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IMPROVED METHOD FOR THE CYANOGEN BROMIDE ACTIVATION OF AGAROSE BEADS

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SUMMARY

The significant new feature of the procedure is the reaction control of the BrCN activation merely by the slow transit of BrCN from a dispersed organic phase to the aqueous phase containing agarose beads in concentrated buffer.

The product thus obtained was applied in a model immunoaffinity chromatographic separation. Experimental conditions are given for the control of the degree of activation and of the multiplicity of attachment of the protein ligand, for optimizing the immunological reactivity of the immunosorbent and for minimizing leakage of covalently bound protein from the resin.

INTRODUCTION

The immobilization of proteins to agarose beads by covalent binding after activation with cyanogen halides is an important technique in solid-state enzymology and affinity chromatography. The original method described for this purpose[1] involves extensive handling of the highly poisonous and volatile cyanogen bromide (BrCN) and the quality of the resulting end product depends on the ability of the operator to control at the same time reaction kinetics, concentration of BrCN, temperature and pH. Modifications of this method have been published[2–4], each avoiding some of the experimental disadvantages of the original technique.

The biological activity of protein ligands can be seriously hampered or even destroyed by multiple point attachment of the protein molecule to the resin, which has been explained by abnormal conformation changes[5]. Thus the opposite situation, namely coupling by as few binding sites per molecule as is feasible, might seem to be the optimal solution, as this should preserve the native conformation and leave the ligand freely accessible and in a state more closely resembling that of a free protein molecule in solution. The experiments described here proved this to be a fallacious assumption, at least as far as immunoglobulins as ligands are concerned. If we controlled the reaction conditions in order to obtain a very small number of binding sites per IgG molecule, a very tight binding of the corresponding antigen was indeed

achieved, presumably by optimal preservation of biological activity. However, this in turn necessitated the application of harsh elution conditions likely to interfere with further maintenance of native protein conformation and resulting in considerable leakage of matrix-bound IgG and deterioration of the IgG column. It is therefore necessary to find a compromise, which combines preservation of the native conformation and hence biological activity, with a mode of binding that allows elution at a less acidic pH. For each protein to be coupled, the degree of activation necessary to meet these requirements can be different. Although BrCN-activated agarose has become commercially available (at about ten times the price of agarose itself), a reliable and simple method that allows precise control of the degree of activation and the multiplicity of attachment of the ligand molecule is still indispensable.

METHODS

Activation

BrCN was dissolved as delivered in the original flask by adding 0.5 ml of acetonitrile per gram of BrCN (final concentration 1 g/ml) under a fume-hood and stored in portions at −20°. In the standard activation procedure 20 g of agarose slurry (Sepharose 4B from Pharmacia Uppsala, Sweden) are washed three times with distilled water in a filter-funnel, sucked dry and added to 30 ml of 3.3 *M* potassium phosphate buffer solution (pH 11.9 at ten-fold dilution) in a 250-ml vessel with a suspending stirring bar (as used for suspension cell cultures), then placed on a magnetic stirrer under the well ventilated hood. A 1-ml volume of BrCN solution is immediately added and the vessel is closed and stirred vigorously for 2 min to suspend the acetonitrile. The agarose beads are apparently unaffected by the vigorous stirring even if a normal beaker and a conventional stirring bar are used, as no deterioration of flow characteristics could be noticed.

Under these reaction conditions there is no need to control the reaction kinetics by cooling or adjustment of the pH or BrCN concentration. The acetonitrile is only partly dissolved and is mainly dispersed in the concentrated buffer. It is an important feature of this technique that the reaction is well controlled by the slow transit of the BrCN from the suspended acetonitrile to the aqueous phase.

Coupling

The mass is transferred to a filter-funnel containing distilled water and washed rapidly under suction with 300 ml of ice-cold 0.25 *M* phosphate buffer solution (pH 6), 500 ml distilled water at ambient temperature and 200 ml of the coupling buffer. The choice of the coupling buffer strongly influences the quality of the end product (see Results and Discussion). The buffer is sucked off, the resulting BrCN-activated gel dispersed in a solution of the protein to be coupled (5 mg per millilitre of coupling buffer) and gently shaken for 46 h at 4°. The gel is then centrifuged (10 min at 250 *g*) and the binding efficiency determined from the protein content of the supernatant. Protein not bound to the Sepharose can be re-used. The washed gel is dispersed in 1 *M* ethanolamine–hydrochloric acid (pH 9) for 2 h and washed on a filter-funnel three times with 50 ml of 0.5 *M* sodium hydrogen carbonate solution, twice with 50 ml of 0.1 *M* sodium borate buffer solution (pH 8.5), 1 *M* sodium chloride solution and twice with sodium acetate buffer solution (pH 4.1), 1 *M* sodium

chloride solution. This alternation between the latter two buffers is repeated twice. Finally, the gel is equilibrated on the funnel with 0.01 *M* sodium orthophosphate solution, 0.15 *M* sodium chloride solution, pH 7 (PBS). The gel can be stored at 4° for several weeks if 0.02% sodium azide is added to prevent microbial growth. If 20 g of agarose (Sepharose 4B) were activated, 80 mg of protein were coupled and the product was packed in a plastic column (13 × 1.5 cm). The technique was applied without alteration also for activation of amounts up to 300 g of agarose. To reduce later bleeding we included a "pre-wash" of the column with 30 ml 0.4 *N* glycine–hydrochloric acid buffer (pH 2) before the first use of the immunosorbent.

The sample protein to be separated on the immunoadsorption column was pumped continuously and very slowly through the column for at least 2 h at 10° to allow optimal saturation of the IgG with the antigen. Elution was performed with PBS (wash peak), then 1 *M* sodium chloride solution and finally 0.1 *M* glycine buffer solution (pH 2) in the standard procedure (elution peak). The acidic eluates were either adjusted to pH 7 with 2 *M* Tris buffer or immediately desalted and neutralized by gel filtration on a Sephadex G-25 column annexed to the immunosorbent column. The absorbance (254 nm) of the eluate was monitored, its radioactivity counted and its immunological reactivity tested by immunoelectrophoresis (IEP). A sample of the resin was measured in the γ-counter without further treatment.

RESULTS AND DISCUSSION

The standard procedure was optimized by making considerable variations in the experimental conditions. We tested the resulting product in comparison with a commercially available BrCN-activated agarose (Pharmacia) with respect to coupling efficiency, biological activity and non-specific elution (leakage) of the covalently linked ligand and non-specific binding of the sample protein. The multiplicity of attachment depends on the degree of activation of the agarose and on the experimental parameters of the coupling reaction [3]. Since the proteins bind to the activated agarose by the unprotonated form of their free amino groups, the use of 0.1 *M* sodium citrate solution (pH 6.5) as a coupling buffer [2,5] leads to a slower and less complete reaction than 0.1 *M* sodium chloride solution (pH 8.5). To control the number of activated sites on the matrix we varied the BrCN concentration during activation. The coupling reaction was regulated by variation of the protein concentration and choice of the buffer system. Rabbit serum and IgG from swine anti-rabbit serum [Sw-a(Ra-s)IgG] were used for the tests. One of these proteins was bound to agarose the other eluted. One or the other reaction partner was labelled with ^{125}I (ref. 6). If we chromatographed labelled protein on the immunosorbent, the absorbance and radioactivity peaks coincided. After all elution steps, less than 0.3% of the total radioactivity applied to the column remained non-specifically bound to the matrix. Leakage, coupling efficiency, capacity and biological activity of the eluates were tested in experiments in which radiolabelled Sw-a(Ra-s)IgG was coupled and whole rabbit serum chromatographed.

Highly activated agarose (450 mg of BrCN per gram of gel) and use of the alkaline coupling buffer leads to a coupling efficiency of 99%, but the resulting IgG–agarose is nearly inactive. This can be seen from the IEP data given in parentheses in Table I. Most of the IEP-reactive protein did not bind to the IgG on the column,

TABLE I

DEPENDENCE OF LEAKAGE FROM THE COLUMN AND BIOLOGICAL ACTIVITY OF THE ELUATE ON THE REACTION CONDITIONS OF ACTIVATION AND COUPLING

^{125}I-Labelled Sw-a(Ra-s)IgG was linked to the column, normal rabbit serum was chromatographed. The coupling efficiency was determined as the percentage of the radioactivity recovered on the agarose after the coupling and pre-wash procedure from the input radioactivity in the coupling reaction. The leakage was calculated correspondingly as the percentage of the radioactivity in the eluate from the radioactivity on the column (1% leakage = $4 \cdot 10^4$ cpm of ^{125}I). The immune reactivity of the eluting proteins was tested with immunoelectrophoresis and evaluated as negative (−), weak (±) or positive (+, ++, +++).

Figure	*mg BrCN/g gel*	*pH of coupling*	*Coupling efficiency (%)*	*Leakage (%) and, in parentheses, IEP reactivity in the eluate*			
				PB	*pH 3*	*pH 2*	*pH 1.5*
—	150*	8.5	98	0.01	0.04	0.08	0.11
—	150	8.5	99	0.02 (++)	0.08 (−)	0.08 (±)	0.10 (−)
1d	150	6.5	81	0.12 (±)	0.19 (−)	0.28 (±)	1.75 (++)
1c	50	8.5	92	0.04 (−)	0.04 (−)	0.11 (+++)	0.15 (−)
1b	25	8.5	70	0.11 (+)	0.21 (−)	0.30 (−)	1.56 (++)
1a	—**	8.5	90	0.05 (−)	0.05 (−)	0.16 (+++)	0.2 (−)

* Elution was carried out only with buffers without sample protein after repeated use of the column.

** Commercially available BrCN-activated Sepharose 4B.

but eluted with the wash peak. The percentage of leakage of agarose-coupled IgG was low in this instance.

If coupling is performed at low pH (citrate buffer, pH 6.5) with highly activated agarose (150 mg of BrCN per gram of gel), or at high pH (sodium hydrogen carbonate buffer, pH 8.5) with weakly activated material (25 mg of BrCN per gram of gel), the resulting product still shows a coupling efficiency of 70%; binding of the corresponding antigen to the IgG–Sepharose is very strong (pH 1.5 or even pH 1 is needed for elution), but the product does not show full capacity as some of the reactive protein of 200 mg of rabbit serum eluted with the wash peak (see Fig. 1b and Table I). The use of an elution buffer of pH 1.5 leads to considerable loss of reactivity of the immunosorbent after repeated use and irreversibility in the renaturation process of the proteins eluted in general becomes more probable. About 1.5% of the ligand is lost by leakage under these conditions. If it is taken into account that 80 mg of IgG had been coupled and that the separation of 200 mg of rabbit serum generally yielded about 20 mg of immunoreactive protein in the elution peak, leakage as high as 1.5% leads to an intolerable amount of impurities.

The use of an acidic coupling buffer to avoid multiple point attachment[2] is unneccessary, however. Multiple point binding can effectively be avoided by a lower although controlled activation as achieved by the described standard procedure, that is, by moderate activation (50 mg of BrCN per gram of gel) and use of 0.1 *M* sodium hydrogen carbonate, 0.5 *M* sodium chloride solution (pH 8.5) as coupling buffer.

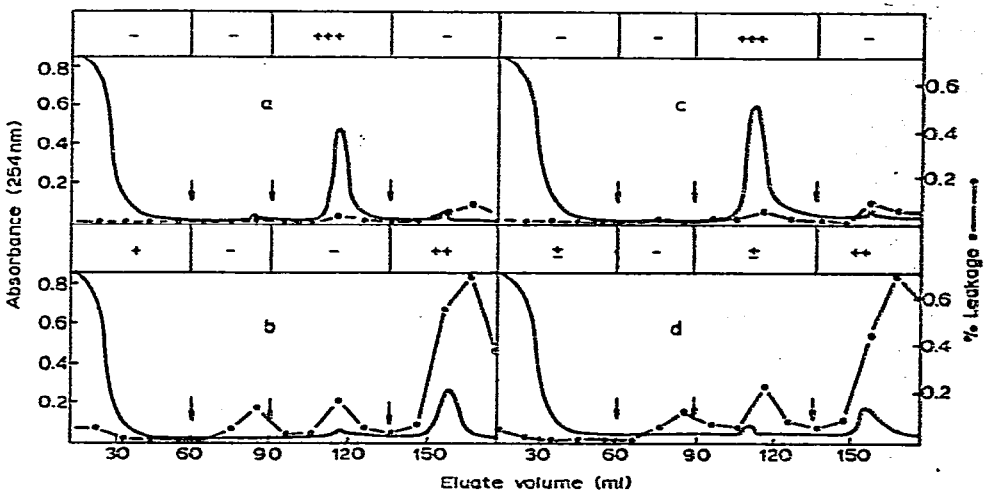

Fig. 1. Elution profiles (absorbance of 254 nm), leakage and biological reactivity (immunoelectrophoresis) in a test elution of rabbit serum on Sepharose-linked swine anti-rabbit serum IgG [Sw-a(Ra-s)IgG]. (a) Commercially available BrCN-activated agarose, coupled at pH 8.5; (b) weakly activated agarose (25 mg of BrCN per gram of gel), coupled at pH 8.5; (c) standard procedure, moderately activated agarose (50 mg of BrCN per gram of gel), coupled at pH 8.5; (d) highly activated agarose (150 mg of BrCN per gram of gel), coupled at pH 6.5. The arrows, from left to right, indicate application of glycine–HCl buffer of pH 3, 2 and 1.5, respectively. 1% of the radioactivity on the column refers to $4 \cdot 10^4$ cpm of ^{125}I. The inserts above the curves in each panel refer to the results of immunoelectrophoresis.

With these conditions we obtained the best results for both IgG (see Fig. 1c) and whole serum (data not shown).

It should be mentioned that elution of adsorbed antigen from the matrix-bound IgG can be achieved with glycine buffer of pH 2.5. Elution at this pH, however, requires low flow-rates and the application of large volumes of elution buffer. This renders optical monitoring of the eluate difficult and necessitates concentration of large volumes. In addition, the eluted proteins should not be in contact with the acidic buffer for extended periods of time. Instead of acidic elution conditions, the use of denaturing agents and chaotropic ions such as 8 *M* urea[7], 5 *M* guanidine[8], 3.5 *M* potassium thiocyanate[8,9] or 3.5 *M* potassium iodide[10] has been proposed. We tried urea and sodium thiocyanate, but could not find any advantage in the use of either. Both agents are more difficult to remove from the eluate than 0.2 *M* glycine buffer and, in agreement with the results of Johnson and Garvey[11] considerable loss of immunological reactivity occurred. Whichever agent is chosen for elution, denaturation has to be expected in any event, as the forces that hold the immune complex together are of the same nature as those which are active in preserving the secondary and tertiary structure of a protein.

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