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A Preliminary Study for Isolation of Catalytic Antibodies by Histidine Ligand Affinity Chromatography as an Alternati ve to Conventional Protein A/G Methods

Elsa Nedonchelle, Olivier Pitiot, and Mookambeswaran A. Vijayalakshmi*

Laboratoire d'Interactions Moleculaires et de Technologie des Séparations (LIMTechS), Unité CNRS UPRES A 6022, Centre de Recherche de Royallieu, BP 20 569, 60200 Compiègne Cedex, France; E-mail: viji@utc.fr

Abstract

Catalytic autoimmune antibodies from the sera of lupus patients were purified using histidyl-aminohexyl-Sepharose gel and compared with the antibodies purified with protein A and protein G affinity chromatography. The IgG preparations from the histidine affinity column had a much higher catalytic activity in hydrolyzing the peptide substrate Pro-Phe-Arg-methyl-coumarinamide compared to the antibodies obtained by the conventional protein A/G method. This preservation of catalytic activity is attributed to the gentle buffer conditions used in the histidine ligand method that allowed the integrity of three-dimensional structure of purified catalytic antibodies. Thus, histidine affinity offer a superior method for isolating autoimmune catalytic antibodies.

Index Entries: Catalytic autoimmune antibodies; activity preservation; immobilized histidine; affinity chromatography.

Introduction

The catalytic function of autoimmune antibodies has been reported by many groups (*1*, for review). In all of these studies, the autoimmune antibodies were purified from patient sera *(2–4)* and culture media of recombinant systems using the conventional protein A/protein G affinity chromatography *(5,6)*.

As with enzymes, the observed catalytic functions of antibodies can be expected to be related to a stable three-dimensional structure. It is pos-

*Author to whom all correspondence and reprint requests should be addressed.

sible that the protein A/protein G methods, which involve eluting IgG using acidic buffers, might induce conformational alterations and partial denaturation *(7)*. Therefore, the observed activity of the purified antibodies may not reflect the actual catalytic efficiency of the native antibodies.

To address this issue, alternative purification methods were studied to recover the pure antibodies in their native-state structure. Among the different chromatographic methods available such as ion-exchange, hydrophobic affinity, and pseudobioaffinity chromatography, immobilized histidine ligand chromatography (a pseudobioaffinity chromatography system) seemed promising in terms of yield, purity, and elution under very mild conditions that are unlikely to cause denaturation *(8)*.

In this preliminary report, we studied the autoimmune antibody preparations from healthy subjects and patient (lupus) sera purified by the conventional protein A/protein G methods (potentially denaturing) and by the immobilized histidine ligand affinity method (nondenaturing). The purity and catalytic activity of the antibodies was determined. Pro-Phe-Arg-methylcoumarinamide (PFR-MCA) was used as substrate, as reported by Sarath et al. *(2)*.

Experimental

Three sera—one from a healthy individual and two (LO and FE) from lupus patients—were chosen for IgG purification. All chromatographic experiments were carried out on "Biorad Polyprep" chromatography columns using gravity-flow. Unless otherwise specified 0.1 mL aliquot of the serum samples were used for purification. Each sample was run in triplicate to check reproducibility and calculate the standard deviations. The mean values are reported.

IgG Purification Using Protein G Sepharose

Two-tenths of a mililiter (0.2 mL) of the proteinG-Sepharose settled gel (Pharmacia) was loaded in a 2 mL Biorad Polyprep chromatography column, and equilibrated in 50 m*M* Tris-HCl, pH 7.4; 0.1 mL of serum to be purified was passed through the gel and after washing with the neutral pH buffer, bound IgG was eluted with 0.8 mL 100 m*M* glycine-HCl, pH 2.7, into collection tubes containing 0.04 mL 1 *M* Tris-HCl, pH 9.0.

IgG Purification Using Protein A Sepharose

We used the commercially available "Immunopure Gentle Antigen/Antibodies Buffers" from Pierce for this purpose; 0.2 mL of the protein A gel was loaded in a 2 mL Biorad Polyprep Chromatography Column, and equilibrated in "Immunopure Gentle Binding Buffer" (a sodium borate buffer, pH 8.0). Serum (0.1 mL) was loaded, and the bound IgG was eluted using "Immunopure Gentle Elution Buffer" (pH 6.55), the composition of which has not been revealed by the manufacturer.

IgG Purification Using Histidyl-Aminohexyl-Sepharose Gel

A negative affinity histidyl-aminohexyl-Sepharose gel was used for IgG recovery under the chromatographic conditions described by Pitiot et al. *(9)*; 1 mL of the settled gel was loaded in a 10 mL Biorad Polyprep chromatography column, and equilibrated in 25 m*M* MOPS, pH 7.5. The serum sample (0.1 mL diluted 1 : 40 in the equilibration buffer) was loaded on the gel and the flow through (unretained fraction) was collected in 1 mL fractions. An additional 10 mL of 25 m*M* MOPS, pH 7.5, was applied and collected. Optical density readings at 280 nm localized fractions containing IgG (first five fractions). The other proteins bound to the gel were eluted using 25 m*M* MOPS, 500 m*M* NaCl, pH 7.5.

Dialysis and Protein Determination

The antibody fraction recovered from each chromatographic runs were dialyzed using "Slide-A-Lyzer Dialysis Cassettes" from Pierce, against 50 m*M* Tris-HCl, 100 m*M* glycine, 0.025% Tween 20, pH 7.7. Protein determination was done according to Lowry *(11)*.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis of antibody preparations treated under nonreducing or reducing conditions (20 m*M* β-mercaptoethanol) with 2.5% SDS (100°C, 5 min) was done using Phastsystem SDS-gradient gels (8–25% and 4–15%; Pharmacia). The gels were stained with silver nitrate (PhastSystem Development Technique File).

Assay of Catalytic Activity

The antibodies were adjusted to the same concentration of 0.6 μ*M* in the reaction buffer (50 m*M* Tris-HCl, 100 m*M* glycine, 0.025% Tween 20, pH 7.7). Using 96-well microplates (MicroFluor, Dynatech), 25 μL of 0.6 μ*M* antibody solution was mixed with 25 μL of increasing concentrations of Pro-Phe-Arg-methylcoumarinamide (PFR-MCA; 0.2 m*M*) substrate (Peptides Institute Inc., Japan) in the reaction buffer. The plates were incubated at 37°C in a humidified incubator for 24 h. (Fluorescence monitoring was performed at time zero and then every 3 h until 12 h, followed by the final reading after 24 h of incubation.) Hydrolysis of the PFR-MCA substrate was determined by estimating the fluorescence of the leaving group (aminomethylcoumarin), using a plate reader (Perkin-Elmer LS 50 fluorometer; λ_{ex} = 360 nm and λ_{em} = 470 nm). On each plate, several concentrations of authentic aminomethylcoumarin were included as standards. A blank to account for the spontaneous degradation of the substrate consisting of 50 μL 0.2 m*M* PFR-MCA in reaction buffer and a positive control consisting of 25 μL PFR-MCA (final concentration 0.2 m*M*) and 25 μL of trypsin (final concentration 200 m*M*) were used.

Results and Discussion

Purification and Catalytic Activities of IgG

The peptide-cleaving catalytic activity using PFR-MCA was measured for the IgG fractions obtained by the conventional protein A or protein G methods, or by histidyl-Sepharose affinity chromatography *(9)* (Fig. 1). The average values for 3 h incubation at 37°C are plotted as a function of substrate (PFR-MCA) concentration. The deviations are marked in the figure representing the values from three independent purifications and catalysis assays.

In the sera from both patients, we noted a much higher catalytic activity in IgG purified by the histidine affinity method compared to the activity observed in IgG purified using protein G or protein A columns. The observation of increased specific activity in the case of histidine affinity purified IgG fractions can be readily attributed to the very mild elution conditions, compared to the harsh conditions used for protein G elution. Note that in the case of protein A column, the commercially available "immunopure gentle elution buffer" at pH 6.55 was used. The manufacturer did not disclose the buffer composition, but stated that some protein denaturation might occur (personal communication).

Having ascertained that the IgG from histidine affinity displayed superior specific activity, the important questions are: How pure are the IgG fractions obtained from histidine column, and is the observed catalytic activity due to antibodies or contaminating proteins?

Electrophoresis of Histidine Affinity Purified IgG

We compared the SDS-PAGE patterns of IgGs purified by the different methods. Under the reducing conditions, two clear bands corresponding to heavy chain (50 kDa) and light chain (25 kDa) of IgG are observed in all cases (Fig. 2). In nonreducing conditions, a single band of 150 kDa was observed in each of the purified IgG fractions. Thus, the SDS-PAGE patterns of the differently purified IgG samples (using protein A, protein G and histidine-Sepharose) were identical. This analysis suggests the absence of contaminants at the nanogram level (detection limit using silver staining) in the IgG purified using histidine-coupled matrix. However, it is possible that traces of a highly active enzyme contaminant that could be present in the sample could have escaped detection by this method.

Rechromatography of Histidine IgG Fractions on Protein G Column

To further investigate the potential contaminants, we applied the IgG fractions (from one patient sera, LO) purified by the histidine gel to a protein G column. The IgG containing fraction was dialyzed against 50 m*M* Tris-HCl, pH 7.4 (protein G equilibration buffer) and chromatographed using protein G column as described in Experimental Methods. Both unretained and eluted fractions were analyzed further.

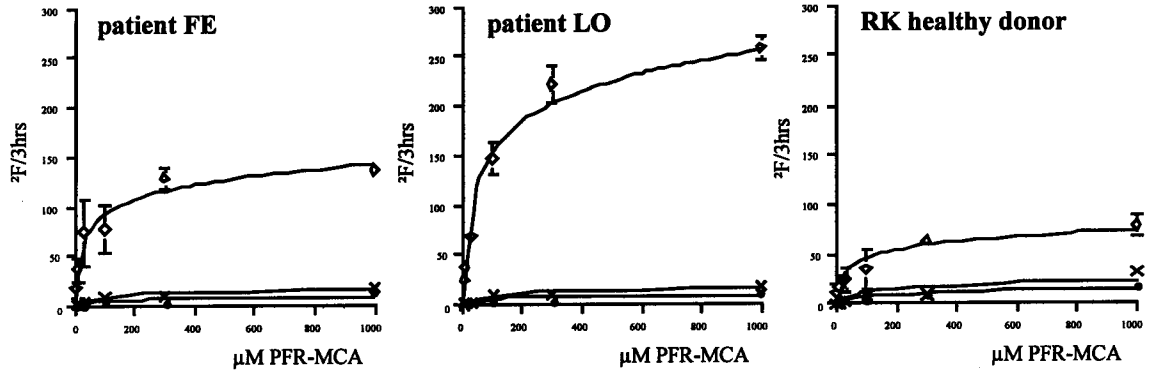

Fig. 1. Catalytic activity of IgG fractions from two lupus patients (LO and FE) and one healthy donor (RK) using protein G, protein A, and histidine-aminohexyl-Sepharose gel purification methods. Increasing concentrations of PFR-MCA were incubated with a fixed concentration IgG (0.6 µM). Reaction time, 3 h. Values are means ± standard deviation from purifications of triplicates samples. ◊ Histidine-aminohexyl-Sepharose gel; ● protein G; x protein A.

Table 1
Protein Recovery and Catalytic Activity of Histidine Column Purified IgG Fractions Rechromatographed on Protein G Column

		Protein G chromatography		
	Protein applied	Nonretained fraction	Retained fraction	Recovery (%)
Protein recovery	37.6 μg	BT[a]	24.1 μg	64.1
PFR-MCA activity (% of initial activity)[b]	100	8.9	9.4	18.3

[a]BT: Below threshold of detection.
[b]% of initial activity was measured as ΔF/6 h in aliquots of the different fractions.

The quantities of applied protein and the protein content of the fractions nonretained and acid-eluted fractions are given in Table 1. No protein could be detected in the protein G nonretained fraction using Lowry's method (Table 1). About 60% of the total applied protein was recovered in the acid-eluted fraction. SDS-PAGE electrophoresis under reducing and nonreducing conditions did not reveal any band in the nonretained fraction, while the eluted fraction contained a single IgG band, confirming the purity of the preparations.

The peptidase activity of the histidine-purified IgG applied to the column, the unbound fraction and the pH 2.7 eluted fractions is shown in Table 1. About 9% of the initial activity applied to the column was recovered in unbound fraction and 9.4% of the activity, in the acid eluate. Thus, more than 80% of the initial activity seemed to be lost during the protein G purification procedure. The use of an acidic elution to recover the IgG from the protein G, could be responsible for the loss of activity.

Conclusion

We conclude that autoantibodies purified through the histidine matrix display much greater catalytic activity than the protein G or protein A purified material. No detectable contaminants were revealed by SDS-PAGE electrophoresis after silver staining. Chromatography of the active IgG preparations on protein G resulted in loss of more than 80% of the catalytic activity of IgG suggesting that the harsh conditions used for elution may account for the lower catalytic activity of IgG purified from serum by protein A/G affinity methods. The histidine purification method thus appears to be a superior tool to study catalytic activities of polyclonal IgG. However, to definitively establish this claim, further characterization of the activity in the unbound fraction should be necessary. This might include protein fractionation by conventional chromatographic methods (gel filtration) and immunoprecipitation to exactly identify the catalytic components. These studies are underway in our laboratories.

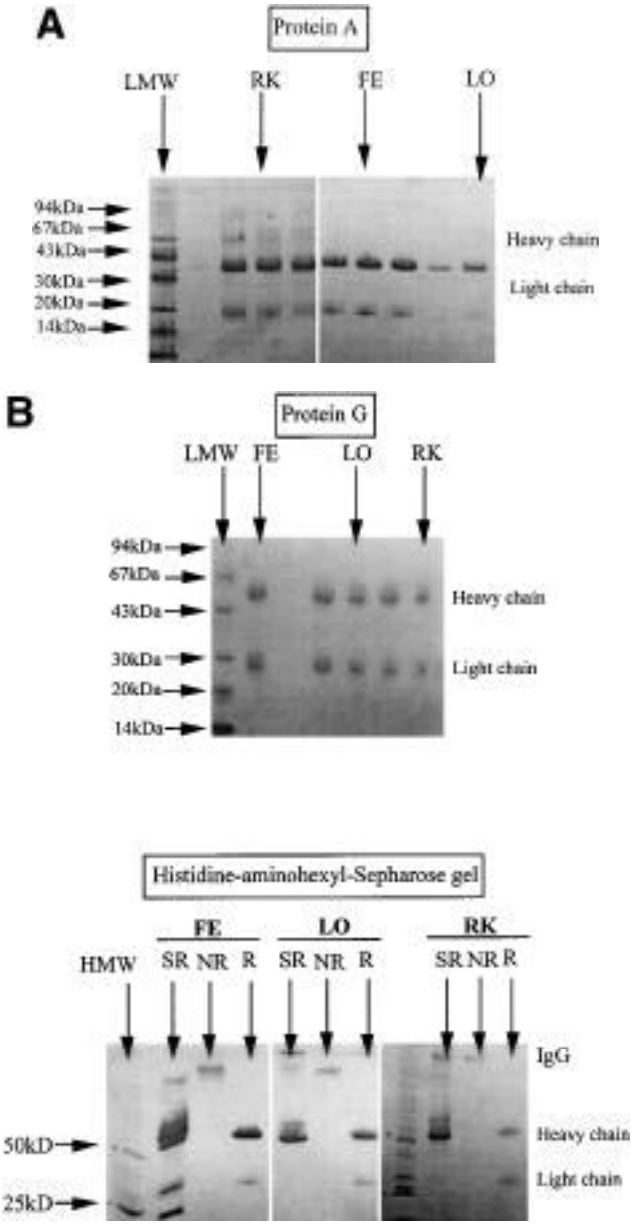

Fig. 2. SDS-PAGE analysis of fractions purified on protein G, protein A, and histidine-aminohexyl-Sepharose gel. Gels were silver stained. LO and FE IgG are from two lupus patients and RK IgG, from a healthy donor. LMW and HMW: low- and high-molecular weight standard markers. SR: Starting material under reducing conditions. R: IgG fraction under reducing condition. NR: IgG fraction under non reducing condition.

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Discussion

Paul: I have a comment about contamination and silver staining of gels. When you overload the gels, you do see bands other that the 150 kDa IgG band. These can be contaminant subunits and H-L dimers. One should not be put off by these bands. You just need to use blotting techniques to figure out if the bands are antibody subunits or not.

Gabibov: I think the easiest way to show that the activity really is due to antibodies is put your purified material on Protein A.

Vijayalakshmi: We have not yet done that control.

FitzGerald: You are hypothesizing that a change in conformation occurs. Have you looked at the antibodies by a spectroscopic method?

Vijayalakshmi: No, but we plan to do this.

Marchalonis: I think this is the beginning of a very important approach. Dr. Vijayalakshmi points out the importance of the native configuration of

the antibodies. Old fashioned immunochemists who eluted antibodies with glycine-HCl or propionic acid had the possibility of denaturation, but we were happy if we got binding. Although measurement of enzymatic activities from three patients does not tell you a whole lot, it would be interesting to see if any sequence patterns are associated with the activity, for example primitive V_H4s and V_H3s.

Gabibov: In regard to contamination, have you done silver staining and immunoblotting of your gels?

Vijayalakshmi: Yes, we have done that and we cannot see contaminants.

Paul: Have you marketed your histidine gel?

Vijayalakshmi: No, not yet. The method is very simple and I will be happy to help anybody who wishes to use it. By the way, the method is also useful for other proteins if you change the chromatography conditions. Contamination is possible because the gel can be washed extensively.

Koentgen: I can't share this optimistic view at the moment even with the silver staining. If a contaminant is responsible for the protease or DNase activity, it might be present only in trace amounts. Now, that means you would have to load a milligram of protein to see a nanogram of your contaminating protease. So, functional evidence is needed that you can remove the activity by taking out all of your immunoglobulins.

Paul: Active-site titration coupled with electrophoresis is a good method to rule out contamination. Dr. Matsuura has estimated that 20% of the protein mass is catalytically active by active site titration, and we have similar results. So, 20% of the preparation is the catalyst, it should easily be visible on the gels, regardless of whether it is an antibody or not.