THE IDENTIFICATION OF A TRANSFORMED CELL GROWTH FACTOR IN LACTALBUMIN HYDROLYZATE AS BIOTIN

Delano V. YOUNG, Stewart D. CHIPMAN* and Stephen LESCARBEAU
Department of Chemistry, Boston University, 685 Commonwealth Avenue, Boston, MA 02215, USA

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1. Introduction

Although serum is the major nutritional supplement needed for the growth of animal cells in culture, synthetic hydrolyzates of naturally occurring protein mixtures have often been used to replace or augment serum [1]. Such digests include peptone and lactalbumin hydrolyzate. Lactalbumin hydrolyzate (LH) is an enzymatic digest of a milk protein fraction, and it has been shown by this laboratory [2] to be a good source of growth activity for simian virus 40-transformed 3T3 (SV3T3) mouse fibroblasts. When added to Dulbecco's modified Eagle's medium (DME) containing a low concentration of calf serum (0.15–0.30%, v/v), LH causes an increase in the final cell density of 5–10-fold over control levels but only a modest reduction in the population doubling time [2]. In contrast, LH does not affect the growth of untransformed 3T3 cells under similar low serum conditions.

The lactalbumin hydrolyzate growth activity is readily water soluble, autoclavable, completely resistant to proteolytic treatment (trypsin, pepsin, pronase) and dissociable into acid-sensitive (pH 2 or 4) and acid-resistant components. Exposure of LH to a pH 2 or 4 for even a brief time causes complete loss of the growth rate stimulating ability, while the activity which increases the final cell density survives unimpaired. Here we show that the acid-resistant growth factor for SV3T3 cells is the vitamin, biotin.

*Present address: Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA

2. Materials and methods

2.1. *Maintenance of stock cells and the cell growth assay*

The Swiss SV3T3 cells were maintained as in [3] in 10% (v/v) calf serum DME (Gibco) and examined for PPLO by autoradiography. The growth activity in LH was assayed, as detailed in [3], by monitoring its ability to increase the number of living SV3T3 cells. Each reported cell count, determined with a Coulter Counter, was the average of 3–5 dishes.

2.2. *Column chromatography*

The Dowex 1 X 2 resin (Bio Rad Labs. 1 X 2, 200–400 mesh) was hydrated, washed, and packed as in [2]. Lactalbumin hydrolyzate (Nutritional Biochemicals Corp., tissue culture grade) (15 g) was dissolved in 100 ml Milli-Q distilled water (distilled water passed through a Millipore reverse osmosis unit), centrifuged to remove undissolved, inactive material, and loaded onto the column (dimensions: 34 X 7 cm). The column was washed with 3–4 ℓ 0.01 M NaCl (pH 7) at 100 ml/h flowrate. Elution was achieved with 0.01 M NaCl (pH 2) during which 20 ml fractions were collected, filter sterilized, and assayed for growth activity.

The Sephadex G-15 (Sigma Chemical Co.) was hydrated, de-gassed, and packed in pH 7 Milli-Q distilled water. Dowex purified growth activity (1 g) dissolved in 10 ml Milli-Q distilled water was loaded onto the G-15 column (dimensions: 2.5 X 140 cm) and eluted at 10 ml/h flowrate with pH 7 distilled water. The 2 ml fractions were assayed as before.

2.3. *Avidin–Sepharose affinity chromatography*

The egg white avidin (Worthington Biochemical)

was coupled to Sepharose 4B (Pharmacia Fine Chem.) as in [4] with the following modifications:

(i) During the Sepharose activation step the cyanogen bromide was dissolved in acetonitrile and added all at once to 10 ml Sepharose;

(ii) Avidin (20 mg) in 10 ml 0.1 M $NaHCO_3$ was added to the activated Sepharose.

Radioactive biotin (D-[*carbonyl*-^{14}C]biotin, Radiochemical Centre, Amersham, diluted with biotin from Calbiochem) was used to assess the binding efficiency of the column.

G-15 purified growth activity (500 mg) in 1 ml of a pH 7.4 Tris-(hydroxymethyl)-aminomethane (Tris)-saline buffer [2] was applied to the affinity column (dimensions: 6 mm × 9 cm, binding capacity: ∿ 39.2 μg biotin/ml). Elution occurred at 14 ml/h flowrate with the Tris–saline buffer. Fractions (3 ml) were collected and assayed as above.

3. Results

The acid-resistant growth activity was partially purified from lactalbumin hydrolyzate by Dowex 1 × 2 and Sephadex G-15 chromatography. At neutral pH the growth activity was adsorbed onto Dowex 1. After extensive washing at this pH the growth activity was eluted by lowering the pH [2]. The active fractions were then pooled, lyophilized, and loaded onto a Sephadex G-15 column. As illustrated in fig.1, the growth activity was retarded on G-15 and eluted near or at the third, sharp, absorbance peak (the second peak was sometimes not present). The dips in the growth assay at fractions 120–135 and around 222 probably revealed the presence of mildly toxic substances. This chromatographic profile differed [2], in which unfractionated LH at neutral PH was found to elute at the G-15 void volume. The presence of the acid-sensitive growth factor plus the fact that the previous G-15 column was eluted using a 0.01 M NaCl solution and not distilled water may have accounted for the results in [2]. The activity recovered from the Dowex 1, G-15 steps exhibited saturating activity between ∿ 10–25 μg/ml.

Preliminary chemical tests were performed on this partially purified material. The Dowex-1 purified growth activity was subjected to the phenol–sulfuric acid test for carbohydrates [5] and found to contain no detectable sugars. The standards employed, which yielded positive results, were methyl–mannose and sucrose. Similarly, material purified through the G-15 step was shown by atomic absorption spectro-

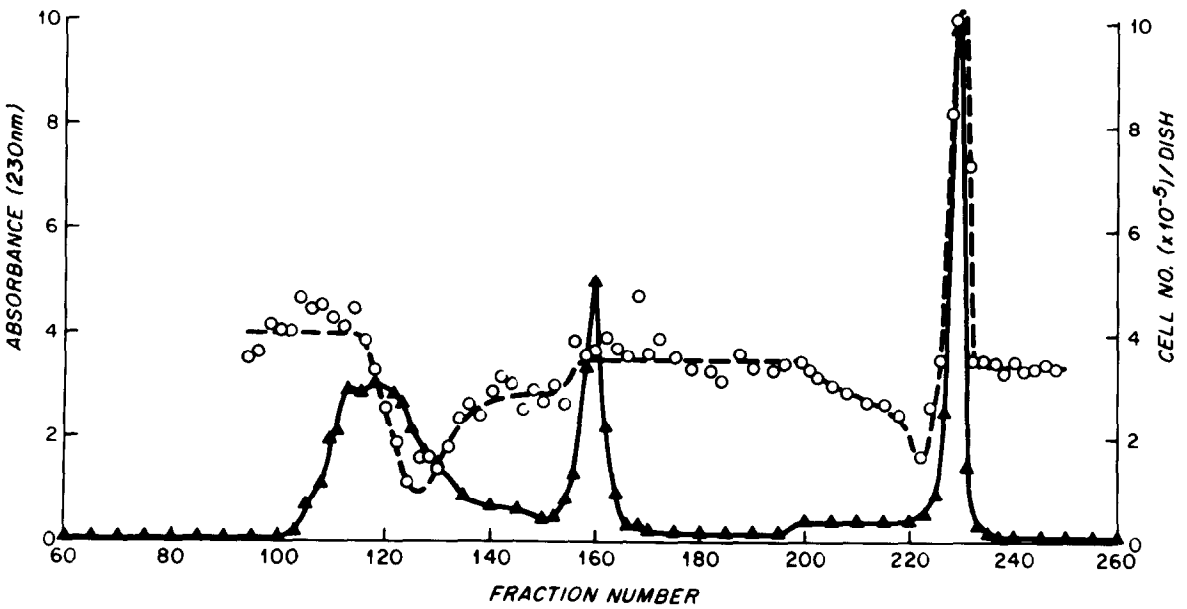

Fig.1 Sephadex G-15 chromatography of the Dowex purified growth activity. Details of the chromatographic procedures and growth assay are presented in section 2 (▲), A_{230}; (○) cell count at 6 days after plating.

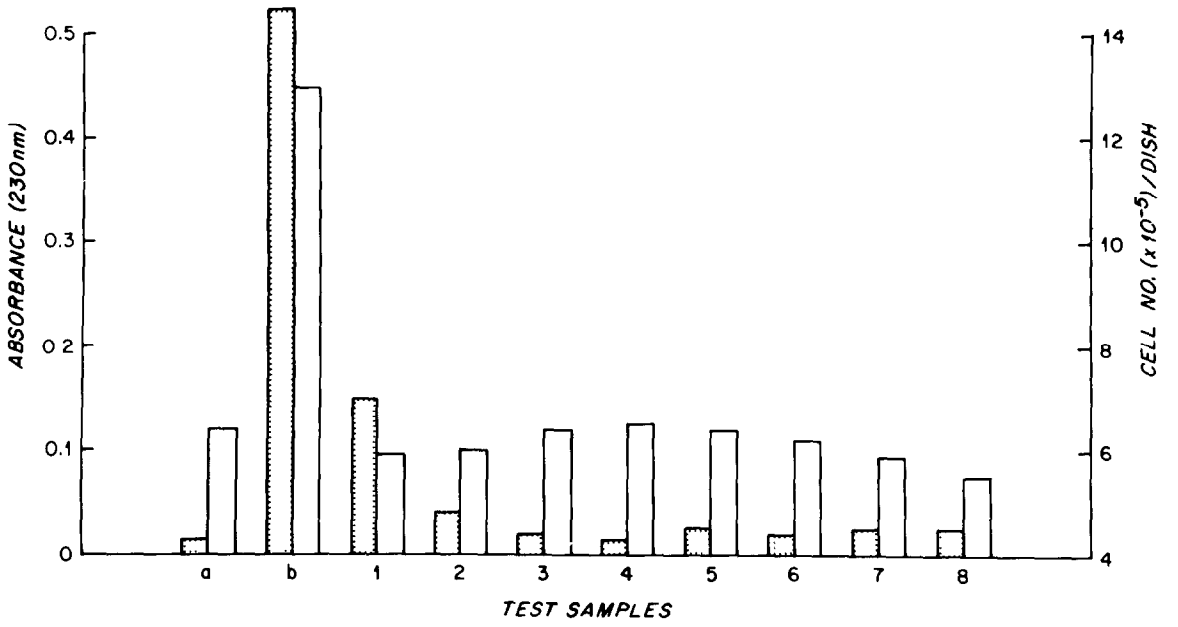

Fig.2. Avidin–Sepharose affinity chromatography of the G-15 purified growth activity. Consult section 2 and the text for the experimental details. Samples tested were: (a) Tris–saline buffer blank; (b) G-15 purified growth activity before loading on the column (absorbance of a 500 mg/ml solution in Tris–saline buffer was measured and 0.2 ml assayed for growth activity); no 1–8, fractions collected from the column (absorbance taken and 0.2 ml assayed for growth activity). Filled bars: absorbance; open bars: cell count on day 4 after plating.

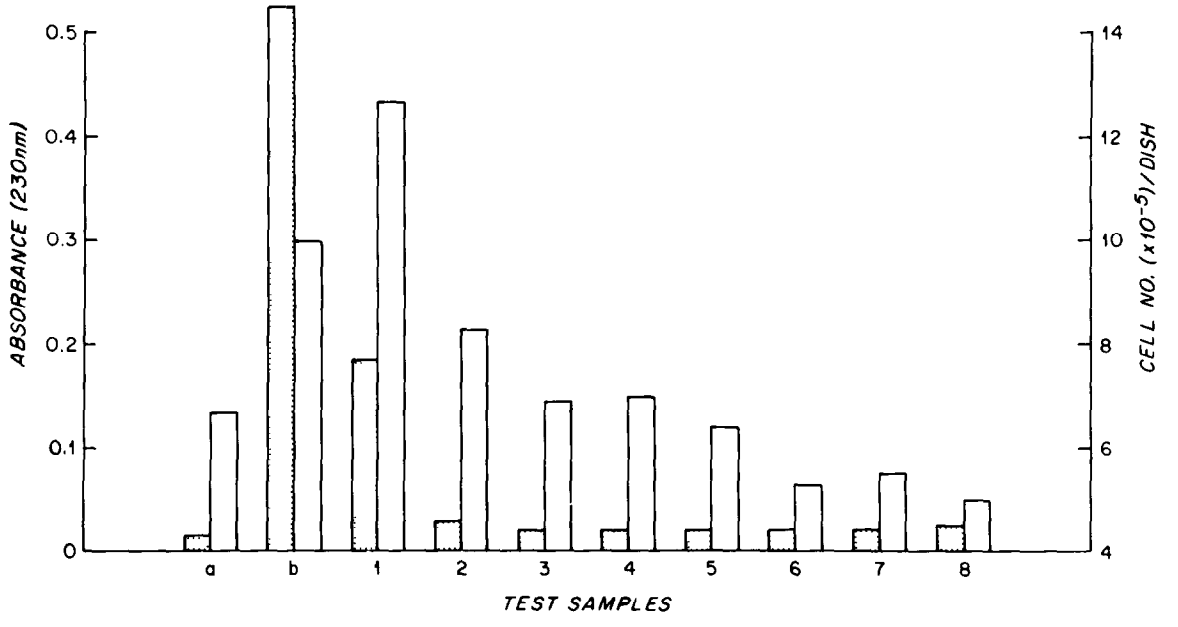

Fig.3. Sepharose 4B chromatography of the G-15 purified growth activity. The experimental procedures and the identities of the samples tested were exactly as in fig.2, with the exception that the Sepharose did not have avidin coupled to it. Filled bars: absorbance; open bars: cell count on day 4 after plating.

photometry to possess <0.5 ppm iron. Iron is known to be a potent growth agent for SV3T3 [6] and other cell lines [7,8]. However, an acid-hydrolyzed G-15 fraction did reveal the presence of several amino acids with aspartic acid, glutamic acid, and glycine predominating. Despite their use as nutritional supplements [9], none of these individual amino acids affected the growth of SV3T3 cells using the standard growth assay conditions.

Because of the importance of biotin as a major growth-limiting agent for SV3T3 cells and the similarity in the biological effects of biotin and this substance [2,10], it was decided that the acid-resistant material should be tested for the presence of this vitamin. Accordingly, a biotin affinity column was prepared by coupling avidin to Sepharose. In our hands this column very efficiently removed biotin from standard solutions. As can be seen in fig.2 when the unknown substance was loaded onto such a column all growth activity was retained by the avidin. However, a Sepharose column without avidin allowed all activity to pass through unretarded (fig.3). The identity of the acid-resistant factor was further confirmed.

(i) By demonstrating that it would stain with the biotin-specific reagent, *p*-dimethyl-aminocinnamaldehyde [11];
(ii) By observing that it migrated with the same R_F as pure biotin on thin-layer chromatography (using a microcrystalline cellulose plate developed with butanol:acetic acid:water, 5:1:1);
(iii) That its biological effect on SV3T3 growth was not additive with the effect exhibited by a saturating level of biotin.

One can estimate the relative amount of biotin present in the G-15 purified fraction by dividing the experimentally determined saturating dose for biotin ($\sim$ 1 ng/ml, [10]) by the saturating dose for the G-15 activity ($\sim$ 10–25 μg/ml). Biotin is probably present in the partially purified fraction as a trace (0.004–0.01%) but highly potent contaminant.

4. Discussion

The identification of one of the growth active components in lactalbumin hydrolyzate as the vitamin, biotin, underscores the possibility that biotin may also be one of the growth agents in other commercial preparations of protein hydrolyzates. Although it had been widely assumed that the growth activity in these hydrolyzates is attributable to mitogenic peptides [12,13], other growth active substances have been found [14,15], and the potency of biotin means that it may go undetected by ordinary chemical analyses. Indeed, it had been concluded in [2] that biotin was probably not responsible for the growth activity found in lactalbumin hydrolyzate. This conclusion was based on the apparent additivity of the growth effects of biotin and unfractionated, neutral LH. In explaining this discrepancy one must remember the variability inherent in tissue culture growth assays and the presence of the poorly-characterized, acid-sensitive growth factor in unfractionated LH.

Biotin is clearly an important growth-limiting agent for SV3T3 cells growing in low serum DME [10]. It has also been shown to be necessary for growth stimulation by iron in this system [6]. For these reasons and because biotin is a vitamin, it should be stressed that in the use of serum-limiting or serum-free media for the cultivation of animal cells the possible inclusion of biotin in the medium should be a primary consideration.

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