Diabetologia 9, 53–55 (1973)

PRELIMINARY COMMUNICATIONS

Insulin Labelled by Coupling with Peroxidase*

J. Biener, F.K. Jansen and D. Brandenburg
Diabetes Forschungs-Institut, Düsseldorf and Deutsches Wollforschungsinstitut, Aachen, Germany (F.R.G.)

Received: September 1, 1972, accepted: October 27, 1972

Summary. By coupling purified horseradish peroxidase with glutaraldehyde and subsequent reaction with insulin, conjugates were obtained. These were partially purified by ion exchange chromatography on DEAE-cellulose. A fraction with an average molar ratio of peroxidase to insulin 1:0.37 was analysed by electrophoresis and gel filtration. The peroxidase activity of this fraction was found to be 19% of normal and the immunological reactivity 0.6% as compared with that of insulin.

Key words: insulin, peroxidase, enzyme marker, immunologic activity, modified insulin.

Enzymes labelled proteins have been introduced by Avrameas [1, 2] and Nakane [11, 12] as useful tools for intracellular detection of antigens and antibodies and for ultrastructural localization in the cell, Shnitka [15].

Peroxidase labelled insulin can be utilized in biological studies of insulin receptors and in immunological studies of the insulin antibody-producing cells. Therefore, we coupled insulin to horseradish peroxidase (HRP) by glutaraldehyde (GA) using a slight modification of the original method described by Avrameas [3]. Coupling products with different ratio of insulin to HRP have been obtained.

Materials and Methods

Peroxidase (AbR = 0.65[1]): Boehringer, Mannheim; crystallized insulin(bovine) and ^{125}I-insulin(porcine): Farbwerke Hoechst, Frankfurt; DEAE and CM-cellulose: Serva, Heidelberg; Sephadex G-25 and G-50: Pharmacia, Frankfurt; glutaraldehyde (GA; 25% solution): Merck, Darmstadt; bovine serum albumin: Behringwerke, Marburg. Antibodies against insulin had been prepared in our laboratories by immunisation of guinea pigs with insulin in complete Freund's adjuvant.

HRP was purified on DEAE-cellulose and subsequently on CM-cellulose according to the method described by Shannon [14]. B and C isoenzymes (AbR usually of 2.9–3.3) were pooled and a concentration reached by ultrafiltration of 50 mg HRP/ml. Crystallized insulin was purified by gel filtration on Sephadex G-50 superfine (column 65 × 1500 mm) in 10% acetic acid. 200 mg of this material was mixed with different quantities of ^{125}I-insulin (usually 200 μU) and applied to a DEAE-cellulose column equilibrated with 0.005 sodium acetate buffer (pH 4.4). A linear gradient of the same buffer and 0.3 M acetate buffer, 250 ml each, was used. The insulin peak eluting at a molarity of 0.2–0.3 was collected and this mixture of insulin and ^{125}I-insulin directly used.

Coupling procedure

The method of Avrameas [3] was used with the following slight modification. A solution was prepared containing 25 mg of HRP and 12.5 mg GA per ml 0.15 M phosphate buffer (pH 6.8). After standing for 8 h at room temperature, the mixture was filtered through a G-25 Sephadex column (14 × 600 mm) in 0.15 M NaCl solution. The activated HRP fraction was mixed, without any further concentration, with insulin (molar ratio 1:1.2) and 0.2 ml M sodium carbonate-bicarbonate buffer was added to bring the solution to pH 9.3. The resulting mixture was allowed to stand for an additional 18 h at room temperature when an excess of lysine was added to block the remaining aldehyde groups. The mixture was dialysed against 7 M urea in 0.005 M Tris buffer (pH 8.45) and applied to a DEAE-cellulose column (14 × 200 mm) equilibrated with the same buffer. Step elution with NaCl (see Fig. 1) was performed. The concentration of HRP in each tube was determined from the absorption at 403 and that of insulin from radioactivity.

The electrophoresis (see Fig. 2) was carried out in a Boskamp Mikrophor apparatus and the enzymatic activity estimated according to Chance [8].

The immunological assessment of products was performed by radioimmunoassay with amberlite according to Meade [9]. One ml of different dilutions of the original chromatographed insulin and of the insulin coupled to HRP, with a final concentration of 3 μU of ^{125}I-insulin and antibodies, were incubated for 18 h.

* Kindly supported by Landesversicherungsanstalt Rheinprovinz, Düsseldorf, and Landesamt für Forschung, Nordrhein Westfalen.

1 AbR = Absorption ratio = $\varepsilon \frac{403\text{ nm}}{280\text{ nm}}$ indicating purity grade.

The antiserum had been diluted to an antibody concentration that bound about 50% of the ^{125}I-insulin. 300 mg amberlite was added and 30 minutes thereafter separated by centrifugation. 0.5 ml of the supernatant containing the antibody-bound ^{125}I-insulin was counted in a gamma counter (Packard). The percentage of antibody bound activity (% experimental CPM minus % CPM ^{125}I-insulin fixed with normal serum) was calculated and the immunological activity assessed at the point where 25% of the radioactive insulin was bound.

Fraction II contained 32% of the recovered HRP and 7.5% of the initial radioactivity. Radioactivity could not be detected in the first tubes eluted by 0.05 M NaCl solution. As indicated by model experiments, these probably contain modified HRP. The next tubes contain insulin coupled to HRP. As calculated from the extinction at 403 nm and the radioactivity, the molar ratio of the coupling products rises to a maximal value of 1 mole of HRP to 3 moles of insulin; the average of this fraction is 1:0.37.

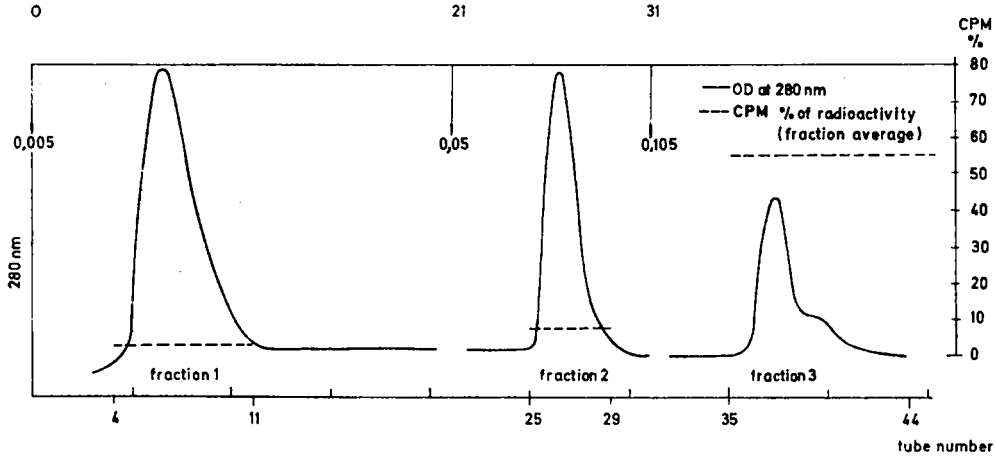

Fig. 1. Elution diagram of coupling products on DEAE cellulose column. buffers: fraction I: 0.005 M Tris pH 8.45/7 M urea. Fraction II: 0.05 M NaCl/ 0.005 M Tris pH 8.45/7 M urea. Fraction III: 0.1 M NaCl/ 0.005 M Tris pH 8.45/7 M urea

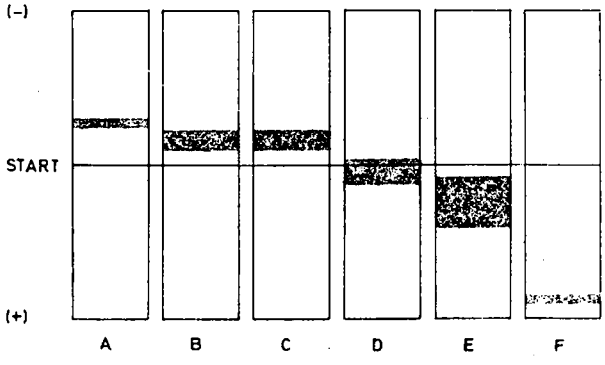

Fig. 2. Cellulose acetate electrophoresis. Buffer: 0.03 M sodium barbital 0.016 M EDTA / 4 M urea, pH 8.8. A) purified HRP; B) GA-activated HRP, treated with lysine; C) fraction I; D) fraction II; E) fraction III; F) insulin;

Results and Discussion

When the resulting products were chromatographed on DEAE-cellulose, the elution pattern shown in Fig. 1 was obtained. In a typical experiment the total recovery of HRP was 21.5 mg from 26 mg HRP, 4.9 mg (4360 CPM) insulin being used for coupling.

Fraction I contained 61% of the recovered HRP eluting at the same NaCl concentration as untreated HRP, and almost no radioactivity.

Quantitative amino acid analysis on a sample after splitting with hydrogen cloride/acetone (Maehly, [9]) and removal of the prosthetic group and subsequent hydrolysis (Spackman, [16]) showed values consistent with this ratio.

Fraction III contained 1.5% of the recovered HRP and 55% of the initial radioactivity, representing a very small amount on insulin-HRP coupling products and largely free insulin.

In order to confirm that covalent binding had occurred between HRP and insulin, electrophoresis and gel filtration were performed. As seen in Fig. 2, the cathodic mobility of HRP is only slightly reduced by treatment with GA and subsequent reaction with lysine. On the other hand, coupling of the negatively charged insulin molecules to HRP brings about a definite change in the mobility of HRP, the extent of which depends on the molar ratio. The heterogeneity of fraction II is obvious from the occurrence of a rather broad band.

HRP treated with GA was eluted from a Sephadex G-150 column at pH 8.4 in 7 M urea in the same position as untreated HRP, which is in agreement with Avrameas [3] and excludes the formation of polymerisation products. When an aliquot of fraction II has been concentrated by ultrafiltration on a PM 10 Amicon membrane and subsequently applied to a Sephadex G-150 column, 95% of all the radioactivity was recovered before and together with the HRP peak.

The enzymatic activity of fraction II was reduced 6 times as compared with that of the purified HRP. Usually, the activity of pure HRP, as used in this study, was about 200 U/mg. By simple "coupling" with GA its activity is reduced to 65 U/mg and after the conjugation and isolation procedure (see above) retained only an activity of 35 U/mg. This loss of activity can be explained by structural changes caused by chemical modification [4, 7].

The immunological analysis was performed on products eluted in fraction II with a molar ratio 1:0.77, as calculated in the basis of radioactivity. The low immunological activity of 0.6% could be explained by steric inhibition of antibody fixation due to coupled HRP rather than by essential alteration of antigenic determinants in the insulin molecule. We based our presumption on the parallelism on both the inhibition curve of insulin-peroxidase and that of insulin. The inhibition curves obtained by Cuatrecasas [6] with insulin coupled to Sepharose are similar to those obtained by us with HRP coupled insulin, although the insulin-sepharose shows a much higher activity.

The reaction of GA with proteins is complex (see Richards [13] and Korn [8]) and chemically well characterized derivatives have, to our knowledge, not yet been isolated. Our preliminary experiments have shown that the reagent can be used to prepare insulin-peroxidase conjugates, possessing both enzymatic and immunological activity, though the latter is slight. Further experiments are in progress to obtain more information on the reactions involved and products formed.

Acknowledgement. We wish to express our thanks to Mrs R. Rusniak and Miss M. Jacobsen for their skilfull technical assistance.

References

1. Avrameas, S., Uriel, J.: Methods de marquage d'anticorps avec des enzymes et son application en immunodiffusion. C.R. Acad. Sci. (Paris) **262**, 2543 (1966).
2. Avrameas, S.: Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry **6**, 43–52 (1969).
3. Avrameas, S., Ternick, T.: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry **8**, 1175–1179 (1971).
4. Brill, A.S., Weinryb, I.: Reactions of horseradish peroxidase with azide. Evidence for a methionine residue al the active site. Biochemistry **6**, 3528–3535 (1967).
5. Chance, B., Maehly, A.C.: In: Colowick S.P. and Kaplan N.O. Methods in enzymologie **2**, p. 769. New York: Acad. Press 1955.
6. Cuatrecasas, P.: Insulin-Sepharose immunoreactivity and use in the purification of antibody. Biochem. biophys. Res. Commun. **35**, 531–537 (1969).
7. Ellis, W.D., Dunford, B.: The effect of ligand binding and acid splitting on the rotatory dispersion of ferric horseradish peroxidase. Canad. J. Biochem. **46**, 1231–1235 (1968).
8. Korn, A.H., Feairheller, S.H., Filachione, E.M.: Glutaraldehyde: Nature of the reagent. J. Mol. Biol. **65**, 515–529 (1972).
9. Maehly, A.C.: Splitting of horseradish peroxidase into prosthetic group and protein as a means of studying the linkages between hemin and protein. Biochim. biophys. Acta **8**, 1–17 (1952).
10. Meade, R.C., Klitgard, H.M.: A simplified method for immunoassay of human serum insulin. J. Nucl.Med. **3**, 407–416 (1962).
11. Nakane, K.P., Pierce, G.B.: Enzyme labeled antibodies: Preparation and application for localization of antigens. J. Histochem. Cytochem. **14**, 929 (1966).
12. Nakane, P.K., Pierce, G.B.: Enzymes labeled antibodies for the light and electron microscopic localization of tissue antigens. J. cell. Biol. **33**, 307–318 (1967).
13. Richards, F.M., Knowles, J.R.: J. Mol. Biol. **37**, 231–233 (1968).
14. Shannon, L.M., Kay, E., Lew, J.Y.: Peroxidase izoenzymes from horseradish roots. J. biol. Chem. **241**, 2166–2172 (1966).
15. Shnitka, T.M., Seligman, A.M.: Some recent electron chemical application of diaminobenzidine (DAB) technic. Ann. rev. Biochem. **40**, 375 see 390–396 (1971).
16. Spackman, D.H., Stein, W.H., Moore, S.: Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chemistry **30**, 1190–1206 (1958).

Jeana Biener
Diabetes Forschungsinstitut
D-4000 Düsseldorf 18
Hospitalstraße 1
Federal Republic of Germany