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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 1/18, 1/34, 1/36, C12N 5/02, A61K 38/18, A23J 1/20, A23C 9/00, 21/10

A1

(11) International Publication Number: **WO 95/29933**

(43) International Publication Date: 9 November 1995 (09.11.95)

(21) International Application Number: PCT/AU95/00237

(22) International Filing Date: 26 April 1995 (26.04.95)

(30) Priority Data:
PM 5347 28 April 1994 (28.04.94) AU

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: MODIFIED MILK GROWTH FACTOR

(57) Abstract

The invention provides a process for preparing a plurality of modified milk growth factors which process includes: providing a milk product extract including a plurality of milk growth factors having basic to approximately neutral isoelectric points and a source of acid; subjecting the milk product extract to transient acidification utilising the acid source; and isolating a plurality of modified milk growth factors from the transiently acidified milk product extract. Also provided are growth promoting compositions, cell culture compositions, and pharmaceutical or veterinary compositions for the treatment of surface wounds, gastrointestinal injuries, diseases or ulcers, each including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30.000, the milk growth factors being modified by transient acidification.

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MODIFIED MILK GROWTH FACTOR

This invention relates to the modification of growth factors in milk or milk extracts and to improved methods for the extraction of such growth factors from milk or milk products and their use in cell culture and pharmaceutical applications.

Animal cells are grown in culture to provide a number of pharmaceutical, diagnostic and veterinary products including human vaccines, lymphokines, hormones, monoclonal antibodies, other pharmaceutically active protein products, veterinary hormones and for research and development and diagnostic purposes.

The growth of animal cells requires a defined isotonic medium that contains salts, nutrients, lipid precursors, nucleic acid precursors, vitamins and amino acids that are formulated to mimic the medium that would normally bathe those cells *in vivo*. Examples in common use include Eagle's Minimal Essential Medium, Dulbecco's modified Eagle's Minimal Essential Medium (DMEM), Medium 199, RPMI 1640 medium and Ham's F12 Medium. However, virtually no animal cells will grow in such media, but require the co-addition of serum. Fetal bovine serum is frequently used as it is more effective than serum obtained from post-natal animals and it contains only minimal concentrations of immunoglobulins which otherwise could have undesirable effects.

The supply of fetal bovine serum is limited by the number of pregnant cows slaughtered. It also has undesirable lot-to-lot variations and may include toxins. Particular concern surrounds its use for the eventual production of recombinant proteins and other pharmaceuticals for human use because the serum may also contain viruses that are harmful to humans and may be carried through a purification protocol that yields the desired product. Principally for these reasons, extensive efforts have been directed towards the replacement of serum by pure ingredients. Examples of such ingredients are growth factors, hormones and cell attachment factors. Unfortunately, the requirements of each cell type being grown are different and are difficult to establish. Frequently it has not proved possible to achieve equivalent growth properties or equivalent yields of cell products with "serum-free" media as can be obtained with medium containing fetal bovine serum.

The limited availability of fetal bovine serum, its lot-to-lot variability, its resultant considerable cost as well as the deficiencies of "serum-free" media

described above have prompted the investigation of other biological fluids as potential replacements in cell culture media. Some progress has been reported in the prior art with bovine milk and bovine colostrum as evidenced by the following selected reports: M. Klagsbrun, *Proc. Natl. Acad. Sci. USA 75*, 5057 (1978); M. Klagsbrun et al. *J. Supramol. Struct. 11*, 349 (1979); M. Klagsbrun, *J. Cell Biol. 84*, 808 (1980); K.S. Steimer et al. *J. Cell Biol. 88*, 294 (1981); A. Sereni et al. *Cell Biol. Intl. Rep. 5*, 339 (1981); A. Khar *Experientia 39*, 534 (1983); O. Damerdji et al. *Biotech. Tech. 2*, 253 (1988); O.T. Ramirez et al. *Lait 70*, 313 (1990); H. Ichiba et al. *Biol. Neonate. 61*, 47 (1992); R. Pakkanen et al. *Appl. Microbiol. Biotechnol. 37*, 451 (1992). These and other publications alluded to herein are incorporated by reference.

The prior art also includes U.S. Patent 4,440,860 to M. Klagsbrun which describes "compositions and methods for promoting cell growth featuring, in one aspect, cell culture media containing milk or colostrum and fibronectin; fibronectin is preferably pre-coated onto the culture substrate" and Japan Patent JP 59166879 to Morinaga "A culture medium for cell incubation - containing milk or milk components". Ultrafiltrates of milk whey have also been used to support the growth of cultured cells, as in European Patent A0219372 to G. Linden et al. "Fractions de lait, Procedee d'obtention de ces fractions et milleux de culturo cellulaires renfermant ces fractions" and application WO 90/06357 to G. Linden et al. "Supplements of animal cell culture media based on products derived from the milk industry". An active fraction from colostrum obtained by hydroxyapatite chromatography has been reported in application WO 92/00014 by B. Quinque et al. "Method for treating colostrum thereby obtained, and a cellular medium containing same" and by cation exchange chromatography in Australian Patent 645589 to F.J. Ballard et al. "Growth-promoting agent". The prior art also includes Australian Patent 598205 to F.J. Ballard et al. "Growth Factor" and European Patent 03 13515 to R.R. Burk et al. "A polypeptide growth factor from milk" in which individual growth factors have been isolated from colostrum or milk in pure form.

This progress has led to the development of formulations that support the growth of some cell types but usually require protein concentrations in the medium that approach those in more general use with fetal bovine serum.

It is accordingly an object of the present invention to overcome or at least alleviate one or more of the deficiencies related to the prior art.

According to the present invention there is provided a process for preparing a plurality of modified milk growth factors which process includes

providing

a milk product extract including

a plurality of milk growth factors having basic to approximately neutral isoelectric points; and

a source of acid,

subjecting the milk product extract to transient acidification utilising the acid source; and

isolating a plurality of modified milk growth factors from the transiently acidified milk product extract.

Preferably the isolation step includes subjecting the transiently acidified milk product extract to a purification step to remove inactive proteins.

By "transient acidification" is meant acidification for a period sufficient to enhance the growth promoting activity of the factors whilst avoiding any significant degradation of the factors.

The present invention further provides a growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification.

Preferably the modified milk growth factors have isoelectric points between approximately 6.0 and 10.5.

The term "milk" as used herein refers to lactational secretions of human or animal origin.

The term "modified milk growth factor" as used herein refers to growth factors of milk that exhibit enhanced growth promoting activity compared to the growth promoting activity of the same growth factors in their occurring state or when extracted from milk, a milk product or a milk product extract.

The term "milk product" as used herein refers to a derivative from human or animal milk in which the fat and/or protein constituents thereof are reduced.

Examples of milk product include milk whey, skim milk, cheese whey and acid (casein) whey. Preferably the milk product is cheese whey.

The term "milk product extract" as used herein refers to an extract of milk product in which the salt and/or main protein constituents thereof are reduced. Examples of milk product extracts are ultrafiltrates of milk products or milk products that have been subjected to adsorption and to elution from chromatography matrices. Preferably the milk product extract is cheese whey that has been subjected to cation exchange chromatography by the method described in Australian Patent 645,589.

Preferably the modified growth factor are obtained by acidifying milk product extract containing growth factors having basic to approximately neutral isoelectric points. Preferably inactive proteins are removed from the modified milk growth factors in accordance with the invention. Molecular sieve chromatography or ultrafiltration under acidic conditions may be used to remove inactive proteins. Molecular sieve chromatography is particularly preferred as it removes higher molecular weight proteins such as growth factor binding proteins that would otherwise recombine with certain growth factors after transient acidification.

The term "enhanced growth promoting activity" as used herein is a measure of increased cellular growth per unit of growth factor compared to the growth promoting activity of the same growth factors in their occurring state or when extracted from milk, a milk product or a milk product extract. Preferably cellular growth per unit of growth factor is increased by 10%, more preferably by 25%.

Accordingly, in a preferred aspect, the purification step in the process of the present invention includes subjecting the transiently acidified milk product extract to a chromatography or ultrafiltration process performed under acidic conditions, preferably performed at a pH of approximately 3.0 or below.

Preferably the chromatography process is a molecular sieve chromatography process and the ultrafiltration process is a controlled pore ultrafiltration process. Preferably the controlled pore ultrafiltration is performed using a membrane that excludes molecules larger than approximately 100 kDa, more preferably approximately 50 kDa, most preferably approximately 30 kDa.

In a further preferred aspect, the process of the present invention further provides the preliminary step of

providing

a source of milk or milk product;

a cationic exchange resin; and

a buffer solution;

contacting the milk or milk product with the cation exchange resin such that the more basic components of the milk or milk product are absorbed thereon;

eluting the cationic exchange resin with the buffer solution; and

collecting a milk product extract therefrom.

The preliminary purification step is more fully described in Australian Patent 645,589 to applicants.

Preferably the milk product extract includes a GFE-2 or like growth promoting agent as hereinafter defined.

Preferably the growth factors are subject to acidification to a pH of approximately 3.0 or below. More preferably the factors are acidified to a pH in the range 2.0 to 3.0. An acidification pH of about 2.5 is particularly preferred.

Preferably acidification is carried out using an inorganic acid, for example, HCl. For example acidification may be achieved by dissolving the milk product extract in water and acidifying with a strong inorganic acid such as 5M HCl and drying. Preferably the growth stimulating factors in the milk product extract are obtained by carrying out cation exchange chromatography on a milk product. The milk product extract may be produced by the method described in Australian Patent Application 645,589.

As stated above, the process of the invention may include the further step of removing inactive proteins after acidification has taken place.

Removal of inactive proteins may be carried out using molecular sieve chromatography or ultrafiltration under acidic conditions. As mentioned previously, molecular sieve chromatography is particularly preferred as it removes high molecular weight proteins that would otherwise recombine with certain growth factors following neutralisation.

The present invention will be more fully described with reference to the milk product extract described in Applicant's earlier Australian application Patent 645,589 (herein incorporated by reference). However, this is illustrative only and should not be taken as a restriction on the generality of the invention.

The milk product extract used in the method of the invention should not previously have been subjected to transient acidification to approximately pH 3.0 or below.

The individual growth factors present in serum, milk or other biological fluids such as epidermal growth factor, insulin-like growth factor I or II, acidic or basic fibroblast growth factor, transforming growth factor alpha or beta, or platelet derived growth factor are all small proteins with molecular weights between 5,000 and 30,000. It was surprising, therefore, that following molecular sieve chromatography under conditions near neutral pH of the growth promoting agent GFE-2 described in Australian patent 645589 to F.J. Ballard et al., essentially all the cell growth stimulating activity was present in the high molecular weight region. Specifically, the growth promoting activity on L6 myoblasts, Balb/C 3T3 fibroblasts and human skin fibroblasts co-eluted with lactoperoxidase, the most abundant protein in GFE-2, which has a molecular weight of 78,000 (see Figure 1, dashed line).

By the term "GFE-2 or like growth promoting agent" as used herein we mean a milk product extract in which a plurality of growth factors having neutral to basic isoelectric points are present and have been isolated as a milk growth factor extract by cation exchange chromatography without exposure to acidic conditions.

The unexpected finding that the growth promoting activity in GFE-2 extracted from cheese whey has a much higher molecular weight than essentially all known growth factors suggested that the growth effects in the GFE-2 were either elicited by unknown factors or by known factors but which were in the form of aggregates. The invention describes two approaches utilised to distinguish between these alternatives and provides methods for activating or enhancing the growth promoting activity of milk product extracts, and in particular enhancing the growth promoting activity of GFE-2 or like growth promoting agents.

In plasma, certain growth factors, for example insulin-like growth factors I and II, occur predominantly in association in binding protein complexes that include an acid-labile subunit (R.C. Baxter et al. *Progress in Growth Factor Research 1*, 49, 1989). Transient acidification of plasma releases the insulin-like growth factors to lower molecular weight forms that may have enhanced activities.

A similar process is known to release transforming growth factor beta from a high molecular weight complex (M.B. Sporn et al. *J. Cell Biol. 105*, 1039, 1987). Applicant has now found that if the growth factors in milk products such as cheese whey remain in complexes through the cation exchange chromatography step required to produce milk product extracts such as GFE-2, then transient acidification releases those factors and hence leads to an increase in the capacity of the milk extract to stimulate the growth of cultured cells. This is confirmed in the experiment shown in Example 2 where the growth of CHO-K1 cells is stimulated at much lower protein concentrations and to a greater extent with transiently acidified GFE-2 than with GFE-2 itself.

Accordingly there is provided in a preferred embodiment a plurality of modified milk product growth factors exhibiting enhanced growth stimulating activity and having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification.

Preferably the plurality of modified milk growth factors is modified GFE-2 or like growth promoting agent.

Whereas transient acidification does lead to enhanced growth-promoting activity in CHO-K1 cells, the total protein concentration of GFE-2 is not markedly reduced by such a process. The protein content of the acidified growth promoting extract may be substantially reduced by molecular sieve chromatography under acid conditions whereby the growth promoting activities are separated from the bulk of the inactive protein. Although the examples in Figures 3, 4 and 5 utilise Superose 12 HR 10/30™ (Pharmacia) and Matrex Cellufine GCL 1000™ (Amicon) molecular sieve column packings, these are illustrative only so that a person familiar with the art would recognise that similar separations could be obtained with other molecular sieve column packings.

In this embodiment of the invention the growth-promoting activity on several cell lines is transferred to the lower molecular weight region and separated from the bulk of extraneous proteins in GFE-2 (see Figure 3). Should the individual fractions collected in the example of Figure 3 instead be collected as a single molecular weight pool, as in example Figure 4, the resultant pool exhibits growth-promoting activity at substantially lower protein concentrations than obtained with

unfractionated GFE-2 (see Figure 5).

In a further aspect of the invention the protein concentration of the acidified growth promoting extract may be reduced by controlled pore ultrafiltration instead of molecular sieve chromatography. When this is accomplished under acidic conditions using an ultrafiltration membrane with a nominal size cut off of, say, 100 kDa, the growth promoting activities may be transferred to the permeate as demonstrated in Figure 6. Moreover, growth promoting activities occur at substantially lower protein concentrations than in unfractionated GFE-2. This example of use with a 100 kDa membrane is illustrative only so that a person familiar with the art would recognise that similar separations could be obtained with other membranes that allow passage of growth factors into the permeate fraction but retard the permeation of proteins with molecular weights larger than approximately 30,000.

In a further aspect the present invention provides a cell culture composition including

an effective amount of a growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a culture medium.

Preferably the plurality of modified milk growth factors is a modified GFE-2 or like growth promoting agent.

More preferably, the culture medium is a substantially protein-free culture medium and/or contains fetal bovine serum.

The present invention also provides a method for culturing human or animal cells which method includes

providing

a source of animal cells; and

a cell culture composition including an effective amount of a growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of

approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a culture medium; and

culturing the cells in the cell culture composition for a time sufficient and at a temperature sufficient to achieve a predetermined cell concentration.

Preferably the cells are cultured at a temperature in the range of approximately 35°C to 40°C for a period of approximately 1 to 5 days.

The processes of this invention that include either transient acidification of milk product or acidification followed by separation to yield a low molecular weight fraction under acid conditions also can be applied to yield products for uses not restricted to the growth of cells in culture. In this context specific examples include growth of more organised structures in culture, such as skin for subsequent transplant to burned individuals or to cover exposed wounds.

The present invention also provides a pharmaceutical or veterinary composition for the treatment of surface wounds, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

Preferably the composition further includes an effective amount of at least one active ingredient selected from antibiotics, antiseptics, other growth promotants, and mixtures thereof.

Accordingly the present invention provides a method of treating surface wounds in animals, including humans, which method includes administering to the patient to be treated an effective amount of a pharmaceutical or veterinary composition, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

Preferably the composition further includes an effective amount of at least one active ingredient selected from antibiotics, antiseptics, other growth promotants, anaesthetics, and mixtures thereof.

The pharmaceutical or veterinary compositions may be adapted for the treatment of gastrointestinal injuries, diseases or ulcers. Accordingly the present invention provides a pharmaceutical or veterinary composition for the treatment of gastrointestinal injuries, diseases or ulcers, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

Preferably the composition further includes an effective amount of at least one active ingredient selected from antibiotics, antiseptics, other growth promotants, anaesthetics, and mixtures thereof.

The present invention further provides a method for the treatment of a human or animal subject, said method including administering an effective amount of a growth promoting composition in accordance with the invention.

An application of this invention is to produce products that can be added directly to surface wounds where the enhanced growth-promoting activities can accelerate the repair of those wounds.

EXAMPLES

The present invention will now be more fully described with respect to the following examples. It should be understood, however, that the description following is illustrative only, and should not be taken in any way as a restriction of the generality of the invention described above.

DESCRIPTION OF FIGURES

Figure 1: Milk product extract GFE-2 as in Example 1 was dissolved in phosphate buffered saline and chromatographed on a Superose 12 HR10/30™ (Pharmacia) column equilibrated with the same buffer. Protein was measured by absorbance at 280 nm (dashed line). Growth promoting activity in each fraction was measured in the 3 cell lines [L6 Myoblasts (Figure 1A), Balb/C 3T3 (Figure 1B), SF1972 (Figure 1C)]

as the percentage of growth achieved when 10% fetal bovine serum was the reference source of growth factor (•).

Figure 2: Growth promoting activity in CHO-K1 cells by milk product extract GFE-2 as in Example 1 (•) and transiently acidified milk product extract GFE-2 prepared as in Example 2(o). Fetal bovine serum (FBS) is shown as a reference source of growth factors (■).

Figure 3: Milk product extract GFE-2 as in Example 1 was acidified and subjected to molecular sieve chromatography as in Example 3. Protein is shown by its absorbance at 280 nm (dashed line). Growth promoting activity of dried and reconstituted fractions was measured in the 3 cell lines [L6 Myoblasts (Figure 3A), Balb/C 3T3 (Figure 3B), SF1972 (Figure 3C)] as the percentage of growth achieved when 10% fetal bovine serum was the reference source of growth factors (•).

Figure 4: Larger scale preparation of a modified milk growth factor by chromatography of acidified GFE-2 on Matrex Cellufine GCL I000™ (Amicon) under acid conditions according to Example 4. The low molecular weight pool (LMW-pool) was collected and dried for subsequent assay of growth promoting activity.

Figure 5: Growth promoting activities of GFE-2 prepared as in Example 1 (•) and the LMW-pool prepared as in Example 4 (o) on 3 cell lines [L6 Myoblasts (Figure 5A), human endothelial cells (Figure 5B), SF3169 (Figure 5C)]. Fetal bovine serum (FBS) is shown as a reference source of growth factors (■).

Figure 6: Growth promoting activities of GFE-2 prepared as in Example 1 (•) and a permeate of acidified GFE-2 obtained by ultrafiltration through a 100 kDa membrane (Sartorius) (o) on 3 cell lines [L6 Myoblasts (Figure 6A), Balb/C 3T3 (Figure 6B), SF1967 (Figure 6C)]. Activities are expressed relative to those achieved when 10% fetal bovine serum was the reference source of growth factors.

EXAMPLE 1

Molecular sieve chromatography of whey extract under neutral pH conditions demonstrates cell growth promoting activity predominantly in the high molecular weight region

GFE-2 was prepared as in Australian Patent 645589 to F.J. Ballard et al. This process involved microfiltration of pasteurised whey to remove solids; adsorption of growth-promoting material to a column of S-Sepharose Fast Flow S™ cation exchange resin (Pharmacia) that had been equilibrated with 50mM sodium citrate buffer at pH 6.5; washing the column with the same buffer to remove unadsorbed material; elution of GFE-2 with 0.4 M NaCl added to 10mM sodium citrate pH 6.5; diafiltration against water, concentration by ultrafiltration and freeze drying.

The GFE-2 was dissolved in Dulbecco's phosphate buffered saline at pH 7.4 (PBS), passed through a 0.22μm filter and 200μl applied to a Superose 12 HR 10/30™ (Pharmacia) column equilibrated with the same buffer and chromatographed at 0.3ml/min. Fractions of 0.9ml were collected into tubes containing 100μl of 10mg bovine serum albumin/ml.

This example utilises the cell lines L6 (rat myoblast), Balb/C 3T3 (mouse fibroblast) and SF 1972 (human diploid skin fibroblast) to test the activities of each fraction.

Each cell line was subcultured on to 96-place tissue culture plates in Dulbecco-Modified Eagle's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum (5% for L6 myoblasts) and left in a 5% CO_2, 37°C, humidified incubator overnight to ensure attachment of the cells. Sterile techniques were used throughout. The plates were thoroughly washed in DMEM to remove any residual serum and the whey fractions or fetal bovine serum (FBS) added at the indicated concentrations. The total volume in each well was 0.1 ml at 37°C, 5% CO_2 and 100% humidity.

After a further 2 days the plates were washed, fixed and the cell numbers quantified using an automated methylene blue method (M.H. Oliver *et al., J. Cell Sci.* 92, 513, 1989). Growth is expressed as the percentage increase in absorbance units relative to the increase in absorbance produced by growing the

cells in DMEM containing 10% fetal bovine serum (Figure 1).

This example shows that in all three cell lines fetal bovine serum and 20µl portions of certain fractions obtained from the column stimulated growth. The fractions with the most activity coincided with the main protein region eluted from the column as shown by its absorbance at 280nm (dotted lines, Figure 1). Activities in Figure 1 are expressed as the percentage increase in growth relative to that obtained with 10% fetal bovine serum.

EXAMPLE 2

Transient acidification leads to enhanced growth promoting activity in CHO-K1 cells

A 50mg sample of GFE-2 prepared as in Example 1 was dissolved in 2ml of water and acidified to pH 1.6 with 5M HCl; adjusted to pH 2.5 with 0.5M NaOH and dried under vacuum. The dried material was dissolved in 1ml of Ham's F12 cell culture medium containing 10mg of bovine serum albumin and serially diluted in the same medium. A sample of GFE-2 at the same initial concentration and fetal bovine serum were similarly diluted in Ham's F12 medium containing bovine serum albumin.

The test solutions were evaluated for growth-promoting activity as described in Example 1 except that the cells were CHO-K1 and the medium Ham's F12 instead of DMEM.

This example (Figure 2) shows that whereas GFE-2 does produce a modest growth effect, much greater growth at lower total protein concentrations is achieved by the transiently acidified preparation.

EXAMPLE 3

Molecular sieve chromatography under acid conditions transfers the growth promoting activity of whey extracts to the low molecular weight region

GFE-2 was prepared as in Example 1. A 25mg portion was dissolved in 1 M acetic acid containing 0.15M NaCl, passed through a 0.22µm filter and 200µl applied to the same Superose 12 HR 10/30 column as in Example 1 except that the column was equilibrated with 1M acetic acid containing 0.15M NaCl. Chromatography and sample collection were as described in Example 1 except that the elution buffer was 1M acetic acid containing 0.15M NaCl. The fractions were dried under vacuum, dissolved in DMEM and the growth promoting activities

evaluated as described in Example 1. The results are shown in Figure 3 with the protein absorbance profile shown as dotted lines.

This experiment showed that acidification of GFE-2 followed by molecular sieve chromatography led to the growth promoting activity in L6 myoblasts, Balb/C 3T3 and SF 1972 cells shifting to later elution volumes than the protein absorbance peak; hence to low molecular weight regions.

EXAMPLE 4

The pooled low molecular weight fraction obtained after molecular sieve chromatography of whey extracts under acid conditions has enhanced specific activity for supporting cell growth.

GFE-2 was prepared as in Example 1. A 10g portion was dissolved in 250ml of 10mM HCl containing 0.2M NaCl and the pH adjusted to 2.5 with NaOH. This solution was used to equilibrate a 2 litre Cellufine GCL I000™ (Amicon) column. 125ml of the dissolved GFE-2 was applied to the column and eluted at 6.8ml/min with the same solution. 675ml was collected from when the absorbance profile at 280nm fell below 0.4 (Figure 4). This pool was diafiltered against 0.1 M ammonium bicarbonate and freeze-dried to yield 385mg of product.

The low molecular weight pool (LMW pool) and GFE-2 were dissolved as described in Example 1 and cell growth measured as in that Example except that the cell lines tested were L6 myoblasts, SF 3169 human skin fibroblasts and human endothelial cells obtained as described by E. Jaffe *et al. J. Clin. Invest.* 52, 2745 (1973).

This experiment shows that lower concentrations of LMW pool stimulated growth than obtained with GFE-2. This increased specific activity was particularly apparent with the human skin fibroblast and human endothelial cells, as shown in Figure 5.

EXAMPLE 5

Controlled pore ultrafiltration under acid conditions transfers the growth promoting activity of whey extract to the permeate.

GFE-2 was prepared as in Example 1. 40g of GFE-2 was suspended in 40L of 10mM tri-sodium citrate and adjusted to pH 2.5 using 0.1M HCl. The acidified protein solution was left overnight at 4°C and then concentrated to 5L against a large pore ultrafiltration membrane (Sartorius, 0.7m^2, polysulfone, 100kDa cut-off

pore size). The concentrate was diafiltered extensively (20:1) with 100L of 150mM NaCl/HCl solution at pH 2.5. Permeate (approx. 130L) collected from the concentration/diafiltration cycle was adjusted to pH 7.0 with 0.1M NaOH and transferred to an Amicon ultrafiltration system equipped with a single 1.8m^2, spiral wound polysulfone membrane (3kDa cut-off pore size). The permeate was concentrated in this system to 10L; diafiltered (5:1) with 50L of 150mM NH_4HCO_3, and then further concentrated to a final volume of 250ml. The concentrated permeate was freeze dried, yielding approximately 1.8g of powder.

The dried permeate and GFE-2 were dissolved in water and the growth promoting activities evaluated as described in Example 1. The results are shown in Figure 6.

This experiment shows that the permeate obtained through ultrafiltration of acidified whey extract supports the growth of L6 myoblasts, Balb/C 3T3 and SF 1967 cells, a human skin fibroblast line similar to the SF 1972 and SF 3169 cells used in Examples 1, 3 and 4. Furthermore, the growth promoting activity in all three cell lines occurs at substantially lower protein concentrations than observed with GFE-2.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

Claims

1. A process for preparing a plurality of modified milk growth factors which process includes

providing

a milk product extract including

a plurality of milk growth factors having basic to approximately neutral isoelectric points; and

a source of acid,

subjecting the milk product extract to transient acidification utilising the acid source; and

isolating a plurality of modified milk growth factors from the transiently acidified milk product extract.

2. A process according to claim 1 wherein the milk product extract is subjected to transient acidification to a pH of approximately 3.0 or below.

3. A process according to claim 2 wherein the isolation step includes subjecting the transiently acidified milk product extract to a purification step under acidic conditions to remove inactive proteins.

4. A process according to claim 3 wherein the purification step includes subjecting the transiently acidified milk product extract to a molecular sieve chromatography process.

5. A process according to claim 3 wherein the purification step includes a controlled pore ultrafiltration process using a membrane that excludes molecules larger than approximately 30 kDa.

6. A process according to claim 1 wherein the milk product extract is a cheese whey extract.

7. A process according to claim 1 wherein the process includes the preliminary step of obtaining a milk product extract by

providing

a source of milk or milk product;

a cationic exchange resin; and

a buffer solution;

contacting the milk or milk product with the cation exchange resin such that the more basic components of the milk or milk product are absorbed thereon;

eluting the cationic exchange resin with the buffer solution; and

collecting a milk product extract therefrom.

8. A process according to claim 7 wherein the milk product extract includes a GFE-2 or like growth promoting agent.

9. A plurality of modified milk growth factors exhibiting enhanced growth stimulating activity and having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification.

10. A plurality of modified milk growth factors according to claim 9 being modified GFE-2 or like growth promoting agent.

11. A plurality of modified milk growth factors according to claim 9 whenever prepared by a process according to any of claims 1 to 8.

12. A growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification.

13. A growth promoting composition according to claim 12 wherein the plurality of modified milk growth factors is modified GFE-2 or like growth promoting agent.

14. A growth promoting composition according to claim 12 including a plurality of modified milk growth factors whenever prepared by a process according to any of claims 1 to 8.

15. A cell culture composition including

an effective amount of a growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a culture medium.

16. A cell culture composition according to claim 15 wherein the plurality of modified milk growth factors is modified GFE-2 or like growth promoting agent.

17. A cell culture composition according to claim 15 including a plurality of

modified milk growth factors whenever prepared by a process according to any of claims 1 to 8.

18. A cell culture composition according to claim 15 wherein the culture medium is a substantially protein-free culture medium.

19. A cell culture composition according to claim 15 wherein the culture medium contains fetal bovine serum.

20. A method of culturing human or animal cells which method includes providing

a source of animal cells; and

a cell culture composition including an effective amount of a growth promoting composition exhibiting enhanced growth stimulating activity including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a culture medium; and

culturing the cells in the cell culture composition for a time sufficient and at a temperature sufficient to achieve a predetermined cell concentration.

21. A pharmaceutical or veterinary composition for the treatment of surface wounds, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

22. A pharmaceutical or veterinary composition for the treatment of gastrointestinal injuries, diseases or ulcers, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

23. A method of treating surface wounds in animals, including humans, which

method includes administering to the patient to be treated an effective amount of a pharmaceutical or veterinary composition, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

24. A method for the treatment of gastrointestinal injuries, diseases or ulcers, which method includes administering to the patient to be treated an effective amount of a pharmaceutical or veterinary composition, which composition includes

a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification; and

a pharmaceutically or veterinarily acceptable diluent, carrier or excipient therefor.

25. A plurality of modified milk growth factors substantially as hereinbefore described with reference to any one of Examples 1 to 6.

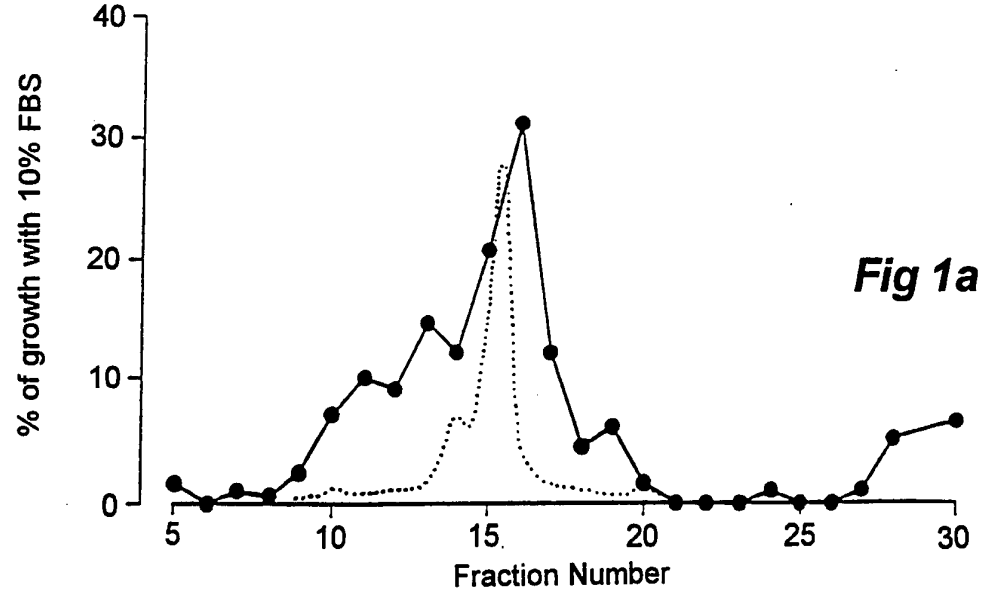

Fig 1a

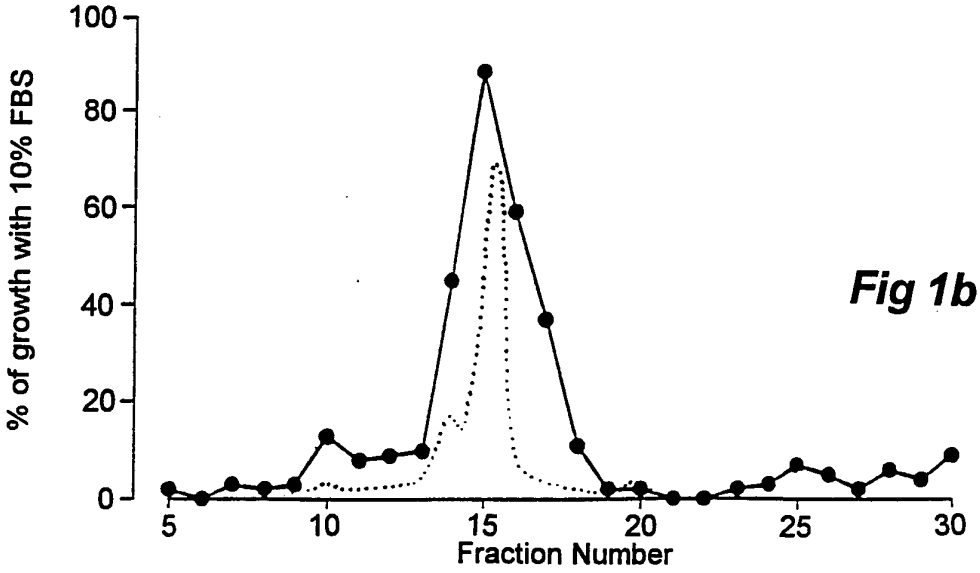

Fig 1b

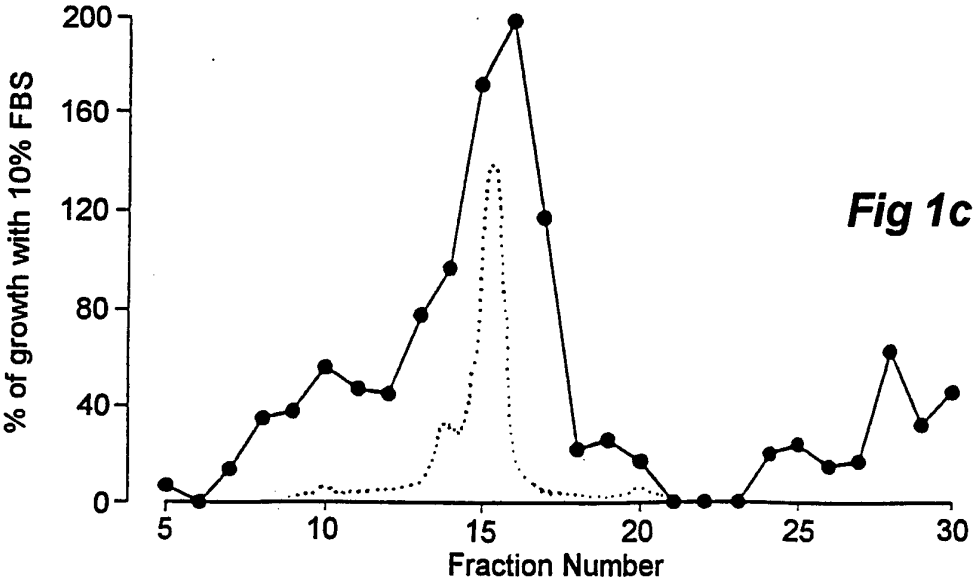

Fig 1c

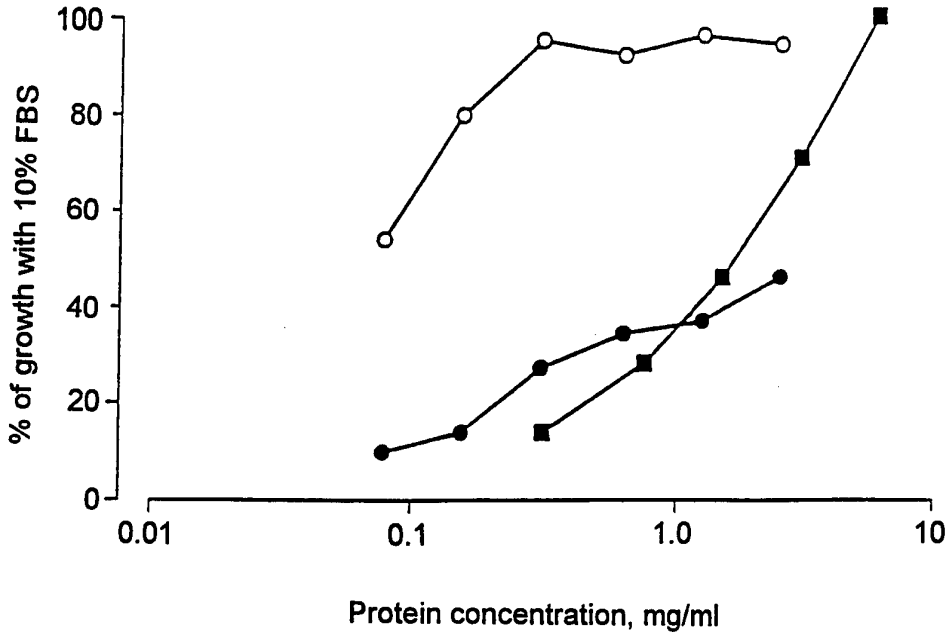
100
80
60
40
20
0
% of growth with 10% FBS
0.01
0.1
1.0
10
Protein concentration, mg/ml

Fig 2

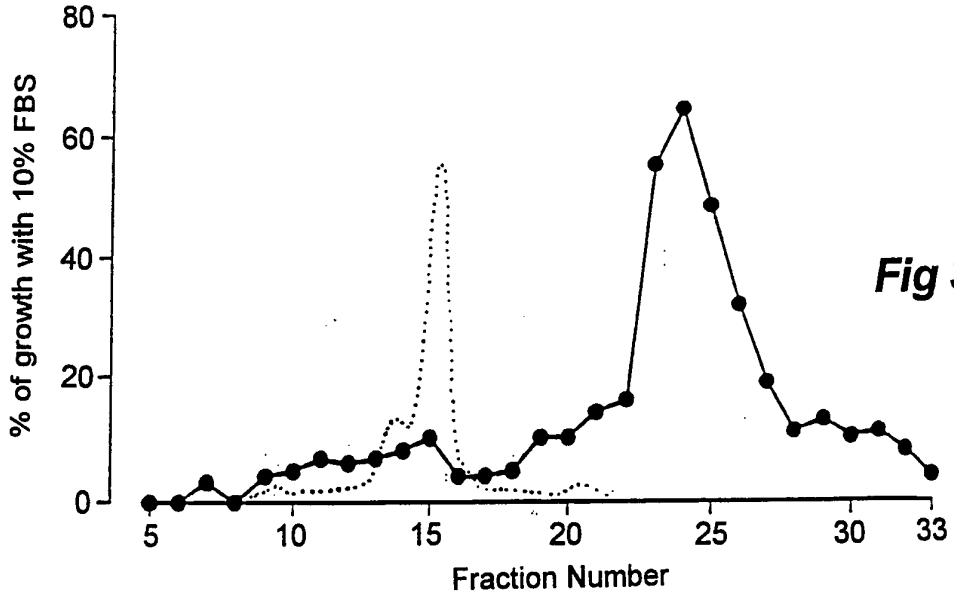

Fig 3a

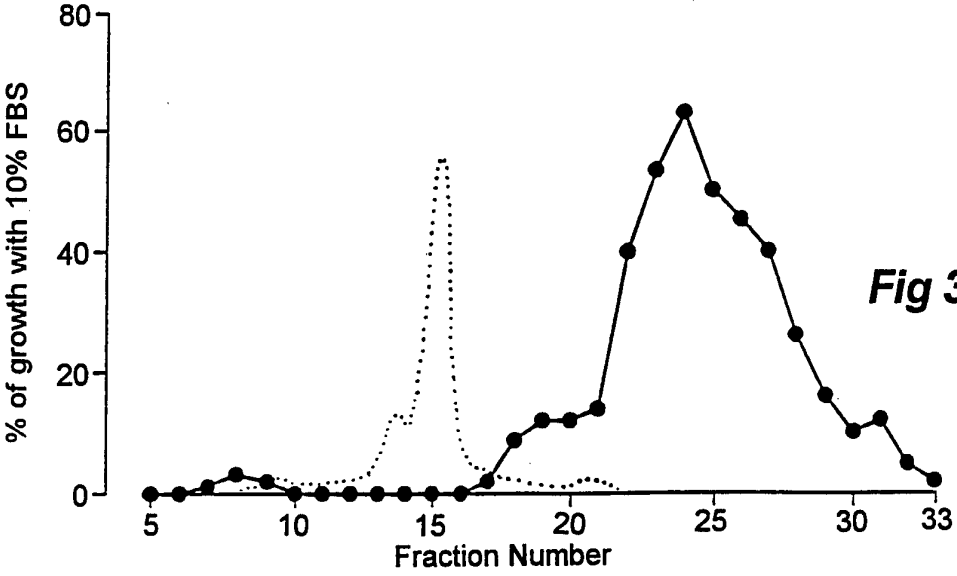

Fig 3b

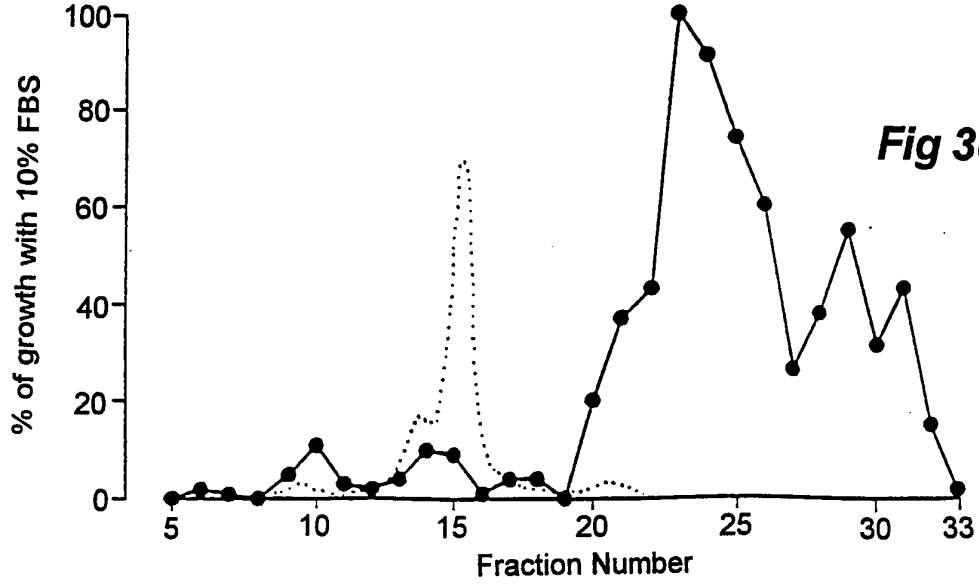

Fig 3c

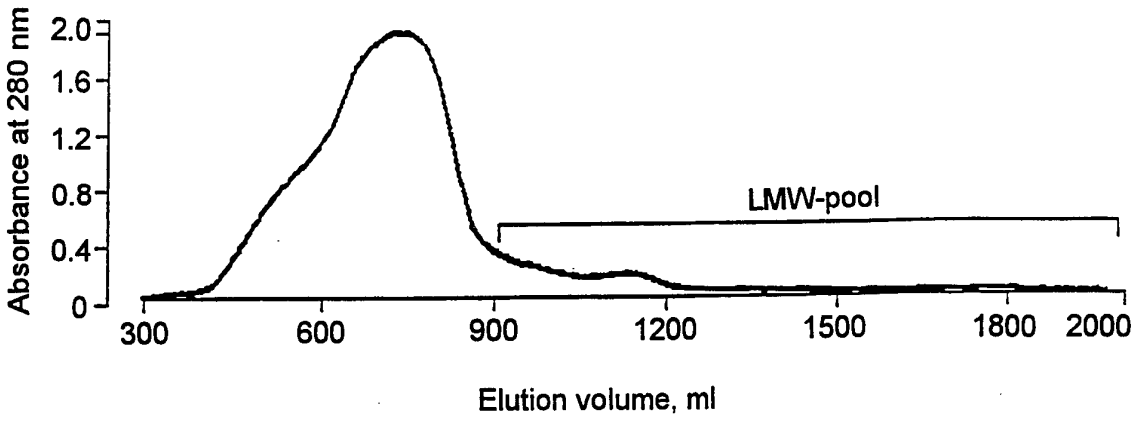
Absorbance at 280 nm
2.0
1.6
1.2
0.8
0.4
0
300
600
900
1200
1500
1800
2000
LMW-pool
Elution volume, ml

Fig 4

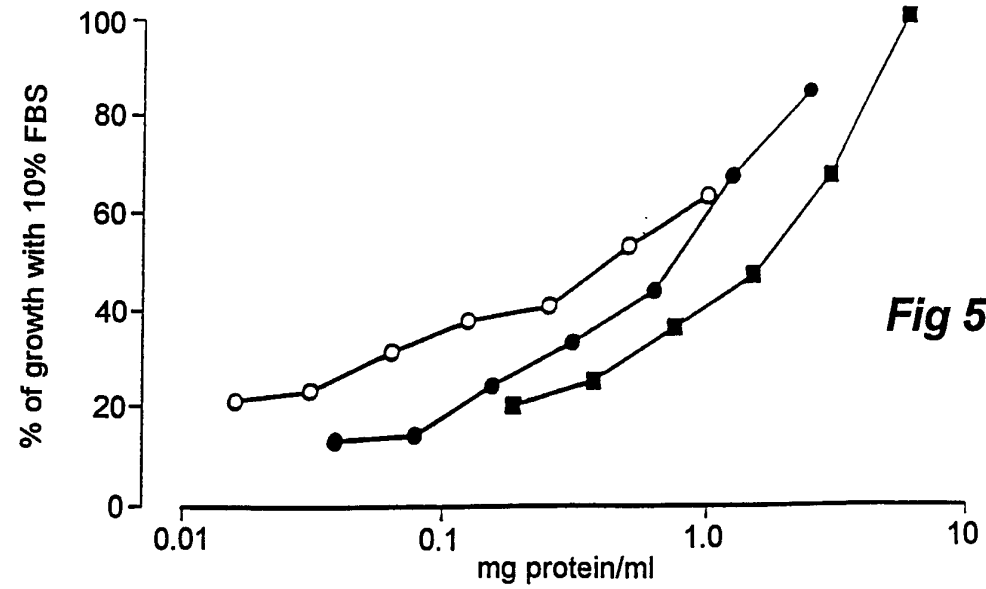

Fig 5a

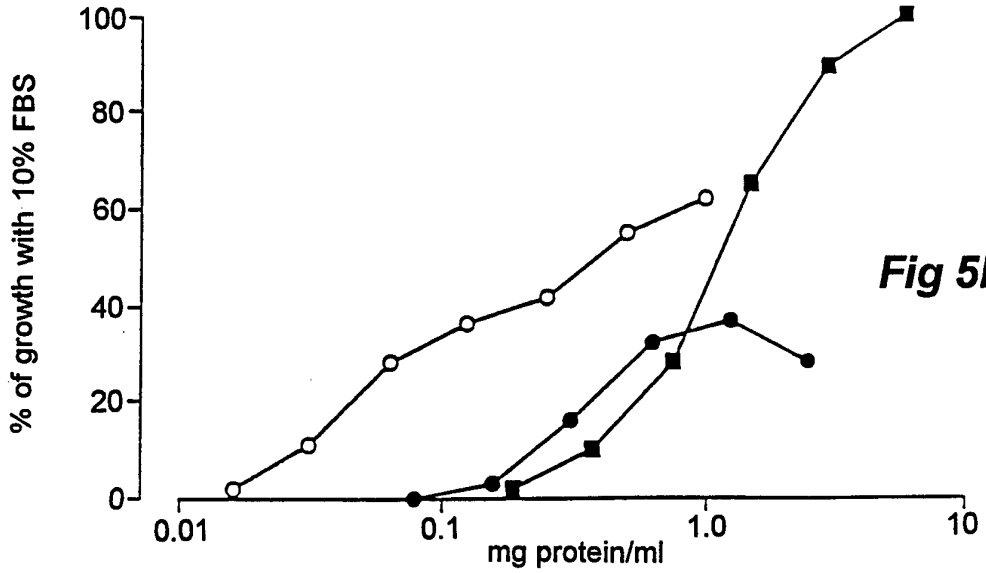

Fig 5b

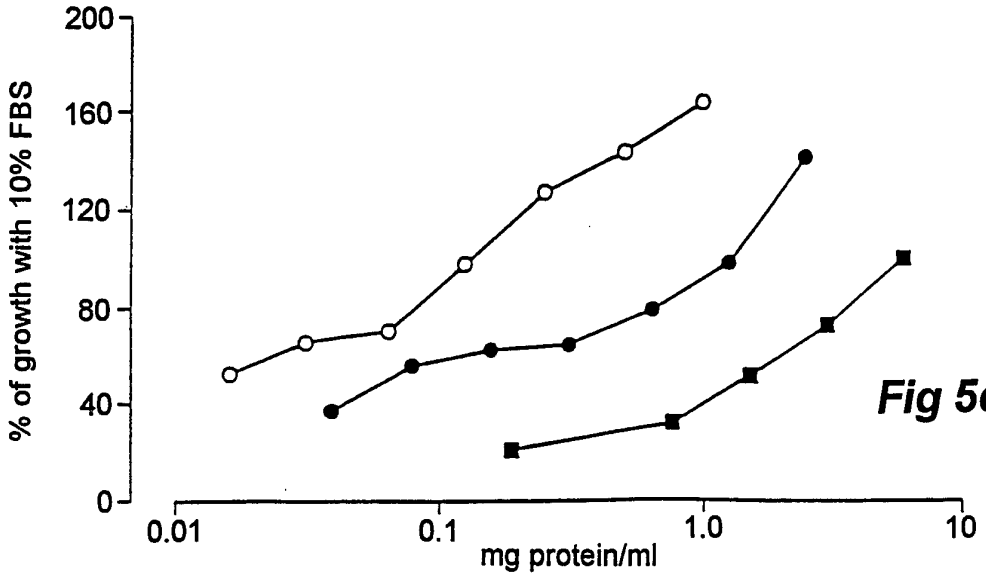

Fig 5c

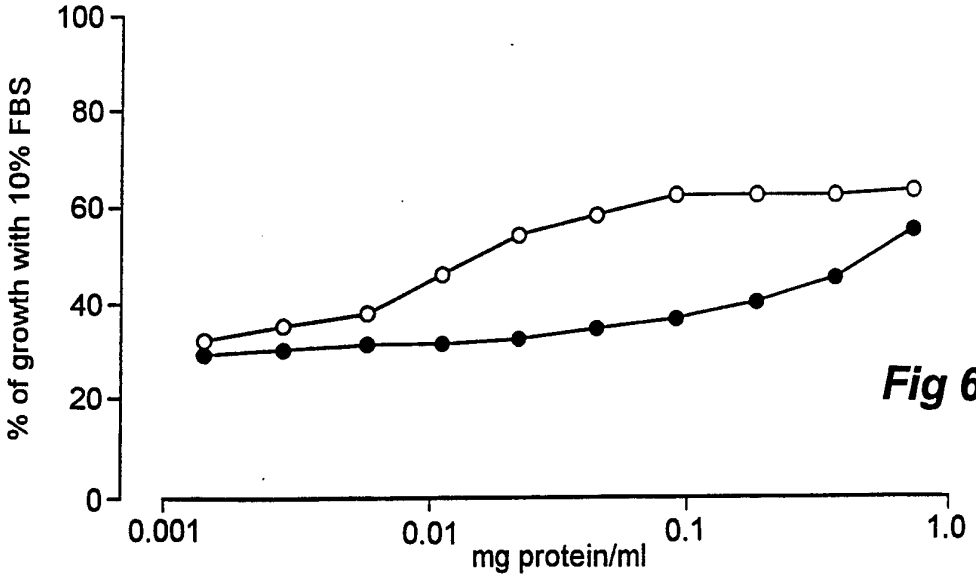

Fig 6a

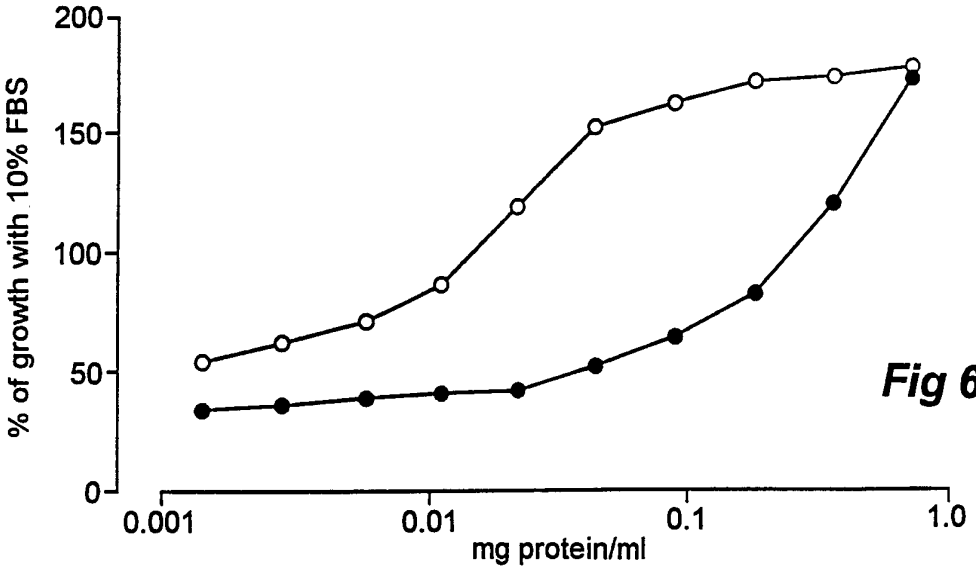

Fig 6b

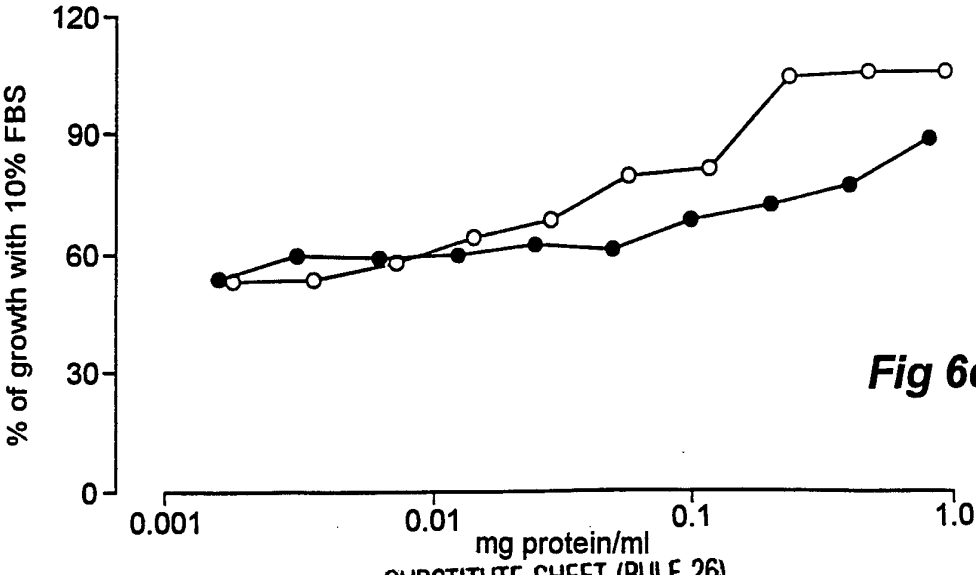

Fig 6c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00237

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] C07K 1/18, 1/34, 1/36, C12N 5/02, A61K 38/18, A23J 1/20, A23C 9/00, 21/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C07K 1/18, 1/34, 1/36, 3/12, 3/22, 3/28, 7/10, 13/00, 15/06, C12N 5/02, A23J 1/20, A23C 9/00, 21/10, A61K 38/18, 37/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT: (MILK OR WHEY OR COLOSTRUM) AND/OR GROWTH FACTOR
CHEMICAL ABSTRACTS: (MILK OR WHEY OR COLOSTRUM) AND GROWTH FACTOR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	AU 26667/92 A (VALIO BIOBRODUCTS LTD, 21 May 1993 (see entire document, in particular page 10 lines 1-4)	1, 9, 12, 15
X	Annu. Rev. Nutr. Vol. 14 (1994) pages 147-167 "Growth Factors in Milk as Mediators of infant development", S.M. Donovan and J. Odle see page 149 lines 35-37 and pages 158-159	1
X	Journal of Dairy Research Vol. 59, (1992) pages 369-380, "Factors in ruminant colostrum that influence cell growth and murine IgE antibody responses" D. L. Watson et al., see entire document (inparticular see page 370 (preparation of bovine colostral extract), page 377 lines 15-17 and page 378 lines 3-5)).	1-4, 7, 12, 14-15, 20

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1995	10 AUGUST 1995 (10.08.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer NORMAN BLOM Telephone No. (06) 2832238

Form PCT/ISA/210 (continuation of first sheet (2)) (July 1992) copkjy

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	Developmental Biology, Vol. 130, (1988) pages 16-27, "Maternal and Neonatal Somatomedin C/Insulin-like Growth Factor-1 (IGF-1) and IGF Binding Proteins during Early lactation in the Pig", F.A. Simmen et al., (entire document, see in particular page 16 column 2 and page 20 column 1).	1-4, 12, 14-15, 20
X Y	Agric. Biol. Chem., Vol. 53 Number 4, (1989) pages 1043-1050, "Occurrence of High Molecular Weight EGF Complexes in Human Milk". N. Azuma et al. (entire document, see inparticular page 1043 column 2, pages 1048-1049 as above	1-4, 9, 12, 15-17, 19-20 1-20
X Y	Eur. J. Biochem., Vol 197, (1991), pages 353-358 "Isolation and characterization of milk growth factor, a transforming-growth-factor-ß2-related polypeptide, from bovine milk" D.A. Cox and R.R. Bürk, see page 353 column 1 and page 357 column 1 as above	1, 3-4, 7, 9, 12, 15, 20 1-20
X	EP 367447 A1 (Smithkline Beecham Corporation) 9 May 1990 see page 3 column 3 line 8 and page 4 column 5 lines 43-45, page 6 column 9 lines 13-20, page 7 column 12 lines 34-43, example 4	1-4, 7
X	EP 313515 A1 (Ciba-Geigy AG) 26 April 1989 see whole document, in particular see page 11 lines 13-14	1, 3-4, 7, 9, 12, 15, 21-24
X	AU 19521/92 A (Societe Des Produits Nestle S.A.) 18 February 1993 see page 1A line 31 to page 2 line 2 and page 6	9, 22
Y	Biochem. J. Vol. 251, (1988) pages 95-103 "Insulin-like growth factors 1 and 2 in bovine colostrum Sequences and biological activities compared with those of a potent truncated form" G. L. Francis et al., page 98 last two lines	1-20
P, X	Life Sciences, Vol. 55, No. 19 (1994) pages 1509-1520, "Growth Factor from Human Milk: Purification and characterization", Y. Kanda et al., see entire document, in particular page 1510 lines 24-25	1
X	Clin. Exp. Immunol. Vol. 94, (1993) pages 220-224, "Transforming growth factor-beta (TGF-ß) in human milk" S. Saito et al., (see page 220 column 2 last 3 lines, page 221 and 222 and the discussion).	1, 2, 12, 15, 20
Y	WO 92/00014 (CLAR (SARL)) 9 January 1992 see entire document	1-20
A	Endocrine Reviews, Vol. 14 No. 1 (February 1993) pages 710-728, "Hormones and Growth Factors in Milk", C.E. Grosvenor et al. (see page 715 column 1 lines 6-16 and column 2 lines 20-21 and pages 714-718)	1, 15
X	AU 62869/86 B (598205) (GROPEP PTY. LTD.) 10 March 1987 see page 8 lines 5-18, example 1	9, 12
A	AU 39035/93 A (BOPA IRELAND LTD) 27 January 1994 whole document	1-7
A	Chemical Abstracts, Vol. 119, No. 14, issued 4 October 1993 (Columbus Ohio), USA; T. Yukihiro et al "Osteoblast growth factor and food and beverages containing it", page 494 column 1, abstract No. 146574n, PCT int. appl. WO 93 12807 Abstract	1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	Regulatory Peptides Vol. 27, (1990), pages 61-74, "Fibroblast mitogens in swine milk include a epidermal growth factor-related peptide" T. J. Tan et al., (see page 72 lines 31-32)	1
A	AU 82072/91 B (645589) (GROPEP PTY. LTD.) 4 February 1992 see entire document	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00237

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
AU 26667/92	EP,A, 610245 WO,A, 9308264	FI,A, 914911 ZA,A, 9207993	NO,A, 941381
WO,A, 9200014	EP,A, 489884	FR,A, 2663816	
EP 237447	FR,A, 2594268		
EP 313515	AU,A, 23326/88 JP,A,01-1121300	DK,A, 5463/88 PT,A, 88626	GB,A, 8723094 US,A, 5221734
AU 19521/92	BR,A, 9203106 PT,A, 100766	EP,A, 527283	JP,A, 5284936
AU 39035/93	WO,A, 9320713		
AU 82072/91	CA,A, 2086681 WO,A, 9200994	EP,A, 545946	NZ,A, 238890
AU,B, 62869/86	EP,A, 235205 FI,A, 930869 NO,A, 930698 WO,A, 9422839	US,A, 5077276 WO,A,9203344 WO,A,9422839	WO,A, 8701038 EP,A, 544659 GB,A, 9306578

END OF ANNEX