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Erythropoietin: Isolation by affinity chromatography with lectin–agarose derivatives

(wheat germ agglutinin/phytohemagglutinin/glycoprotein)

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ABSTRACT **Affinity chromatography using agarose-bound lectins was used to isolate erythropoietin from crude preparations of sheep plasma and human urinary erythropoietin. On the basis of previous estimates of the sugar content of the hormone, six lectins (wheat germ agglutinin, phytohemagglutinin, *Ricinus communis* 120, soybean agglutinin, concanavalin A, and limulin) were chosen for study. Only wheat germ agglutinin–agarose and phytohemagglutinin–agarose derivatives had significant affinity for erythropoietin. By use of wheat germ agglutinin–agarose columns, erythropoietin could be separated from over 95% of the initial starting protein, resulting in an 8- to 100-fold purification and a recovery of at least 40% depending on the source of the hormone. Affinity chromatography with agarose-bound lectins provides a simple rapid method for isolating erythropoietin from crude preparations of the hormone.**

The exact mechanisms by which the polypeptide hormone erythropoietin (EP) regulates red blood cell division and differentiation are not well understood. Part of the difficulty in assigning specific effects on responsive cells to EP *per se* stems from the necessity of using the crude preparations of the hormone currently available. Small quantities of highly purified EP have been isolated from sheep plasma (1) and human urine (2) by conventional techniques for separating proteins on the basis of size and charge. In each instance, however, large quantities of starting material were required, yields were low (0.4–18.0%), and only tentative claims for homogeneity were considered prudent (2, 3). Because of our interest in studying the interaction of EP with its target cells, we sought a simpler and more efficient method for the isolation of EP from crude starting material. We now report that, in a single step, affinity chromatography with lectin–agarose derivatives can, depending on the source of the starting material, provide up to a 100-fold purification of erythropoietin with yields of 40% or greater.

MATERIALS AND METHODS

Erythropoietin. Sheep plasma EP (2–4 units/mg of protein) was from Connaught Laboratories; human urinary EP (Pool H-11-TaLSL; approximately 50–70 units/mg of protein) was from the National Heart and Lung Institute. Crude EP was labeled with ^{125}I by using a water-insoluble oxidizing agent, 1,3,4,6-tetrachloro-3α,6α-glycoluril (4), coated as a film (40 μg in 20 μl of chloroform) in 12 × 75-mm glass tubes. The reaction of 100 μg of crude EP with 1.5 mCi of iodine-125 (Amersham) in a total volume of 200 μl of 125 mM phosphate buffer, pH 7.4, was allowed to proceed for 30 sec, at which time the solution was transferred to a column (1 × 16.5 cm) of Sephadex G-25 and the protein fraction was eluted with 0.1 M phosphate buffer, pH 7.4. On average, 30% of the radioactivity was incorporated into protein.

The erythropoietic activity of column fractions was measured by the exhypoxic polycythemic mouse assay (5). In this assay, the ability of test substances to stimulate erythropoiesis is determined by measuring their effect on the incorporation of ^{59}Fe (as citrate, Mallinckrodt) into newly formed red cells. A positive response is indicated by a value for ^{59}Fe incorporation of greater than 4%. The second International Reference Preparation of Human Urinary Erythropoietin obtained from the World Health Organization was used as a standard.

Chromatography with Lectin–Agarose Derivatives. Except for concanavalin A–agarose (Pharmacia, 10 mg/ml of gel), lectin–agarose derivatives (Sepharose 4-B, Pharmacia) were prepared by a simplified cyanogen bromide–agarose activation method (6). Coupling of lectins (2–10 mg of lectin per ml of activated agarose) was allowed to proceed for 20 hr at 4°, followed by the addition of 1 M glycine (4 hr at 24°) to mask unreacted groups on the agarose. Soybean and *Ricinus communis* 120 agglutinins were from Miles; phytohemagglutinin (PHA) (P form) was from Difco; limulin was prepared from the hemolymph of *Limulus polyphemus* (a gift of J. Levin) by the technique of Roche and Monsigny (7); and wheat germ agglutinin (WGA) (kindly provided by Joel Shaper) was prepared as described (8). The columns, which did not yield detectable free lectin in the effluent, were washed extensively before use. Chromatography was performed at 4° on small columns (0.5 × 9 cm; 2-ml bed volume) of lectin–agarose derivatives; adsorbed protein was eluted with buffers containing the appropriate pure sugars (from Pfanstiehl). Protein in the effluent and in purified samples was monitored by ultraviolet adsorption (9) and with the Folin–Ciocalteu reagent (10).

RESULTS

All of the lectin–agarose derivatives were able to bind an appreciable amount of protein, which could be eluted with the appropriate lectin-specific sugar. However, of the six lectins studied, only WGA and PHA yielded agarose derivatives capable of binding substantial amounts of EP. The results for human urine EP are shown in Table 1. Similar results were also obtained for sheep plasma EP.

The addition of small amounts of ^{125}I-labeled crude EP as a tracer greatly facilitated monitoring the chromatographic procedures; the measurement of radioactivity provided the most convenient estimate of protein content in the sugar eluate, since the presence of amino sugar interfered with the direct measurement by spectrophotometric methods of the small amounts of protein in this eluate. As indicated by the elution

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Abbreviations: EP, erythropoietin; PHA, phytohemagglutinin; WGA, wheat germ agglutinin; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

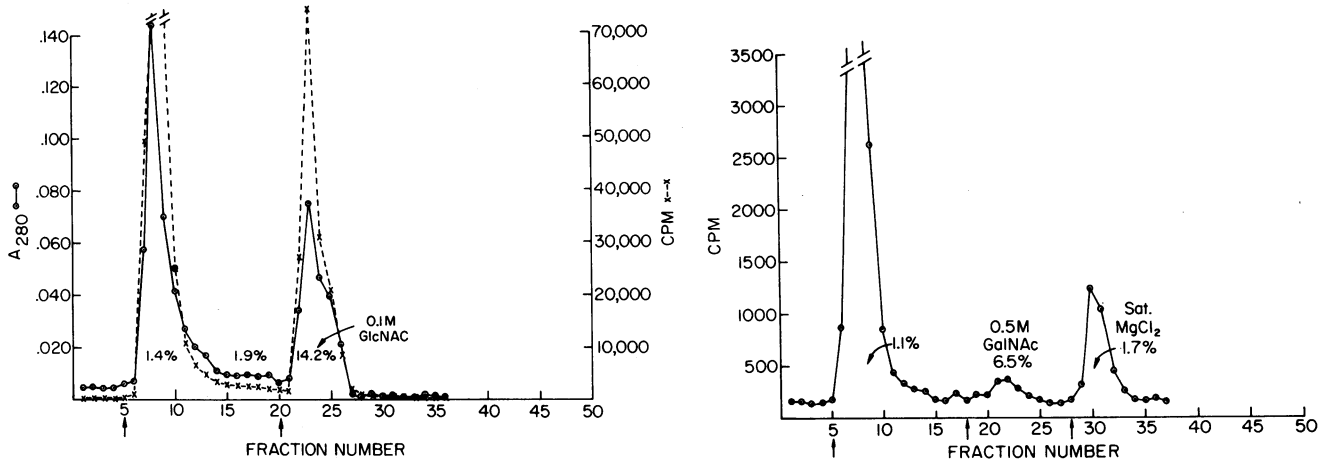

FIG. 1. Affinity chromatography of EP on lectin–agarose derivatives. Crude human urine EP was applied (first arrow) to columns (0.5 × 9 cm) of either WGA–agarose or PHA–agarose derivatives and eluted at 4° with Dulbecco's phosphate-buffered saline, pH 7.0, until the eluate was free of protein. Elution was then begun (second arrow) with buffer containing the sugar specific for the particular lectin. Aliquots of pooled samples from the unretarded fraction (void volume, 6 ml), from the buffer eluate (5 ml), and from the fraction eluted with sugar (5 ml) were assayed for EP activity. The numbers represent the percent of injected ^{59}Fe that was incorporated into newly formed red cells. (*Left*) WGA–agarose eluted with 0.1 M *N*-acetylglucosamine (GlcNAc). Aliquots of 0.5 ml from the void volume and buffer eluate and 0.1-ml aliquots from the sugar eluate were assayed individually for erythropoietic activity. Fifty units of erythropoietin (1 mg of protein) were applied to this column. (*Right*) PHA–agarose eluted with 0.5 M *N*-acetylgalactosamine (GalNAc) followed by elution with saturated $MgCl_2$ (third arrow). Approximately 5 units of EP (0.1 mg of protein) were applied to this column. Aliquots of 0.5 ml were assayed individually for erythropoietic activity.

profiles for human EP (Fig. 1), a large proportion of the protein, devoid of appreciable erythropoietic activity, was recovered in the void volume from columns of WGA or PHA. Once the eluate became free from protein (as monitored by either radioactivity or UV absorption), elution of WGA or PHA derivatives with sugar-containing solutions yielded erythropoietic activity in a single peak. In view of a report (11) that appeared during the course of these studies, indicating that saturated solutions of $MgCl_2$ can displace EP adsorbed to PHA-agarose derivatives, sequential elution of EP adsorbed to PHA–agarose was performed first with *N*-acetyl-D-galactosamine (GalNAc) and then with saturated $MgCl_2$. From Fig. 1 *left*, it is evident that for human EP, while the hormonal activity is eluted with the sugar, subsequent treatment of the PHA column with saturated $MgCl_2$ eluted additional protein devoid of erythropoietic activity.

The sugar specificity of the lectin–human urine EP interaction with WGA and PHA was examined with *N*-acetylglucosamine (GlcNAc), GalNAc, and D-galactose. Of the hexosamines, only GlcNAc competed for the binding of EP to WGA (Fig. 2 *left*) while only GalNAc competed for the binding of EP to PHA (Fig. 2 *right*). EP was not displaced from PHA by galactose.

Table 1. Affinity of agarose-bound lectins for human urine erythropoietin

Lectin	Specific sugar	EP activity* Void volume	EP activity* Sugar eluate
Limulin	Sialic acid	16.0 ± 1.0	3.0 ± 0.2
Concanavalin A	Mannose	15.0 ± 2.0	3.0 ± 1.0
Ricinus communis 120	Galactose	13.0 ± 2.0	1.0 ± 0.3
Soybean agglutinin	GalNAc	21.0 ± 4.0	1.0 ± 0.3
PHA	GalNAc	2.0 ± 0.5	11.0 ± 2.0
WGA	GlcNAc	1.0 ± 0.3	16.0 ± 2.0

Crude human urine EP (0.1 mg, approximately 5–7 units) with a trace amount of ^{125}I-labeled EP was applied to each agarose–lectin column and eluted at 4° with Dulbecco's phosphate-buffered saline (pH 7.0) until the eluate was free of significant amounts of labeled material. Elution was then begun with buffer containing sugar specific for the particular lectin. Fractions of 1 ml were collected. Equal aliquots of pooled fractions from the void volume and the sugar eluate were assayed for erythropoietic activity.

* % ^{59}Fe incorporated into red cells; mean ± SEM.

In the above experiments, too little protein was present in the fractions containing EP to permit a reliable estimate of specific activity; larger amounts (1–7 mg) of both human urinary and sheep plasma EP were, therefore, purified with the WGA–agarose derivative (Table 2). With both preparations, over 95% of the total protein was recovered in the void volume, leading to an 8- to 100-fold purification of EP subsequently eluted with GlcNAc. Forty percent of the erythropoietic activity initially present in the crude preparation of human urinary EP was recovered in the sugar eluate. With crude sheep plasma EP, the apparent recovery of EP in the sugar eluate was greater than 100%. This reflects the removal during chromatography of inhibitors that were present in the crude starting preparation (12).

As indicated by the specific activity of the human and sheep EP recovered and by the electrophoretic analysis of protein from the sugar eluates (data not shown), the material purified by affinity chromatography, while comprising markedly fewer constituents than the starting material, still contained a number of proteins in addition to EP.

DISCUSSION

EP constitutes only a minor fraction of the protein present in concentrates of urine and plasma obtained from anemic subjects. Attempts to isolate EP by techniques based on differences in size and charge have not been completely successful since many of the contaminating proteins, of which albumin constitutes a major component, share similar physicochemical properties with the hormone.

EP is a glycoprotein. Chemical analyses of purified sheep (3) and human (2) EP reveal a carbohydrate content of 24 and 29%, respectively. For sheep plasma EP, the carbohydrate content is accounted for by sialic acid, mannose, galactose, glucose, and GlcNAc; 55% of the carbohydrate of human EP consists of sialic acid and GlcNAc. The identity of the other sugars is uncertain. We decided, therefore, to exploit differences in carbohydrate

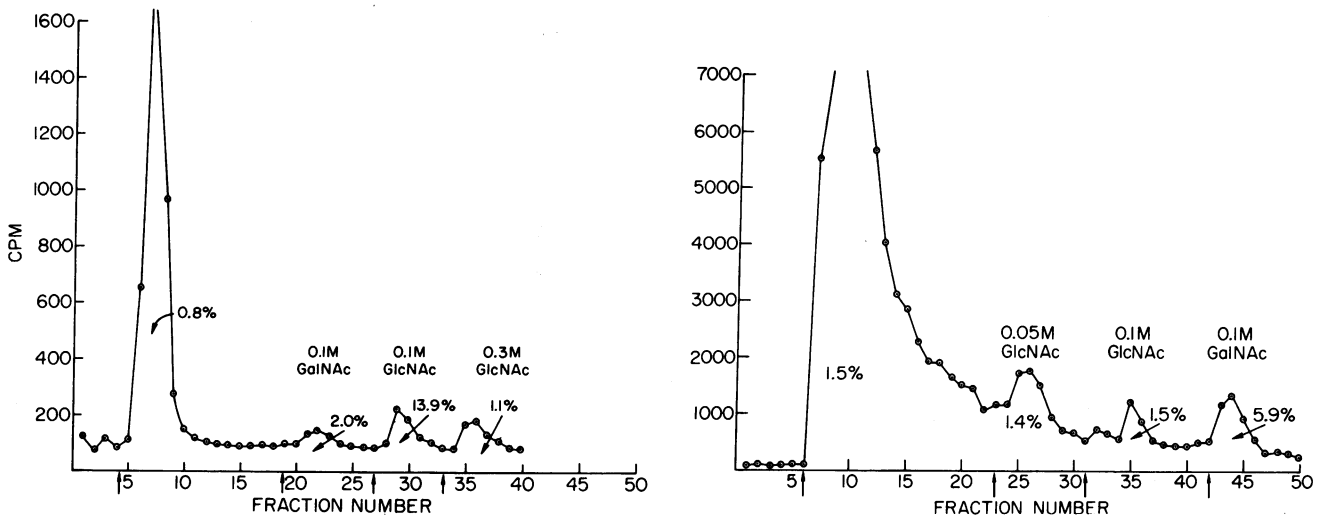

FIG. 2. Sequential elution of human urine erythropoietin from (*Left*) WGA–agarose and (*Right*) PHA–agarose with GlcNAc and GalNAc. The arrows represent the point of application of the EP (5 units) or the amino sugars and the numbers represent the percent of injected ^{59}Fe that was incorporated into newly formed red cells after injection of aliquots from the pooled fractions of the void volume and sugar eluates into exhypoxic polycythemic mice.

configuration using affinity chromatography with agarose-bound lectins to separate the hormone from other proteins in crude starting preparations. With a single chromatographic procedure, over 95% of contaminating proteins could be removed. In addition, the ability of both human urinary and sheep EP to absorb to WGA–agarose derivatives and to elute in the presence of GlcNAc confirms the presence of this amino sugar in EP from both species in a manner independent of chemical analysis. Similarly, although the sugar specificity of PHA is less certain than that of WGA, the ability of GalNAc to elute both human urinary and sheep EP from PHA–agarose derivatives suggests the presence of this sugar amongst the total hexosamines present.

Given the suspected sugar content of EP and the known sugar specificities of the lectins (Table 1), it was somewhat surprising that only two (PHA and WGA) of the six lectin–agarose derivatives were capable of binding EP. In contrast, all of the lectin derivatives were capable of binding substantial amounts of nonerythropoietically active glycoproteins, with an appropriate specificity for certain sugars (e.g., binding by limulin–agarose of sialic acid-containing ceruloplasmin). The ability of only certain lectin derivatives to adsorb EP may relate to the accessibility of particular sugar moieties within the oligosaccharide chain of EP. Although the identities of many of the sugars in the carbohydrate chain of human EP are unknown, the similar behavior of both sheep and human EP suggests that the sugar configuration of the two hormones is similar.

The technique we have described provides a convenient method for substantially purifying EP for biochemical studies from the small quantities of crude starting material currently available. The preliminary observations of Sieber (11), as well as our own, suggest that this technique can also be applied to isolation of EP directly from urine without prior manipulation. When large quantities of EP-rich urine or plasma become available, affinity techniques using agarose-bound lectins should provide a new avenue for the isolation of the hormone.

Table 2. Purification of sheep plasma and human urine EP by WGA affinity chromatography

Material tested	Protein, mg: Total	Protein, mg: For assay	Activity, units	Specific activity, units/mg	Purification factor	Recovery, %
Sheep EP step III	7.0	0.05	0.1	2	—	—
WGA sugar eluate	0.2	0.0005	0.1	200	100	>100
Human urine EP	1.0	0.008	0.4	50	—	—
WGA sugar eluate	0.05	0.001	0.4	400	8	40

Seven milligrams of sheep EP, step III, (specific activity, 2 units/mg of protein) or 1 mg of human urine EP (specific activity, 50 units/mg of protein) was applied to an agarose–WGA column and eluted with 0.1 M GlcNAc. Aliquots of the void volume and sugar eluates were assayed for erythropoietic activity. The recovery of activity in excess of 100% for sheep EP is accounted for by the removal of inhibitors from the crude preparation during affinity chromatography.

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