DAIRY FOODS

Extraction from Cheese Whey by Cation-Exchange Chromatography of Factors that Stimulate the Growth of Mammalian Cells

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ABSTRACT

Bovine cheese whey was investigated as a source of growth-stimulating factors that might replace or supplement fetal bovine serum in cell culture. Although some cell growth activity was demonstrated in whey or whey ultrafiltrates, enrichment on the basis of molecular size was not useful because the most abundant whey proteins, β-lactoglobulin and α-lactalbumin, have molecular masses that are similar to most known growth factors. Instead, cation-exchange chromatography was selected as an enrichment process because, in contrast to the major whey proteins, growth factors generally have basic isoelectric points. Adsorption to and elution from Sepharose Fast Flow-S resin yielded an extract containing only 1 to 2% of whey protein but substantial growth-promoting activities on Balb/c 3T3 cells, L6 myoblasts, and human skin fibroblasts. The growth activity could be separated from lactoferrin, one of the prominent basic proteins present, through a stepwise elution from the resin. The resultant fraction, which contained lactoperoxidase as the most abundant protein stimulated the growth of the three cell lines at protein concentrations that were 2- to 20-fold lower than observed with fetal bovine serum. Immunoglobulin G could be removed by affinity chromatography, or lactoperoxidase could be inactivated by heat, without significant losses to the growth-promoting capacity of the fraction. These results suggest that enrichment of growth factors by cation-exchange chromatography offers a practical method for the large-scale isolation of an extract from cheese whey that stimulates cell growth. (Key words: whey, cell growth, lactoperoxidase, cation-exchange chromatography)

Abbreviation key: FBS = fetal bovine serum.

INTRODUCTION

Bovine milk and colostrum contain several growth factors, including IGF-I, IGF-II, and des(1-3)IGF-I (7), transforming growth factor-β (3), and platelet-derived growth factor (16). Heparin-binding growth factors have also been reported (14) that may be examples of the fibroblast growth factor family. Epidermal growth factor, although abundant in human milk, has not been unequivocally detected in bovine colostrum or milk (9).

In view of the limited availability of fetal bovine serum (FBS), as well as variability and safety concerns of serum in pharmaceutical manufacturing processes, attention has been directed toward milk or colostrum as a replacement for FBS (10, 15). More recently, this research has focused on the use in cell culture of ultrafiltrates of colostrum, milk, and cheese whey that have had the protein concentration reduced by removal of casein and large molecular mass proteins (4, 5, 13). One difficulty with this approach is the similarity in molecular mass between most growth factors and α-lactalbumin (14 kDa) and β-lactoglobulin (18.5 kDa), the two most abundant proteins present in cheese whey or in casein-depleted milk.

In this investigation, we explored a different strategy to enrich the growth factors in whey. Although all the growth factors identified in bovine milk or whey have basic isoelectric points, α-lactalbumin, β-lactoglobulin, and bovine serum albumin are acidic. Hence, cation-
exchange chromatography can be utilized to adsorb and elute the growth factors under conditions in which the major whey proteins are not adsorbed. We demonstrate that the separation can be further enhanced without significant loss of cell growth activity by the separation of the growth factors from lactoferrin on the same chromatography column. Accordingly, this one-step chromatographic enrichment of growth factors may have considerable advantages in the development of serum replacements or extenders for the growth of mammalian cells in culture.

MATERIALS AND METHODS

Ultrafiltration Size Exclusion

Pasteurized bovine Cheddar cheese whey (30 L), preclarified by microfiltration, was acidified with HCl to pH 3.0 and then processed using an Amicon DC-to ultrafiltration unit (Amicon, Danvers, MA) equipped with a .1-μm hollow fiber cartridge. The permeate from this treatment (25 L) was concentrated to 3 L using the same unit equipped with a 3-kDa exclusion membrane and then diafiltered with 10 L of acidic buffer (a solution containing 10 mM HCl and 150 mM NaCl; pH 3.0). This retentate (2 L) was again diafiltered with 10 L of acidic buffer but against a 10-kDa exclusion membrane and in turn against 30- and 100-kDa exclusion membranes. A 5-ml sample of each retentate was collected for assay. The retentate volume was maintained at 2 L throughout each diafiltration.

Cation-Exchange Chromatography

Fresh Cheddar whey (pH 6 to 6.5) was either passed through a .1-μm hollow fiber cartridge in an Amicon DC-10 ultrafiltration unit or pasteurized (72°C for 15 s) prior to microfiltration with a .2-μm pore membrane fitted to a Sartorius Sartocon II crossflow filtration unit (Sartorius, Göttingen, Germany). The permeate from either procedure was essentially fat-free (.01%, wt/wt), and recovery of the major whey proteins from this stream was 70 to 80%. Chromatography was performed using either a Bio-Rad 5-cm diameter column (Bio-Rad, North Ryde, New South Wales, Australia) packed with 100-ml of Sepharose Fast Flow-S resin (Kabi-Pharmacia, Uppsala, Sweden) equilibrated in 50 mM sodium citrate buffer, pH 6.5, or a Pharmacia BPG 10-cm diameter column (Kabi-Pharmacia) packed with 500 ml of the resin. In the first method, 5 L of the preclarified whey were passed over the 100-ml ion-exchange bed at a flow rate of 70 ml/min. The whey effluent was collected, and the resin was washed with .25 L of 10 mM sodium citrate buffer, pH 6.5. The bound protein was eluted with .25 L of a solution containing 1 M NaCl plus 250 mM NH₄OH at pH 11 to yield the growth factor extract. In an initial experiment with this first method, the flowthrough fraction was reapplied to the column after it had been reequilibrated and any adsorbed protein eluted with .25 L of 1M NaCl containing 250 mM NH₄OH at pH 11.

In the second method, 30 L of preclarified whey were applied to the 500-ml column. The protein was eluted stepwise with 1.6 L of a solution containing 400 mM NaCl and 10 mM sodium citrate at pH 6.5, followed by elution of a second fraction with 1.6 L of 1 M NaCl containing 250 mM NH₄OH at pH 11. The alkaline fractions from both methods were adjusted to pH 7.4 with HCl and all were diafiltered against deionized water using an ultrafiltration unit equipped with a 3-kDa exclusion membrane. The endpoint for dialysis was determined by measuring the conductivity of the permeates. Following dialysis, retentates were concentrated 10-fold in the same apparatus. The desalted concentrates were then filtered through a 1-μm glass filter, freeze-dried, and stored at 4°C.

Heat Treatment to Inactivate Lactoperoxidase

The 400 mM NaCl eluate from method 2 was heated for 10 s at either 80 or 100°C using a UHT apparatus (Tetra-Laval, Melbourne, Victoria, Australia). The treated samples were centrifuged before the diafiltration, ultrafiltration, and freeze-drying steps just described.

Affinity Chromatography for the Removal of IgG

A freeze-dried sample (100 mg) of the 400 mM NaCl eluate from method 2 was dissolved in 20 mM sodium phosphate buffer, pH 7.0 (running buffer), and applied to a 5-ml column
of Protein G Sepharose Fast Flow (Kabi­Pharmacia) at a flow rate of 1 ml/min. The column was washed with running buffer until the absorbance at 280 nm had returned to baseline, after which the IgG was eluted with 100 mM glycine adjusted to pH 2.7 with HCl. The unadsorbed fraction was dialyzed exhaustively against water and freeze-dried.

**Total Protein**

Total protein in the extracts was measured by the method of Lowry (11) using a bovine serum albumin standard.

**Chromatography of Proteins In Whey Extracts**

The protein composition of the whey extracts was analyzed using a Mono-S HR 5/5 cation-exchange column (FPLC®; Kabi-Pharmacia). The column was equilibrated in 50 mM sodium citrate buffer, pH 6.5, and loaded with approximately 4 mg of protein in 200 µl of the citrate buffer. Elution was achieved using a 0 to 1 M NaCl linear gradient in the same buffer over 30 min at a flow rate of 1 ml/min. The elution of protein was monitored by its absorbance at 280 nm.

**SDS-PAGE**

Protein solutions for electrophoresis contained 1 to 3 mg/ml of protein, 1% (vol/vol) 2-mercaptoethanol, 4% (wt/vol) SDS, 200 mM Tris, and 5% bromphenol blue and were dissociated by heating at 90°C for 5 min. A 0.5-µl aliquot of the dissociated protein was analyzed with a Pharmacia Phastsystem (Kabi-Pharmacia) on an 8 to 25% preformed slab gel and stained with Coomassie blue according to the manufacturer’s instructions.

**Lactoperoxidase Assay**

Lactoperoxidase was assayed spectrophotometrically using 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]-diammonium salt (Boehringer, Mannheim, Germany) substrate. The assay mixture comprised 2 ml of 100 mM sodium citrate buffer (pH 5.5), 200 µl of substrate (11 mg/ml in buffer), and 200 µl of H₂O₂ (25 µl of 30% H₂O₂/100 ml of H₂O). Enzyme (20 µl) was added to start the reaction, which was followed at 405 nm. One unit of enzyme activity produced a change in absorbance of 1 per min at 25°C.

**Methylene Blue Cell Proliferation Assay**

Rat L6 myoblasts (provided by J. M. Gunn, Texas A&M University, College Station), Balb/c 3T3 mouse fibroblasts (CSL Ltd., Parkville, Victoria, Australia), and human diploid skin fibroblasts (SF 1972, Women’s and Children’s Hospital, North Adelaide, South Australia, Australia) were subcultured in 96-well tissue culture plates in Dulbecco’s modified Eagle’s minimal essential medium containing antibiotics (I) and 5% FBS (not inactivated by heat; HyClone Laboratories, Logan, UT) and left in a humidified incubator equilibrated with 5% CO₂ overnight to ensure attachment of cells. The cell densities used were 1 x 10⁴ per well for L6 myoblasts and 2 x 10⁴ per well for the other two cell lines. The plates were thoroughly washed with Dulbecco’s minimal essential medium to remove any residual serum after which the whey extract or FBS was added at the indicated concentrations. The total volume in each well was 100 µl. After further incubation for 2 d, the plates were washed twice with 150 mM NaCl, the monolayers of cells fixed, and the cell mass quantified by absorbance at 600 nm using an automated methylene blue staining method (12).

**Protein Synthesis In Rat L6 Myoblasts**

Stimulation of protein synthesis in rat L6 myoblasts grown in 24-well dishes was measured as described previously (7). Briefly, the whey samples were diluted with Dulbecco’s phosphate-buffered saline, and the stimulation of protein synthesis was measured as the incorporation of [³H]-labeled leucine into total cell protein over an 18-h incubation period. Results were expressed as the percentage of increase relative to a serum-free control.

**RESULTS**

In an initial attempt to purify the growth factor activity in whey on the basis of molecular mass, we fractionated acidified whey by ultrafiltration using membranes of porosity be-
The stimulation of protein synthesis in rat L6 myoblasts was measured using these whey concentrates. However, all activities in retentates were substantially less than those achieved with FBS and were reduced in parallel to total protein as the pore size was increased (data not shown).

Subsequent experiments were directed toward the separation of a growth factor mixture from the most abundant whey proteins by cation-exchange chromatography. In preliminary studies, a number of cation-exchange resins were evaluated for the adsorption of cell growth-promoting factors at neutral pH and elution with 1 M NaCl containing 250 mM NH₄OH. Although some activity was recovered under these conditions with Trisacyl LS SP and Spherisol S (IBF Biotechnics, Villeneuve-la-Garenne, France), SP Sephadex G-25 (Kabi Pharmacia) and Macro-Prep 50S (Bio-Rad, Richmond, CA) (data not shown), recoveries were substantially higher with Sepharose Fast Flow-S.

The protein recoveries from this experiment are shown in Table 1, method 1. Of 19.9 g of protein that were applied to the column, 249 mg were recovered in the 1 M NaCl, 250 mM NH₄OH eluate, and a further 20 mg were recovered when the unadsorbed material was chromographed and eluted a second time. The first eluate contained larger proteins but very little material in the 10- to 20-kDa range that was characteristic of the major whey proteins (Figure 2). Accordingly, the procedure successfully separated the growth-promoting activity from the most abundant whey proteins. The 70- to 80-kDa proteins evident from SDS-PAGE were likely to be lactoferrin or lac-

Figure 1. Growth-stimulating activity of whey fractions after chromatography on a Sepharose Fast Flow-S (Kabi-Pharmacia, Uppsala, Sweden) resin. Cheese whey was microfiltered through a .1-μm hollow fiber cartridge, adsorbed to Sepharose Fast Flow-S, and eluted with 1 M NaCl containing 250 mM NH₄OH. The unadsorbed fraction was reapplied on the column, and a second eluate was obtained with 1 M NaCl containing 250 mM NH₄OH. All fractions were dialyzed exhaustively before addition to monolayers of Balb/c 3T3 cells in the presence of Dulbecco's minimal essential medium. Cells were grown in diluted test solutions or in the presence of fetal bovine serum (FBS) for 2 d, after which growth was measured as absorbance at 600 nm (ΔA600) using the methylene blue staining procedure (12). The protein concentrations of the undiluted test solutions were FBS, 42 mg/ml (●); microfiltered whey, 4.0 mg/ml (○); nonadsorbed fraction, 3.7 mg/ml (□); first eluate, 1.66 mg/ml (▲); and eluate after rechromatography of the nonadsorbed fraction, .16 mg/ml (△). Values are means of three determinations; standard errors are indicated when they are larger than the symbols.
TABLE 1. Recovery of protein after cation-exchange chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Protein (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td>5800</td>
<td>5.83</td>
<td>33.8</td>
</tr>
<tr>
<td>Microfiltered whey</td>
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<td>3.98</td>
<td>19.9</td>
</tr>
<tr>
<td>Column flow-through</td>
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<td>19.3</td>
</tr>
<tr>
<td>First eluate with 1 M NaCl plus 250 mM NH₄OH</td>
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<td>1.66</td>
<td>2.49</td>
</tr>
<tr>
<td>Second column flow-through</td>
<td>5500</td>
<td>2.99</td>
<td>16.4</td>
</tr>
<tr>
<td>Second eluate with 1 M NaCl plus 250 mM NH₄OH</td>
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<td>.16</td>
<td>.020</td>
</tr>
<tr>
<td>Method 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>ND³</td>
</tr>
<tr>
<td>400 mM NaCl eluate</td>
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<td>.682</td>
</tr>
<tr>
<td>1 M NaCl plus 250 mM NH₄OH eluate</td>
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<td>1.31</td>
<td>2.10</td>
</tr>
</tbody>
</table>

¹Cheese whey was microfiltered and chromatographed on a 100-ml column of Sepharose Fast Flow-S (Kabi-Pharmacia, Uppsala, Sweden) with elution by 1 M NaCl containing 250 mM NH₄OH. The unadsorbed material was reapplied after regeneration of the column and eluted as before to obtain the second eluate.

²Cheese whey was microfiltered and chromatographed on a 500-ml column of Sepharose Fast Flow-S with elution batchwise by 400 mM NaCl, followed by 1 M NaCl containing 250 mM NH₄OH.

³Not determined.

toperoxidase, basic proteins that could be expected to bind to and elute from the cation-exchange resin (6, 17). Analysis of the preparation by Mono-S cation-exchange chromatography indicated the presence of both these proteins (Figure 3a). Accordingly, the elution conditions were modified for the 500-ml preparative column to produce an initial 400 mM NaCl eluate, followed by a second eluate obtained with 1 M NaCl containing 250 mM NH₄OH (Method 2). The first eluate contained lactoperoxidase (Figure 3b), and the second, lactoferrin (Figure 3c). The identity of these proteins was confirmed by their characteristic green and pink colors, respectively, and by chromatography of pure standards under the same conditions. There was no detectable contamination of lactoferrin in the first eluate or lactoperoxidase in the second.

Stepwise elution of the cation-exchange column according to method 2 gave 682 mg of protein in the first eluate and 2.1 g in the second eluate from 30 L of whey (Table 1). These fractions were evaluated for growth-promoting activities in Balb/c 3T3 cells, L6 myoblasts, and human skin fibroblasts (Figure 4). Compared with the single eluate described as method 1, the 400 mM NaCl eluate produced higher growth rates on a protein basis in all three cell lines. Moreover, this fraction induced cell growth with a somewhat higher potency than FBS in L6 myoblasts and was 5- to 20-fold more potent than FBS in the other two cell lines. The fraction eluted by 1 M NaCl plus 250 mM NH₄OH in which lactoferrin was the predominant protein (Figure 3c) exhibited some growth-promoting activity, but...

Figure 2. The SDS-PAGE separations of whey that were chromatographed on the 100-ml Sepharose Fast Flow-S column (Kabi-Pharmacia, Uppsala, Sweden) (cation-exchange chromatography, method 1). Lane 1, molecular mass standards of 116, 97, 67, 45, and 29 kDa (Sigma Chemical Co., St. Louis, MO); lane 2, microfiltered whey; lane 3, the first eluate obtained with 1 M NaCl plus 250 mM NH₄OH; lane 4, the second eluate obtained after application of the unadsorbed material to the column after reequilibration followed by a second elution with 1 M NaCl plus 250 mM NH₄OH; and lane 5, the unadsorbed fraction from the first application of whey to the column.
Figure 3. Mono-S chromatography of a) the 1 M NaCl plus 250 mM NH₄OH eluate from whey applied to Sepharose Fast Flow-S (Kabi-Pharmacia, Uppsala, Sweden; method 1), showing absorbance peaks for lactoperoxidase (8-ml elution volume) and lactoferrin (22-ml elution volume); b) the 400 mM NaCl eluate that contains lactoperoxidase; and c) the 1 M NaCl plus 250 mM NH₄OH eluate of whey applied to Sepharose Fast Flow-S (method 2) that contains lactoferrin. Protein was monitored by absorbance at 280 nm (A₂₈₀).

Figure 4. Growth-stimulating activities of the three fractions isolated together with fetal bovine serum (FBS) in a) Balb/c 3T3 cells, b) L6 myoblasts, and c) human skin fibroblasts. Values are means of three determinations for the following samples: FBS (●), 1 M NaCl plus 250 mM NH₄OH eluate from method 1 (○); 400 mM NaCl eluate from method 2 (△); and 1 M NaCl plus 250 mM NH₄OH eluate from method 2 (△). Growth is expressed as absorbance at 600 nm (A₆₀₀) according to the methylene blue technique (12) and is related to the final protein concentration. Standard errors are indicated when they are larger than the symbols.
in all cell lines, the responses were much lower than those obtained with the method 1 material or with the 400 mM NaCl eluate. The recovery of protein in the 400 mM NaCl eluate in the experiment shown in Table 1 was 22.7 mg/L of microfiltered whey. In a series of seven separations using Cheddar cheese whey from three different dairies, the yield obtained in the 400 mM NaCl fraction was 24.4 ± 2.1 mg/L (X ± SEM).

Lactoperoxidase is the most abundant protein in the 400 mM NaCl eluate obtained in method 2 (Figure 3b). To test whether this protein contributed to the growth-promoting activity of cells, 3.16 g of this fraction isolated from 150 L of whey were heated at temperatures that inactivate lactoperoxidase. Treatment at 80°C for 10 s led to a 40% loss of protein through precipitation and a 75% reduction of both lactoperoxidase activity and lactoperoxidase protein. When the transient heat treatment was increased to 100°C, 60% of the total protein was lost, and less than 2% of the initial lactoperoxidase protein or lactoperoxidase activity remained. The heat-treated and control samples were evaluated for growth-promoting activity in Balb/c 3T3 cells, L6 myoblasts, and human skin fibroblasts (Figure 5). When the extracts were reconstituted at concentrations that allowed for the protein losses, no loss of growth-stimulating activity could be detected in any of the cell lines after the 80°C treatment, and only slight losses occurred when the extract was heated to 100°C. Indeed, because the heated samples had been dissolved at lower protein concentrations to determine overall recoveries, the experiment demonstrated that the specific activities had been increased.

Analysis by SDS-PAGE of the 400 mM NaCl eluate (Figure 6a, lane 2) indicated two major protein bands in addition to lactoperoxidase. Because the bands at approximately 50 and 25 kDa are likely to represent the heavy and light chains of IgG (17), a sample of the dialyzed and freeze-dried 400 mM NaCl eluate was passed through a Protein G affinity column to establish whether removal of the IgG reduced the growth-promoting activity. This chromatography resulted in the removal of the 50-kDa band (Figure 6a, lane 3), although some residual staining occurred at 25 kDa, presumably because of the presence of proteins other than IgG, which could be casein fragments. Dialysis of the unadsorbed fraction, followed by freeze-drying, showed that 30% of the protein had been removed by the Protein G chromatography. Accordingly, the sample was

![Graph](https://example.com/graph.png)

Figure 5. Growth-stimulating activities of the 400 mM NaCl eluate from Sepharose Fast Flow-S (Kabi-Pharmacia, Uppsala, Sweden) chromatography of whey (§), or the same extract heated at 80°C for 10 s (○) or at 100°C for 10 s (△) when added to a) Balb/c 3T3 cells, b) L6 myoblasts, and c) human skin fibroblasts. Each fraction was added so that the volumes tested were equivalent to the unheated extract.
reconstituted for testing at 70% of the initial protein concentration. A comparison of these solutions in the Balb/c 3T3 cell growth assay (Figure 6b) showed essentially no loss of activity associated with the removal of IgG. A similar situation occurred with L6 myoblasts (data not shown).

**DISCUSSION**

The presence of cell growth-promoting activity in whole bovine milk is well established (10, 15), and, more recently, such activity has been demonstrated in the whey fraction (4, 5). In this context, several mitogenic factors have been identified in bovine colostrum, including insulin (1), members of the IGF family (7), transforming growth factor-β (3), and platelet-derived growth factor (16). However, the mitogenic activity and the growth factors present in cheese whey have not been examined. We report here that the isolation of growth factors from whey by cation-exchange chromatography is more effective than by size separation, thus allowing for a substantial purification of the mitogenic whey components from other proteins in this dairy fluid.

Separation of whey proteins by diafiltration against membranes rated at 10-, 30-, and even 100-kDa exclusion suggested that membrane filtration could not efficiently partition those growth factors acting on L6 myoblasts from the major whey proteins. Variations in the specified pore size of the ultrafiltration cartridges and secondary membrane effects that reduce protein permeability and allow for the retention of smaller proteins probably accounted for part of the overlap of stimulatory responses given by the ultrafiltered retentates. A second factor contributing to the apparent spread of activities across a wide range of molecular mass might have been the need for several growth factors of different molecular mass to act together to achieve an optimal stimulation of cell growth.

Cation-exchange chromatography was evaluated as a method to provide a mixture of growth factors suitable for replacing serum in culture media. The rationale for this approach was that most growth factors have basic isoelectric points, a property that could lead to the generation of a growth factor concentrate. This property was especially relevant when the biological source contained predominantly acidic proteins, a situation applicable to whey, in which the acidic proteins β-lactoglobulin, α-lactalbumin, casein fragments, and serum albumin together account for about 98% of the total protein (6, 17).

For the ion-exchange process used in this investigation, whey clarity was a requirement for reproducible chromatography and reduced column fouling. Microfiltration was therefore employed as a pretreatment to remove fat without substantial loss of the major whey

![Figure 6](image-url)

Figure 6. a) The SDS-PAGE of lane 2 400 mM NaCl eluate from whey chromatographed on Sepharose Fast Flow-S (Kabi-Pharmacia, Uppsala, Sweden; method 2; lane 3, the same eluate after passage through a protein G affinity column; and lane 4, IgG eluted from the protein G column. The molecular masses of standards in lane 1 are 94, 67, 45, 30, 20, and 14.4 kDa. b) Growth-stimulating activities of the 400 mM NaCl eluate from whey chromatographed on Sepharose Fast Flow-S (•) and the unadsorbed fraction after passage of that eluate through the protein G column (○) when added to Balb/c 3T3 cells. Each fraction was added so that equivalent volumes were tested.
proteins. Adsorption of the microfiltered whey on to Sepharose Fast Flow-S at neutral pH, followed by elution at high pH and high salt, led to the isolation of a fraction that contained only 1 to 2% of the initial protein but retained substantial growth-promoting activity in Balb/c 3T3 cells. Indeed, 10 µl of this fraction, containing 17 µg of protein, elicited a much higher growth rate than did 20 µl of microfiltered whey, containing 80 µg of protein and having a growth rate equal to 1 µl of FBS, which contained 42 µg of protein (see Figure 1). The column eluate completely replaced the need for FBS over a 2-d growth period once the cells had been attached to the dish. Analysis of the fraction by SDS-PAGE indicated a major protein band at 70 to 80 kDa that could be resolved by Mono-S chromatography into lactoperoxidase and lactoferrin. This observation suggested a strategy to refine further the growth factor preparation using stepwise elution from the preparative Sepharose Fast Flow-S column into separate samples rich in lactoperoxidase and lactoferrin. When this concept was tested, virtually all the growth-stimulating activity on Balb/c 3T3 cells, L6 myoblasts, or human skin fibroblasts had eluted with the lactoperoxidase component; hence, the specific activity of the preparation had been further increased. In relation to this point, lactoferrin preparations have been shown to stimulate the growth of some cells, including the L6 myoblast line used (2, 8). In view of the very poor stimulation of growth for all three cell lines exhibited by the fraction containing lactoferrin compared with the eluate containing lactoperoxidase plus lactoferrin (Figure 4), the previously reported activity of lactoferrin is unlikely to be due to this protein, but, rather, to copurified growth factors.

Because lactoperoxidase and IgG are the most abundant proteins present in the 400 mM NaCl eluate from the Sepharose Fast Flow-S column (Figure 6a), one or more of these components could conceivably contribute to the growth-promoting activity of the fraction; however, we eliminated this possibility by demonstrating that essentially no effect on residual growth activity occurred following the removal of IgG by affinity chromatography or by heat inactivation of lactoperoxidase. Indeed, removal of these proteins increased the specific activity of the preparation.

CONCLUSIONS

The isolation procedure used in the present study yielded a fraction from whey capable of significant growth stimulation in three different mammalian cell lines. The growth response from this lactoperoxidase-rich fraction was superior to that provided by FBS at equivalent protein concentrations, especially with Balb/c 3T3 cells and human skin fibroblasts. Such a whey extract might be considered for use as a replacement for FBS for the culture of these and other cell types.

ACKNOWLEDGMENTS

This work was supported by a grant from the Dairy Research and Development Corporation of Australia. We acknowledge helpful discussions with G. Smithers and I. Mitchell at the CSIRO Dairy Research Laboratory, Highett, Victoria, Australia; the provision of whey by their laboratory; and the gift of lactoperoxidase and lactoferrin standards from D. Dionysius, International Food Institute, Hamilton, Queensland, Australia.

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Journal of Dairy Science Vol. 78, No. 6, 1995