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EPO immunoaffinity columns for purification

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Introduction

The current detection methodology for recombinant erythropoietin (EPO) in urine (Lasne et al 2002) relies on a non-selective extraction procedure followed by a very specific detection procedure. Our aim was to develop an inexpensive selective extraction procedure which could separate EPO from the other proteins present. Immunoaffinity purification was our choice but to do this a supply of appropriate antibodies was needed in relatively large quantity and at a low cost. To achieve this it was decided to prepare polyclonal antisera by the immunisation of sheep. Sheep were chosen as they have a much larger blood volume than rabbits which are commonly used for such studies. It was hoped that the sheep could have been kept as an ongoing source of antibodies however the animals response to their immunisation with human recombinant EPO prevented this.
It was hoped to prepare immunoaffinity columns from the antibodies which would selectively extract EPO from urine. In this way it should be possible to use simpler detection procedures and in the future apply such techniques as HPLC coupled with tandem mass spectrometry (LC/MS/MS) to characterise recombinant human EPO. The initial studies reported here relate to the preparation of the antisera, the incorporation of the antibodies into immunoaffinity columns, and the evaluation of these columns in the extraction of EPO from urine.

Experimental

Three vaccines were prepared. The first antigen was recombinant human EPO (Eprex, 40,000 IU or 336 µg/mL), and was kindly supplied by Janssen-Cilag (North Ryde, NSW Australia). The second antigen was a peptide fragment of EPO, corresponding to the first 26 amino acids from the N-terminal. This was synthesised by AusPep (Parkville, Vic). To increase its antigenicity, the peptide was also conjugated to a large protein - Keyhole Limpet Haemocyanin (KLH-peptide), which is commonly used in antigen construction. Vaccines were prepared by dissolving either 42 µg EPO, or 500 µg KHL-peptide, in 1 mL of 0.9% saline. This aqueous component was then mixed thoroughly with 1 mL of Freund's adjuvant, until a stable emulsion was formed that did not disperse readily when a drop was placed in water. A control vaccine was made also, using saline and adjuvant, but no antigen.

All procedures that involved animals were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (6th Edn, National Health and Medical Research Council, 1997) and with approval from the Animal Care and Ethics Committee of Charles Sturt University (Protocol number 03/061).

The sheep used for the study were Merino wethers housed at Charles Sturt University in rural NSW. Eighteen Merino wethers were divided into three groups:

- One group received a vaccine containing recombinant EPO
- One group received a vaccine containing a peptide consisting of the first 26 amino acids of EPO
- One group received a vaccine containing no antigen

Additional inoculations were made on days 29 and 62. The experiment finished on day 90.

All reagents were of AR or HPLC grade and unless otherwise stated were from Sigma-Aldrich. Water was from a Milli-Q water purification system.

The antibody titre of the serum from each sheep was measured using ELISA. For the purpose of initial screening, a series of double dilutions of serum was made, commencing at 1/100. Antibody titre was defined as the maximum dilution of serum that produced an absorbance reading of at least 0.2 units higher than the corresponding control sample. Control serum samples were those collected from the same animal before its first vaccination, and were tested at the same dilution rates as the test samples.

To map the various regions of EPO that might have been recognised by the sheep antibodies (B-cell epitopes), a series of overlapping fragments of the molecule was synthesised (Mimotopes, Clayton South,Vic.). Each peptide comprised a 15 amino acid fragment of

erythropoietin, plus a 4 amino acid motif at the C-terminal (S-G-S-G) which served both as a spacer and a means of linking the peptide to biotin. The spacer was designed to allow each peptide to adopt its natural conformation and reduce any steric hindrance that would have occurred if the peptide had been bonded directly to the ELISA plate. The biotin group afforded a convenient way to immobilise the peptides, via bonding to ELISA plates that were pre-coated with streptavidin (Figure 1). As each peptide overlapped the next by 2 amino acids, 77 peptides were synthesised to cover the entire 166 amino acid sequence of erythropoietin (Figure 2).

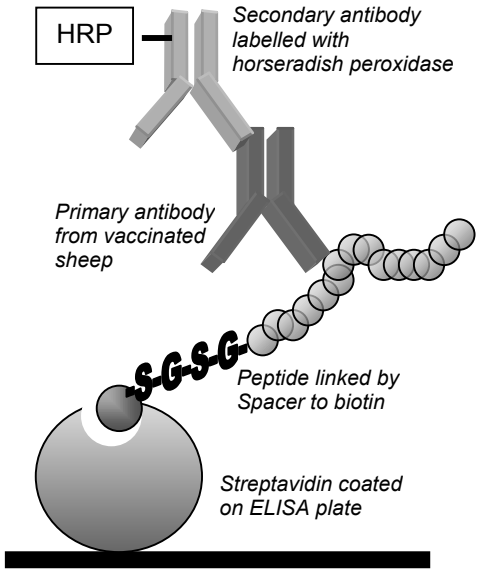

Figure 1. Principle of epitope mapping using ELISA.

NH_2-A-P-P-R-L-I-C-D-S-R-V-L-E-R-Y-L-L-E-A-K-E-A-E-N-I-T-T-G-C-A-E-H-C-S-L-N-E-N-I-T-V-P-D-T-K-V-N-F-Y-A-W-K-R-M-E-V-G-Q-Q-A-V-E-V-W-Q-G-L-A-L-L-S-E-A-V-L-R-G-Q-A-L-L-V-N-S-S-Q-P-W-E-P-L-Q-L-H-V-D-K-A-V-S-G-L-R-S-L-T-T-L-L-R-A-L-G-A-Q-K-E-A-I-S-P-P-D-A-A-S-A-A-P-L-R-T-I-T-A-D-T-F-R-K-L-F-R-V-Y-S-N-F-L-R-G-K-L-K-L-Y-T-G-E-A-C-R-T-G-D-R—COOH

Figure 2. Amino acid sequence of EPO.

Immunoaffinity columns have been prepared using two different methods using the serum from the sheep B55, which had been immunised with EPO. Columns using cyanogen bromide activated Sepharose gel were prepared using polyclonal antibodies from B55 which were precipitated by the addition of saturated ammonium sulfate and reconstituted in phosphate buffered saline (PBS). Bio-Rad Affigel Hz Hydrazine gel were prepared using

polyclonal antibodies from B55 purified using the Bio-Rad Econo-Pac Serum IgG Purification Kit.

Urine samples used for column evaluation were prepared by concentration with Centricon Plus 20 30kDa filters. The concentrates were reconstituted in 2 mL of glycine loading buffer (50 mM pH 8) and applied to the columns. The columns were rotated for three hours and then the loading buffer was drained off. The columns were washed with 4 x 2 mL of loading buffer. The EPO was washed off the column by the addition of 10 x 1 mL of glycine elution buffer (0.1 M pH 2.5). The eluted samples were buffered back to pH 7.4 with Tris buffer (1 M pH 9).

EPO concentrations were measured using a DPC Immulite. The total protein concentrations were measured using a Bio-Rad protein assay. EPO isoforms were determined using the double blotting method (Lasne et al 2002).

Results and Discussion

The only sheep to produce antibodies and to display a significant reduction in packed cell volume (PCV) after immunisation were those treated with the recombinant EPO. As the PCV fell from an average of 35 to below 20 and previous experience with horses indicated that such anaemia was likely to be fatal (Schwarzwald and Hinchcliff 2004), it was decided to euthanize the sheep on day 90 and collect the sera. The results from the epitope mapping along with the total activity of each serum is shown in Figure 3. The highest activity serum was from sheep B55. It can be seen that epitopes 5, 12, and 58 are common to all. Epitope 21 was common to five sera but was not dominant in B55 which had the strongest response to EPO overall. The 21 region of EPO is highly conserved in mammals. The differences in the response of the various sera may be useful in discriminating between different forms of EPO. During the epitope mapping it was observed that the affinity of some sera to EPO fragments was very much higher than to entire EPO.

Initially three types of columns were evaluated :-

A commercially available column from Affiland

cyanogen bromide columns prepared using serum B55

Bio-Rad prepared columns using serum B55

All three column types had similar but quite low and variable recoveries with urines spiked with rhEPO. Subsequent work has concentrated on the cyanogen bromide columns as they were the simplest to prepare and the cheapest. Figure 4 shows the results obtained from two different cyanogen bromide columns each run twice on the same day. Typically less than half the EPO added was in the desired fraction. As the total recovery was always less than 100% and often much lower, experiments were carried out to investigate possible losses of EPO. No losses were observed after 50 hours storage in either 1 mL microfuge tubes or 15 mL Falcon tubes. EPO was also found to be stable in the pH 8 glycine buffer used for column loading. However significant losses did occur within 15 minutes with EPO in the pH 2.5 glycine elution buffer. The EPO is stable when the pH is raised to 7.4 with Tris buffer. As a result for subsequent experiments the pH of the eluted EPO was raised to 7.4 immediately after elution. Figure 5 shows the results obtained from two columns run on four different days. In these experiments the total EPO recovery was approximately 50% with over 70% of the recovered EPO being in the desired elution fraction. There is always some EPO in the initial and final washes. The lower recoveries shown in Figure 5 compared to Figure 4 may be due to the higher spiking level used in Figure 5 (15 mIU/mL compared to 7.5 mIU/mL). Whilst it is clearly important to get good recovery of EPO from the columns it is equally important to have separated the EPO from the other much more abundant urinary proteins. To investigate this the total protein content of the urines and the eluted fractions were measured. The results are shown in Table 1 where it can be seen that column 1 has been much less effective in removing urinary proteins than has column 2. There is no obvious explanation for the differing behaviour of the two columns as both were prepared in the same way.

	Column 1		Column 2	
	Protein (mg)	EPO (mIU)	Protein (mg)	EPO (mIU)
Amount loaded	0.8	300	0.78	370
Initial wash	0.14	11	0.4	8
Elution	0.17	57	0.02	72
Final wash	0.28	13	0.12	14

Table 1. Protein and EPO measurements in urine before and after immunoaffinity purification.

Another important aspect of the immunoaffinity columns behaviour is whether the isoform distribution of EPO is altered by passage through the column. Two separate urines spiked with rhEPO were prepared and passed through the columns. The results are shown in Figure 6. The recombinant EPO bands are clearly visible and in the same position as the standard. However the urinary EPO which was present in both samples before extraction is barely visible.

Conclusions

By using sheep we have been able to generate large amounts of sera containing polyclonal antibodies to EPO. Using CnBr we have been able to readily prepare immunoaffinity columns for extracting EPO from urine. The columns prepared from serum B55 give a recovery of EPO in the elution fraction of up to 50%. Preliminary indications are that such extracts have much of the other more abundant urinary proteins removed. The recombinant EPO recovered has the same isoform distribution as that added, however the urinary EPO isoforms are not recovered with the same efficiency as is the recombinant EPO.

References

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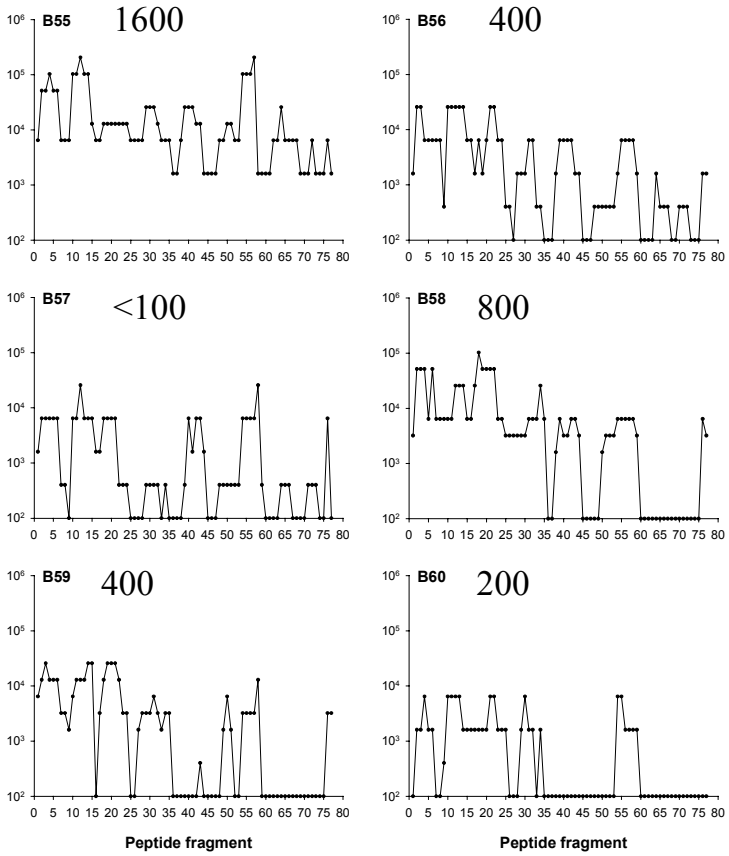

Figure 3. Antibody titres of sheep sera and results of epitope mapping.

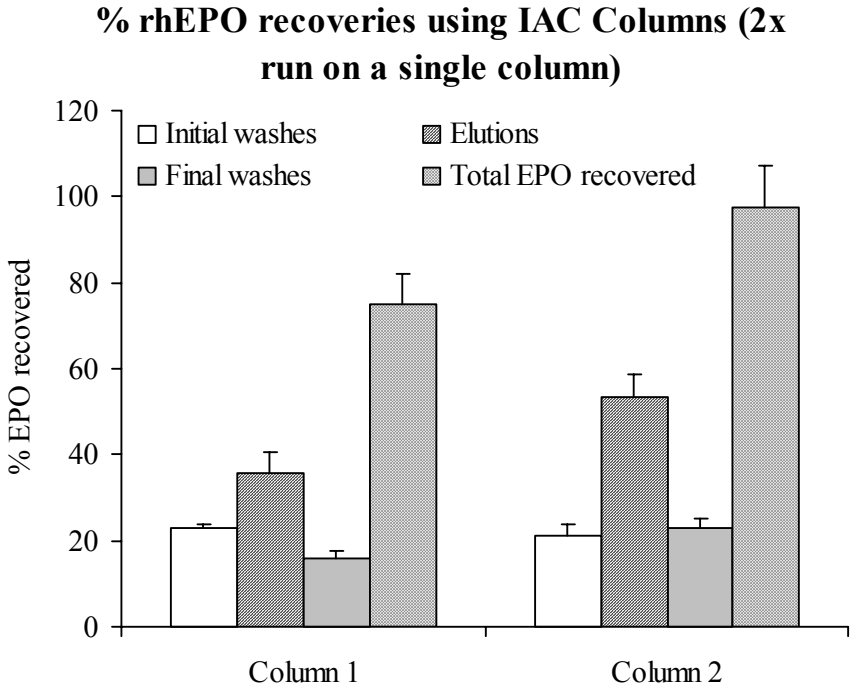

Figure 4. Recoveries of EPO from early CnBr IAC columns.

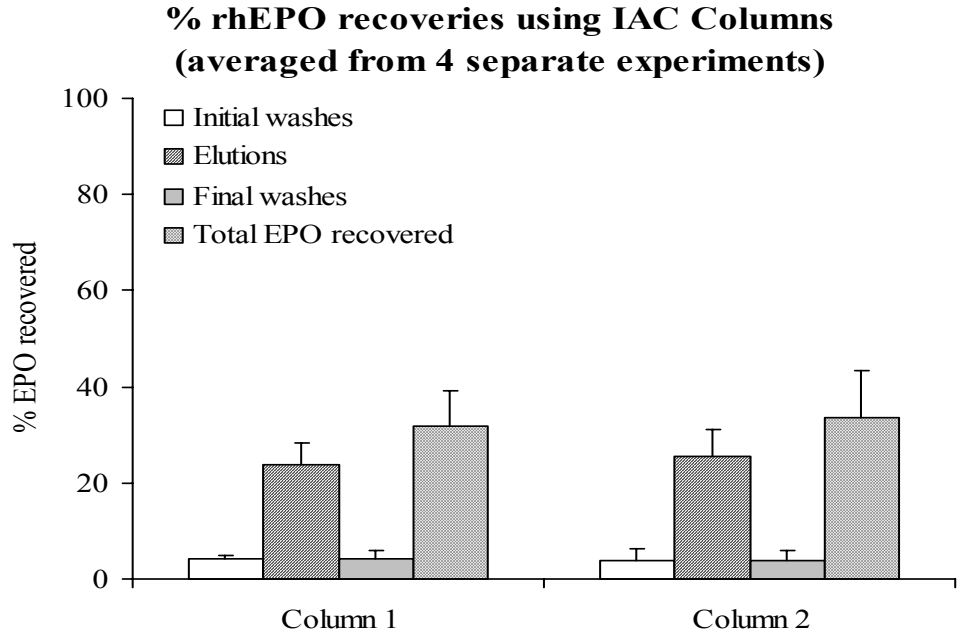

Figure 5. Average EPO recoveries from two CnBr columns run on four separate days.

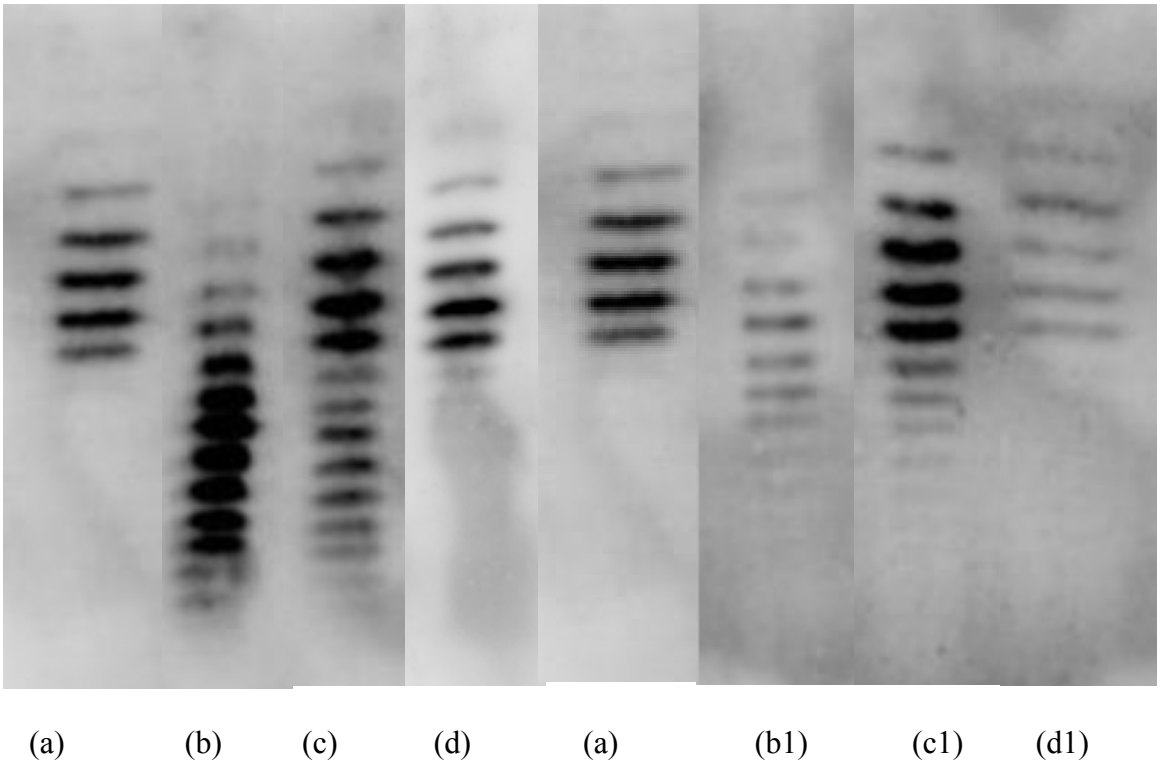

Figure 6. Isoform distributions from (a) standard rhEPO, (b)and (b1) urine samples, (c) and (c1) urine samples spiked with rhEPO, and (d) and (d1) elution fractions after immunoaffinity purification.