PREPARATION OF PURIFIED ERYTHROPOIETIN FROM RABBIT BLOOD PLASMA

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Erythrocyte formation by the bone marrow is regulated by the protein hormone erythropoietin. This explains the enormous interest in the obtaining of purified preparations of erythropoietin and the study of its properties. Because of the low concentration of the hormone in mammalian blood, its isolation is very difficult, and for that reason the urine of patients with aplastic anemia [4, 8], in whom the erythropoietin concentration is higher in the urine than in the blood plasma, is most frequently used to obtain purified preparations. The most highly purified preparation of erythropoietin from sheep blood plasma was obtained in Goldwasser's laboratory [6]. Mainly rabbits are used in laboratory practice to study the regulation of erythropoiesis. During work with these animals, to reduce undesirable side effects due to injection of foreign proteins, an erythropoietin preparation with sufficiently high activity obtained from the blood of rabbits themselves must be available. This preparation can also be used to create a convenient laboratory standard. Highly purified erythropoietin is necessary for research connected with the study of the early stages of differentiation of red blood cells.

The object of this investigation was to develop a method of obtaining partially purified erythropoietin from rabbit blood plasma.

EXPERIMENTAL METHOD

Noninbred rabbits weighing 3-5 kg, kept in a pressure chamber at 0.5 atm for 20 h to stimulate erythropoietin production, were used to obtain blood plasma. Erythrocytes and other blood cells were removed by centrifugation at 600g for 30 min. As the first step in purification, ion-exchange chromatorgraphy was carried out on DEAE-cellulose (DE-32 from Serva, West Germany) as described in [5]. For the second stage of purification affinity chromatography was carried out on immobilized phytohemagglutinin P [11]. The phytohemagglutinin was obtained by the method in [10] and immobilized on sepharose 4B (from Pharmacia, Sweden) by means of cyanogen bromide as described in [9]. Erythropoietin, adsorbed on phytohemagglutinin P, was eluted with 4 M guanidine hydrochloride solution. Subsequent purification was carried out on Ultragel ACA-44 (from LKB, Sweden). Before application to the

TABLE 1. Purification of Erythropoietin from Rabbit Blood Plasma

Stage of purification	Order of purification	Volume, ml	Protein concentration, mg/ml	Total protein, mg	Incorporation of ^{59}Fe, % (M ± m)	Activity, units/ml	Specific activity, units/mg protein	Total activity, units	Yield, %	Purification factor
1. Chromatography on DE-32 cellulose	Original plasma	135	88,0	11 880	2,4±0,2 $P<0,001$	0,26	0,003	35,6	80,0	80
	Product	130	0,9	117	2,3±0,1 $P<0,001$	0,22	0,24	28,6		
2. Chromatography on phytohemagglutinin P	Taken after stage 1	50	0,9	45	2,3±0,1	0,22	0,24	11,0	91,0	3 330
	Product	40	0,025	1	2,4±0,3 $P<0,001$	0,25	10,0	10,0		
3. Chromatography on Ultragel ACA-44	Taken after stage 2	10	0,025	0,25	2,4±0,3	0,25	10,0	2,5	96,0	25 000
	Product	8	0,004	0,032	2,8±0,4 $P<0,001$	0,30	75,0	2,4		

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Ultragel column, the active fraction obtained in the preceding stage was concentrated on an FM-02-10 ultrafiltration apparatus. A CM-20 membrane was used for concentration. The active fraction was eluted with 0.1 M phosphate buffer, pH 6.8. All chromatographic procedures were carried out at 4°C. Elution of protein from the column was monitored by means of a Uvicord III photometer (from LKB, Sweden). The protein concentration was determined by Lowry's method [1]. Erythropoietic activity of the fractions was determined as incorporation of ^{59}Fe into erythrocytes by the method in [3] and estimated quantitatively against International Standard B [2]. Female CBA mice weighing about 20 g, kept in a pressure chamber at 0.5 atm for 20 of the 24 h daily, were used for biotesting. The test material was injected intraperitoneally, once in a volume of 0.5 ml. Each sample was tested on seven mice.

EXPERIMENTAL RESULTS

Ion-exchange chromatography on DE-32 cellulose is characterized by smaller losses and a higher degree of purification than other methods [7], as shown by the high efficiency of this stage of hormone purification (Table 1). To study the regulation of erythropoiesis and to obtain a standard, more purified specimens of erythropoietin than those obtained on cellulose are usually used, and for that reason the possibility of purifying the hormone further by affinity chromatography on sepharose with immobilized phytohemagglutinin P was studied. The method of affinity chromatography is currently the most promising method of biopolymer purification, for it provides a high degree of purification together with high yield of the product. As a result of this stage a hormone with specific activity of 10 units/mg protein was obtained with a yield of 91%. The erythropoietin obtained after this stage of purification can be used as a laboratory standard. Further purification was carried out on Ultragel ACA-44. Erythropoietic activity was found in the protein fraction with molecular weight of 20,000-40,000. The specific activity of the erythropoietin obtained as a result of this stage was estimated to be 75-100 units/mg protein.

A three-stage scheme for obtaining partially purified erythropoietin hormone from rabbit blood plasma, with a specific activity of 75-100 units/mg protein and with a purification factor of about 25,000, is thus suggested.

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