Purification and Characterization of Cell Growth Factor in Bovine Colostrum

Yoshihiro KISHIKAWA, Toshi WATANABE, Tasuku WATANABE[1)], and Shuichiro KUBO

Laboratories of Physiological Chemistry and [1)]Fish Pathology, College of Agriculture and Veterinary Medicine, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252, Japan

(Received 10 May 1995/Accepted 13 September 1995)

ABSTRACT. Bovine colostrum has growth factor activity for stimulating DNA synthesis in calf kidney epithelial cells (CKT-1), Madin-canine kidney epithelial cells (MDCK) and rat L6 myoblasts (L6), of which the DNA stimulation level and the activity change with the time elapsed after the birth of a calf varied with their respective cells. The growth factor activity of colostrum for CKT-1 was stable regardless of the collection time of colostrum, and it was purified about 3,650-fold in an overall yield of 1.2% from colostrum obtained 30 min after the birth of a calf. The purified growth factor had a molecular weight (MW) of 5,000 and an isoelectric point of pH 9.7, and the amino acid composition was: Asx_5, Thr_2, Ser_4, Glx_{14}, Pro_2, Gly_4, Ala_4, Val_2, Ile, Leu_2, Tyr, Phe, Lys, His and Arg. The stimulated DNA synthesis in CKT-1 and L6 by the addition of purified growth factor at a final concentration of 16 *ng/ml* was as the same extent as calf serum at a final concentration of 1.52 mg/m*l*, and the relative activity for CKT-1 was even greater than that for L6. — KEY WORDS: bovine colostrum, calf kidney epithelial cell, characterization, epithelial growth factor, purification.

J. Vet. Med. Sci. 58 (1): 47–53, 1996

Milk contains cell growth factors in addition to nutrients, minerals and vitamins, and can replace serum for the growth of sparse cells in culture [6, 9, 10–11]. Human milk is capable of stimulating DNA synthesis and cell division in cultured mouse and human fibroblasts, and the growth factor is a polypeptide with a molecular weight (MW) 14,000–18,000 and an isoelectric point pH 4.4–4.7 [10]. On the other hand, another evidence was stressed that epidermal growth factor (EGF) is a major growth-promoting agent in human milk, because the antibody against human EGF blocks the mitogenic activity of human milk. In bovine colostrum, colostrum obtained within 8 hr after the birth of a calf has been the most effective in supporting proliferation for Madin-canine kidney epithelial cells (MDCK) [11]. However, the major growth factor in bovine colostrum, which was purified as to mouse BALB/c3T3 fibroblasts (BALB/c3T3) by the same author [18], was platelet-derived growth factor (PDGF). Furthermore, growth-promoting activity in bovine colostrum has been detected as insulin-like growth factor-1 (IGF-1) and the capacity to stimulate protein synthesis in rat L6 myoblasts (L6) [8].

Thus, with regard to cell growth factors in bovine colostrum and milk, there are various reports that the cell growth factor promotes the growth of only epithelial cells, only fibroblasts and both, and so there is no consensus regarding susceptibility for cell species. The disagreement is thought to be caused by the activity change with the time elapsed after the birth of a calf and the presence of cell growth factor but mentioned above. Therefore, in this study, we examined the activity change of cell growth factor derived bovine colostrum using four kinds of cultured cells, and furthermore purified cell growth factor and then investigated the molecular characters.

MATERIALS AND METHODS

Cells: CKT-1, MDCK, L6 and BALB/c3T3 were kindly provided by Japanese Cancer Resources Bank. The cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 584 mg/*l* of L-glutamine in 5% CO_2-humidified atmosphere.

Bovine colostrum: Colostrums from Holstein cows were collected at various times and centrifuged at 14,300 × g for 30 min, the infranatants were quickly frozen and stored at −20°C until use.

Acid precipitation fraction of colostrum for cell culture: The presence of casein micelles and other particles in colostrum makes it difficult to filtrate the colostrum for obtaining the sterile medium, or in some cases is likely to disturb the establishment of confluent monolayers of cells. Therefore, the colostrum was treated with acid to remove casein, fats and cellular debris.

The infranatant described above was thawed and adjusted to pH 2.8 with 4 N HCl and stirred for 24 hr at 0°C. After centrifuged at 14,300 × g for 30 min to remove fat and cell debris, the supernatant was adjusted to pH 4.5 with 4 N NaOH and again centrifuged to remove the large amount of casein precipitates. The supernatant was dialyzed against 2% $NaHCO_3$ by means of Spectra/por 3 membrane (SPECTRUM) with molecular weight cutoff 3,500.

Cell growth factor assay: Growth factor activity was measured by the DNA synthesis method of Shing *et al.* [18] with slight modifications. Cells, which were grown in DMEM supplemented with 10% FBS at 37°C in 5% CO_2-humidified atmosphere, were detached by the incubation with 0.1% (v/v) trypsin and 0.02% (w/v) EDTA made up in phosphate buffered saline lacking calcium and magnesium. The cells were resuspended in DMEM supplemented with 10% calf serum (CS) at a concentration of 6×10^4 cells/m*l*.

Aliquots of 200 μl cell suspension were placed into 96-well microtiter plates. The cells were incubated for 7 days at 37°C in 5% CO_2-humidified atmosphere without medium change to deplete the serum of growth factors, thereby establishing confluent monolayers of quiescent cells. Aliquots of 40 μl of test samples in DMEM and 10 μl of [^{3}H] thymidine (25 μCi/ml) were added to each well, and further incubated for 1, 2 and 3days. The medium was removed, and the labeling cells were rinsed twice with serum-free DMEM. The cells were harvested with trypsin-EDTA onto filter paper with a semi-automatic multiple cell harvester. After placing the dried filter paper into vial, 2 ml of ACS-II was added to each vial to count radioactivity with a liquid scintillation counter (LS-3801 , Beckman). A unit of growth factor specific activity was defined as the amount of growth factor required to elicit half-maximum DNA synthesis as indication by addition of 50 μl of 10% CS (final concentration: 1.52 mg protein/ml) according to the method of Shing *et al.* [18] and calculated by the following equation: U/mg = [(A - C)/ 1/2 (B - C)] × 1/D, where A is [^{3}H] thymidine incorporated into cells by the addition of test sample in medium, B is [^{3}H] thymidine incorporated into cells by the addition of 10% CS in medium, C is [^{3}H] thymidine incorporated into cells by the addition of serum-free-DMEM in medium and D is the protein concentration (mg) in test sample.

Adsorbent for column chromatography: DEAE-Sephacel and Bio-Rex 70 were equilibrated by 20 mM Tris-HCl at pH 8.0 and the same buffer at pH 7.0, respectively, and each of the resin was packed into a column (2.6 × 40 cm). Blue Sepharose CL-6B gel was prepared from Cibacron 3B dye and Sepharose CL-6B by the method of Lowe and Pearson [14], then the affinity resin equilibrated by 20 mM Tris-HCl-0.4 mM EDTA at pH 8.0 was packed into a column (1.0 × 20 cm).

Electrophoresis: Isoelectrofocusing was carried out in an LKB column of 110 ml volume at 0°C using 1% Pharmalyte (pH 8–10.5) -8M urea and a sucrose gradient. All solutions and gradients were prepared by the method of Vesterberg [21]. Polyacrylamide gel electrophoresis was carried out by the method of Laemmli [13] using a PhastSystem (Pharmacia) equipped with reverse polarity electrode assembly [12] and 50 mM β-alanine-acetic acid at pH 4.2 as a running buffer. The gel was stained with 0.03% Coomassie dye [22].

Molecular weight and protein determinations: The MW of purified cell growth factor was determined by the method of Andrews [1] using gel filtration. A 0.1 mg of purified factor and molecular weight maker (Pharmacia) were loaded on a column (1 × 100 cm) of Sephadex G-100 equilibrated with 0.1 M CH_3COOH and eluted with the same buffer at a flow rate of 10 ml/hr, respectively. The protein was routinely estimated by the method of Lowry *et al.* [15] with bovine serum albumin as a standard. For purified cell growth factor, a value of $A^{1\%}_{280nm}$ = 0.268 was used (calculated from the results of amino acid analysis).

Determination of amino acid composition: Purified cell growth factor was hydrolyzed by 4 M methanesulfonic acid at 115°C for 24, 48 and 72 hr [16]. The hydrolysate was neutralized with 3.5 N NaOH and applied to a Hitachi-835 amino acid analyzer. The amino acid composition was determined by the average of analytical values obtained from 24, 48 and 72 hr-hydrolysates.

RESULTS

Change of growth factor activity in colostrum with the time elapsed after birth of a calf: Colostrums from the same Holstein cow 1, 8, 32, and 68 hr after the birth of a calf (1, 8, 32, and 68-colostrums, respectively) were used. After thawing the frozen colostrum, an acid precipitation fraction was obtained by the acid treatment of the infranatant layer of colostrum as described above.

In a preliminary experiment, the stimulation of DNA synthesis was affected by the addition of varying concentration of the acid precipitation fraction (at final concentrations of 1–10%) in quiescent cells, and the optimal concentration of the acid precipitation fraction of 1 hr-colostrum was 5% (1.84 mg protein/ml) for the growth of CKT-l, MDCK and L6. Therefore, this concentration was used for the detection of change of growth factor activity in colostrum with the time elapsed after the birth of a calf.

The stimulated DNA synthesis in CKT-1, MDCK and L6 by the addition of acid precipitation fractions obtained from 1, 8, 32, and 68 hr-colostrums was compared with that of CS 10% (Fig. 1). The DNA synthesis in CKT-1, MDCK and L6 was stimulated by the addition of acid precipitation fraction, but the level and the activity change with the time elapsed after the birth of a calf varied with their respective cells. The maximum stimulation of DNA in CKT-1 occurred at approximately 48 hr, and the stimulation levels by the addition of acid precipitation fraction of 1–68 hr colostrums were almost the same. In the case of MDCK, the maximum stimulation was observed with 8 hr-colostrum and the stimulation level was about 0.01% of that of CKT-1. Furthermore, the stimulation level was decreased in colostrum with the time elapsed after birth of a calf. In the case of L6, the maximum stimulation occurred at approximately 30–48 hr with 1 and 8-colostrums and the stimulation level was about 60–80% of that of CKT-1, however the stimulation level was decreased in colostrum with the time elapsed after the birth of a calf. The stimulating effect of the acid precipitaion fraction in BALB/c3T3 was not observed.

Purification procedure of cell growth factor for CKT-1: The growth factor activity in colostrum to CKT-1 was stable regardless of the collection time of colostrum described above, then CKT-1 was used for purification. The colostrum obtained 30 min after the birth of a calf (30 min-colostrum) was used, and all procedures were carried out in a cold room. The frozen colostrum was thawed and treated with 4 N HCl to obtain the acid precipitation fraction described above.

The acid precipitation fraction was applied to a column

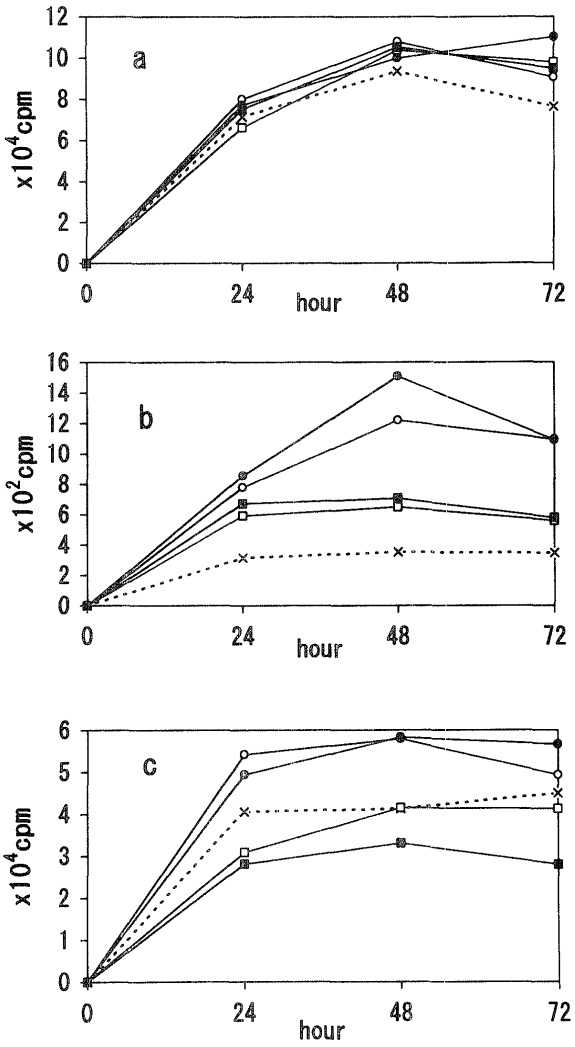

Fig. 1. Stimulation of DNA synthesis in calf kidney epithelial cells (CKT-1) (a), Madin-canine kidney epithelial cells (MDCK) (b), and rat L6 myoblasts (L6) (c) by acid precipitation fraction of colostrum obtained 1 (○), 8 (●), 32 (□) and 68 hr (■) after the birth of a calf. The fraction of 1 hr-colostrum (37 mg protein/m*l*) was added to the confluent monolayer of quiescent cells at a final concentration of 5% (1.84 mg protein/m*l*), and the fraction of other colostrum to the quiescent cells at the same final protein concentration as 1 hr's. Calf serum was added to quiescent cells at a final concentration of 10% (1.52 mg protein/m*l*) (×).

of DEAE-Sephacel equilibrated with 20 mM Tris-HCl at pH 8.0. After washing the column with 2 column volumes of the same buffer, the break-out fraction was collected and adjusted to pH 7.0 with 4 N HCl, then the fraction was applied to a column of Bio-Rex 70 equilibrated with 20 mM Tris-HCl at pH 7.0. After washing the column with 2 column volumes of the same buffer, the absorbed protein was eluted with a linear gradient of 0–1.0 M NaCl containing 20 mM Tris-HCl at pH 7.0. Each fraction was pooled and dialyzed against 2% NH_4HCO_3, then the activity was determined with the dialyzates and lyophilized (Fig. 2b). A growth factor activity was also found in the adsorbed fraction of DEAE-Sephacel column, but the activity could not purify in this study (Fig. 2a).

The lyophilized fraction containing the activity was dissolved in 1% glycine solution, and then isoelectrofocused in a column of 110 m*l* capacity in 1% Pharmalyte (pH 8–10.5) containing 8 M urea. The activity focused at pH 9.4–10.0 and the fraction was dialyzed against 2% NH_4HCO_3 and lyophilized (Fig. 3).

The lyophilized fraction was dissolved in 20 mM Tris-HCl-0.4 mM EDTA at pH 8.0 and applied to a column of Blue-Sepharose CL-6B equilibrated with the same buffer. After washing with 2 column volumes of the equilibration buffer, the activity was eluted with 5 mM ATP in the equilibration buffer, collected, dialyzed against 2% NH_4HCO_3 and lyophilized.

The result of this purification procedure is summarized in Table 1. The cell growth factor for CKT-1 was purified by these procedures to approximately 3,600 fold from infranatant layer of colostrum in an overall yield of 1.2% to a final specific activity of 6,570 units per mg of protein. The purified cell growth factor showed a single protein band in acrylamide gel electrophoresis (Fig. 4).

Some properties of cell growth factor for CKT-1: Sephadex G-100 gel filtration of the purified growth factor yielded a single peak of an estimated MW of 5,000, based on its elution volume relative to marker proteins (Fig. 5). The amino acid composition of the purified growth factor was found to be Asx_5, Thr_2, Ser_4, Glx_{14}, Pro_2, Gly_4, Ala_4, Val_2, Ile, Leu_2, Tyr, Phe, Lys, His, Arg and no Trp. The content of Cys or CySH was not measured for having no available amount of sample. Table 2 shows the amino acid composition of bovine colostrum-derived growth factor, human milk EGF (h-EGF) [17] and bovine IGF (IGF-1) [8]. A percentage number of each amino acid residue to total amino acid residues was calculated. Small differences were observed in each amino acid content between the purified growth factor and h-EGF, however, the rates of polar and nonpolar amino acid in the purified growth factor were similar to those in h-EGF, respectively. But there was not similarity between the purified growth factor and IGF-1.

The stimulated DNA synthesis in CKT-1 and L6 by the addition of purified growth factor and 10% CS were compared (Fig. 6). The maximum stimulation in CKT-1 occurred at approximately 24 hr, whereas at 48 hr in L6. The purified growth factor at a final concentration of 16 *ng*/m*l* was as active as calf serum at a final concentration of 1.52 mg/m*l* (10% CS), and the relative activity of purified growth factor for CKT-1 was even greater than that for L6.

DISCUSSION

Bovine colostrum contained growth factor activity for developing CKT-1, MDCK and L6, of which the DNA stimulation level and the activity change with the time elapsed after the birth of a calf varied with their respective

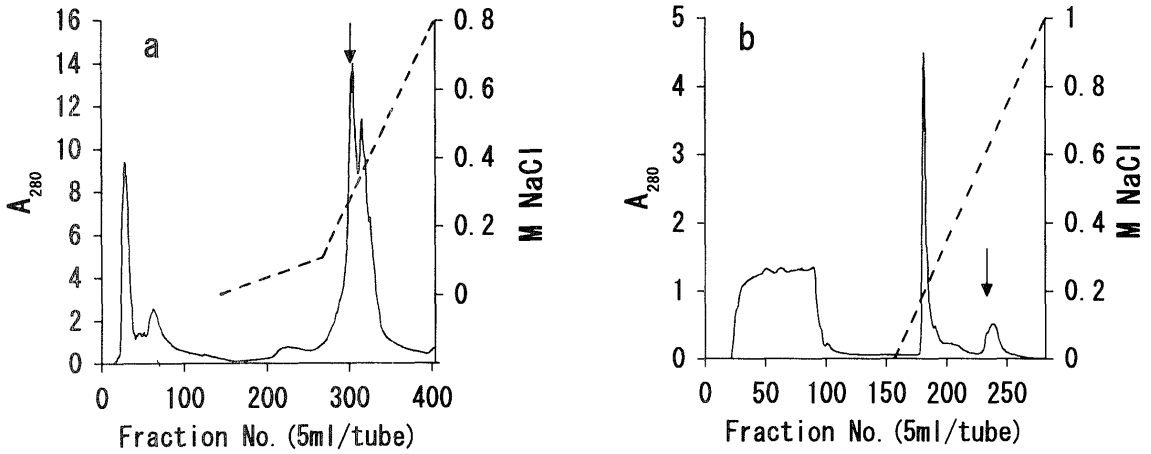

Fig. 2. Ion exchange chromatography profiles in DEAE-Sephacel (a) of the acid precipitaion fraction of colostrum obtained 30 min after the birth of a calf, and in Bio-Rex 70 (b) of non-adsorbed fraction of (a). Fractions of 5 m*l* were collected and elution was at a flow rate of 30 m*l*/hr. ——— : A_{280}, ----- : NaCl concentration in linear gradient. Arrow shows growth factor activity.

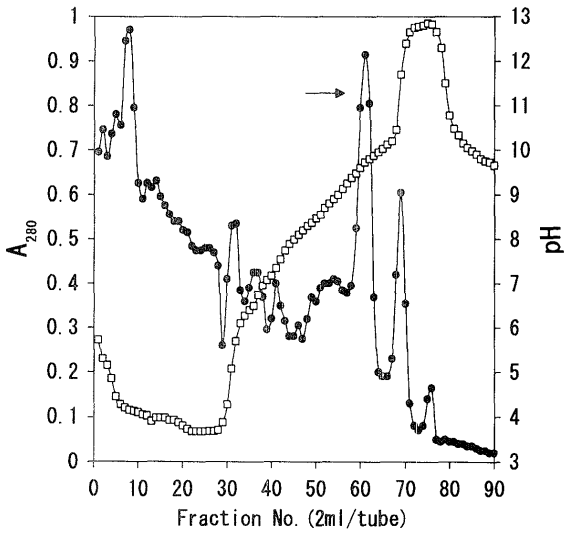

Fig. 3. Isoelectrofocusing profile of cell growth factor-active fraction in Bio-Rex 70 chromatography. The fraction (900 mg protein) was applied to a 110 m*l* column, and electrophoresis was carried out at 1,000 volt and 0°C until maintaining a constant current. Fractions of 1m*l* were collected. ●: A_{280}, □: pH. Arrow shows growth factor activity.

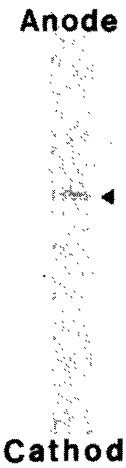

Fig. 4. Polyacrylamide gel electrophoresis of purified cell growth factor. Growth factor (140 *n*g) was applied on a PhastSystem Homogenate 20 gel.

Table 1. Purification of bovine colostrum-derived growth factor

Step	Total volume (m*l*)	Total protein (mg)	Specific activity (U/mg)	Total activity (Units)	Yield (%)	Purification (-fold)
Infranatant	357	61,013	1.8	109,823	100	1
Acid precipitation	67.4	4,563	2.7	12,320	11	1.5
Bio-Rex 70	21.5	900	10.4	9,360	8.5	5.8
Isoelectric focusing	8.4	20	448.1	8,962	8.2	249
Affinity chromatography	1.5	0.2	6,570	1,314	1.2	3,650

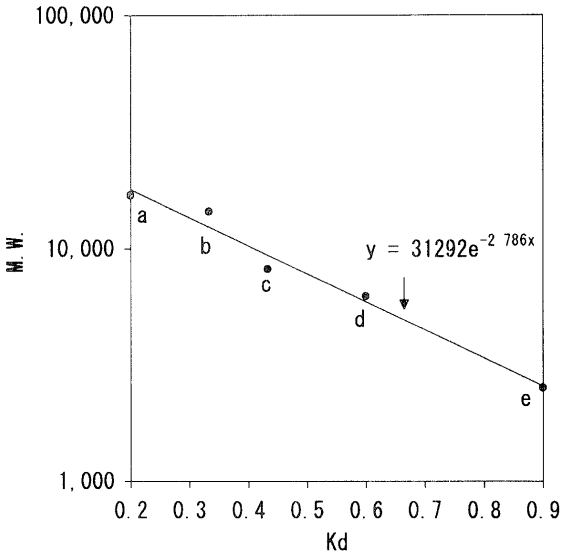

Fig. 5. Determination of molecular weight (MW) of purified growth factor by Sephadex G-100 gel filtration. Distribution coefficient (Kd) was calculated by the method of Andrew [1] as follows: Kd = (Ve - Vo)/(Vt - Vo), where Ve is the elution volume of maker protein or purified growth factor, Vo is the void volume obtained by the elution of 0.2% blue dextran, and Vt is the total bed volume obtained by the elution of 0.2% K_2CrO_4. a: myoglobin (MW 16,900), b: myoglobin I & II (MW 14,400), c: myoglobin I (MW 8,100), d: myoglobin II (MW 6,200), e: myoglobin III (MW 2,500). Arrow shows the molecular weight of growth factor.

cells. The mechanism of action of the growth factor can be divided into three steps, binding with the receptors of target cell membrane, internalization into the cell and development of activity at the intracellular site [2–5, 7]. If this mechanism is applied to our results, we can explain that three kinds of growth factor activity in colostrum for CKT-1, MDCK and L6 would be caused by one kind of growth factor having different affinity against CKT-1, MDCK and L6. However, it is reasonable that bovine colostrum have three kinds of growth factor, because each of their activity changes with the time elapsed after the birth of a calf. Klagsbrun showed that bovine colostrum obtained within 8 hr after the birth of a calf supports the serum-free proliferation of MDCK but not of fibroblasts and this proliferation activity decreases with the time elapsed after the birth of a calf [11]. The growth factor activity in human milk also decreases with the collection time after the birth of a newborn baby [9].

The growth factor for CKT-1 was purified from colostrum obtained 30 min after the birth of a calf, because bovine milk contains cysteine protease having high activity with the time elapsed after the birth of a calf [19]. In purification procedures, blue dye conjugated with Sepharose was very effective, which was very useful in purifying enzymes having a dinucleotide fold structure [14]. Burwen and Jones suggested that growth factors may have nuclear sites of action [2], and the purified growth factor may have high affinity site for nucleotide in the structure .

Table 2. Amino acid composition of bovine colostrum-derived growth factor, human milk EGF and bovine IGF-1

	Bovine clostrum-derived GF		Human milk EGF		Bovine IGF-1	
Amino acid	Assumed	% to total residues	Assumed	% to total residues	Assumed	% to total residues
Lys	1	2.2	0	0	1	3.3
His	1	2.2	1	1.9	0	0
Arg	1	2.2	4	7.5	1	3.3
Cys	0	0	6	11.3	2	6.7
Asx	5	11.1	7	13.2	3	10
Thr	2	4.4	2	3.8	2	6.7
Ser	4	9.0	6	11.3	0	0
Glx	14	31.1	3	5.7	3	10
Pro	2	4.4	2	3.8	2	6.7
Gly	4	9.0	6	11.3	5	16.6
Ala	4	9.0	0	0	2	6.7
Val	2	4.4	2	3.8	2	6.7
Met	0	0	1	1.9	0	0
Ile	1	2.2	2	3.8	0	0
Leu	2	4.4	4	7.5	3	10
Tyr	1	2.2	5	9.4	1	3.3
Phe	1	2.2	0	0	3	10
Trp	0	0	2	3.8	0	0
Total residues	45		53		30	
Polar amino acid		66		66		53
Acidic and neutral basic		8		9		7
Nonpolar amino acid		26		25		40

The purified growth factor had a MW of about 5,000 and an isoelectric point of about pH 10, and amino acid composition was Asx_5, Thr_2, Ser_4, Glx_{14}, Pro_2, Gly_4, Ala_4, Val_2, Ile, Leu_2, Tyr, Phe, Lys, His and Arg. Considering the isoelectric point, the basic amino acid content was a small amount. For this reason, amidation of β and γ-carboxyl group in acidic amino acid might occur. The ability of growth factor at final concentrations of 1.6–16 *ng/ml* for CKT-1 and L6 was as active as that of calf serum at a final concentration of 1.52 mg/m*l* in stimulating DNA synthesis. Bovine colostrum contains two kinds of growth factor, one of which is platelet-derived growth factor (PDGF) having a MW of 30,000 and an isoelectric point of pH 10 and stimulates DNA synthesis in fibroblasts [18], and the other is IGF-1 having a MW of 7,800 and stimulates protein synthesis in L6 [8]. On the other hands, human milk contains EGF, which has a MW of 6,000 and an isoelectric point of pH 4.5 and stimulates DNA synthesis of epithelial cells and fibroblasts [18]. Though the isoelectric point of our growth factor is near to that of bovine PDGF, the rates of polar and nonpolar amino acid and the MW of our purified growth factor are similar to those of h-EGF, respectively. Furthermore, the action of our growth factor appears to be similar to h-EGF, because it stimulates DNA synthesis in epithelial cells and fibroblasts. EGF has a broad specificity for target cells in stimulating DNA synthesis [4]. Not only differences of the method of purification but also those of species between bovine and human might be due to differences of an isoelectric point and target cells in stimulating DNA synthesis. Further studies including determination of amino acid sequence are needed to clarify the molecular structure of our growth factor.

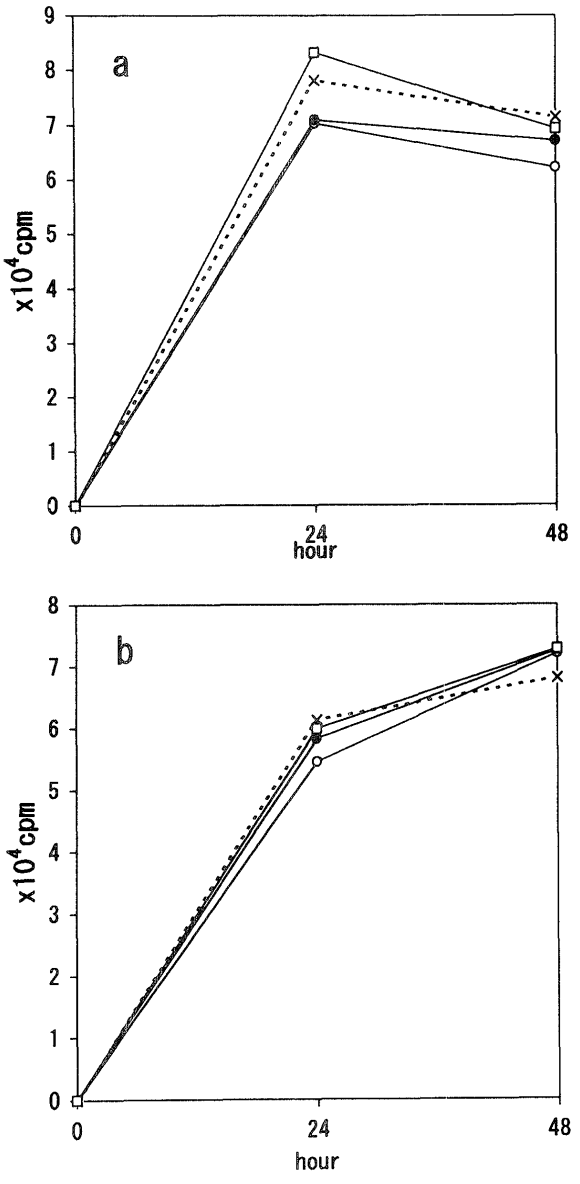

Fig. 6. Stimulation of DNA synthesis in calf kidney epithelial cells (CKT-1) (a) and rat L6 myoblasts (L6) (b) by purified cell growth factor. The purified cell growth factor was added to confluent monolayer of quiescent cells at final concentrations of 1.6 (○), 8 (●) and 16 *ng* /m*l* (□). Calf serum was added to quiescent cells at a final concentration of 1.52 mg/m*l* (DMEM supplemented 10% calf serum) (×).

REFERENCES

1. Andrews, P. 1965. The gel-filtration behavior of protein related to their molecular weights over a wide range. *Biochem. J.* 96: 596–606.
2. Burwen, S. J. and Jones, A. L. 1987. The association of polypeptide hormones and growth factors with the nuclei of target cells. *Trends Biochem. Sci.* 12: 159–162.
3. Carpenter, G. and Cohen, S. 1975. Characterization of the binding of ^{125}I-labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* 250: 4297–4304.
4. Carpenter, G. and Cohen, S. 1976. ^{125}I-Labeled human epidermal growth factor. *J. Cell. Biol.* 71: 159–171.
5. Carpenter, G. and Cohen, S. 1979. Epidermal growth factor. *Ann. Rev. Biochem.* 48: 193–216.
6. Carpenter, G. 1980. Epidermal growth factor is a major growth-promoting agent in human milk. *Science* 210: 198–199.
7. Cohen, S., Carpenter, S., and King, L. Jr. 1980. Epidermal growth factor-receptor-protein kinase interaction: Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J. Biol. Chem.* 255: 4834–4842.
8. Frances, G. L., Read, L. C., Ballard, J., Bagley, C. J., Upton, F. M., Gravestock, P. M., and Wallace, J. C. 1986. Purification and partial sequence analysis of insulin-like growth factor-1 from bovine colostrum. *Biochem. J.* 233: 207–213.
9. Hirata, Y. 1983. Human epidermal growth factor in milk. *Jpn. J. Endocrinol.* 59: 601–606. (in Japanese)
10. Klagsbrun, M. 1978. Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 75: 5057–5061.
11. Klagsbrun, M. 1980. Bovine colostrum supports the serum-free proliferation of epithelial cells but not of fibroblasts in long-term culture. J. *Cell. Biol.* 84: 808–814
12. King, T. P. 1979. Immunochemical properties of some atopic allergens. *J. Allergy Clin. Immunol.* 64: 159–163.

13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* (Lond.) 74: 680–685.
14. Lowe, C. R. and Pearson, J. C. 1984. Affinity chromatography on immobilized dyes. *Methods Enzymol.* 104: 97–113.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
16. Moor, A. T. 1972. Amino acid analysis. p. 629. *In*: Chemistry and Biology of Peptides (Meinhober, ed.), Arbor Sci. Publisher, U. S. A.
17. Savage, C. R. Jr., Inagami, T., and Cohen, S. 1972. The primary structure of epidermal growth factor. *J. Biol. Chem.* 247: 7612–7621.
18. Shing, Y., Davison, S., and Klagsbrun, M. 1987. Purification of polypeptide growth factors from milk. *Methods Enzymol.* 146: 42–48.
19. Suzuki, T. and Katoh, N. 1990. Cysteine protease in bovine milk capable of hydrolyzing casein as the substrate and elevation of the activity during the course of mastitis. *Jpn. J. Vet. Sci.* 52: 947–954.
20. Thompson, S. T., Cass, K. H., and Stellwagen, E. 1975. Blue dextran-Sepharose: An affinity column for the dinucleotide fold in proteins. *Proc. Natl. Acad. Sci. U.S.A.* 72: 669–672.
21. Vesterberg, O. 1971. Isoelectric focusing of proteins. *Methods Enzymol.* 22: 389–412.
22. Wilson, C. M. 1979. Studies and critique of amido black 10B, coomassie blue R and fast green FCF as stains for protein after polyacrylamide gel electrophoresis. *Anal. Biochem.* 96: 268–278.