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Highly active enzyme preparations immobilized via matrix-conjugated anti-Fc antibodies

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ABSTRACT

A novel method for enzyme immobilization to achieve highly active enzyme preparations is described. The enzyme is first reacted in solution with a specific monoclonal antibody (mAb) which does not interfere with its enzymic activity and which possesses a high affinity towards the enzyme. The immunocomplex is then reacted with immobilized anti-Fc antibodies which are coupled to the matrix by methods that preserve maximum immunological activity. Horseradish peroxidase (HRP) immobilization on epoxy-activated beads (CB6200) was chosen as a model system for this approach. Coupling of HRP via its mAbs to anti-Fc antibodies immobilized yielded the best results, corresponding to binding of 325 ng of fully active enzyme per bead. Binding of the enzyme to anti-HRP mAb directly immobilized on the beads was considerably lower.

INTRODUCTION

During the last two decades, reactions which involve catalysis by immobilized enzymes have been widely used on both the laboratory and industrial scales with preparative, analytical and diagnostic applications. Many matrices and covalent binding methods have been developed in order to achieve highly active enzyme–matrix conjugates [1]. However, enzymes often tend to lose their biological activity on coupling to a matrix owing to masking of their active sites or multi-point attachments which restrict local movements necessary for the activity of the enzyme. In such cases, coupling of the enzymes via monoclonal antibodies (mAbs) which do not affect their activity and possess a high binding affinity towards the enzyme may be advisable [2].

Previously we have shown that the binding of such antibodies to insoluble carriers, *e.g.*, Eupergit C, yields matrix-conjugated mAb preparations which efficiently bind the corresponding enzymes to yield highly active immobilized enzymes [3]. In such preparations, the enzyme is bound via specific preselected and well defined sites. Apparently, in this instance, mAbs form an extended spacer separating the enzyme from the carrier. In addition, the mAbs bind the respective enzymes via epitopes remote from the active site so that the active sites face the outer solution. Multi-point attachments are also eliminated and the enzymes are relatively free to move to express higher activity.

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Since, in principle mAbs, which do not affect enzymatic activity and which bind the enzymes at high affinity, may be prepared against almost any enzyme, this technique may represent a general solution to the preservation of high activity of immobilized enzymes. At present, the main restriction on the use of mAbs is their high cost, but improvements of the techniques for their large-scale production is expected to lead to a marked decrease in price [4].

We expected that the formation of soluble complexes of enzymes with specific mAbs, followed by immobilization via reaction with anti-Fc antibodies, should lead to a higher capacity for enzyme binding than direct coupling of the enzyme to immobilized antibodies. Further, optimization of anti-Fc immobilization onto the solid carrier with respect to antigen binding activity should eventually lead to even higher activity of the immobilized enzyme. We have tested various methods for antibody immobilization in order to achieve the highest antigen binding activity.

The carrier used in this study was Eupergit C1Z-coated poly(methyl methacrylate) beads (6 mm, CB6200). These beads are especially suitable for application in immunodiagnostic assays. Immunoassays performed with such beads are expected to be more reproducible and reliable than those performed with enzyme-linked immunosorbent assay (ELISA) microtitre plates owing to the covalent binding, rather than hydrophobic adsorption, of the first antibody to the surfact of the matrix. Nevertheless, assays based on these beads have to be optimized with respect to protein-binding capacity, antigen-binding activity of the immobilized antibodies and reproducibility of the results.

As a model system we chose immobilization of horseradish peroxidase (HRP) via specific mAbs. Two approaches were examined and compared; (a) binding of HRP to immobilized mAb and (b) binding of a soluble complex of HRP with its mAb to immobilized anti-Fc antibodies. This enzyme, which is commonly used in immunodiagnostics as a reporter enzyme, contains only a small number of lysine residues and a blocked N-terminus [5] and, therefore, does not react directly with the oxirane groups of the matrix. Thus any matrix-associated enzymatic activity may be attributed to antibody-modulated immobilization of the enzyme.

EXPERIMENTAL

Materials

Eupergit C1Z-coated poly(methyl methacrylate) beads (6 mm, CB6200) were obtained from Rohm-Pharma (Darmstadt, Germany). Horseradish peroxidase (HRP) was purchased from Sigma (St. Louis, MO, U.S.A.).

Chemical modification of CB6200 beads

Fifty beads were incubated with 25 ml of 0.1 *M* adipic dihydrazide (ADH), hexamethylenediamine (HMD) or hydrazine hydrate (HH) in 0.2 *M* phosphate buffer (pH 8.8) for 16 h at room temperature. The beads were then extensively washed and unreacted oxirane groups were blocked with 0.2 *M* β-mercaptoethanol. The modified beads were extensively washed and kept for further use in phosphate-buffered saline (pH 7.4) (PBS) at 4°C.

Assay of the enzymic activity of horseradish peroxidase

HRP (10–100 μl, 0.1–10 ng) was incubated in ELISA microtitre plates with 100 μl of HRP substrate solution [2 mg/ml of *o*-phenylenediamine (Fluka, Buchs, Switzerland) and 0.008% hydrogen peroxide in 50 m*M* citrate buffer (pH 5.0)] for 1 min at room temperature. Colour development was stopped by the addition of 4 *M* hydrochloric acid (50 μl) into each well. The intensity of the colour developed was measured with an ELISA reader (SLT, Grodig, Austria) at 492 nm (with a reference beam at 405 nm).

Preparation and purification of monoclonal antibodies

The preparation of anti-HPR antibodies has been described previously [6]. A monoclonal antibody (HRP2) which did not interfere with the enzymic activities of HRP was selected using the microassay described above. This antibody was purified by high-performance immunoaffinity chromatography using Eupergit C-bound HRP as described previously [7]. The apparent binding constant of this antibody was determined according to the procedure of Pinckard and Weir [8], *i.e.*, as derived from the reciprocal of the free monoclonal antibody concentration at which 50% of the maximum binding to HRP was achieved.

Immobilization of anti-Fc antibodies on CB6200 beads

Polyclonal anti-Fc antibodies were coupled to oxirane groups of the beads either directly or after reversible protection of their amino groups by reaction with dimethylmaleic anhydride (DMA). By the direct immobilization procedure the antibodies (0.1 mg) were incubated with ten beads in 5 ml of 1 *M* potassium phosphate buffer (pH 7.4) for 16 h at 4°C. Blocking of unreacted oxirane groups was achieved by incubation with 0.2 *M* β-mercaptoethanol (pH 8.0) for an additional 4 h at room temperature. By the DMA procedure the antibodies (0.1 mg) were incubated first with DMA for 1 h and then coupled to the beads as described previously [9].

Alternatively, oriented immobilization of anti-Fc antibodies was achieved by coupling of periodate-oxidized antibodies to ADH-, HH- or HMD-modified CB6200 beads as described previously [6].

Anti-Fc antibodies were also coupled to chemically modified CB6200 by incubation of the antibodies (0.1 mg) with ten beads in the presence of 10% glutaraldehyde in water for 16 h at room temperature.

Immobilization of the monoclonal antibody HRP2 on CB6200 beads

Monoclonal antibody HRP2 was coupled to CB6200 beads either directly (as described above for the anti-Fc antibodies) or via its immunological reaction with the immobilized anti-Fc antibodies. Beads containing anti-Fc antibodies were incubated with increasing amounts of mAb HRP2 (1–10 μg per bead) for 1 h at 37°C. The amount of antibodies bound to the beads was determined by the difference in the amounts of protein measured before and after coupling by a microassay described by Bradford [10].

Immobilization of horseradish peroxidase on CB6200 beads

Two approaches were used for the coupling of HRP to CB6200. First, HRP was coupled to matrix-conjugated anti-HRP mAb (see above). Alternatively, the enzyme

was reacted with the antibody in solution and then the immunocomplex was immobilized onto the CB6200 beads via reaction with immobilized anti-Fc antibodies.

Coupling of HRP to the beads by immunological reaction with immobilized mAb HRP2 was carried out by incubation of the enzyme (5 μg in 200 μl of low-fat milk) with the beads containing increasing amounts of antibody (0.3–1000 μg pr bead) for 1 h at room temperature with gentle agitation. For coupling of HRP to soluble antibody, 5 μg of the enzyme were incubated with 10 μg of antibody in PBS for 1 h at 37°C. The immunocomplex formed was the incubated with immobilized anti-Fc for a further 1 h in PBS at 37°C with gentle agitation. In a control experiment, the direct binding of HRP to the beads was tested. A 5-μg amount of HRP was incubated with each bead in 1 *M* potassium phosphate buffer (pH 7.4) for 16 h at 4°C, followed by washing with 1% Triton X-100 in PBS. The enzymic activity of bound HRP was determined by incubation of each of the beads with 0.5 ml of *o*-phenylenediamine [2 mg per ml of 0.1 *M* phosphate buffer (pH 8.0) containing 0.008% hydrogen peroxide for 1 min at room temperature. Colour development was stopped by the addition of 4 *M* hydrochloric acid (0.5 ml) to each test-tube. The intensity of the colour developed was measured with the ELISA reader using a calibration graph based on the activity of known amounts of soluble HRP measured under the same experimental conditions.

RESULTS AND DISCUSSION

Binding of HRP via specific antibodies immobilized on CB6200 beads

Immobilization of HRP on CB6200 beads via its mAb HRP2, which does not interfere with its enzymic activity and binds to the enzyme with a high binding constant (10^9 l/mol), provides an efficient model system for immobilization of enzymes. In order to determine the protein-binding capacity of CB6200 beads, increasing amounts of ^{125}I-labelled immunoglobulin G (IgG) were incubated with the beads. The beads were then extensively washed with a solution of 10% SDS in 8 *M* urea in order to remove any non-covalently bound protein. The amount of covalently linked antibodies was determined by measuring the gamma irradiation of the beads. As shown in Table I, the amount of bound IgG increased with protein input up to 2.2 μg bound to each bead.

Increasing amounts of anti-HRP antibody (HRP2) were coupled to the CB6200

TABLE I

BINDING OF HORSERADISH PEROXIDASE ON mAb HRP2 DIRECTLY IMMOBILIZED ON CB6200 BEADS

mAb HRP2			HRP bound (μg)	HRP/mAb HRP2 (mol/mol)
Input (μg)	Bound			
	μg	%		
0.3	0.3	100	0.055	0.73
1.0	0.9	90	0.110	0.49
3.0	1.3	43	0.175	0.54
10.0	1.8	18	0.100	0.22
30.0	2.2	7	0.025	0.05

beads and its immunological activity was determined by the ability to bind HRP. As shown in Table I, when HRP was immobilized via bound mAb HRP2, the amount of enzyme bound to the beads increased with the amount of matrix-conjugated antibody up to 1.3 μg per bead. The specific antigen-binding activity of the immobilized antibody decreased markedly with increasing amount of antibody bound to the beads (Table I). Even at a low antibody concentration (0.3 μg per bead) the molar ratio of bound antigen to antibody did not exceed 0.73, compared with 2.0 mol/mol obtained for the same antigen–antibody complex in solution (see below). At higher antibody concentrations, the amount of bound enzyme decreased, apparently owing to steric hindrance effects (see Fig. 1).

In order to improve the antigen-binding capacity of the bound antibodies and increase the activity of the immobilized enzyme, different binding methods were attempted. Binding of mAb HRP2 via its carbohydrate moieties [6] or after reversible blocking of its most reactive amino groups [9] did not improve the antigen-binding activity of the immobilized antibody.

Binding of HRP–anti-HRP immunocomplex to anti-Fc antibodies immobilized on CB6200 beads

Oriented immobilization of mAb HRP2 via anti-Fc antibodies led to a considerable enhancement of the enzymic activity of immobilized HRP. Anti-Fc antibodies were immobilized on CB6200 beads using various immobilization methods. Immunocomplexes between mAb HRP2 and HRP were formed in solution to achieve the highest antigen-binding capacity (2 mol enzyme/mol antibody) and then incubated with anti-Fc immobilized on the beads by various methods, as presented in Table II. By this procedure the enzymic activity of immobilized HRP was expected to be dependent on the immunological capability of the immobilized anti-Fc antibodies to bind the immunocomplex between HRP and its mAb. The enzymic activity of bound HRP was determined by a specific microassay using a calibration graph constructed with soluble enzyme under the same experimental conditions. As shown in Table II, immobilization of anti-Fc antibodies via a small spacer (hydrazine hydrate) failed to improve their immunological activity. The activity of anti-Fc antibodies immobilized via large

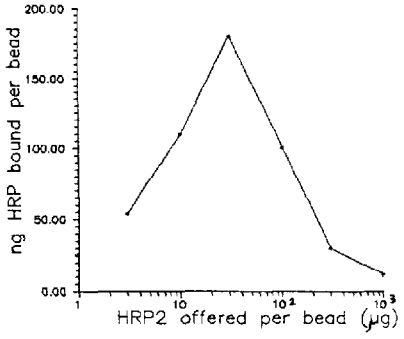

Fig. 1. Effect of amount of antibody offered per bead on the activity of the immobilized antibody. Increasing amounts of HRP2 (anti-HRP mAb) were offered to the beads. Following washing, the beads were incubated with HRP and the amount of immobilized HRP was determined as described under Experimental.

TABLE II

ENZYMIC ACTIVITY OF THE COMPLEX OF HORSERADISH PEROXIDASE WITH mAb HRP2 IMMOBILIZED ON CB6200 BEADS VIA ANTI-Fc ANTIBODIES

Immobilization method[a]	HRP	
	Enzymic activity (O.D. 492)	Bound enzyme (ng)
Oxirane (no anti-Fc)	0.020	2
Oxirane + anti-Fc	1.060	132
Oxirane + DMA–anti-Fc	1.949	244
Hydrazine hydrate + oxidized anti-Fc	0.728	91
Hydrazine hydrate + GA + anti-Fc	1.083	135
HMD + GA + anti-Fc	2.600	325
ADH + oxidized anti-Fc	1.145	143
ADH + GA + anti-Fc	2.596	324

[a] Abbreviations: ADH, adipic dihydrazide; HMD, hexamethylenediamine; DMA, dimethylmaleic anhydride; GA, glutaraldehyde.

spacers, *e.g.*, glutaraldehyde coupled to ADH or HMD, was found to be considerably higher than the activity of antibodies bound direclty via oxirane groups.

Binding of anti-Fc antibodies to oxirane groups of the matrix by using the reversible blocking with DMA led also to preparations which possessed high immunological activity. The method [9] is based on reversible blocking of most reactive amino groups of the antibodies, usually located on Fab regions, with DMA prior to the coupling reaction. After coupling to matrix via another amino group that remained unblocked during reaction with DMA, the blocking agent was removed by changing the pH and the active amino groups were available for the immunological reaction with the antigen.

The amount of HRP coupled to the various preparations of CB6200-conjugated anti-Fc varied between 90 and 325 ng per bead, depending on the immunological activity of the anti-Fc antibodies.

We have shown that enzymes may be immobilized via anti-Fc antibodies by a two-step reaction. First, the enzyme is allowed to react in solution with a properly selected specific monoclonal antibody, which binds it with high affinity and which does not interfere with its activity. Then the immunocomplex formed is bound to highly active anti-Fc antibodies. The resulting preparation of immobilized enzyme should possess high enzymic activity.

The soluble immunocomplex of enzymes and its monoclonal antibodies function as markers to quantify and purify the antibodies, and also to determine small amounts of enzymes present in the biological fluids. The use of other soluble immunocomplexes, such as glucose oxidase–antibodies for the immunohistochemical detection of antigen [11], alkaline phosphatase–antibodies in quantitative immunoassay [12] or β-galactosidase–antibodies for the purification of enzymes or antibodies [13], has been reported previously. The smaller amounts of antibodies or enzymes detected in these systems depend on the affinity of the antibody tested and on the location of the antigenic site on

the enzyme molecule. The method is applicable using crude preparations of enzymes and unpurified ascitic fluids containing the desired antibodies which decrease the cost of the test considerably. The immobilized immunocomplex of monoclonal antibodies and enzymes offers a streamlined purification system for the commercial-scale isolation of specific proteins (enzymes) found in complex mixtures. Utilization of other Eupergit C derivatives, such as the 30 N type, which possess high flow-rates and operating pressures suitable for high-performance liquid chromatography, for binding of enzymes and antibodies, provides an excellent chromatographic system [7]. The isolation of the soluble immunocomplex from the reaction mixtures or other biological fluids, using the highly active anti-Fc antibodies immobilized on the above system, is under investigation.

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