent (10×) according to supplier's recommendations, and heated prior to loading on a Novex BOLT 4–12% Bis–Tris electrophoresis gel. Novex prestained protein standards (Life Technologies) were run in one lane for calibration. After electrophoresis the gels were washed in MQ-water 3 × 5 min and then stained with SimplyBlue SafeStain (Invitrogen, Auckland, New Zealand) according to supplier's instructions.

2.4. Ion exchange chromatography

Aliquots (50 mL) of clarified sheep cheese whey, pre-filtered using a cellulose acetate syringe filter unit (13 mm diam. 0.45 μm) (Axiva, USA) were adjusted to pH 3.8 with 1 M HCl and initially subjected to anion exchange chromatography on two × 5 mL HiTrap Q-FF cartridges linked in series (GE Healthcare), equilibrated in 20 mM sodium citrate, 40 mM NaCl, pH 3.8, installed on an AKTAprime FPLC (GE Healthcare, Auckland, New Zealand). Unbound protein was retained for subsequent HiTrap SP-FF chromatography. Bound protein was eluted from the HiTrap Q-FF with two column volumes of citrate buffer containing 1 M NaCl. The unbound protein from the HiTrap Q-FF was adjusted to pH 7.0 and loaded onto two 5 mL HiTrap SP-FF cartridges linked in series (GE Healthcare), equilibrated in 20 mM sodium phosphate, pH 7.0. The unbound protein eluting from the HiTrap SP column was retained for subsequent HiTrap Q-FF chromatography at a different pH. Bound protein was eluted stepwise with 2 column volumes each of phosphate buffer containing (i) 0.1 M NaCl, (ii) 0.4 M NaCl and (iii) 1.0 M NaCl. The unbound protein from the HiTrap SP-FF chromatography was adjusted to pH 6.5 with 1 M phosphoric acid, then loaded onto the HiTrap Q-FF column equilibrated with 20 mM sodium phosphate, pH 6.5 (buffer A), and the bound protein was eluted with a gradient (0–25%) of buffer B (20 mM phosphate, 0.25 M NaCl, pH 8.0) over 60 min. All buffers were pre-filtered (Advantec cellulose acetate membrane, 47 mm diam., 0.45 μm) (Toyo Roshi Kaisha Ltd., Japan). Fractions were collected and pooled corresponding to the peaks in the chromatograms and the whey proteins in each peak pool were concentrated and desalted using Vivaspin 20 10 kDa molecular weight cut off (MWCO) centrifuge filter units (GE Healthcare) to a final volume of 2 mL, from which aliquots were displayed on 1D-PAGE. Subsequently, the Ig and Lp whey proteins fractionated by ion exchange chromatography were subjected to fluoroappatite chromatography (BioRad, CA, USA).