Chromatographic purification of immunoglobulin G from bovine milk whey

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Abstract

We used thiophilic chromatography on T-gel, a resin of the structure agarose–O–$CH_2CH_2SO_2CH_2$ $CH_2SCH_2CH_2OH$, to purify immunoglobulin G from "sweet" cheese whey. The purity of immunoglobulin G, as indicated by radial immunodiffusion, was 74% after a single chromatography on T-gel. Preparation of samples for adsorption onto thiophilic gels requires only the addition of salt (sodium/potassium sulfate) to the samples. Thus, this method may be suitable for large-scale whey IgG isolation.

1. Introduction

Thiophilic gel (T-gel) introduced by Porath and co-workers [1,2] has proved to be a useful tool for selective purification of immunoglobulins (including monoclonal antibodies) from various sources, mainly mammalian sera and ascites fluid [3–9]. One chromatography step was sufficient to obtain the desired purity, although T-gel can be used in tandem with other purification methods such as hydrophobic chromatography [1] or phase partitioning [10]. Thiophilic gel did not display a marked selectivity for human immunoglobulin G (IgG) subclasses [11]. Thiophilic affinity is not restricted to immunoglobulins, and T-gel could be employed also for purification of certain other proteins, such as α_1-macroglobulin

[1], papain, trypsin [4] and sweet potato β-amylase [12].

The thiophilic interactions are salt promoted, but distinct from hydrophobic interactions that also require the presence of lyotropic (water-structure-forming) salts. The thiophilic adsorption of immunoglobulins and some other proteins is based on an interaction with a sulfone group in close proximity to a thioether group of the T-gel ligand. Other nucleophiles (X), such as nitrogen or oxygen, may take the place of sulfur in the general thiophilic ligand structure, matrix–O–CH_2CH_2–SO_2–CH_2CH_2–X–R, where R is usually an alkyl function. The postulated mechanism of thiophilic interactions involves either an electron-donor/acceptor or a proton-transfer process [4,13,14].

Adsorbents with sulfone–aromatic ligands and those with 3-(2-pyridyldithio)-2-hydroxypropyl ligand also have thiophilic properties and are useful for purifying immunoglobulins [15–17].

Among the lyotropic salts, sulfates provide the

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best environment for thiophilic-type adsorption. Ammonium, sodium and potassium sulfates are used extensively. Bound proteins can be easily eluted by lowering the salt concentration. The yield is generally high and up to 85–90% immunoglobulins are recovered from T-gels at low-salt conditions. Thus, only the addition of salt is required prior to chromatography [1,2,4,5].

Immunoglobulins are minor whey protein components [18–20] but have a high potential economic value. For example, whey immunoglobulins have been suggested as a dietary supplement for newborn calves instead of colostrum or colostrum-derived immunoglobulin-rich preparations [21–23]. The treatment provides prophylactic and/or therapeutic benefits by enhancing the calves immune response. The concentration of immunoglobulins in "sweet" cheese whey is ca. 0.4–0.6 g/l, of which about 90% is IgG (mainly IgG1) [20,24]. The "sweet" whey is derived from manufacture of ripened cheeses (Swiss, Cheddar, Provolone, etc.) that are prevalent in cheese production.

So far, purification of IgG from cheese whey has been attempted using various methods, usually membrane filtration/ultrafiltration techniques and/or chromatography, although low-resolution salt ($(NH_4)_2SO_4$, $FeCl_3$) fractionation was also used [25,26]. Membrane separation according to molecular mass (ultrafiltration) does not give high resolution and results in a rather impure mixture of proteins, that is enriched in immunoglobulins [22,27]. Chromatographic methods employed for immunoglobulin purification from whey provide significantly better resolution but are usually not easily scaled-up.

Gel permeation chromatography yielded 92% pure IgG from sweet cheese whey [28] but would not be feasible in pilot and/or large-scale whey processing. On the other hand it can be used for analytical purposes [29,30], along with reversed-phase chromatography [31]. Ion-exchange chromatography is not frequently used to purify cheese whey immunoglobulins, despite a common use in the purification of immunoglobulins from sera [32], in the routine preparation of whey protein concentrates [33] and also in the isolation of other whey proteins (β-lactoglobu-

lin, α-lactalbumin, lactoferrin) [34]. Anion-exchange chromatography is not suitable because immunoglobulins are not retained on the adsorbent [30,35], and it has only been employed to enrich whey protein preparations in immunoglobulins [27], or for analytical purposes [30]. Other chromatographic methods used include metal chelation chromatography [36,37], silica adsorption chromatography [38] and immunoaffinity chromatography with immobilized specific monoclonal antibodies [21]. Protein G used as an affinity ligand in combination with membrane microfiltration yields a 90% pure IgG [39]. A review of the previous research shows that preparation of a pure immunoglobulin requires the application of a selective purification method in order to permit economic scale-up.

We present the use of a thiophilic gel for one-step purification of IgG from "sweet" cheese whey. Thiophilic gel is less costly than protein G (A) type adsorbents, yet providing a high specificity for IgG. The procedure requires a very simple pretreatment of the whey, the addition of an appropriate salt, which might be subsequently removed by ultrafiltration of the IgG-depleted whey and possibly even reused. The resulting IgG is 74% pure after one-step thiophilic chromatography, compared to 81% purity for IgG prepared by protein G chromatography.

2. Experimental

2.1. Materials

Protein G-Sepharose 4FF was obtained from Pharmacia LKB (Alameda, CA, USA), 40% acrylamide-N,N'-methylenebisacrylamide (37.5:1, premixed solution); N,N,N',N'-tetramethylethylenediamine and ammonium persulfate were purchased from Bio-Rad (Hercules, CA, USA), sodium sulfate and potassium sulfate were from Mallinckrodt (Paris, KY, USA), thiophilic gels (the same thiophilic ligands attached to cross-linked 4 and 6% beaded agarose, respectively), bovine γ-globulin, bovine IgG, α-lactalbumin, β-lactoglobulin, Coomassie Brilliant Blue R-250,

molecular mass standard kit SDS-7, glycine and all other chemicals (research-grade purity) were purchased from Sigma (St. Louis, MO, USA), Spectrapor dialysis membrane was from Spectrum Medical Industries (Los Angeles, CA, USA); SP-filter paper, grade 391 was purchased from Baxter (McGaw Park, IL, USA), 0.45-μm ultrafilters Acrodisc 13 were from Gelman (Ann Arbor, MI, USA), Electrophoresis duplicating paper EDP was purchased from Kodak (Rochester, NY, USA), Centrex filtration cartridges were from Schleicher & Schuell (Keene, NH, USA), radial immunodiffusion kit (bovine IgG, low) was purchased from ICN Biochemicals (Costa Mesa, CA, USA), "sweet" cheese whey was obtained from Gossner Foods (Logan, UT, USA).

Low-pressure chromatographic columns of 1.5 cm I.D. were obtained from Kontes (Vineland, NJ, USA), low-pressure modular chromatograph Econo System, MiniProtean II vertical gel electrophoresis and PowerPac 3000 power supply were purchased from Bio-Rad.

2.2. Methods

Salt effects on thiophilic adsorption of bovine IgG

Thiophilic gel was suction-dried and 0.5-g aliquots were equilibrated with 0.05 M sodium/potassium phosphate buffer, pH 7.5 containing various salts at 0.5 M concentration: potassium sulfate, sodium sulfate and ammonium sulfate. After 30 min equilibration, the buffer was removed by centrifugal filtration in Centrex cartridges and gel samples were incubated batchwise with 2 ml of IgG solutions (1 mg/ml) in appropriate 0.5 M sulfate buffers at 20°C for 30 min with gentle mixing. The protein solution was removed by filtration and the gels were washed with 2-ml aliquots of 0.5 M sulfate buffers until the absorbance of washings was less than 0.01 at 280 nm. Subsequently bound IgG was eluted with a low-salt elution buffer, 0.05 M sodium/potassium phosphate buffer, pH 7.5 and quantified spectrophotometrically at 280 nm.

The same procedure was used to determine the effect of salt concentration and temperature on binding efficiency of T-gel for bovine IgG. When the effect of temperature on adsorption was tested, the adsorption was performed at 20 and 40°C, respectively, while elution was carried out at 20°C.

Determination of capacity of adsorbents for IgG

Bovine γ-globulin or IgG (both commercial products, Sigma) (1 mg/ml) in 0.5 M sodium sulfate/0.05 M sodium/potassium phosphate buffer, pH 7.5 was applied to 2.3-ml gel beds in chromatographic columns (1.5 cm $\times$ 1.3 cm) at flow-rate 1.0 ml/min. The sample application proceeded until a 10% breakthrough was detected by UV monitor (280 nm) at the outlet of the column. (The 10% breakthrough is defined as 1/10 of the absorbance of the IgG solution applied. This parameter would be an important factor in process-scale purification). At this point the column was washed with the binding buffer (0.5 M sodium sulfate/0.05 M sodium/potassium phosphate, pH 7.5) and the bound IgG was eluted with 0.05 M sodium/potassium phosphate, pH 7.5 (elution buffer).

The dynamic capacity at 10% breakthrough was calculated from the volume of the IgG solution applied to the column (until 10% breakthrough reached) minus the volume of the IgG solution retained in the inlet/outlet tubings and in the void volume of the column. The dynamic capacity was compared with the amount of IgG eluted from the column after extensive washing (eluted capacity), which was determined spectrophotometrically at 280 nm.

Bovine γ-globulin (electrophoretic purity *ca.* 99%) was used instead of much more expensive IgG to determine the capacity as well as the effects of salts and temperature on thiophilic adsorption.

Pretreatment of whey prior to chromatography

Fresh whey (pH 6.2–6.6 in various batches) was cooled to 4–6°C and centrifuged at 10 000 g for 30 min. Residual milk fat floating on top of the liquid was removed and the resulting supernatant was collected and passed first through a SP-glass fiber filter and subsequently through

0.45-μm ultrafilter. The clarified whey was then ready for chromatography.

Sodium azide was added as a preservative to 0.02% final concentration. The clarified, sodium azide treated whey can be stored for over 30 days at 4°C without any signs of a bacterial growth. Presence of the preservative did not affect subsequent chromatography.

Chromatographic purification of IgG from cheese whey

Solid sodium sulfate was added to clarified cheese whey (see pretreatment) to a final concentration of 0.5 M. The pH of the resulting solution was not adjusted. The treated whey (85 ml) was then applied to a chromatographic column (1.5 cm × 1.3 cm) with the appropriate adsorbent equilibrated with 0.5 M sodium sulfate/0.05 M sodium/potassium phosphate buffer, pH 7.5 (binding buffer) at a flow-rate of 1.0 ml/min (linear flow-rate 34 cm/h). The protein elution was detected by UV monitor at 280 nm. The column was thoroughly washed with the binding buffer until absorbance reached baseline and then the IgG was eluted with a low-salt 0.05 M sodium/potassium phosphate buffer, pH 7.5, at the same flow-rate. The adsorption of whey IgG was performed at both 20 and 40°C (the column, sample solution and buffers were placed in a thermostated incubator).

In a similar fashion, IgG was purified from whey on protein G-Sepharose 4FF column equilibrated with 0.1 M sodium/potassium phosphate buffer, pH 6.9. The clarified whey (85 ml) was applied to the column at flow-rate 1.0 ml/min, the column was washed with the binding buffer, and the adsorbed material was eluted with 0.1 M glycine–HCl buffer, pH 2.8.

Eluates containing IgG were dialyzed against distilled water overnight, frozen and lyophilized.

Protein concentration assay

Total protein concentration of column effluents was determined spectrophotometrically at 280 nm using highly purified bovine IgG as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Chromatographically purified IgG fractions were analyzed for purity by vertical SDS-PAGE using 12% resolving gel and 4% stacking gel under denaturing conditions [40]. Protein staining was performed with 0.1% Coomassie Brilliant Blue R-250 solution in 10% acetic acid–40% methanol–50% water. Positive photographic images of the stained protein patterns were obtained using the Kodak EDP direct duplication method.

Radial immunodiffusion

Immunochemical analysis was carried out using radial immunodiffusion. The lyophilized IgG preparations were dissolved in 0.9% sodium chloride (*ca.* 1 mg/ml) and 5-μl aliquots were pipetted into wells of the radial immunodiffusion gel. Highly purified bovine IgG was used as a standard. The concentrations of IgG were compared with total protein concentration.

3. Results and discussion

3.1. Salt effects on thiophilic adsorption of IgG

Among the salts tested, sodium sulfate most efficiently promoted IgG adsorption on T-gel; 94% of the IgG was bound to the gel at 0.5 M salt concentration. The amount of IgG eluted with sodium sulfate was also higher (75%) than with potassium or ammonium sulfates (Fig. 1). IgG from T-gels was only partially eluted by three batchwise elutions (most IgG, 44–52%, was recovered in the first eluate). Since all the T-gel aliquots were treated identically, the amount of eluted IgG accurately represented the effects of different salts. These results indicated that sodium sulfate should be employed in subsequent experiments.

3.2. Effect of salt concentration on thiophilic adsorption of IgG

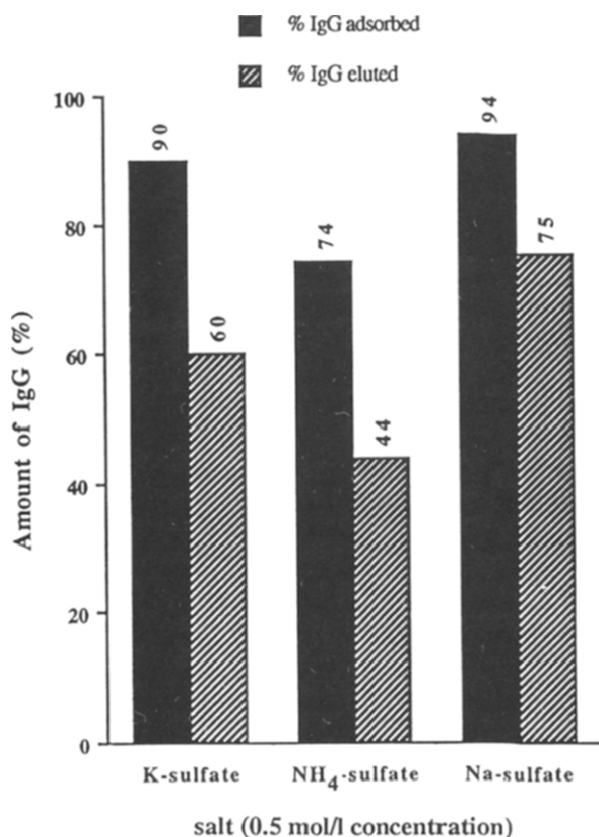The 0.5 M salt concentration was used as described previously since it was reported to be

Fig. 1. Effect of salts on thiophilic adsorption of bovine IgG. Aliquots (0.5 g) of suction-dried T-gel (matrix: cross-linked 4% beaded agarose) were equilibrated with 0.5 M salt solutions in 0.05 M sodium/potassium phosphate buffer, pH 7.5, then treated with 2 ml of IgG solutions (1 mg/ml) for 30 min. The gels were washed with the appropriate salt/buffer, then adsorbed IgG was eluted with 0.05 M sodium/potassium phosphate buffer, pH 7.5. IgG concentration in both washings and elution fractions was quantified. The experiment was done in a batchwise mode. Values reported are the average of five determinations, the standard deviation being less than ± 4%.
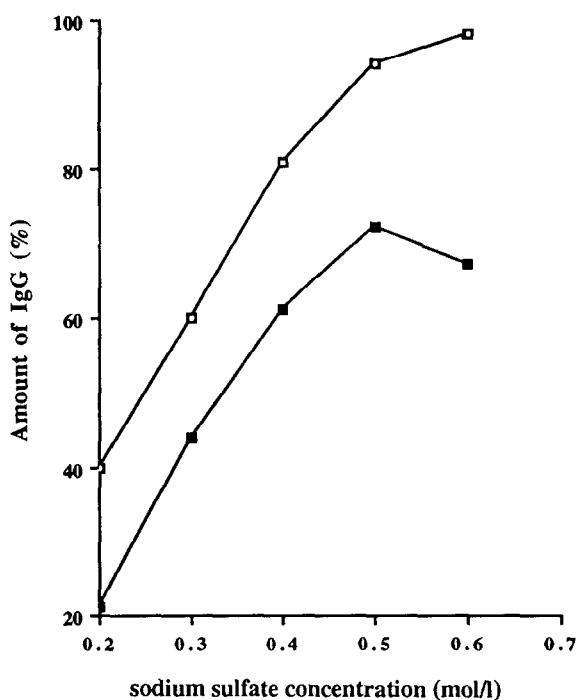

Fig. 2. Effect of salt concentration on thiophilic adsorption of bovine IgG. T-gel was equilibrated with sodium sulfate solutions of 0.2–0.6 M concentration and then treated as described (Methods, Fig. 1). The amount of IgG in washing and elution fractions was determined for various salt concentrations. Values reported are the average of five determinations, the standard deviation being less than ± 4%. □ = IgG adsorbed; ■ = IgG eluted.

optimal for potassium sulfate, the most frequently used salt in thiophilic chromatography [1,3,5]. We demonstrated that this was also the optimal concentration for sodium sulfate in thiophilic chromatography of bovine IgG (Fig. 2). The amount of bound IgG continued to increase above 0.5 M sodium sulfate concentration. However, the yield of eluted IgG was lower indicat-

ing that part of the protein adsorbed at the high salt concentration cannot be recovered using low-salt elution. That portion of the IgG could only be released by using 8 M guanidine–HCl during regeneration of the T-gel. Also, increasing ionic strength during adsorption was not desirable because the salt must be removed from IgG-free whey before subsequent processing.

The experimental procedure was basically the same as in the previous experiment and thus IgG was only partially eluted by three batchwise elutions. Nevertheless, total amount of IgG eluted as well as the amount of IgG in individual eluates showed that the yield of IgG was the highest at 0.5 M sodium sulfate.

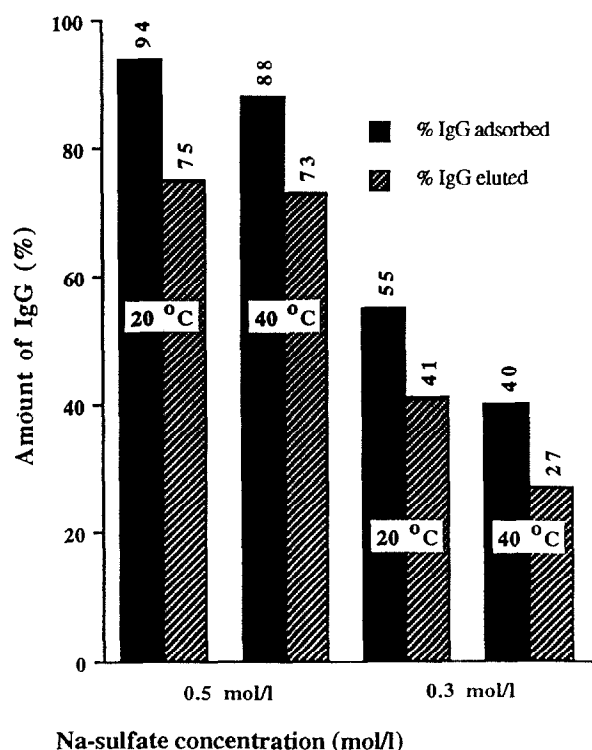P. Konecny et al. / J. Chromatogr. A 673 (1994) 45–53

Fig. 3. Effect of temperature on thiophilic adsorption of bovine IgG. Adsorption of IgG to T-gel (0.5-g aliquots of suction-dried gel) equilibrated with sodium sulfate solutions of 0.3 and 0.5 M concentrations (in 0.05 M sodium/potassium phosphate buffer, pH 7.5) was performed at 20 and 40°C. Elution was done as described (Methods, Fig. 1) at 20°C and amounts of IgG in all fractions were quantified spectrophotometrically. Values reported are the average of five determinations, the standard deviation being less than ± 4%.

3.3. Effect of temperature on thiophilic adsorption of bovine IgG

The amount of IgG eluted from T-gel after adsorption (batchwise experiment) of the protein at 40°C was comparable to that carried out at 20°C (Fig. 3). Although slightly less IgG was bound at 40°C (88%) than at 20°C (94%), the amounts of eluted protein were essentially the same (73 vs. 75%). These results are consistent with similarities in adsorption isotherms of IgG adsorption on T-gel at 4 and 20°C [3].

However, at a salt concentration of 0.3 M, less IgG was adsorbed and eluted at 40 than at 20°C. This confirms the differences between thiophilic and hydrophobic chromatography. Unlike hydrophobic interactions, thiophilic interactions are not promoted at higher temperatures.

3.4. Capacity of adsorbents for bovine IgG

Under the conditions described above (see Methods), we determined the capacity of commercially available T-gels (with the same thiophilic ligand attached to cross-linked 4% beaded agarose and 6% beaded agarose, respectively) and protein G-Sepharose 4FF (Table 1). The capacity of the T-gel based on 6% beaded agarose was approximately the same at 20 and 40°C and was only about 20% lower than the capacity of protein G-Sepharose 4FF.

The capacities for bovine IgG determined in our experiments differed from capacities for

Table 1
Capacity of T-gels and protein G-Sepharose 4FF for bovine IgG

	Temperature (°C)	Dynamic capacity at 10% breakthrough (linear flow-rate 34 cm/h) (mg IgG/ml gel)	Eluted capacity (mg IgG/ml gel)
T-gel (cross-linked 4% beaded agarose)	20	12.1 ± 0.3	12.0 ± 0.4
T-gel (6% beaded agarose)	20	18.2 ± 0.3	19.0 ± 0.4
	40	17.3 ± 0.5	18.3 ± 0.5
Protein G-Sepharose 4FF	20	22.1 ± 0.4	19.0 ± 0.6

human IgG as reported by the manufacturer (cross-linked 4% beaded agarose T-gel: 1–3 mg/ml gel, 6% beaded agarose T-gel: 30–45 mg/ml gel). The capacities for bovine and human IgG are significantly different for the T-gel with cross-linked 4% agarose matrix, with bovine IgG giving higher yields. In contrast, the reported capacity for human IgG of the other T-gel, based on 6% agarose, is about twice that found for bovine IgG.

The observation that the eluted capacity of protein G-Sepharose 4FF was lower (ca. 15%) than the dynamic capacity at 10% breakthrough could be linked with leakage of a small portion of the bound IgG during the washing phase. That implies that the adsorption of IgG onto the adsorbent might not be strong enough under the typical washing conditions.

3.5. Chromatographic purification of IgG from cheese whey

IgG was purified from whey by chromatography on the two thiophilic gels described above. Adsorption was carried out at 20 and 40°C, while elution was performed at 20°C. In a typical T-gel chromatography (Fig. 4) the yield was 0.29–0.32 mg IgG/ml whey. The bulk flow-through fraction contained less than 0.08 mg IgG/ml. Protein G-Sepharose 4FF, which was chosen as a reference adsorbent for the IgG purification, yielded 0.38–0.42 mg IgG/ml whey. Radial immunodiffusion indicated that the flow-through fractions were essentially IgG-free. Fractions containing IgG were assayed for total protein concentration (A_{280} assay), and purity by radial immunodiffusion and SDS-PAGE under denaturing conditions (Fig. 5). The two quantitative assays show that the IgG prepared by thiophilic chromatography was 74% pure, while the IgG preparation from protein G-Sepharose 4FF was 81% pure. Thus, the purity of IgG prepared by T-gel chromatography was 91% of that prepared by protein G-chromatography. The electrophoretic pattern suggests a higher purity of the IgG purified by protein G chromatography than determined by radial immunodiffusion. The reason could be that part of the
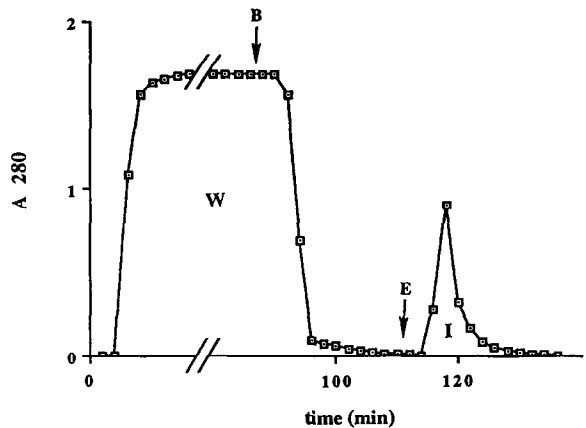

Fig. 4. Purification of IgG from whey by thiophilic chromatography. Clarified sweet whey (85 ml), with solid sodium sulfate added to a concentration of 0.5 M, was applied to a column (1.5 cm × 1.3 cm) of T-gel (matrix: 6% beaded agarose) equilibrated with 0.5 M sodium sulfate/0.05 M sodium/potassium phosphate, pH 7.5. After washing with the same buffer (B), adsorbed IgG was eluted with the elution buffer (E) of 0.05 M sodium/potassium phosphate, pH 7.5. The chromatography was performed at a flow-rate of 1.0 ml/min. Fractions from the chromatography (W = IgG-depleted whey, I = IgG fraction) were characterized by SDS-PAGE (see Fig. 5).
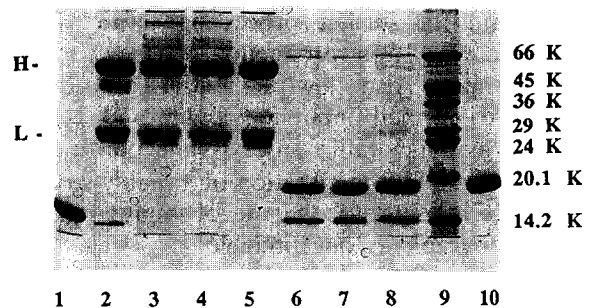

Fig. 5. SDS-PAGE (denaturing conditions) of fractions from chromatographic purifications of sweet whey on T-gel and protein G-Sepharose 4FF. SDS-PAGE was run on a 8 cm × 7 cm × 0.75 mm gel (stacking gel: 4% acrylamide, separating gel: 12% acrylamide), using the Laemmli buffer system [40] at constant voltage 200 V. Lanes: 1 = α-lactalbumin; 2 = bovine IgG (standard; H = heavy chain, L = light chain); 3 = bovine whey IgG from T-gel chromatography at 20°C (see Fig. 4, fraction I); 4 = bovine whey IgG from T-gel chromatography at 40°C; 5 = bovine whey IgG from protein G chromatography; 6 = IgG-depleted whey after T-gel chromatography (see Fig. 4, fraction W); 7 = IgG-depleted whey after protein G chromatography; 8 = whey; 9 = molecular mass standards (K = kilodalton); 10 = β-lactoglobulin.

protein lost its antigenic activity, possibly during the low-pH elution.

Whey was applied on T-gel without prior adjustment of pH, although thiophilic chromatography usually requires pH 7.5–8.0 to reach the highest yield, and possibly also to obtain the maximum purity of the product [3,6]. We did not adjust the pH (6.2–6.6) to avoid another procedure to the sample preparation, which might also negatively affect further processing of the IgG-free whey. If pH adjustment is feasible on a large scale, it might increase the adsorption and the yield of IgG.

4. Conclusions

The purification of IgG from whey requires a selective purification method. Affinity chromatography on IgG-binding adsorbents is one such method. Protein G-adsorbents would theoretically fit the concept of a highly specific adsorbent but they are rather vulnerable to harsh conditions (including higher temperatures) and may also leak small amounts of protein G which could be undesirable in both whey IgG preparations and for further processing of the IgG-depleted whey (e.g. production of whey protein concentrate). Also, low-pH elution may denature the purified immunoglobulin and is not viewed as a favorable process-scale purification feature. Unless a preparation of a highly specific antibody is required, the use of immunoaffinity chromatography is questionable.

At this point the thiophilic gel, which is stable, sturdier and less expensive than most of other commercially available affinity chromatography materials, seemed to be worth studying. The thiophilic gel proved to be useful for the isolation of IgG, giving IgG with a purity comparable to that obtained by protein G chromatography. The capacity of a randomly chosen commercially available thiophilic gel was found to be reasonable for bovine IgG. It is likely that other thiophilic gels might be more suited for this purpose, including other than "classical" T-gel [1,2], e.g. gels with sulfone–aromatic ligands [15] or 3-(2-pyridyldithio)-2-hydroxypropyl ligands [16].

The gel proved to be capable of binding bovine IgG efficiently at 40°C and should easily withstand even higher temperatures without a substantial decrease in binding capacity. The IgG purified in one step by T-gel chromatography from "sweet" whey was slightly less pure (74%) than that prepared by protein G chromatography (81%), also in one step. The purity of whey IgG from single T-gel chromatography was significantly higher than that prepared by metal chelation chromatography, which yielded a 53% pure product [36], but it was lower than the purity of whey IgG (90%) obtained from a combined use of ultrafiltration and protein G-adsorbent [39]. Higher purity of IgG could be achieved by rechromatography. Another option would be a combination of an ultrafiltration preseparation step and thiophilic chromatography which might also lead to a purer product. Sodium sulfate (at 0.5 M concentration) was found to be the most effective among the salts tested for IgG adsorption on T-gel. The use of less costly sodium sulfate instead of potassium sulfate would have an additional advantage in lower production expenses.

The ability to use T-gel at elevated temperatures would not require cooling the whey to room temperature. The preparation of whey prior to thiophilic chromatography is simple, only the addition of salt (preferably sodium sulfate) is needed. This would fit a large-scale purification process, although it might cause a need for additional post-column treatment of the IgG-free whey. Concentrating whey by ultrafiltration (which is routinely used in whey processing [20]) could reduce the amount of whey to as little as 10%, and thus reduce the amount of salt added. That would also reduce the throughput and the amount of chromatographic adsorbent required. Similarly, separation membrane techniques (ultrafiltration combined with reverse osmosis) could be used to desalt washings from the T-gel column.

5. Acknowledgements

Utah Agricultural Experiment Station, Utah State University, Logan, UT, USA. Approved as journal paper No. 4566.

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