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Purification of antibodies against *N*-homocysteinylated proteins by affinity chromatography on *N*ω-homocysteinyl-aminohexyl-Agarose

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Abstract

Modification with homocysteine (Hcy)-thiolactone leads to the formation of *N*ε-Hcy-Lys-protein. Although *N*ε-Hcy-Lys-proteins are immunogenic, pure antibodies have not yet been obtained. Here we describe synthesis and application of *N*ω-homocysteinyl-aminohexyl-Agarose for affinity purification of anti-*N*ε-Hcy-Lys-protein antibodies. *N*ω-homocysteinyl-aminohexyl-Agarose was prepared by *N*-homocysteinylation of ω-aminohexyl-Agarose with Hcy-thiolactone. Immune serum was obtained from rabbits inoculated with *N*ε-Hcy-Lys-keyhole limpet hemocyanine and IgG fraction prepared by chromatography on protein A-Agarose. Anti-*N*ε-Hcy-Lys-protein IgG was adsorbed on *N*ω-homocysteinyl-aminohexyl-Agarose column at pH 8.6 and eluted with a pH 2.3 buffer. Enzyme-linked immunosorbent assays demonstrate that the antibody recognizes specifically *N*-homocysteinylated variants of hemoglobin, albumin, transferrin, and antitrypsin.

Keywords: Homocysteine-thiolactone; Proteins; Immune tolerance

1. Introduction

Elevated levels of plasma homocysteine (Hcy) are associated with cardiovascular disease [1,2], Alzheimer's disease [3] and cancer [4]. Because of structural similarity of Hcy to methionine (Met), Hcy is misactivated by methionyl-tRNA synthetase (Met-RS). However, Met-RS possess an editing mechanism, which does not allow Hcy to be incorporated into protein. As a product of editing reaction, Hcy-thiolactone is produced [5]. It was shown that the production of Hcy thiolactone is enhanced in human cells with deregulated Hcy metabolism. Hcy-thiolactone mediates incorporation of Hcy into protein, which occurs by *N*-homocysteinylation of ε-amino groups of protein lysine residues and leads to formation of *N*ε-Hcy-Lys-protein adducts, as shown in Fig. 1 [6–8]. *N*-homocysteinylation leads to protein damage and a loss of function [7]. Because *N*ε-Hcy-Lys-proteins are structurally distinct, they could be recognized as neo-self antigens and induce immune response [9]. Immune activation is known to modulate atherogenesis [10]. Hyperhomocysteinemia is associated with elevated levels of *N*ε-Hcy-Lys-protein [11,12] and immune activation [13].

Serum from rabbits immunized with *N*ε-Hcy-Lys-LDL has been shown to contain anti-*N*ε-Hcy-Lys-protein antibodies [14]. However, anti-*N*ε-Hcy-Lys-protein antibodies have never been obtained in a pure form. The present study describes synthesis of *N*ω-homocysteinyl-aminohexyl-Agarose and its application as an affinity matrix for the purification of anti-*N*ε-Hcy-Lys-protein antibodies.

2. Experimental

2.1. Reagents

Keyhole limpet hemocyanine (KLH), complete Freund's adjuvant, Protein A immobilized on 6% fast-flow bead Agarose, ω-aminohexyl-Agarose, L-homocysteine-thiolactone, anti-rabbit IgG alkaline phosphatase conjugate,

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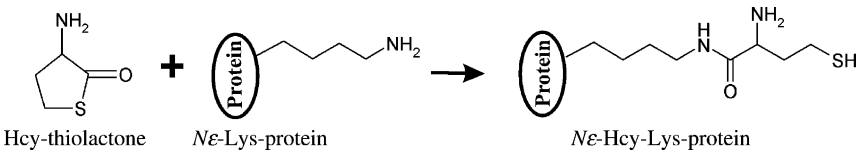

Hcy-thiolactone *N*ε-Lys-protein *N*ε-Hcy-Lys-protein

Fig. 1. Formation of *N*ε-Hcy-Lys-protein adduct by homocysteinylation of ε-amino groups of protein lysine residues with Hcy-thiolactone.

p-nitrophenyl phosphate, diethanolamine, human hemoglobin, albumin, transferrin and antitrypsin, bovine serum albumin, EDTA, potassium phosphate, Tween 20, glycine, ammonium persulfate were obtained from Sigma. Biomax-30K NMWL membranes were purchased from Millipore. Nunc Maxi-Sorp 96-well plates were purchased from Fisher.

2.2. Protein modification

Proteins used as antigens or competitors in solid phase ELISA were modified at concentration 10 mg/ml with 5 mM L-Hcy-thiolactone in 0.05 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA. The reaction was allowed to proceed to completion (16 h at 37 °C). Under these conditions, ~25% of protein lysine residues are modified [7]. No other amino acid residues in protein are known to be modified by Hcy-thiolactone [7,12].

In order to obtain an antigen with blocked thiol groups, human *N*ε-Hcy-Lys-albumin was reduced with 2.5 mM dithiothreitol (DTT) and blocked by alkylation with 10 mM iodoacetamide (IAA). The reaction with IAA was carried out for 1 h in the dark at room temperature.

2.3. Preparation of immune serum

KLH was dissolved at 20 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, 0.9 M NaCl, and modified with 40 mM L-Hcy-thiolactone HCl for 16 h at 37 °C. The extent of modification was 131 mol Hcy/mol KLH (72% lysine residues modified), as determined from parallel reactions with [^{35}S]Hcy-thiolactone [7]. 500 μg of *N*ε-Hcy-Lys-KLH adduct was dissolved in 0.5 ml phosphate-buffered saline and emulsified with 0.5 ml complete Freund's adjuvant and injected intradermally into several sites in New Zealand White rabbits. Blood was collected before and 8 weeks after inoculation. After clotting, serum was separated by centrifugation for 15 min at 1000 × *g*, and stored at −80 °C.

2.4. Protein A-Sepharose chromatography

Rabbit IgGs were isolated by affinity chromatography on 1 ml protein A-Sepharose column (binding capacity for IgG 37–43 mg/ml) equilibrated with 0.05 M Tris–HCl buffer, pH 8.6, 0.15 M NaCl. Procedure was carried out at room temperature. 1 ml serum was diluted five-fold with 0.05 M Tris–HCl buffer, pH 8.6, 0.15 M NaCl and applied onto the column at a flow rate 0.5 ml/min. After washing off unbound proteins, bound IgGs were eluted with 4 column volumes of glycine–Cl buffer, pH 2.3 (0.05 M glycine, 0.15 M NaCl) into tubes containing 0.5 M disodium phosphate, pH 7.7, as a neutralizing buffer in an amount equal to one-quarter of the collected volume. Concentration of protein in collected fractions was monitored by measuring the column effluent at A_{280} on a Varian Cary 50 UV/Vis spectrophotometer. IgG-containing fractions exhibiting A_{280} above 0.1 were pooled, concentrated using 30 kDa molecular mass cut-off Millipore spin filters and washed with Tris–HCl buffer, pH 8.6.

2.5. Preparation of Nω-Hcy-aminohexyl-Agarose

Hcy was attached via its carboxyl group to the ω-amino group of commercially available *N*ω-aminohexyl-Agarose pre-washed with 0.05 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA. This was accomplished by an overnight incubation at 37 °C of *N*ω-aminohexyl-Agarose (0.5 ml packed gel) in 1 ml of 18 mM Hcy-thiolactone, 0.05 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA. The 7.38 μmol Hcy was incorporated per millilitre packed gel. This degree of Hcy incorporation did not change after additional treatment with 18 mM Hcy-thiolactone, indicating that all available ω-aminohexyl groups had reacted to completion with Hcy-thiolactone. The schematic structure of *N*ω-Hcy-aminohexyl-Agarose is shown in Fig. 2.

2.6. Nω-Hcy-aminohexyl-Agarose chromatography

Purification of anti-*N*ε-Hcy-Lys-protein antibodies from the IgG fraction was performed at room temperature.

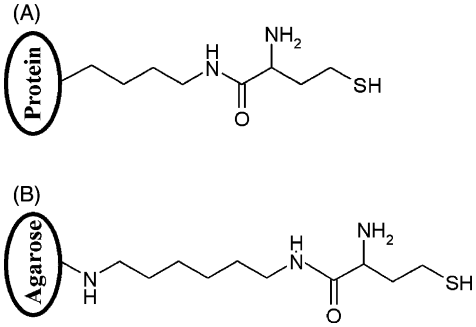

Fig. 2. Schematic comparison of structures of (A) *N*ε-Hcy-Lys-protein and (B) *N*ω-Hcy-aminohexyl-Agarose.

*N*ω-Hcy-aminohexyl-Agarose column (0.5 ml) was washed with Tris–HCl buffer, pH 8.6, treated with 2 ml 6 mM DTT, washed with five column volumes of glycine–HCl buffer, pH 2.3, and equilibrated with 2.5 ml Tris–HCl buffer, pH 8.6. IgGs from protein A-Agarose column (concentrated to 0.5 ml) were applied on *N*ω-Hcy-aminohexyl-Agarose column at a flow rate 2 ml/min. Unbound IgGs were washed off with equilibrating buffer. Bound IgGs were eluted with 4 column volumes of glycine–HCl buffer, pH 2.3 directly into tubes containing 0.5 M sodium phosphate neutralizing buffer (pH 7.7) in an amount equal to one-quarter of the collected volume. Concentration of protein in collected fractions was determined by measuring A_{280}. IgGs bound to column, eluted in two 1 ml fractions, was concentrated to 100 μl using 30 kDa molecular mass cut-off Millipore spin filters and washed with Tris–HCl buffer, pH 8.6.

2.7. Determination of anti-*N*ε-Hcy-Lys-protein antibodies by ELISA

Solid phase indirect antibody capture technique was used to determine the amount of anti-*N*ε-Hcy-Lys-protein antibodies in rabbit serum before and after immunization, as well as in fractions obtained after purification on *N*ω-Hcy-aminohexyl-Agarose column, and to demonstrate the specificity of isolated antibodies. Ninty six-well plates were initially coated with *N*ε-Hcy-Lys-albumin, IAA-*N*ε-Hcy-Lys-albumin, native hemoglobin or *N*ε-Hcy-Lys-hemoglobin (200 μl, 20 μg/ml). For antibody titer determinations and for competitive ELISA experiments, rabbit sera and affinity-purified antibodies, respectively, were diluted as indicated in Section 3. Alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:2000 was used as a secondary antibody. *p*-Nitrophenyl phosphate was used as a substrate for alkaline phosphatase. After a 30 min incubation the absorbance was measured at 405 nm on a 96-well plate reader.

3. Results and discussion

3.1. Rationale

Previous studies suggest that anti-*N*ε-Hcy-Lys-protein antibodies recognize *N*ε-Hcy-Lys epitope on *N*-homocysteinylated proteins [14]. If such epitope, or a close analogue, could be immobilized on a solid support, it would serve as an affinity matrix for selective binding of anti-*N*ε-Hcy-Lys-protein antibodies. To prepare such an affinity matrix, we have modified *N*ω-aminohexyl-Agarose with Hcy-thiolactone, which resulted in the attachment of Hcy via its carboxyl group to amino group of *N*ω-aminohexyl residue, yielding *N*ω-Hcy-aminohexyl-Agarose. Similarities between structures of *N*ε-Hcy-Lys epitope and *N*ω-Hcy-aminohexyl residue are illustrated in Fig. 2.

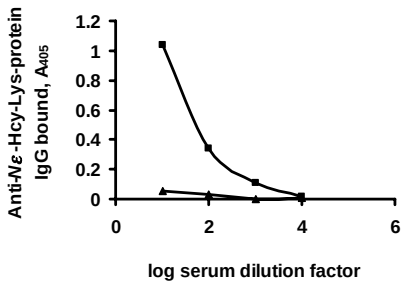

Fig. 3. (■) Antibody dilution curves for immune and (▲) pre-immune rabbit sera. *N*ε-Hcy-Lys-albumin was used as an antigen. Nonspecific binding, determined with IAA-*N*ε-Hcy-Lys-albumin as an antigen was subtracted from the binding observed with *N*ε-Hcy-Lys-albumin.

3.2. Purification of anti-*N*ε-Hcy-Lys-protein antibodies

As a source of anti-*N*ε-Hcy-Lys-protein antibodies for purification, immune serum collected 8 weeks after

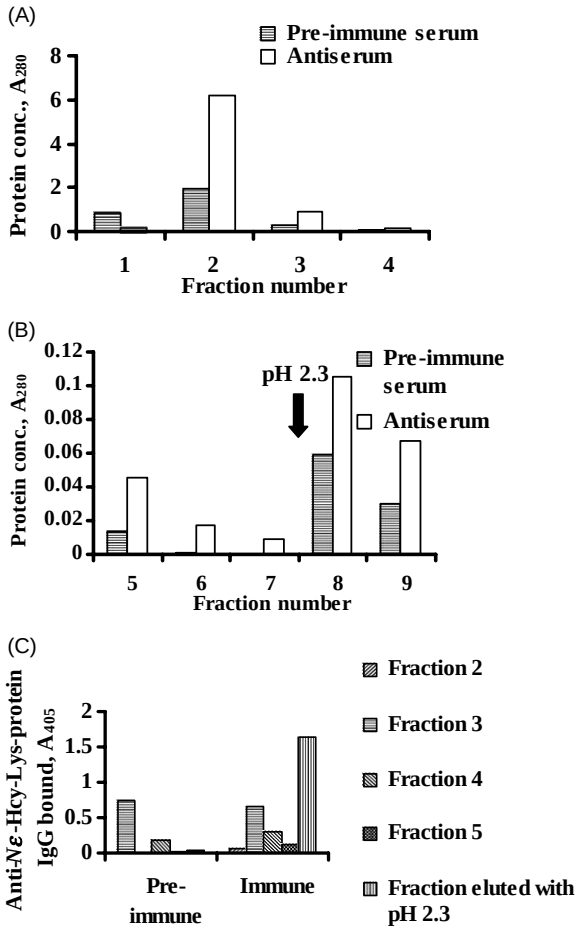

Fig. 4. Chromatography of rabbit IgGs on *N*ω-Hcy-aminohexyl-Agarose. Protein (A, B) and anti-*N*ε-Hcy-Lys-protein antibody (C) profiles for IgGs from immune and pre-immune sera are shown. Panel A shows unbound IgG protein, fractions 1–4. Panel B shows unbound IgGs, fractions 5–7, and bound IgG protein eluted with glycine–Cl buffer, pH 2.3, fractions 8, 9. Panel C shows anti-*N*ε-Hcy-Lys-protein antibody activity of unbound IgG, fractions 2–5, and of bound IgG eluted with glycine–Cl buffer, pH 2.3 (combined fractions 8, 9).

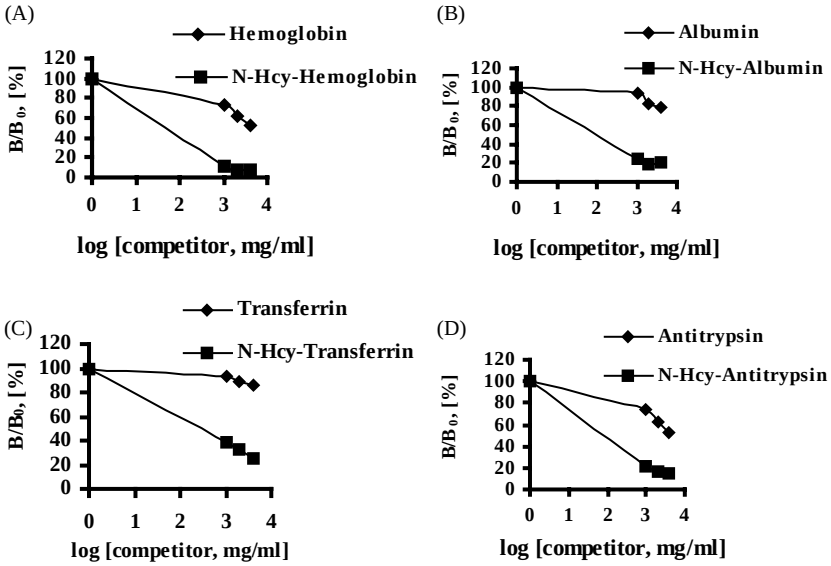

Fig. 5. Specificity of anti-*N*ε-Hcy-Lys-protein IgG antibody purified by affinity chromatography on *N*ω-Hcy-aminohexyl-Agarose column. Microtiter wells were coated with *N*ε-Hcy-Lys-hemoglobin (200 μl, 20 μg/ml) as an antigen and incubated with 1:500 dilution of affinity-purified antibody with and without competitors. After an overnight incubation, bound IgG was quantified as described in Section 2 section. Results are presented as B/B_0, in where B is the amount of IgG bound in the presence of competitor and B_0 that without competitor.

inoculation of rabbits with *N*ε-Hcy-Lys-KLH was used. As shown in Fig. 3, immune serum exhibited elevated levels of anti-*N*ε-Hcy-Lys-protein antibodies, compared with pre-immune serum.

IgG fractions were prepared from immune and pre-immune rabbit sera by chromatography on protein A-Agarose. When IgG from immune serum were subjected to chromatography on *N*ω-Hcy-aminohexyl-Agarose, a small fraction of IgG protein was bound, and could be eluted from the column with pH 2.3 buffer (Fig. 4A and B). Although it contained very little protein, the bound IgG fraction contained most of the anti-*N*ε-Hcy-Lys-protein antibody activity (Fig. 4C). The unbound IgG fraction contained most of the protein, but much less antibody activity than the bound IgG fraction.

When IgG from pre-immune serum were subjected to chromatography on *N*ω-Hcy-aminohexyl-Agarose (Fig. 4A and B), there was very little anti-*N*ε-Hcy-Lys-protein antibody activity in fractions eluted with pH 2.3 buffer (Fig. 4C). The unbound IgG fraction contained most of the protein and most of the antibody activity. These results show that anti-*N*ε-Hcy-Lys-protein antibody present in immune serum has a specific affinity for the *N*ω-Hcy-aminohexyl-Agarose column.

3.3. Specificity of anti-*N*ε-Hcy-Lys-protein antibody

To determine the specificity of anti-*N*ε-Hcy-Lys-protein antibody retained on *N*ω-Hcy-aminohexyl-Agarose and eluted with pH 2.3 buffer, competitive ELISA experiments were performed using human *N*ε-Hcy-Lys-hemoglobin, *N*ε-Hcy-Lys-albumin, *N*ε-Hcy-Lys-transferrin, and *N*ε-Hcy-Lys-antitrypsin as competitors. Native proteins were also tested as competitors. Each dilution of a competitor was added to the wells (prepared as described in Section 2) in the presence of a constant dilution (1:500) of anti-*N*ε-Hcy-Lys-protein antibodies. As shown in Fig. 5, each *N*ε-Hcy-Lys-protein was a good competitor. Native proteins essentially did not compete (albumin, transferrin) or were poor competitiors (hemoglobin, antitrypsin), >100-fold weaker than *N*ε-Hcy-Lys-protein.

Competitive ELISA experiments were also performed to determine the specificity of IgG fraction not retained on *N*ω-Hcy-aminohexyl-Agarose column. As shown in Fig. 6, *N*ε-Hcy-Lys-albumin and *N*ε-Hcy-Lys-transferrin, as wells as native albumin and transferrin were not competitors of IgG binding to *N*ε-Hcy-Lys-hemoglobin. *N*ε-Hcy-Lys-hemoglobin and native hemoglobin were

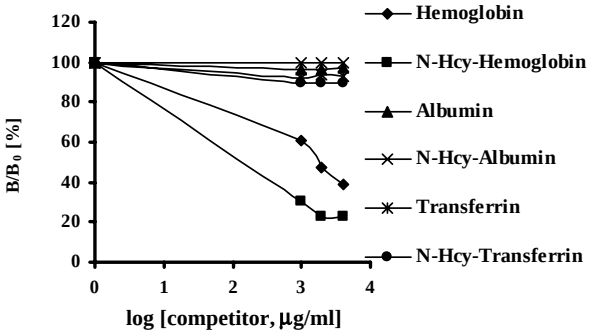

Fig. 6. Specificity of IgG fraction not retained on *N*ω-Hcy-aminohexyl-Agarose column. Microtiter wells were coated with *N*ε-Hcy-Lys-hemoglobin (200 μl, 20 μg/ml) as an antigen and incubated with a 1:600 dilution of affinity-purified antibody with and without competitors. After an overnight incubation, antibody bound was quantified as described in Section 2 section. Results are presented as B/B_0, in where B is the amount of IgG bound in the presence of competitor and B_0 that without competitor.

Table 1
Binding of rabbit anti-*N*ε-Hcy-Lys-protein antibodies to native human proteins

Assay conditions	Anti-*N*ε-Hcy-Lys-protein antibody binding (A_{492}) to			
	Hemoglobin	Albumin	Transferrin	Antitrypsin
Native protein	0.237 ± 0.002	0.069 ± 0.001	0.001	0.002
+*N*ε-Hcy-Lys-albumin, 2 mg/ml	0.049 ± 0.001			
+*N*ε-Hcy-Lys, 5 mM	0.094 ± 0.002			
IAA-protein	0.007 ± 0.001	0.011 ± 0.001		

Microtiter plate wells were coated with indicated human proteins (200 μl, 20 μg/ml), incubated with a 1:100 dilution of rabbit anti-*N*ε-Hcy-Lys-protein IgG antibodies, and the bound anti-*N*ε-Hcy-Lys-protein IgG was determined with a horseradish peroxidase-conjugated goat anti-rabbit IgG.

competitors, with the former being 10-fold stronger than the latter.

Taken together, the competive ELISA experiments show that IgG fraction retained on *N*ω-Hcy-aminohexyl-Agarose and eluted with pH 2.3 buffer contains anti-*N*ε-Hcy-Lys-protein antibodies which specifically recognize the *N*ε-Hcy-Lys epitope on Hcy-thiolactone-modified proteins. IgG fractions not retained on *N*ω-Hcy-aminohexyl-Agarose contain nonspecific antibodies, which recognize both *N*-homocysteinylated and native human hemoglobin.

3.4. Detection of N-homocysteinylated species in native human proteins

Our previous data indicate that small amounts of *N*-homocysteinlated variants occur in native human proteins [11,12]. To determine whether anti-*N*ε-Hcy-Lys-protein antibodies would detect such variants in native proteins, microtiter plate wells were coated with native hemoglobin, albumin, transferrin, or antitrypsin and assayed by ELISA. As shown in Table 1, anti-*N*ε-Hcy-Lys-protein antibodies bind to wells coated with native hemoglobin or albumin, containing 0.6 and 0.3 mol% *N*-linked Hcy [11], respectively. However, anti-*N*ε-Hcy-Lys-protein antibodies do not bind to native transferrin or antitrypsin, which contain ∼10 times less *N*-linked Hcy [11] than native hemoglobin. Treatment of the native hemoglobin or albumin with IAA prevented the antibody binding. In addition, the binding to native hemoglobin was competed out by *N*ε-Hcy-Lys-albumin or *N*ε-Hcy-Lys (Table 1). These observations suggest that the binding was specific for the *N*ε-Hcy-Lys epitope.

4. Conclusion

This communication demonstrates that *N*ω-Hcy-aminohexyl-Agarose is a useful affinity support for the purification of anti-*N*ε-Hcy-Lys-protein antibodies. Availability of such antibodies is essential for development of tools necessary for studies of the role of *N*ε-Hcy-Lys-protein in human disease.

Acknowledgements

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