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Note

Purification of human urinary erythropoietin

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Erythropoietin (EPO) is an acidic glycoprotein that regulates erythrocyte formation by promoting erythroid differentiation and initiating haemoglobin synthesis [1,2]. Purification of EPO to homogeneity, from urine of severely anaemic patients, has been reported by Miyake et al. [3]. This purification procedure involved ethanol precipitation and a complex series of chromatographic steps, ion-exchange chromatography, gel permeation and adsorption chromatography. Immobilized lectins and immunoadsorbent columns have been reported to be more effective for purification of EPO [4,5]. This paper describes a simplified purification procedure by combination of conventional techniques and reversed-phase high-performance liquid chromatography (HPLC) as a final step.

EXPERIMENTAL

Starting materials for purification

Urine from patients with aplastic anaemia was concentrated ca. ten- to twenty-fold by ultrafiltration using a Model ACL-1010 hollow-fiber apparatus (Asahi Chemical Industry, Tokyo, Japan) with a nominal molecular mass cut-off of 13 000. The concentrate was then heated for 1 min in boiling water, and the precipitate formed was removed by centrifugation at 10 000 *g*.

Purification of EPO

All steps except reversed-phase HPLC were carried out at 4°C. The heated concentrate was fractionated with ethanol in the presence of lithium chloride [3]. The precipitate with 70–90% ethanol was dissolved in Dulbecco's phosphate-buffered saline (PBS). The solution was applied to a Con A-Sepharose

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(Pharmacia, Uppsala, Sweden) column equilibrated with PBS, and the flow-through fraction was then loaded onto a wheatgerm lectin-Sepharose (Pharmacia) column equilibrated with PBS. EPO activities were eluted with 0.5 *M* N-acetylglucosamine in PBS. Bed volumes used per 1 mg of protein of the ethanol precipitate were 2 ml for Con A-Sepharose and 0.5 ml for wheatgerm lectin-Sepharose. The eluate from wheatgerm lectin-Sepharose was dialysed extensively against 0.5 m*M* phosphate buffer, pH 7.1. The dialysate containing ca. 2 mg of protein was applied to a hydroxyapatite column (3.5×1.5 cm I.D., Bio-Gel HTP, Bio-Rad, Richmond, CA, U.S.A.) equilibrated with 0.5 m*M* phosphate buffer (pH 7.1). The column was washed with ten column volumes of the buffer, and EPO activities were eluted stepwise with 2 and 100 m*M* phosphate buffer (pH 7.1). The eluate with 2 m*M* phosphate, which contained the majority of EPO activity, was concentrated by lyophilization in order to perform further purification by HPLC. Reversed-phase HPLC was carried out using a μBondapak C_{18} column (300×3.9 mm I.D., 10 μm, Waters Assoc., Milford, MA, U.S.A.) equilibrated with 10 m*M* ammonium formate as starting solvent. The chromatography was developed by running a 60-min linear gradient to 60% *n*-propanol in 10 m*M* ammonium formate. The flow-rate was 1 ml/min. Eluted protein was monitored by UV absorption at 280 nm. Protein peaks were collected manually and evaporated to dryness.

Protein determination and electrophoresis

Protein concentration was determined by the method of Bradford [6]. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli [7]. Protein was visualized by silver staining, according to the manual of the supplier (Bio-Rad).

Amino acid sequence analysis

Sequence analysis was carried out with a gas-phase sequencer, Applied Biosystems Model 470A. A sample was dissolved in 1% SDS solution. Phenylthiohydantoin (PTH) amino acid derivatives were identified as previously described [8], with slight modifications.

Assay of EPO

EPO activity was determined according to Dunn et al. [9] by measuring ^{59}Fe incorporation into newly synthesized haem in cultured fetal mouse liver cells. One unit of EPO is defined as the activity contained in one tenth of the contents of an ampoule of the second International Reference Preparation obtained from the World Health Organization (WHO).

RESULTS AND DISCUSSION

A summary of a typical purification of EPO from concentrate of ca. 70 l of urine is shown in Table I.

After treatment with hydroxyapatite column chromatography, EPO was detected as a major diffuse band with a molecular mass of 36 000 on SDS-PAGE

TABLE I

PURIFICATION OF HUMAN ERYTHROPOIETIN FROM CONCENTRATE OF URINE

Starting material was 70 l of urine; the details of the procedure are described in the text.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Heated concentrate	1560	92 100	59	100
Ethanol precipitate	37.0	65 300	1770	70.9
Wheatgerm lectin-Sepharose	1.98	42 100	21 300	45.7
Hydroxyapatite	0.198	14 300	72 200	15.5
Reversed-phase HPLC	0.064	7540	118 000	8.2

and had a sufficiently high specific activity compared with the value of pure EPO reported previously [3,5]. However, from the results of sequence analysis, it was found that this preparation of EPO yielded a number of N-terminal amino acids derived from impurities.

In order to perform further purification, a reversed-phase HPLC technique was adopted. By testing the stability in various organic solvents, EPO was found to be stable in aqueous *n*-propanol at neutral pH, but unstable in trifluoroacetic acid–acetonitrile, which is frequently used for purification of proteins.

EPO was injected onto a μBondapak C_{18} column equilibrated with 10 m*M* ammonium formate, and elution of EPO was carried out by increasing the concentration of *n*-propanol. A typical chromatographic pattern is shown in Fig. 1. EPO activity eluted as a broad peak at an *n*-propanol concentration of ca. 40%. This step generally resulted in 50–60% recovery of activity. The finding that EPO was precipitated with 70–90% ethanol suggests that EPO has rather hydrophobic properties in spite of its large content of sugar moieties. Therefore, a hydrophobic solvent should be required as an organic solvent to elute EPO. *n*-Propanol can meet this criterion.

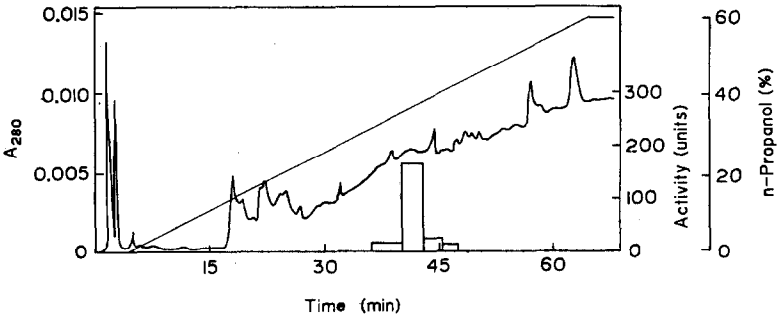
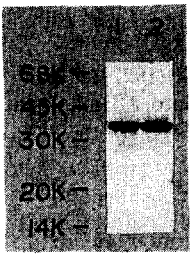


Fig. 1. Purification of EPO by reversed-phase HPLC. Approximately 400 U of EPO purified through the hydroxyapatite step were injected onto a μBondapak C_{18} column equilibrated with 10 m*M* ammonium formate. The flow-rate was 1 ml/min, protein was eluted with a linear gradient of *n*-propanol (0–60%) in 10 m*M* ammonium formate and continuously monitored at 280 nm. Protein peaks were collected manually and the EPO activities were determined (column).



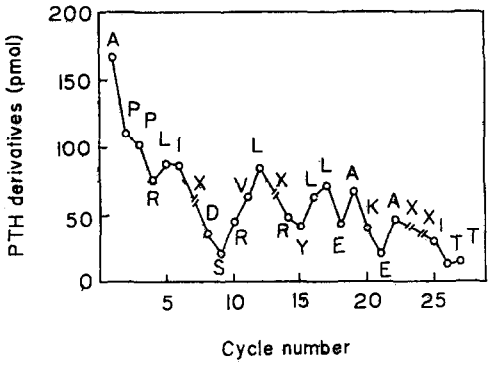


Fig. 2. Analysis of human erythropoietin on SDS-PAGE. EPO purified by reversed-phase HPLC was analysed under (1) non-reducing and (2) reducing conditions.

Fig. 3. Yield of PTH amino acid derivatives identified during sequence of purified EPO; × = unidentified residues.

EPO obtained by this step gave a single diffuse band with a molecular mass of 36 000 on SDS-PAGE (Fig. 2) and also gave a unique N-terminal amino acid, alanine, showing that EPO was purified to homogeneity on the level of polypeptide chain by reversed-phase HPLC. However, some heterogeneity in carbohydrate chains is suggested from the elution pattern on reversed-phase HPLC and the diffuse band on SDS gels. Asialo-EPO, which was generated by treatment of purified EPO with N-acetylneuraminidase, also gave a diffuse band at a position of 31 000 (data not shown), indicating that the carbohydrate heterogeneity is not only due to the difference of sialic acid contents in EPO molecules.

The result of sequence analysis for 160 pmol of purified EPO is shown in Fig. 3. This partial amino acid sequence coincides with the published sequence deduced from cloned cDNA for human EPO [10].

The proposed method is essentially simpler and shorter than the method previously described [3]. The results presented here demonstrate the advantages of using reversed-phase HPLC as a final step for purification of EPO. Although only ca. 1.5-fold of purification was achieved in comparison with the specific activity of the sample obtained in hydroxyapatite column chromatography (Table I), this final step yielded homogeneous EPO. The proposed reversed-phase HPLC method can also be applied to purification of EPO produced by the recombinant DNA technique.

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REFERENCES

1 S.B. Krantz and L.O. Jacobson, Erythropoietin and the Regulation of Erythropoiesis, University of Chicago Press, Chicago, IL, 1970, p. 118.
2 P.A. Marks and R.A. Rifkind, Ann. Rev. Biochem., 47 (1978) 417.
3 T. Miyake, C.K.-H. Kung and E. Goldwasser, J. Biol. Chem., 252 (1977) 5558.
4 J.L. Spivak, D. Small and M.D. Hollenberg, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 4633.
5 S. Yanagawa, K. Hirade, H. Ohnota, R. Sasaki, H. Chiba, M. Ueda and M. Goto, J. Biol. Chem., 259 (1984) 2707.
6 M. Bradford, Anal. Biochem., 72 (1976) 248.
7 U.K. Laemmli, Nature, 227 (1970) 680.
8 M.W. Hunkapiller and L.E. Hood, Methods Enzymol., 91 (1983) 486.
9 C.D.R. Dunn, J.H. Jarvis and J.M. Greenman, Exp. Hematol., 3 (1975) 65.
10 K. Jacobs, C. Shoemaker, R. Rudersdolf, S.D. Neil, R.J. Kaufman, A. Mufson, J. Seehra, S.S. Jones, R. Hewick, E.F. Fritsch, M. Kawakita and T. Miyake, Nature, 313 (1985) 806.