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Research Paper

Edible films based on milk proteins release effectively active immunoglobulins

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Abstract

Objectives: The goal of this study was to develop novel compositions of edible protein coatings based on immunoglobulin (Ig) fraction from bovine milk. Protein coatings can be used to protect foods against microbial, chemical, and physical damage. We developed novel compositions of edible protein coatings based on immunoglobulin (Ig) fraction from bovine milk. A lot of Ig could be obtained from under-utilized side streams of dairy industry. To the best of our knowledge, such use of the Ig fraction has not been published earlier.
Materials and Methods: Bovine colostral Ig's were incorporated in edible films based on various milk proteins and investigated the characteristics of the films including solubility of Ig's and nisin and on technological properties of films. Ig's specific to cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus* were produced to colostrums by hyperimmunizing cows before parturition.
Results: The milk Ig rich fraction suited well as a component of milk protein films. The Ig's dissolved from the films very rapidly. Nisin, commoly used for food protection, was used as a model of antimicrobial peptide. Nisin was released biologically active from both β-lactoglobulin (β-Ig) and β-Ig/Ig films. Nisin exerted its bactericidal effect at clearly lower concentrations in the β-Ig/Ig films when compared with β-Ig film. Nisin also retained its activity better in film containing Ig-enriched whey. Incorporating Ig-enriched whey into films enhanced adhesion and tensile strength of the films. The Ig-enriched whey also affected strongly on the appearance of films based on commercial whey protein concentrate in a dose-dependent way by making the films more smooth, transparent, and clear which are all favoured properties in most food and pharmaceutical applications.
Conclusions: Biologically active Ig's can be successfully incorporated in and released from milk protein based edible films. The content of Igs in films affected considerably technological properties of these films. Composition of other proteins in films had effect on preservability and release of Igs.

Key words: edible films; immunoglobulins; whey proteins; β-lactoglobulin; caseinates; nisin; dental caries.

Introduction

Edible films and coatings composed of naturally occurring food compounds such as proteins, polysaccharides, lipids, and their combinations have aroused a great interest during the last two decades. Coatings can serve as barriers against moisture, gases, aromas, and microbial contamination or give mechanical protection. In specialized food products and pharmaceuticals, edible films may also contain and release biologically active compounds such as antibiotics and antioxidants and are thus specified as active films (Cuppett, 1994; Thatiparti et al., 2010; Espitia et al., 2014; Beristain-Bauza et al., 2016; Alkan and Yemenicioglu, 2016).

To the best of our knowledge, milk immunoglobulin (Ig) fraction has not yet been used as a structural component or as a biologically active substance in edible films. Ig's can be effectively enriched in a large scale from, e.g., cheese whey. Bovine colostrum is not commercially used in most countries. In the first milkings *post partum*, the Ig contents are as high as 20–200 g/l which is about 100 times higher than in normal milk (~0.5 g/l) (Korhonen et al., 2000). A lot of milk Ig's could be obtained from dairy industry side streams. As structural component, the milk Ig fraction could be useful in modifying technological properties of coatings and films. Other milk proteins are frequently studied as promising materials for edible films and coatings because of their good water solubility, emulsifying, and gelation properties (Gennadios et al., 1994; Gounga et al., 2010; Schmid et al., 2015). Milk protein–based films are potent barriers of oxygen and carbon dioxide, but have high permeability to water vapour (McHugh and Krochta, 1994). Whey proteins are globular in shape, and thus, heat denaturation is essential for the film formation. Heating unfolds globules to a random coil conformation (Brown, 1988) and promotes the formation of intermolecular covalent disulphide bonds (McHugh et al., 1994). The addition of plasticizers, often polyols, into whey protein films is necessary, because otherwise films will be brittle. It is possible to regulate tensile strength and elongation properties of films by varying plasticizer concentrations (Myllärinen et al., 1997).

Biologically active substances have been incorporated into films made of various materials such as milk proteins, caseins and whey proteins, corn, wheat, soy and fish proteins, polysaccharides and their derivatives (starch, cellulose, agar, alginate, and chitosan), and protein-polysaccharide composite films. As to other active substances naturally present in milk, Barbiroli et al. (2012) succeeded to incorporate lysozyme and lactoferrin into paper containing carboxymethyl cellulose. The released proteins retained their antimicrobial activity and displayed a synergistic action against *Listeria*.

If milk Ig's which are specific against spoilage microbes or pathogens could be incorporated and released from film materials in biologically active form, then such materials could be used in products for passive immunization against harmful microbes. Passive immunization with bovine milk or colostral Ig's, i.e. oral administration of specific antibodies, has proven effective in prevention of various orally mediated microbial infections [for reviews, see Korhonen et al. (2000) and Lilius and Marnila (2001)]. Hyperimmune milk or colostral preparations containing high concentrations of specific antibodies against cariogenic streptococci have been studied in local passive immunization for prevention of dental caries [for a review, see Koga et al. (2002)] because humans cannot be immunized actively against caries bacteria due to risk of cross-reactions of antistreptococcal Ig's with human tissues. Orally administered Ig's have diminished the colonization of mutans streptococci in animal models (Van Raamsdonk et al., 1993) and in humans (Shimazaki et al., 2001) and the development of caries (Otake et al., 1991; Mitoma et al., 2002) in experimental rat models.

We have previously made bovine colostral immune preparations (IPs) containing specific antibodies against *Streptococcus mutans* and *Streptococcus sobrinus* (Loimaranta et al., 1997, 1999a). The specific antibodies in our IP inhibited *in vitro* the activities of glucosyltransferase and fructosyltransferase enzymes produced by these bacteria. The enzymes form extracellular polysaccharides needed in adherence of bacterial cells to tooth enamel (Loimaranta et al., 1997). The IP also had a synergistic effect with lactoperoxidase–hypothiocyanate–hydrogen peroxide system (Loimaranta et al., 1998a), inhibited adherence of bacteria to saliva-coated hydroxyapatite particles simulating tooth enamel, promoted aggregation of *Str. mutans* (Loimaranta et al., 1998b), and supported phagocytosis and killing of mutans streptococci by human leukocytes *in vitro* (Loimaranta et al., 1999b). When the IP used in the present edible film study was applied as a mouth rinse by adult volunteers for 3 days, it resulted in a higher resting pH in dental plaque and decreased the relative number of mutans streptococci significantly in the test group when compared with the control group (Loimaranta et al., 1999a). Thus, controlled release of specific anti-caries Ig's from lozenges or mouth care products could provide feasible novel ways in caries prevention.

One of the frequently studied antimicrobial compounds incorporated in edible films is nisin. Its antimicrobial activity has been widely studied, alone or in combinations with other active compounds, in different film materials such as whey proteins (Pintado et al., 2009, 2010), sodium caseinate (Cao-Hoang et al., 2010), and chitosan (Ce et al., 2012). Nisin is licensed as GRAS, and in the European Community, it is defined as a safe preservative for food contact, coded as E234 (FSA, 2010).

The objectives of this study were to test the effects of adding colostral Ig fraction into caseinate and whey protein–based films on solubility of main proteins, and on release of specific Ig's in the IP used in the above study by Loimaranta et al., (1999a). Also technological properties of the films were observed. Nisin was applied as a model antimicrobial substance to represent an additional biologically active compound in a whey protein film.

Materials and Methods

Production of the colostral immune and control antibody preparations

To prepare the colostral whey immune preparation (IP), 83 pregnant Friesian cows were immunized with a formalin-killed *Str. mutans* ATCC 25175 (serotype c)/*Str. sobrinus* ATCC 33478 (serotype d) (1:1) vaccine with Al(OH)3 adjuvant as described in Loimaranta et al. (1999a). The first four milkings after calving were collected and frozen. For control preparation (CP), colostra were also collected from nonimmunized cows. The Ig's were enriched in dairy factory by using chromatographic and membrane technologies. Ig concentrates were lyophilized to obtain CP and IP powders. (For details see Supplementary Text S1, online supplementary material.)

Whey protein fraction enriched with β-lactoglobulin

Whey protein fraction enriched with β-lactoglobulin (WBlgFr) was prepared at MTT (now Luke). WBlgFr, originated from bovine Edam cheese whey, was manufactured according to the modified method of Pearce (1983). Lipoproteins were removed by precipitation and microfiltration: $CaCl_2$ and NaOH were added and pH was adjusted to 7.3. The solution was heated to 73°C for 12 s and cooled down to 30°C. The final step was microfiltration (APV filter 0.22 µm). The permeate was ultrafiltrated (9000 Kd cutoff) to gain protein-enriched whey. pH of the retentate was adjusted to 3.6 and it was incubated at 55°C for 30 min. Under these conditions, α-lactalbumin (α-la) became precipitated and was removed by centrifugation (3000 x *g* for 20 min). β-lactoglobulin (β-lg) remained in the solution, was harvested by concentrating (10 000 Kd cutoff) and diafiltering, and was finally freeze-dried. (For protein composition, see Supplementary Table S2, online supplementary material.)

Commercial milk protein preparations

Two commercial whey protein concentrates were used. WPC-75 was obtained from Juusto Kaira Oy, Finland. DSE1908 was manufactured by New Zealand Milk Products. Sodium caseinate was supplied by Kainuun Osuusmeijeri, Finland. (For the protein

compositions, see Supplementary Table S2, online supplementary material.)

Other reagents

Nisin from *Lactococcus lactis* was acquired from Sigma (N-5764, 2.5%, specific activity ≥1 000 000 UI/g). Glycerol was supplied by Oriola, Finland. Water used in film-forming solutions and in reagents was ultrapure water purified by a Milli-Q Integral Water Purification System (Merck Millipore, Darmstadt, Germany). Other reagents are specified in the methods at issue.

Determination of total soluble protein, α-lactalbumin, β-lactoglobulin, IgG, and antibody titres

The total protein contents of IP and CP and DSE1908 were determined by the Kjeldahl method (IDF 1986). IgG, α-la, and β-lg contents were determined with a fast protein liquid chromatography (FPLC) method with Superdex 200 HR filtration column (Pharmacia Biotech., Uppsala, Sweden) by using 50 mM Tris-HCl buffer containing 0.15 KCl (pH 7.8) as described by Syväoja and Korhonen (1994). For determination of α-la and β-lg contents of WBlgFr and WPC75 powders, FPLC with column Mono Q HR 5/5 (Amersham Biosciences, NJ) connected with Waters 2487 detector (Waters, Milford, MA) was used as described in Humphrey and Newsome (1984). The whey proteins were fractionated with piperazine buffer (pH 6.5) in NaCl gradient.

In solubility and storage tests soluble protein, α-la, β-lg, and IgG contents were measured from the water flushed over the films in the Petri dishes. Total protein was measured by the Kjeldahl method. α-la and β-lg contents of films containing DSE 1908 and IP were measured by FPLC using the column Mono Q HR 5/5 (Amersham Biosciences, NJ) as described above. α-la and β-lg contents of films containing sodium caseinate and IP were measured by FPLC using the column Superdex 200HR as described above. The titres of *Str. mutans*-specific colostral IgG antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) as described by Loimaranta et al. (1997). The antibody titres were determined as a cut-off value at 0.5 OD_{405} using the IP as a standard.

Composition of colostral IgG preparations

The dry weight of the IP was 98.5% (w/w), total protein content 89.75%, fat 1.15%, galactose 0.08%, glucose 0.17%, lactose 0.36%, and ash 2.70% (w/w). IgG content was 52% (w/w), α-la 5%, and β-lg 16% (w/w) of total proteins. The rest of protein comprises probably peptides, casein, and other whey proteins.

The specific IgG titres of the IP were for *Str. mutans* 2 900 and for *Str. sobrinus* 650 as measured from 5% (w/v) of preparation in water with ELISA. In CP, the titres were <50 both for *Str. mutans* and *Str. sobrinus*. The composition of control preparation was essentially the same as in IP (Loimaranta et al., 1999a). The most prevalent Ig class in bovine colostrum is IgG, comprising over 80% of the total Ig's (Mehra et al., 2006).

Films and methods for solubility and storage tests

Solubility of Ig's from films and coatings was tested using IP in combination with different whey protein preparations or with sodium caseinate as a film-forming material. The whey protein preparations were a fraction enriched with β-lg (WBlgFr) and a commercial whey protein concentrate DSE 1908. One important difference between them was the proportion of β-lg, an effective film-forming agent:

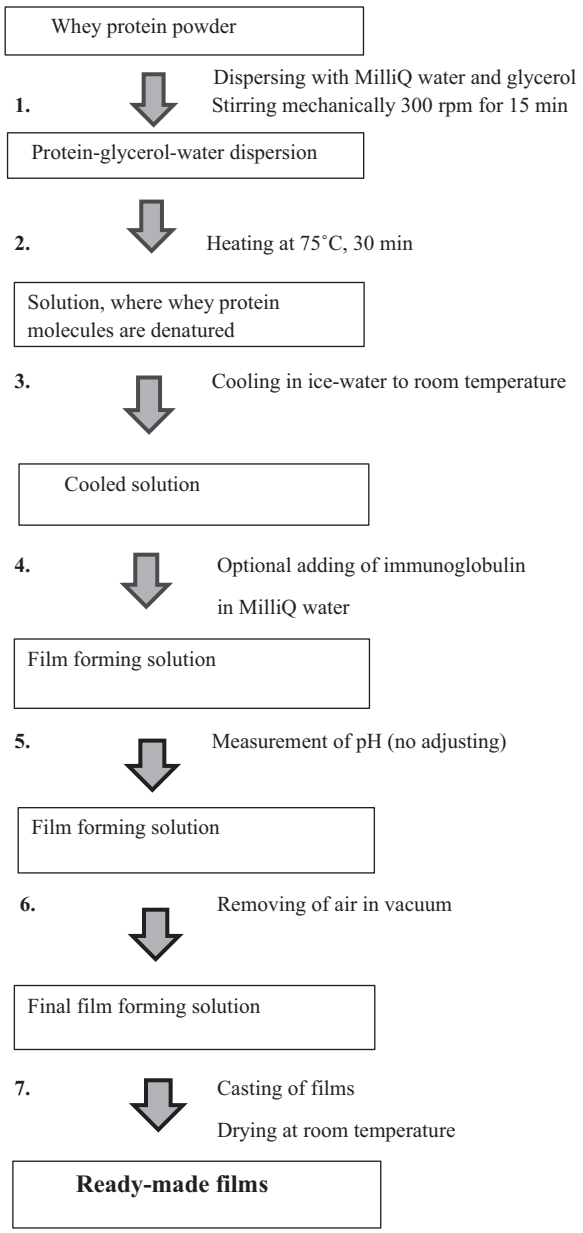


Figure 1. Main preparation steps of whey protein and immunoglobulin films.

in DSE 1908 62% and in WBlgFr more than 90% (Supplementary Table S2, see online supplementary material). The main steps of film preparation are presented in Figures 1 and 2. Heating procedure of whey protein concentrates (75°C for 30 min) was accomplished according to Banerjee and Chen (1995). The procedures of the tests are explained in detail in Supplementary Text S3 (see online supplementary material). The protein preparations used and the measured compounds are briefly described below. In all the film- and coating-forming solutions below, the protein and glycerol contents were 8% (w/v):

1. Protein preparations in films were DSE 1908 and IP. The IP consisted 22.2% (w/v) of the total protein. Films were dissolved in MilliQ water. In addition to total nitrogen and the titres of *Str. mutans*-specific Ig's, also dissolving of α-la, β-lg, and total Ig's was measured (Figure 4a–4d).

2. DSE 1908 and IP. IP consisted 20% of the total protein. The films were incubated in human saliva. Scanning electron microscope pictures were taken before incubation and during the process (Figure 5a–5c).
3. DSE 1908 and IP. As above, IP consisted 20% (w/w) of protein. Pieces of films were immersed in simulated saliva consisting of saline solution (0.9%) containing amylase 100 U/ml and 1 mM $CaCl_2$. Dissolved *Str. mutans*-specific Ig's were determined (Figure 6).
4. Sodium caseinate and IP. IP consisted 22.2% of the total protein content. Films were dissolved in MilliQ water. The Ig's, total nitrogen, α-la, and β-lg were determined (Figure 7a–7d).
5. WBlgFr and IP. The final concentration of protein was 8% (w/v) as before, but a growing gradient of IP from 0% to 100% of total whey protein was tested. Films were dissolved in MilliQ water. The solubility of active *Str. mutans*-specific Ig's and total nitrogen was determined (Figure 8a and 8b). (For the ratios of WBlgFr and IP in different film preparations, see Supplementary Table S4, online supplementary material.)
6. Films containing (a) IP and DSE 1908 and (b) IP and sodium caseinate, storage/solubility test. IP consisted 22.2% of the protein concentrations. The time points for analyses were 0 and 7 days and 5 weeks. Films were dissolved in sterilized Ringer solution and the active *Str. mutans*-specific Ig's were determined (Figure 9a and 9b).

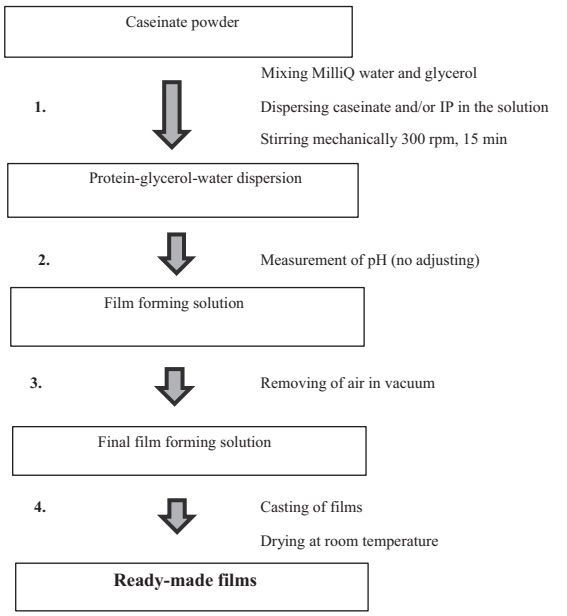


Figure 2. Main steps in preparation of caseinate and immunoglobulin films.

Preparation of films for measuring technological properties

Tensile strength, elongation at the break, as well as stickiness and adhesiveness were tested from films composed of either sodium caseinate and IP or of whey protein concentrate WPC-75 and IP. Both protein and glycerol concentrations in the film solutions were 8%. Films made of sodium caseinate and IP were prepared according to the procedure in Figure 2 so that 20% of the protein originated from IP and 80% from sodium caseinate. No heat treatment was included in the procedure. Three different films were made of WPC-75 and IP according to the procedure in Figure 1. The ratios of IP, IgG, and other whey proteins in films as well as details of manufacturing the

Figure 3. Films with various WPC-75 and IgG contents cast on Petri dishes ready for measurement of tensile strength and the elongation at the break (left). Films were fastened in materials testing machine by pipette tip (right).

films are presented in Supplementary Table and Text S5 (see online supplementary material).

For testing the tensile strength of the films, an attachment point was needed. For this, 1 ml pipette tips were put to stand on the bottom of dishes (Figure 3, left). Tips adhered in the film during hardening and were then used for attaching the mechanical sensor (Figure 3, right). All films were dried in a conditioned room at 20–22°C and the RH 50%–55% for 2 days.

Solubility of total protein, α-lactalbumin, β-lactoglobulin, and IgG

For solubility tests, the dried films were weighed and flushed with selected solvents. Solvents used were saliva, simulated saliva [saline solution (0.9%) containing amylase 100 U/ml and 1mM $CaCl_2$], sterilized water, or Ringer solution [made by dissolving Ringer tablet (1.15525.0001 by Merck KGaA Darmstadt, Germany) to MilliQ water]. The typical procedure was as follows. The dry films were weighed (a typical weigh about 1.6 g) and sterilized water was pipetted at the room temperature on each film the volume that was 4 times the weight of the film (a typical volume about 6.2 ml) so that the film material content was 20% (w/v) when the film was completely dissolved. Petri dishes with films were placed in a rocking table (4RT, Luckham) turning dishes to different directions in a few-second intervals. The whole water solution on films was poured into Falcon tubes at indicated time points after adding the water. Samples were taken typically at time points 1, 3, 10, and 30 min after adding the solvent. Exceptions for this general procedure are described in Materials and methods 'Films and methods for solubility and storage tests'. Contents of total protein, α-la, β-lg, IgGs, and the titres of *Str. mutans*-specific antibodies were measured as described earlier.

Incorporation of nisin into films and determination of its antimicrobial action

A *Lactococcus lactis* MG1299 strain was used as a test organism to measure bactericidal effect of nisin with the agar diffusion technique (Wolf and Gibbons, 1996). Feasibility of agar diffusion method in measuring antibacterial effect of nisin against *L. lactis* was tested. The diameters of inhibition areas were dependent on concentration of nisin dissolved in Ringer. The control did not inhibit growth (data not shown).

The activity of nisin was measured in films composed of enriched β-lg fraction (protein content 76.4%) and in films composed of enriched β-lg fraction and IP (50% and 50% w/w as protein basis). The solution containing whey protein (WBlgFr) and IP was prepared as described in Figure 1. The pH of the final solution was 6.6. The whole solution was shared into smaller portions where after nisin was added in concentration of 0, 10, 20, 40, and 80 mg/100 g (w/w). Four films were cast per every nisin concentration. Films were air dried at the room temperature (22°C) for 3 days the RH being 23%.

The activity of nisin in β-lg film was tested immediately after drying and after the storage of the film for 1, 7, and 13 days at room temperature. Discs in diameter of 6 mm were cut out of the films and they were set on M17 agar plates seeded with *L. lactis*. Plates were incubated at +30°C for 2 days. The antimicrobial effect of nisin was seen as a clear area without bacterial growth around the disc. Tests were made in duplicates. The activity of nisin in films containing both β-lg and IP (see above) was tested. The testing arrangement was the same as with β-lg film except that the storage times were 0, 1, 9, and 16 days.

Tensile strength of films

The films containing only commercial WPC-75 were too brittle for measuring tensile strength by traditional attachment methods like ISO 1184–1983 (E). Therefore, the tensile strength was measured using the films cast together with pipette tips as described above (2.4.2.). The measurements were made by a materials testing machine (Lloyd LR 10K, Fareham, England). The pipette tip was fastened to the grips of load cell and the dish was tightly kept on the table (Figure 3). The pipette tip was drawn apart from the film using the speed of 100 mm/min. The force used was monitored as well as the changes of the force during the drawing, so called 'drawing profile'.

The tensile strength of sodium caseinate/IP (80%/20% of total protein, respectively) films was measured using the method ISO 1184–1983 (E). Measurements were done as seven parallels. A 75 mm long bone-shaped piece was cut out of the film. The piece was fastened to the materials testing machine (Lloyd LR 10K, Fareham, England) and was drawn so long that the structure was broken. The tensile strength was expressed as megapascals (MPa) at the time point of breaking.

Effect of IP on adhesiveness of films

Stickiness and adhesiveness of edible films are properties which have no established determination methods, as far as we know. The effect of Ig content on adhesiveness of the films was demonstrated by adding nonpareils (3.5 g) or silver granules (9.0 g), usually used for decorating a cake, on Petri dishes embedded with film in the bottom. The control films were made of only commercial WPC-75 and in test films 50% or 100% of WPC-75 was replaced with IP (see Supplementary Table S5, online supplementary material). The dishes were closed by a lid and turned around for 30 s. The nonpareils or silver granules, which were loose, were removed and dishes were weighed and photographed. The weights of the particles adhered in the films were measured. The test was performed on days 2 and 7 after casting the films.

Results and Discussion

Solubility of immunoglobulins and other proteins

As shown in the Figures 4a–4d and 6, the release of all detected proteins began immediately after addition of water on films. α-la and total IgGs were released linearly during 30 min, but β-lg followed two phase kinetics (Figure 4b and 4c). It is possible that during the first minutes β-lg molecules, which have not formed covalent bindings, were transferred to water. After 30 min, 14% of the total IgGs had dissolved.

Scanning electron microscope pictures were taken of IP/DSE 1908 films (Figure 5a–5c) in order to visualize the fine structure and dissolving process. On the basis of Figure 5a and 5c, it is obvious that human saliva is effective in dissolving the films. The structure of film had decomposed as if pieces of film surface would have broken away. It suggests that film consists of areas, where different proteins are dominant. In cross section and in surface of partly dissolved film, it seems that the film consists of globular proteinicious structures.

Figure 6 presents the test, where dissolving of active immunoglobulins has been monitored for 60 min. The rate of dissolving diminished clearly after 20 min. The films were stored for 10 days at RT before the test. This storage time did not destroy the activity of immunoglobulins or prevent their release from the film.

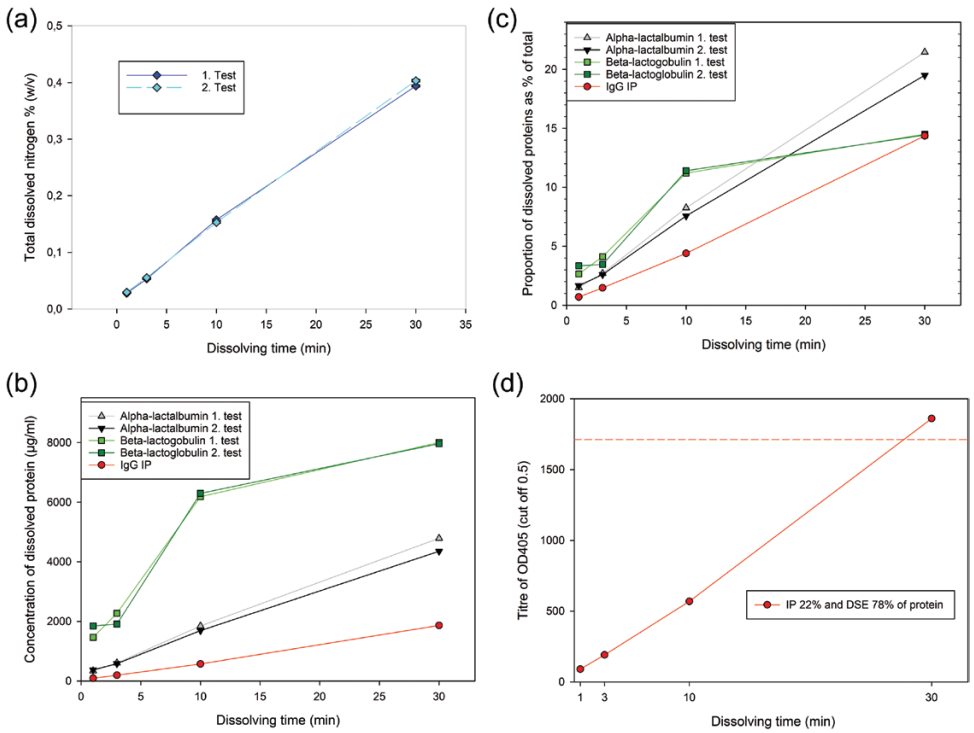


Figure 4. (a–d) Film containing IP and DSE 1908, solubility test. The main steps of film solution making are described in Figure 1 (see composition, preparation, and dissolving in 2.4.1. a). Two parallel film solutions were prepared and from both solutions; three parallel films were dissolved for each time point and pooled. The solubility of active Ig's (the titres of *Str. mutans*-specific colostral antibodies, ELISA), total Igs, total nitrogen, α-lactalbumin, and β-Ig was determined.

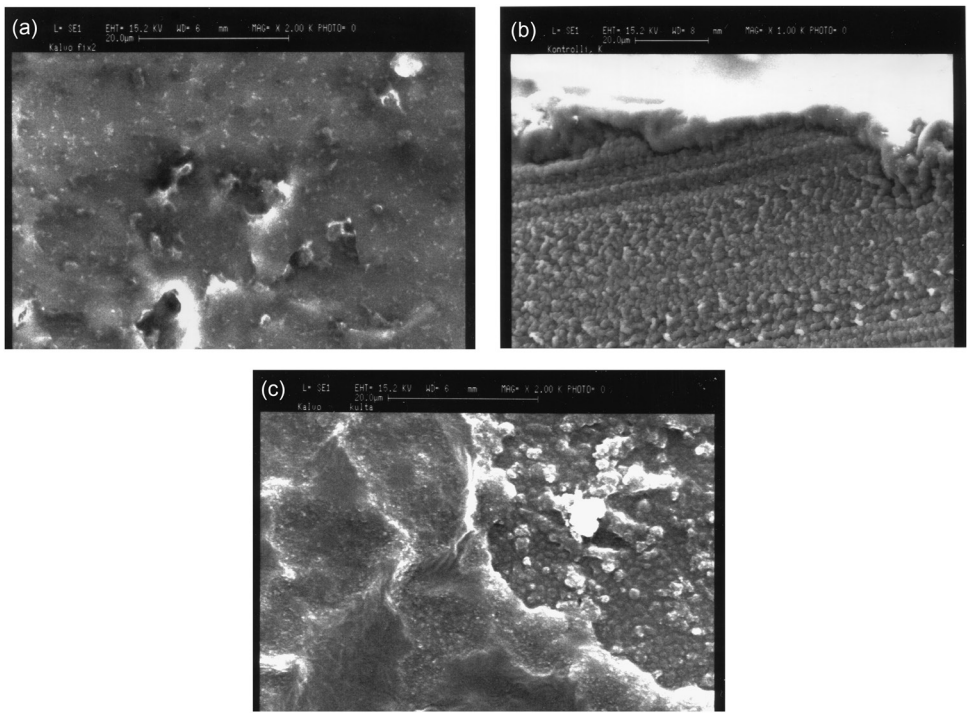


Figure 5. (a–c) Scanning electron microscope pictures of gold molds made from IP/DSE 1908 films before (a and b) and during (c) dissolving in human saliva *in vitro* (see composition, preparation, and dissolving in 2.4.1. b). The line segments 20 µm in upper part of pictures present 20 µm. (a) shows the surface of the film before dissolving (magnification × 2000). (b) shows the cross section of film before dissolving (magnification × 1000). In (c), the surface of film is seen after dissolving for 30 min at 37°C in human saliva (magnification × 2000).

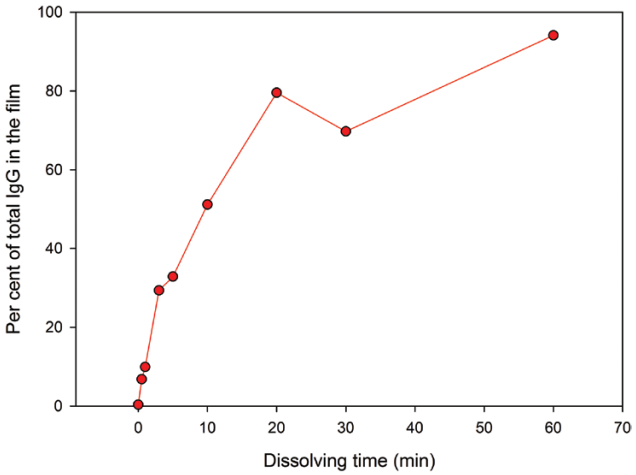
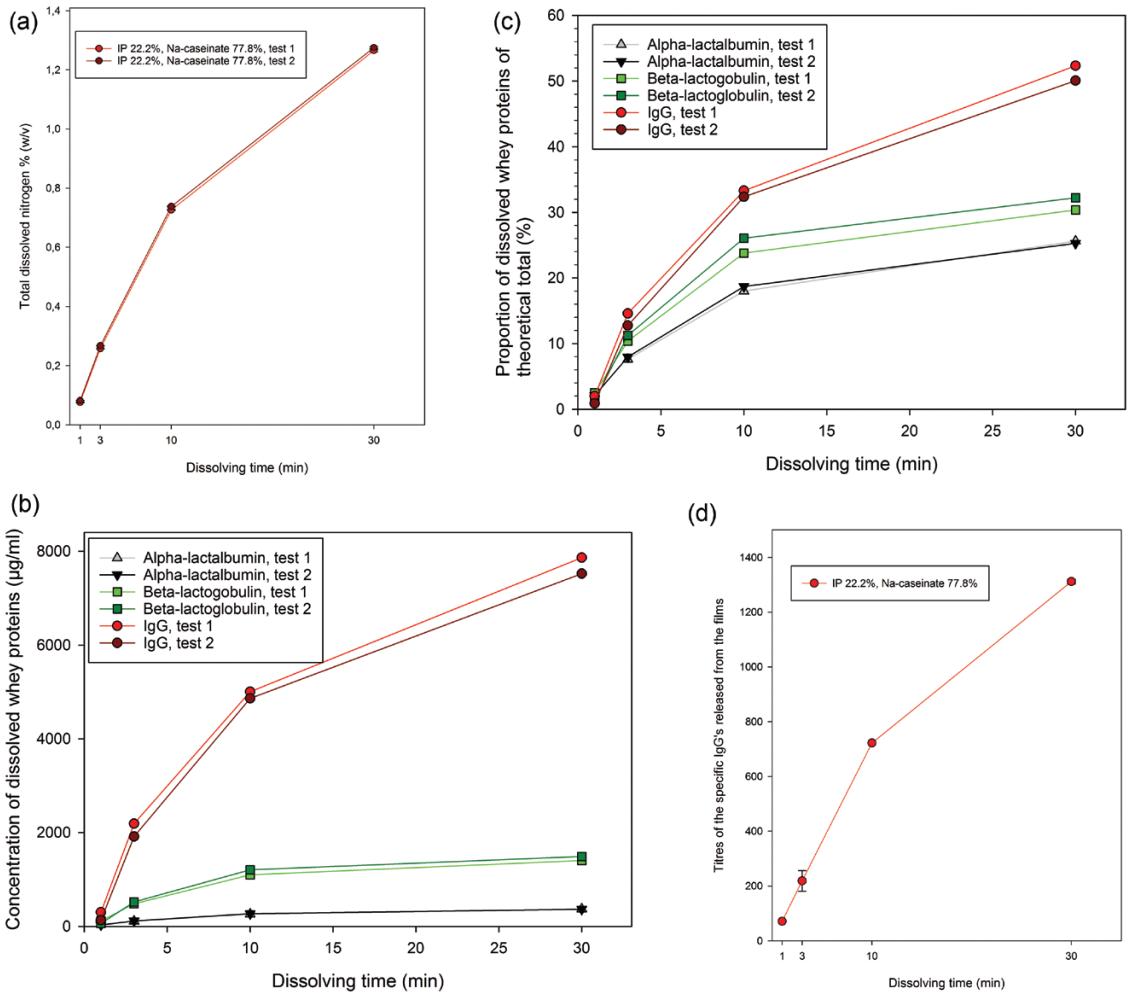


Figure 6. Film containing IP and DSE 1908 (stored at room temperature for 10 days, $n = 1$), solubility test (see composition, preparation, and dissolving in 2.4.1. c). The titres of dissolved *Str. mutans*-specific colostral active immunoglobulins were determined by ELISA.

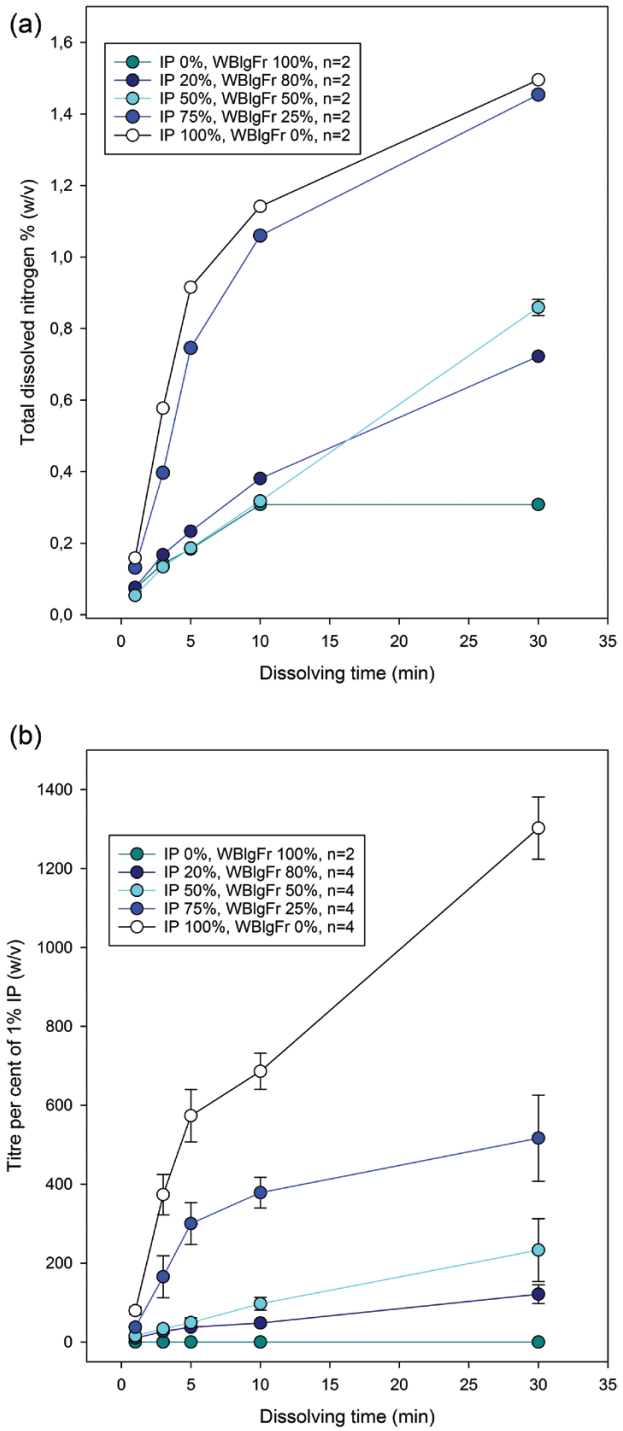
The behaviour of Igs in whey protein and caseinate films seems different from the basis of the results presented in Figures 4c and 7c. Fourteen percent of total Ig was released of whey protein films in 30 min while even 50% was dissolved of sodium caseinate films at the same time. This result is in agreement with Elzoghby et al. (2011). In their review, it was concluded that casein-based formulations are excellent for release of various bioactive substances.

Figure 8a displays that dissolving of nitrogen was accelerated progressively when the portion of immunoglobulin fraction in the film material was higher than 50%. It seems that in films containing high concentration of Igs, the dominant bonds were more easily loosened and more rapidly dissolved than in a typical β-lg containing film or less covalent disulphide bonds were originally formed. This result can be important regarding the release of various biologically active compounds from edible films.

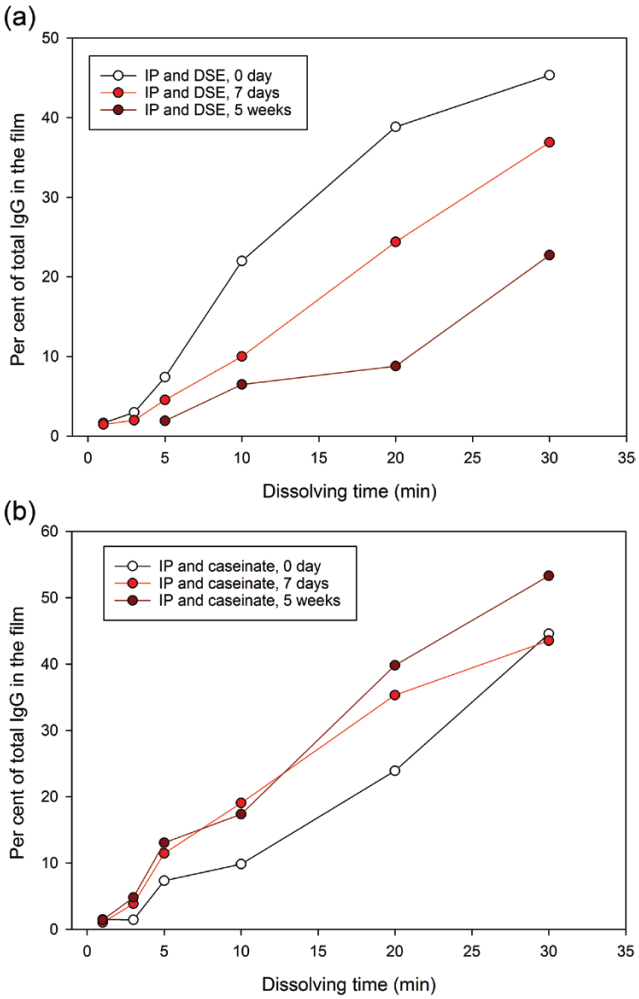
In whey protein films (Figure 9a), the solubility of Ig's from the films decreased in the course of storage time. After 5 weeks of storage, the dissolving velocity in Ringer had declined to about half when compared with the sample of day zero after 30 min dissolving. On the other hand, in sodium caseinate films, this kind of trend was not seen (Figure 9b). Schmid et al. (2015) concluded that cross-linking of



Figures 7. (a–d) Films containing IP and sodium caseinate, solubility test (see composition, preparation, and dissolving in 2.4.1. d). Two parallel solutions were prepared. In each time points, four films of both solutions (test 1 and 2) were dissolved and liquids pooled. The active Ig's, total nitrogen, α-lactalbumin, and β-lactoglobulin were determined.



Figures 8. (a and b) Films containing different proportions of IP and whey protein fraction enriched with β-lactoglobulin (WBIgFr), solubility test (composition, preparation, and dissolving in 2.4.1. e). The solubility of total nitrogen (a) and active *Str. mutans* specific Igs (b) was determined. Error bars represent standard deviation of *Str. mutans*-specific Ig titres (Elisa) between four parallel films in each time point (only two in WBIgFr 100%).



Figures 9. (a and b) Films containing (a) IP and DSE 1908 and (b) IP and sodium caseinate, storage/solubility test (composition, preparation, and dissolving in 2.4.1. f). Three (DSE 1908 and IP) and four (sodium caseinate and IP) films were dissolved in day 0, next 3 and 4 films after 7 days, and 3 and 4 more after 5 weeks, respectively. Liquids from parallel films of each time points were pooled. The active *Str. mutans*-specific Ig's were determined by ELISA.

proteins by disulphide and hydrogen bonds are the most important bonds forming soon after casting whey protein isolate–based films. Later the importance of hydrogen bonds increases. This could explain the results in Figure 9a and 9b. During storage the amount of bonds which keep Ig's tightly in film could increase. This prevents dissolving of Ig's. Elzoghby et al. (2011) presented advantages of casein in delivering biologically active compounds. For instance, casein is able to interact with other macromolecules in forming complexes and conjugates with them. These complexes may have new synergistic properties. Casein can also protect other more sensitive molecules. This property can be used to improve the bioavailability of active components. Results shown in Figure 9b are consistent with that interpretation of the advantages of casein.

In the solubility tests presented above, the films were easily dissolved in water, Ringer (physiological buffer solution), simulated saliva, and real human saliva. The specific Ig's were released as measured by ELISA. The sandwitch ELISA system requires specific binding of ab-parts in antibodies into bacterial antigens and binding of secondary monoclonal antibody to the intact Fc-part of the dissolved antibody. Thus, the bovine milk Ig's were still in active form.

When the effects of storage on Ig release were compared between whey protein and caseinate films, the preservability was better in caseinate-based films. Higher proportion of Ig's increased solubility (Figure 8a). In all our solubility experiments, the surface area/mass ratio was high. It is possible to influence on the release kinetics by modifying the composition and shape of material (the ratio of surface area/mass).

Activity of nisin in β-lactoglobulin and β-lactoglobulin/immunoglobulin films

Nisin is a bacteriocin, produced by some strains of *Lactococcus lactis*, effective against several Gram-positive bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* (Ko et al., 2001). By chemical structure, nisin is a hydrophobic polycyclic peptide with 34 amino acid residues and molecular weight of 3500 Da.

Feasibility of agar diffusion method for measuring nisin activity was tested. Nisin dissolved in Ringer inhibited the growth of *L. lactis* on agar which was seen as a clear area around the wells (data not shown).

Nisin activity in β-lg film is shown in Table 1. The smallest nisin concentration showing inhibition was 400 µg/g of film-forming solution. When the storage period of the film was extended, the inhibition area decreased. Nisin in the films kept most of its antibacterial activity during the 13 days storage period. The antibacterial activity of nisin seemed to be higher in β-lg/IP–based films than in β-lg–based films (Tables 1 and 2). In β-lg/IP films, the inhibition areas appeared in remarkably lower nisin concentrations than in the β-lg films. The activity of nisin seemed to keep better in Ig-containing films.

The results suggest that Ig's might, as a matrix, enable higher activity of many types of bioactive compounds. On the other hand, nisin may have some special properties which improve its activity in Ig-containing films. Dawson et al. (2003) showed that protein type affected the release of nisin, when they compared wheat gluten and corn zein films, the release being higher than wheat gluten film. Redl et al. (1996) suggested that the strong hydrophobicity of protein causes a higher retention of nisin because also nisin is hydrophobic. Rossi-Márquez et al. (2009) studied the release of nisin from whey protein isolate edible films to aqueous solution and its antimicrobial efficacy against *Brochotrix thermosphacta*. Because of the high pI value of nisin (pI ~ 10), its diffusion was favoured at low pH when the experimental pH area was 7–4. Nisin has a positive net charge below pH10, whereas whey proteins have negative net charges until their pI values (α-la 4.2, β-lg 5.3, and IgG1 5.5–6.8). At pH 4, whey proteins have also positive net charge and the repulsion forces between nisin and other proteins improve diffusion of nisin. In our experiment (Tables 1 and 2), the pH of the film solution was altered from 6.5 to 7.2, when the Ig-content in the film was increased. The most prevalent protein in bovine colostral whey is IgG1 which has pI value at 5.5–6.8. When the portion of Ig in the film increased, antibacterial effect of nisin increased as well. It seems that in this case the enhanced activity of nisin cannot be explained by the difference of pI values but by the change of protein composition and hence the structure of the film. In the heat denaturation of whey proteins, intermolecular disulphide bonds are formed leading to polymerization. The Ig fraction was kept at room temperature in order to preserve its activity. Therefore, the bonds formed were weaker than in the heated fraction. In these conditions, nisin was also more weakly attached to the film matrix than in the β-lg film.

Tensile strength and appearance of films

When the tensile strengths of the WPC-75–based films containing different amounts of Ig's were compared with each other (Table 3), the lowest values (1.3 ± 0.3 N) were measured in the film 1, which did not contain IP. The highest tensile strength (10.0 ± 1.6 N) was observed in the film no. 3, which was prepared of 100% IP. Thus, its tensile strength was 7.7-fold compared with the film no. 1. The film no. 3 also had the highest elongation at the break. The tensile strength of sodium caseinate/immunoglobulin films was 1.93 ± 0.16 MPa measured as a mean of seven films. Ko et al. (2001) found that nisin increased significantly tensile strength of whey protein isolate film and thereby adding nisin in the IP film could increase the tensile strength even more.

Effect of IP on adhesiveness and appearance of films

Two days after casting of the films (Table 4), the nonpareils or the cake granules did not adhere to the control film at all, but adhered immediately and covered all the free surfaces of films containing both 50% and 100% IP. Seven days after casting, the adherence of films containing 50% of IP to the particles had decreased but 100% IP films were still adhesive (Supplementary Figure S6, see online supplementary material for a colour version of this figure). The weights of the nonpareils and the cake granules attached to the film surfaces are presented in Table 4.

Incorporating IP into WPC-75 films also affected strongly on their appearance in a dose-dependent way. Films made of WPC-75 were turbid and the surface was grainy. Replacing 50% or 100% (w/w) of WPC-75 with IP made the films smoother and more transparent (Figure 3) which are favoured properties in most food and pharmaceutical applications. The presence of Ig's during the cooling may prevent uneven aggregation of other compounds.

Potential applications of edible immunoglobulin containing films

In most published studies, bovine hyperimmune milk preparations are reported to be effective in the prevention of various orally mediated microbial gastroenteric infections [for reviews, see Korhonen et al. (2000), Lilius and Marnila (2001), Hurley and Theil (2011), and

Table 1. The effect of concentration and storage time on the activity of nisin against *Lactococcus lactis* MG1299 as measured using agar diffusion method

The area of inhibition ring (mm²)*				
Nisin concentration in film solution	Film 0 days	Film 1 day	Film 7 days	Film 13 days
0 µg/g	0	0	0	0
100 µg/g	0	0	0	0
200 µg/g	0	0	0	0
400 µg/g	74.0 ± 6.1	71.8 ± 6.7	63.6 ± 0	60.1 ± 0.7
800 µg/g	113.9 ± 13.7	84.2 ± 4.0	75.0 ± 9.9	74.7 ± 3.8

Nisin was incorporated in β-lactoglobulin films.

*The mean inhibition area of two plates ± minimum and maximum are presented.

Table 2. The effect of concentration and storage time on the activity of nisin against *Lactococcus lactis* MG1299 as measured using agar diffusion method

Area of inhibition ring (mm^2)*				
Nisin concentration in film forming solution	Film 0 days	Film 1 day	Film 9 days	Film 16 days
0 µg/g	0	0	0	0
100 µg/g	58.8 ± 2.0	63.6 ± 0.7	0	0
200 µg/g	86.7 ± 6.6	84.7 ± 6.9	59.8 ± 0.3	68.3 ± 0.3
400 µg/g	92.9 ± 4.7	105.7 ± 1.8	77.7 ± 0.8	90.8 ± 2.5
800 µg/g	125.2 ± 6.4	131.7 ± 1.0	100.7 ± 0.9	119.3 ± 0.4

Nisin was incorporated in the film containing β-lactoglobulin and immunoglobulin-enriched whey protein fractions (50%/50% protein basis).
*The mean inhibition area of two plates ± minimum and maximum are presented.

Table 3. The tensile strength (mean ± *SD*) and the elongation at the break of the whey protein (WPC-75) films with and without added immunoglobulin fraction (IP)

Film	IP* (%)	IgG (%)	Analysed films (n)	Force at break (N)	Elongation at break (mm)
1.	0	0	2**	1.3 ± 0.3	0.2 ± 0.04
2.	50	26	6	6.5 ± 0.9	1.1 ± 0.7
3.	100	51	5	10.0 ± 1.6	1.4 ± 0.2

The force is displayed as Newton (N).
*where of 51% is IgG
**The film was so brittle that a pipette tip broke apart when the film was installed in the materials testing machine. Therefore, results of only two samples are included.

Table 4. The effect of IP/WPC-75 ratio on adherence of nonpareils and cake decoration granules to the films

Material	IP 0% IgG 0%	IP 50% IgG 26%	IP 100% IgG 51%
Nonpareils 2 days	0 g	2.18 g	2.19 g
Cake granules 2 days	0 g	5.54 g	5.79 g
Nonpareils 7 days	0 g	1.59 g	2.11 g
Cake granules 7 days	0 g	0.98 g	2.26 g

Adhesion was tested on days 2 and 7 after casting by pouring nonpareils and cake granules on the films for 30 s. The amount of adhered nonpareils and cake granules were weighted immediately and photographed (Supplementary Figure S6, see online supplementary material online supplementary material for a colour version of this figure.).

Marcotte and Hammarström (2015)]. The protective activity of specific Ig's is significantly reduced by the low pH in stomach and proteolytic enzymes in GI tract. Bovine IgGs are at least partially degraded in stomach by pepsin and in ileum by trypsin, chymotrypsin, carboxypeptidase, and elastase into $F(ab')_2$, Fab, and Fc fragments [for a review, see Reilly et al. (1997)]. However, specific Ig's might be protected against too quick proteolysis and their activity in GI tract retained better if Ig's are embedded in edible whey proteins matrix, e.g. as pills made of the whey protein materials described in this study.

The rapid development of industrial fractionation technologies and progress in understanding the mechanisms of Ig-mediated immune functions have raised interest in developing formulations supplemented with bovine colostral or cheese whey–derived Ig's. Most of the current commercial Ig products are prepared from colostrum of nonimmunized cows and are usually in the form of spray dried and freeze-dried powders or in the form of filtered colostral whey liquids or concentrates (Tripathi and Vashishtha, 2006; Hurley and Theil, 2011; Korhonen and Marnila, 2013; Marcotte and Hammarström, 2015).

The normal colostrum or milk-derived nonspecific Ig preparations are in most countries regarded as food or dietary supplement, whereas the Ig's containing preparations from immunized cows are classified as pharmaceuticals, in the USA and EU (Hoerr and Bostwick, 2002; Mehra et al., 2006). In the case of diseases that can be controlled also by conventional means, e.g. dental caries, this regulatory approach has slowed down the development of immune milk preparations in many countries. However, the emergence of antibiotic resistant pathogen strains will in near future put emphasis on the need to develop alternative ways to prevent and treat oral and gastrointestinal infections. The US Food and Drug Administration (FDA) have accepted the safety of hyperimmune milks on the basis of clinical studies which show no adverse health effects from these products (Gingerich and McPhillips, 2005; Krissansen, 2007). However, to the best of our knowledge, there are no regulatory limitations to utilize Ig fraction from normal cheese whey or colostrum in edible coatings to improve their properties.

Conclusion

The results of this study show that biologically active Ig's can be successfully incorporated in and released from milk protein–based edible films. Also, the content of Ig's in films affected considerably technological properties of these films. Addition of bovine colostral Ig fraction seems to increase stickiness, adhesion, and tensile strength of the films. Composition of other milk proteins in films had effect on preservability and release of Ig's.

Colostrum is normally produced in amounts which exceed the need of newborn calf. At present, dairy industries do not exploit colostrum in large scale. Ig's can also be enriched from side streams

of cheese making. Milk Ig fraction could be utilized in various food technological processes to improve the quality, shelf life, and safety of food products.

The emergence of antibiotic-resistant pathogen strains will in near future put emphasis on the need to develop alternative ways to prevent and treat oral and gastrointestinal infections. The rapid development of industrial fractionation technologies and progress in understanding the mechanisms of Ig-mediated immune functions have raised interest in developing formulations supplemented with bovine colostral or cheese whey–derived Ig's (Korhonen and Marnila, 2009). Since the IgGs are liberated easily to water solvents like saliva, the described material with specific antibodies against cariogenic bacteria could be used as a coating in several products used in caries prevention, e.g. dental floss, teething rings, toothpicks, lozenges, candies, and chewing gums.

In this study, nisin was more active in WBlgFr-IP film than in film made of WBlgFr only. Nisin also retained its activity better in the WBlgFr-IP film. It is possible that also some other bioactive compounds are released easily from Ig films in active form. Milk Ig's have also been found to exert a synergistic effect on the activity of some nonspecific antimicrobial factors in milk, such as lactoferrin, lysozyme, and lactoperoxidase–hypothiocyanate–hydrogen peroxide system (Loimaranta et al., 1998a; Bostwick et al., 2000). Thus, in addition to milk Igs' own bioactivities, they could also be useful as additional ingredient to increase the activities of other bioactive compounds in edible protein films.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

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Conflict of interest statement

None declared.

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