# ISOLATION OF LAMININ BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED *GRIFFONIA SIMPLICIFOLIA* I LECTIN

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## 1. Introduction

Laminin is a non-collagenous glycoprotein which occurs as a major component of basement membrane [1]. It consists of two polypeptide chains ($M_r$ 200 000 and $M_r$ 400 000) joined to each other by disulfide bonds [1]. Various cultured cells including epithelial cells elaborate laminin and it is believed that this glycoprotein may mediate epithelial cell attachment [2]. The EHS sarcoma is a transplantable mouse tumor which produces an extracellular matrix of basement membrane and which provides a rich source of laminin [3]. Here, we describe a one-step purification of laminin extracted from the EHS sarcoma.

## 2. Materials and methods

The EHS sarcoma was grown in C57 black female mice, 4–6 weeks old, and transplanted with a trocar into the femoral muscle. After 5 weeks, tumors were harvested, suspended in PBS which was 5 mM in EDTA and 4 mM in *N*-ethylmaleimide and homogenized on ice in a rotary Dounce homogenizer for 1 min. A mixture of *Griffonia simplicifolia* I isolectins was prepared as in [4]. CNBr-activated Sepharose 4B was from Pharmacia (Uppsala). Coffee bean α-galactosidase (EC 1.2.3.22), galactose dehydrogenase (EC 1.1.1.48), and NAD were purchased from Boehringer-Mannheim.

*Abbreviations:* EDTA, ethylenediamine tetraacetic acid; FITC, fluorescein isothiocyanate; GS I, *Griffonia simplicifolia* I lectin; GS I-$B_4$, *Griffonia simplicifolia* $B_4$ isolectin; NAD, niacin adeninedinucleotide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate

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All chemicals used were of reagent grade or the best quality available. Phosphate-buffered saline (PBS) consisted of 0.3 M NaCl, 0.1 mM $CaCl_2$, 0.1 M sodium-phosphate (pH 7.2) containing 0.02% sodium azide and the protease inhibitors *p*-hydroxymercuribenzoate (50 μg/ml), *N*-ethylmaleimide (50 μg/ml) α-toluene-sulfonyl-fluoride (50 μg/ml) and trypsin inhibitor from soy bean (100 μg/ml) (Sigma Chemical Co., St Louis MO).

### 2.1. *Preparation of affinity adsorbent*

*Griffonia simplicifolia* I lectin was immobilized onto CNBr-activated Sepharose 4B as in [5].

### 2.2. *Immunochemical methods*

Three adult New Zealand white rabbits were immunized with purified laminin (1 mg/ml PBS) homogenized in Freund's complete adjuvant (Difco Labs, Detroit MI), in a Virtis microblender. The emulsion was injected subcutaneously in the back (0.1 ml containing 100 μg laminin) on 3 successive weeks. Blood was drawn from the marginal ear vein at weekly intervals beginning 2 weeks after last injection of laminin. Antisera were stored frozen without addition of preservative. Ouchterlony plates were prepared as in [6]. Wells were 3 mm diam. and 10.5 mm apart and with a capacity of ~12 μl were formed with a gel punch. Affinity-purified laminin was added to the central wells and antisera from the 3 rabbits were added to the peripheral wells. Plates were incubated at room temperature.

### 2.3. *Digestion of affinity-purified laminin by coffee bean α-D-galactosidase*

One unit of coffee bean α-D-galactosidase was added to affinity-purified laminin (500 μg) dissolved



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in 0.05 M citrate buffer (pH 6.1) in a total volume of 5 ml. The digest was incubated at 37°C for 6 h, concentrated at 4°C in a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton OR) and loaded onto a Sephacryl S-200 column (1.5 × 30 cm) equilibrated with PBS (pH 7.2) to separate α-D-galactosidase from laminin which eluted at the void volume. The α-D-galactosidase-digested laminin was added to a *Griffonia simplicifolia* I lectin affinity column.

### 2.4. *'Staining' SDS–polyacrylamide gels with FITC–GS I-$B_4$ isolectin*

Laminin was dissolved in Laemmli sample buffer containing 2% β-mercaptoethanol and applied in triplicate to a 6% Laemmli slab gel [7] 1.5 mm thick. After fixing the slab in methanol–acetic acid–water (5/1/5), it was cut into 3 pieces, each piece bearing one of the triplicate samples. One piece of the slab was stained in Coomassie blue while the remaining 2 pieces were equilibrated in PBS. One piece was immersed in 20 ml FITC–$B_4$ solution (100 μg/ml, prepared as in [8]), and the other piece, serving as control, was treated identically except that methyl α-D-galactoside (2.5 mg/ml) was added to the lectin solution. After 2 days at 4°C, the gels were rinsed in PBS and photographed under long wavelength ultraviolet illumination (C-62 Blak-Ray Transilluminator, Ultraviolet Products, San Gabriel CA).

Glycoconjugates known to contain α-galactosyl groups, such as earthworm cuticle collagen [9] or melibionate–bovine serum albumin conjugate [10], were effectively labeled by this protocol; glycoconjugates known to lack α-galactosyl groups but to contain terminal β-galactosyl units, such as asialofetuin [11,12], bovine asialoprothrombin [13] or lactobionate–bovine serum albumin conjugate [10] were not labeled with the FITC–$B_4$ probe.

### 2.5. *Terminal α-galactosyl group content of laminin*

Laminin (140 μg) was treated with coffee bean α-galactosidase (0.05 U) at 37°C in 1.0 ml McIlvaine's phosphate-citrate buffer (pH 5.1) containing 0.04% sodium azide. Duplicate 50 μl aliquots were taken for the assay of D-galactose at 5 h, 21 h and 94 h incubation. Of the α-galactosidase activity initially present in the digestion mixture 70% remained after 94 h incubation. Relative to the α-galactosidase activity, other glycosidase activities were ≤0.03% as determined with *p*-nitrophenylglycoside substrates [8]. Control mixtures lacked either the α-galactosidase or the laminin.

The D-galactose liberated by α-galactosidase was assayed by a fluorescence modification of the galactose dehydrogenase method in [14]. Samples containing 30–200 ng D-galactose in 50 μl were mixed with 60 μg NAD and 12 μg *Pseudomonas fluorescens* galactose dehydrogenase in 1.5 ml 0.1 M sodium phosphate buffer (pH 7.8). After 4 h at ambient temperature the NADH evolved in the reaction was measured with an Aminco-Bowman fluorometer at an excitation wavelength of 340 nm and an emission wavelength of 465 nm. Blanks were prepared by mixing buffer, NAD reagent and either galactose or galactose dehydrogenase.

### 2.6. *The total galactose content of laminin*

Laminin (140 μg) was acid hydrolyzed as in [15] at 100°C in 1.0 ml 0.02 N HCl containing a suspension (40%, v/v) of freshly regenerated and washed cation exchange resin (AG 50-X2 (200–400 mesh)–hydrogen form). At 2 h, 6 h and 48 h, duplicate 25 μl aliquots of the supernatant solution were taken for the galactose determination as above.

## 3. Results

### 3.1. *Purification of laminin by affinity chromatography on* Griffonia simplicifolia *I–Sepharose 4B*

A clarified crude extract (containing 6 ml, 51 mg protein) from a murine EHS sarcoma, processed as above, was applied at a flow rate of 5 ml/h to the *Griffonia simplicifolia* I lectin–Sepharose 4B column

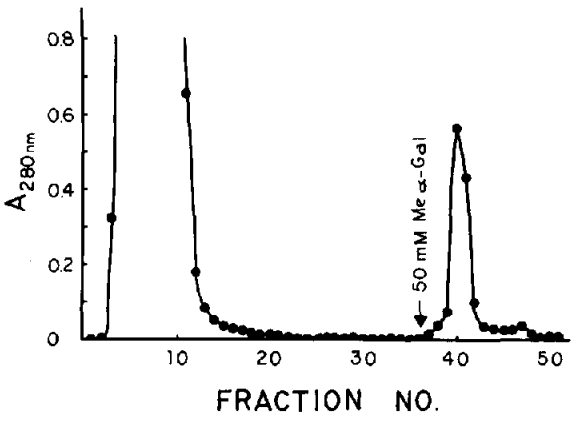


Fig.1. Elution profile of crude extract from a mouse EHS sarcoma tumor on a *Griffonia simplicifolia* I affinity column.

(1.5 × 6 cm), equilibrated with PBS. The column was washed with PBS until the absorbance at 280 nm decreased to 0.02. At this point, addition of 50 mM methyl α-D-galactopyranoside in PBS displaced the laminin (fig.1). A yield of 3.4 mg laminin was obtained based on Lowry assay [16] using bovine serum albumin as standard. This represents a 30% yield of immunoreactive laminin relative to the crude extract as determined by single radial immunodiffusion [17]. The quantity of extract applied was sufficient to fully saturate the column. Application of limited extract (0.5 ml) resulted in essentially quantitative binding of laminin as shown by the absence of laminin bands on 5% SDS–PAGE gels of the wash fractions. Haptenic galactoside was removed by dialysis against PBS.

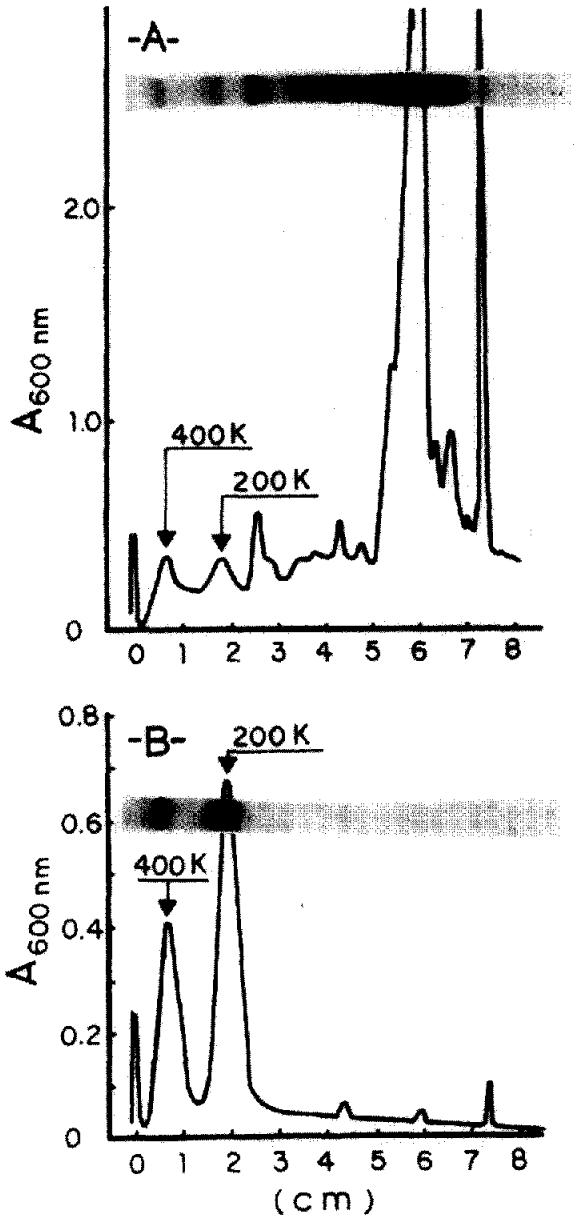


Fig.2. SDS–PAGE and densitometer scans of: (A) crude extract; (B) affinity purified laminin.

### 3.2. *Properties of purified laminin*

The laminin, purified by affinity chromatography, was examined by gel electrophoresis and immunodiffusion. Disc gel (5%) electrophoresis in the presence of SDS and 2-mercaptoethanol followed by Coomassie blue-staining showed 2 bands of $M_r$ 400 000 and 200 000 (fig.2A). This pattern is identical to that reported for laminin purified by a conventional protein purification procedure [1]. Densitometer scans (fig.2B) of the gels of affinity-purified laminin gave 2 major peaks and very small amounts of 2 minor components ($M_r$ 120 000 and 60 000). However, laminin constituted >98% of the material which bound to the affinity column. The ratio of the $M_r$ 400 000 and 200 000 components was 1:2.

Both laminin subunits, separated by SDS–PAGE, were 'stained' with FITC–GS I-$B_4$ indicating that α-galactosyl end groups are substituents of both polypeptide chains (not shown).

SDS–polyacrylamide gel (5%) electrophoresis of affinity-purified laminin, after storage at −20°C for 2 months in the absence of protease inhibitors, revealed breakdown of both polypeptide chains to smaller fragments (60 000–80 000 $M_r$). The 400 000 $M_r$ unit was particularly susceptible to proteolysis.

Coffee bean α-galactosidase liberated 19% of the galactose content of laminin (table 1) and abolished its ability to precipitate the GS I-$B_4$ lectin (but not *Ricinus communis* I agglutinin) and to bind to the GS I–Sepharose column (not shown).

Table 1
The α-D-galactosyl group content of laminin

| Treatment | D-Galactose liberated (g/100 g laminin)[a] |
|---|---|
| α-Galactosidase (94 h)[b] | 1.20 |
| Acid hydrolysis (48 h) | 6.24 |

[a] Laminin concentration was determined by Lowry assay employing a bovine serum albumin standard

[b] At the digestion times shown, the release of D-galactose had reached a maximum value

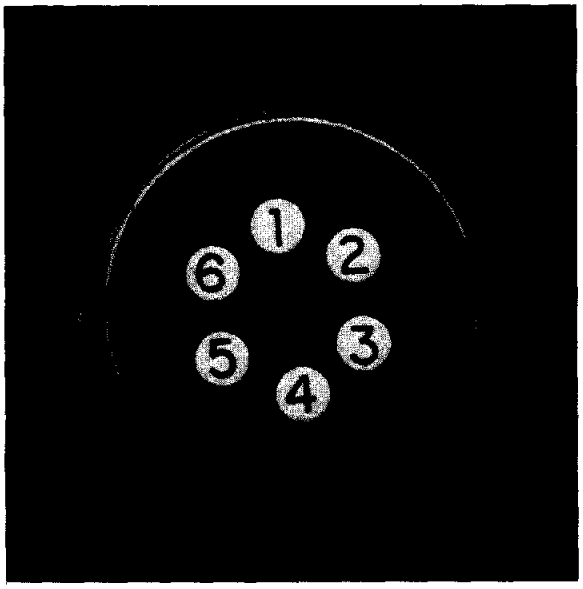


Fig.3. Immunodiffusion of 3 rabbit anti-laminin antisera against affinity-purified laminin. Central well contains purified laminin (10 μl). Peripheral wells 1–3 contain antisera from 3 different rabbits 2 weeks after final injection of laminin. Wells 4–6 contain preimmune sera from the same 3 rabbits.

Ouchterlony analysis of affinity-purified laminin against rabbit antisera raised to the laminin is presented in fig.3. The affinity-purified laminin gave single, sharp, confluent precipitin lines against the 3 rabbit antisera.

## 4. Discussion

Here we show the facile purification of laminin by affinity chromatography on immobilized *Griffonia simplicifolia* I lectin. The basis of the procedure depends on the presence in laminin of terminal α-D-galactopyranosyl groups as revealed by the formation of a specific precipitate with the GS I-$B_4$ isolectin, and by the action of highly purified α-D-galactosidase on laminin, liberating free galactose and destroying its ability to react with GS I-$B_4$.

Fluoresceinated-$B_4$ isolectin was shown to bind in a specific manner to basement membrane structures in a variety of murine tissues [8]. We now believe that laminin is the constituent of basement membrane to which the $B_4$ isolectin binds.

Purified laminin binds to various glycosaminoglycans (immobilized on Sepharose 4B) and may be displaced by gradient elution with NaCl [18]. However, the utility of such an approach for the purification of laminin from a crude extract was not demonstrated.

A serious problem involved in extraction and purification of laminin from the murine EHS sarcoma is the presence of protease activity in the digest. Even after affinity purification, residual proteolytic activity results in the degradation of laminin. The action of a series of proteases (e.g., plasmin and thrombin) on laminin is documented in [19]. Interestingly, SDS–PAGE of our affinity-purified lectin, after long storage, gave a pattern similar to those obtained in [19]. Therefore, it would appear prudent to add several types of protease inhibitors to systems containing laminin in order to prevent proteolytic degradation.

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