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Purification of antibodies labelled with monomeric ferritin, and use in the localisation of *Salmonella* flagellar antigens

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Introduction: In immuno-electron microscopy, electron-dense labels are required to permit localisation of the bound antibody molecules. One of the most successful is ferritin [1–3].

If conjugated antibody is present in the immunolabelling mixture, it must have the effect of diminishing sensitivity, perhaps critically, by competing for the antigenic sites. Therefore, it is important to remove free antibody from the conjugate preparation. Unconjugated ferritin may also interfere by giving a high background of non-specific 'staining', so that this should be removed.

Conventional preparations of ferritin, *e.g.* from horse spleen, are heterogenous in that some of the spherical units exit as dimers, trimers and higher oligomers [4–6]. In addition, isoelectric focusing has demonstrated a further type of heterogeneity in ferritin; multiple band patterns being observed in ferritins from single organs [7, 8]. This latter phenomenon is not significant for the present work.

The presence of oligomers in the conjugate preparation may give rise to errors in interpretation because of a false impression of close grouping of bound antibody molecules. In summary, therefore, only monomer should be used for conjugation and the conjugated product should be rigorously separated both from free ferritin and from free antibody.

Attempts at purification of ferritin-antibody conjugates by chromatography on DEAE-cellulose and on sephadex columns have not been entirely successful [2, 9]. Purification has also been attempted by curtain electrophoresis and by electrophoresis on agar [10, 11]. Neither method has been successful in fully resolving the crude mixture of monomeric and polymeric ferritin, ferritin antibody and uncoupled antibody globulin.

Polyacrylamide gel electrophoresis, however, offers excellent separation of monomer ferritin from oligomers and we have earlier shown that preparative electrophoresis can give essentially pure monomer in a single step [5]. In the present report we describe the use of preparative electrophoresis for the purification of antibody-(monomeric) ferritin conjugate, from the conjugation mixture.

Materials and methods: Bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd, Eastbourne, UK. Horse spleen ferritin (HSF, twice-crystallised from cadmium sulphate), was from Koch-Light Laboratories, Colnbrook, UK. Ferritin monomer was purified as described previously [5].

Ponceau S was from G.T. Gurr Ltd, Poole, UK. A 0.2% solution in 3% trichloroacetic acid was used. The gels were stained for 30 min with differentiation by soaking in repeated changes of 2% acetic acid.

Perl's reagent was used to demonstrate the presence of iron and was made fresh each time, as follows. To a 2% solution of potassium ferrocyanide in distilled water was added an equal volume of 0.25 mol L^{-1} hydrochloric acid and the mixture poured over the gels. The gels were allowed to stain for 30 min at room temperature and then washed in 1% acetic acid for 1 h.

The bifunctional conjugating reagent, p, p′-difluoro-m, m-dinitrodiphenyl sulfone (FNPS) was obtained from General Biochemicals, Columbus, Ohio, USA. Ferritin monomer, prepared as described previously [5], was conjugated with antibody globulin using FNPS according to the method of Ram *et al.* [11]. The final solution containing the conjugate was dialysed against a large excess of buffer, 25 mmol L^{-1} sodium borate (*p*H 8.4).

The conjugate was purified by means of preparative polyacrylamide gel electrophoresis in an apparatus which provides for automatic collection of fractions as they emerge from the end of the gel [12]. Gels were 5% T (5 g total monomers/100 mL solution) and 5% cross-linked (5% C); *i.e.*, 5 g N,N′-methylene-bis-acrylamide/100 g total monomers. The other

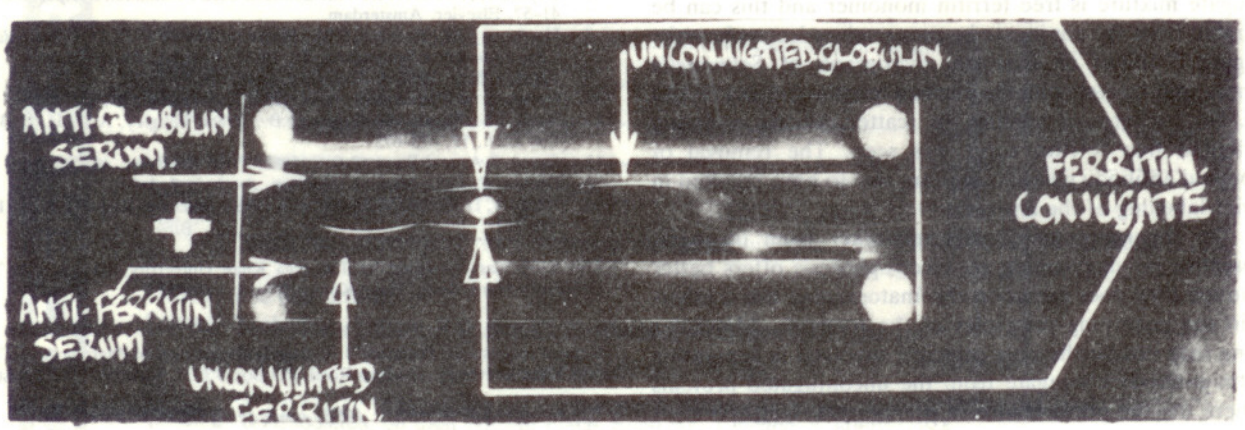

Figure 1: Immunoelectrophoresis: The sample was horse-spleen ferritin monomer conjugated with Rabbit anti-enx-globulin (crude reaction mixture) and the antisera used were respectively anti-(horse) ferritin and anti-(rabbit) globulin.

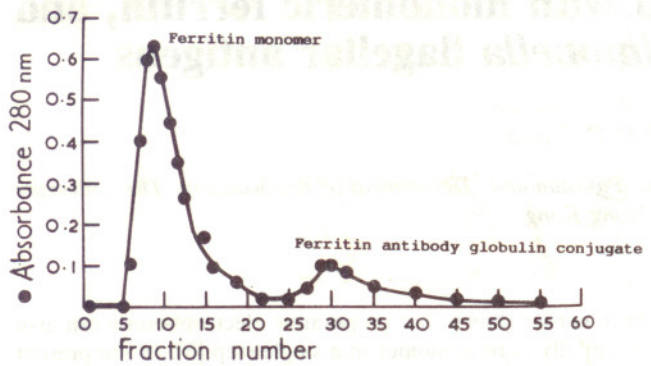

Figure 2: Separation of ferritin-antibody conjugate by preparative polyacrylamide gel electrophoresis.

conditions used were: gel cross-section 5.9 × 1 cm, buffer 25 mmol L^{-1} sodium borate (*p*H 8.4), voltage gradient 4 V cm^{-1}, path length 4 cm, 5°C. 15 mg of conjugate was pipetted into the sample slot in a warm (37°C) solution of agarose, to prevent electrodecantation. The purified product was freed of buffer by dialysis against water and concentrated by ultrafiltration.

Rabbit anti-horse ferritin serum and antisera against the *Salmonella* flagellar antigen *enx* were kindly provided by Professor M.G. McEntegart, Department of Medical Microbiology, University of Sheffield, Sheffield, UK.

Immunoelectrophoresis was carried out according to Grabar [13]. Antisera were allowed to diffuse at room temperature and precipitation lines were stained with Perl's reagent for iron and then for porteins with Ponceau S.

Results and discussion: Figure 1 shows the results of immunoelectrophoresis conducted on the product of conjugation of ferritin monomer and rabbit anti-*enx* antiserum. The leading (anodic) zone reacted only with the anti-ferritin serum, a trailing zone reacting only with anti-rabbit IgG serrum and an intermediate zone reacting with both anti-sera. This is presumably identifiable as the conjugate.

Figure 2 shows the elution profile obtained by preparative polyacrylamide gel electrophoresis of the crude conjugate mixture. Two distinct peaks are seen: free antibody was of no interest and so the electrophoresis run was not continued further. Fractions 5–20 contained free ferritin monomer (not illustrated). Fractions 25–45 were identified as monomer ferritin-antibody conjugate: this pool was found to be homogenous by analytical polyacrylamide gel electrophoresis.

It is evident that the bulk of the material in the crude conjugate mixture is free ferritin monomer and this can be separated from the conjugate by preparative electrophoresis.

Figure 3 shows an electron micrograph of *Salmonella abortus-equi*, treated with the purified ferritin-anti-*enx* conjugate. No background staining or scatter due to randomly distributed free ferritin cores was seen. The removal of uncoupled antibody globulin appears also to have resulted in heavier labelling of the antigen.

In the separation of ferritin monomer from oligmers, preparative polyacrylamide gel electrophoresis offers better resolution than does exclusion chromatography: this is inherent in the governing principles of the methods. Molecular sieving may not be the only principle involved in separating ferritin-antibody conjugate from the unconjugated proteins but here too the performance of preparative electrophoresis

Figure 3: Localisation of Salmonella *flagellar antigens with purified ferritin-antibody conjugate by electron microscopy (× 80,000). The electron micrograph shows four flagellae labelled with purified ferritin-antibody conjugate. Electron microscopy was carried out according to the method of Gregory and Williams [14].*

is excellent, and only this one step is required. In these two cases, preparative electrophoresis seems to be the method of choice in the purification of ferritin monomer-antibody globulin conjugate.

1. Howe, C., Morgan, C. and Hsu, K.C. 1969. *Progr. Med. Virol.*, **11**, 307–353
2. Kishida, Y., Olsen, B.R., Berg, R.A. *et al.* 1967. *J. Cell. Biol.*, **64**, 331–339
3. Briggman, J.V. and Widnall, C. 1983. *J. Histochem Cytochem.*, **31**, 579–590
4. Harrison, P.M. and Gregory, D.W. 1965. *J. Molec. Biol.*, **14**, 626–629
5. Saeed, S.A. and Boyde, T.R.C. 1981. *FEBS Lett.*, **123**, 111–114
6. Theil, E.C. 1987. *Ann. Rev. Biochem.*, **56**, 289–315
7. Drysdale, J.W. 1977. *Iron Metabolism: Ciba Foundation Symposium*, **51**, 41–57. Elsevier, Amsterdam
8. Harrison, P.M., Banyard, S.M., Hoare, R.J. *et al.* 1977. *Iron Metabolism, Ciba Foundation Symposium*, **51**, 19–35. Elsevier, Amsterdam
9. Amstey, M.S. 1967. *J. Lab. Clin. Med.*, **69**, 997–1002
10. Borek, F. and Silverstein, A.M. 1961. *J. Immunol.*, **87**, 555–561
11. Ram, J.S., Tawde, S., Pierce, G.B., Jr and Midgley, A.R. Jr. 1963. *J. Cell Biol.*, **17**, 673–675
12. Saeed, S.A. and Boyd, T.R.C. 1980. *Prep. Biochem.*, **10**, 445–462
13. Grabar, P. 1957. *Methods Biochem. Analysis*, **7**, 1–38
14. Gregory, D.W. and Williams, M.A. 1967. *Biochem. Biophys. Acta.*, **133**, 319–332

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