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Considerations in producing preferentially reduced half-antibody fragments

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Abstract

Half-antibody fragments are a promising reagent for biosensing, drug-delivery and labeling applications, since exposure of the free thiol group in the Fc hinge region allows oriented reaction. Despite the structural variations among the molecules of different IgG subclasses and those obtained from different hosts, only generalized preferential antibody reduction protocols are currently available. Preferential reduction of polyclonal sheep anti-digoxin, rabbit anti-*E.coli* and anti-myoglobin class IgG antibodies to half-antibody fragments has been investigated. The mild reductant 2-mercaptoethylamine (2-MEA) and a slightly stronger reductant tris (2-carboxyethyl)phosphine (TCEP) were used and the fragments obtained were quantitatively determined by SDS-PAGE analysis. It has been shown that fragment yields of half-antibody fragments could be increased by lowering the pH of the reduction mixtures. However, antibody susceptibility to the reductants varied. At pH 4.5 the highest yield of sheep anti-digoxin IgG half-antibody fragments was obtained with 1 M 2-MEA. Conversely, rabbit IgG half-antibody fragments could only be obtained with the stronger reductant TCEP. Preferential reduction of rabbit anti-myoglobin IgG antibodies was optimized and the highest half-antibody yield was obtained with 35 mM TCEP. Finally, it has been demonstrated that produced anti-myoglobin half-IgG fragments retained their binding activity.

Keywords: half-antibody fragments; reduced antibody; 2-mercaptoethylamine; tris(2-carboxyethyl)phosphine; hinge region thiols

1. Introduction

Employing antibodies for surface modification has become a staple technique in the development of immunosensors (Virzonis et al. 2014, Chrouda et al. 2015), drug-delivery systems (Wu et al., Shahbazi et al. 2014) and high-sensitivity biological labels (Jaiswal et al. 2003, Park et al. 2014). The simplest method of coupling antibodies to surfaces is physical adsorption. Its main advantages are low-cost and straightforward procedure. However, adsorption has significant drawbacks, especially if antibody modified surfaces are subjected to biological substances. First, adsorption might induce structural changes, which result in reduced antibody activity (Butler et al. 1993, Buijs et al. 1996, Nakanishi et al. 2001). Second, proteins present in biological samples can displace the adsorbed antibodies and decrease the efficiency of functionalization (Vroman et al. 1980). While antibody molecules can be stabilized by covalent immobilization *via* amine groups of the antibody lysine residues, this approach results in heterogeneity of the antibody layer, as molecules are deposited in random orientations. Uncontrolled antibody deposition may lead to decreased antigen binding efficiency due to steric hindrance or immobilization occurring *via* antigen-binding sites (Kausaite-Minkstimiene et al. 2010, Balevicius et al. 2011). To circumvent these problems substantial effort has been put into developing site-directed antibody immobilization methods (Makaraviciute and Ramanaviciene 2013). Immobilization of antibody fragments has been one of the most widely used in site-directed coupling techniques applied in biosensor development (Dulay et al. 2014, Wu et al. 2014), nanostructural modification (Hu et al. 2010, Quarta et al. 2015) and labeling applications (Pereira and Lai 2008, Cho et al. 2010).

Antibody fragments can be produced by genetic engineering or chemical reduction. Recombinant antibodies are attractive due to controlled selection of domains, purity and homogeneity. However, they also have the disadvantages of high cost, varying stability, and challenging production and purification (Worn and Pluckthun 2001, Quintero-Hernandez et al. 2007, Malpiedi et al. 2013). Antibody fragments obtained by chemical reduction are an

2-MEA – 2-mercaptoethylamine
EDTA – ethylenediaminetetraacetic acid disodium dihydrate
TCEP – (2-carboxyethyl)phosphine

attractive alternative as their production is inexpensive and uncomplicated. Preferential reduction of the disulfide bonds in the antibody hinge region yields monovalent components with free thiol groups that can be employed for site-directed immobilization or conjugation. Reduced antibody fragments can be prepared by reacting gentler reducing agents, such as 2-mercaptoethylamine (2-MEA), dithiotreitol, mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP), and full antibody molecules or their $F(ab')_2$ fragments obtained after pepsin digestion. Although reducing $F(ab')_2$ to Fab' fragments is commonly employed, it has been reported that the antigen binding activity of Fab' fragments might be decreased as opposed to $F(ab')_2$ and intact antibody molecules (Lu et al. 1995). It has also been shown that the structural unfolding of the Fab part of the intact antibody is significantly slower than that of the isolated Fab' fragment. These findings suggest that the structure of the Fab fragment within the intact antibody molecule is stabilized by interactions, presumably with the Fc part missing from the isolated Fab' (Lilie 1997).

Therefore, half-antibody fragments obtained by preferential reduction of intact antibody molecules and consisting of a heavy (H) and a light (L) chain are attractive due to their higher stability and less complicated production (Hermanson 2008, Makaraviciute and Ramanaviciene 2013). The half-antibody fragment consists of a heavy and a light chain joined by a disulfide bond and has a free sulfhydryl group (HL, ~75 kDa).

Reduction of rabbit polyclonal IgG to half-antibody fragments was first demonstrated by Palmer and Nisonoff in 1963 (Palmer and Nisonoff 1963) followed by a report of higher inter-heavy chain disulfide bond susceptibility to reduction (Hong and Nisonoff 1965). The currently used techniques are in principle based on these studies and they have been applied to a variety of IgG molecules (Kato et al. 1976, Karyakin et al. 2000, Cho et al. 2007, Pereira and Lai 2008, Cho et al. 2009, Billah et al. 2010, Ho et al. 2010, Hu et al. 2010, Kausaite-Minkstimiene et al. 2010, Wu et al. 2014). Another of their advantages is that in cases when the amount of antibody is limited, it enables the reduction and/or conjugation procedures to be tested using other IgG antibodies at first.

However, although class IgG antibodies are quite a homogeneous population, there are structural differences among the subclasses in one species (Montano and Morrison 2002) and among antibodies derived from different hosts. Interestingly, the biggest structural variations have been observed in the hinge region and the Fc fragment (Kabat 1967). These

differences have also been reflected in the behaviour of different IgG molecules to reduction. Varied susceptibility to complete reduction of different subclasses of human (Virella and Parkhouse 1973) and rat antibodies (Rousseaux et al. 1979) have been reported. In addition, it has been shown that the higher liability of inter-heavy chain bonds can only be observed in some types of antibody molecules and in the rest the disulfide bond susceptibility to reduction varies (Sears et al. 1977, Liu et al. 2010).

Despite these findings, it is interesting to note that there are two main approaches to half-antibody production. One of them is following the established protocol (Hermanson 2008) not taking the antibody type into consideration (Karyakin et al. 2000, Billah et al. 2010, Ho et al. 2010, Hu et al. 2010). The second one is tailoring the reduction conditions to particular antibodies resulting in many rather widely distributed adaptations of the original protocol (Kato et al. 1976, Cho et al. 2007, Pereira and Lai 2008, Kausaite-Minkstimiene et al. 2010). Although it is sometimes implied that the reduction protocol has to be adapted for particular antibodies used, it is not clear how the reduction products differ when different antibodies are used. Moreover, it is not known what parameters are critical in the reaction and how they can be changed when adapting the preliminary reduction conditions in order to obtain the half-antibody fragments.

Therefore, in this study we sought to investigate preferential reduction of IgG obtained from two different species aiming to obtain the highest yield of half-antibody fragments and determine the parameters influencing this reaction. Both rabbit and sheep IgG have a single interheavy chain disulfide bond in the hinge region and one disulfide bridge between each of the H and L chains (Clarkson et al. 1993, Rayner et al. 2013). A mild reductant, 2-MEA and a slightly stronger reductant, TCEP were used for preferential antibody reduction and the obtained products were analyzed by SDS-PAGE. 2-MEA is the mildest reductant used primarily for the reduction of the disulfide bonds in the antibody hinge region as opposed to stronger reductants that are primarily used for complete protein reduction. TCEP was chosen out of the stronger reductants because of a different mechanism of action, which does not involve a disulfide-thiol exchange reaction, so TCEP does not have to be separated from the reduction products if they are to be exposed to maleimide chemistry (Getz et al. 1999).

2. Materials and methods

2.1 Materials

Protein A-purified rabbit polyclonal anti-*E.coli* IgG class antibodies were obtained from GenScript (UK). Protein A-purified rabbit polyclonal anti-myoglobin IgG class antibodies were acquired from GenScript (UK). Protein G-purified sheep anti-digoxin IgG class antibodies were obtained from Therapeutic Antibodies Ltd. (UK). Myoglobin from equine heart, PBS tablets, sodium chloride, sodium acetate trihydrate, acetic acid, hydrochloric acid, and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were purchased from Sigma (UK). 2-mercaptoethylamine was acquired from Alfa Aesar (UK). Tris(2-carboxyethyl)phosphine hydrochloride was obtained from Thermo Scientific (UK). Amicon Ultra-0.5 mL centrifugal filters (50KDa MWCO) were acquired from Merck Millipore (UK). All chemicals were of analytical grade or better and the solutions were prepared in UHQ water. G:BOX equipped with GeneSys image acquisition software and analyzed using GeneTools analysis software (SynGene, UK).

2.2 Methods

2.2.1 Antibody reduction

Prior to reduction the buffer in antibody solutions was changed to either 100 mM PBS with 10 mM EDTA, pH 7.4 or 150 mM sodium acetate buffer with 10 mM EDTA and 100 mM NaCl, pH 4.5, using a 50KDa centrifugal filter unit. The final IgG concentrations of reduction mixtures were 5 mg/mL. 2-MEA concentrations used varied from 53 mM to 1 M, and TCEP concentrations varied in the range of 5 mM to 125 mM. The reduction mixture was incubated for 90 min at 37°C and then was immediately placed into an ice bath to limit the reduction reaction.

2.2.2 SDS-PAGE analysis

The SDS-PAGE analysis was performed under non-reducing conditions. After reduction, samples were mixed with an equal amount of 125 mM Tris-HCl, pH 6.8, containing 50%

(v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), and 0.006% (w/v) bromophenol blue. The Mini-Protean Tetra cell equipped with Mini-Protean precast gels of 4 - 20% was used for analysis (BioRad, UK). 12.5 µg of sample was loaded to each well and gels were run at 30 mA for 1h. The gels were stained with Quick Coomassie Stain (Generon, UK) overnight then destained in deionized water overnight.

2.2.3 Dot blot immunoassay

Rabbit polyclonal anti-myoglobin IgG half-antibody fragments obtained after reduction with 35 mM TCEP were analyzed by a dot blot immunoassay. 2 µL of 1 mg/mL myoglobin solution were spotted on nitrocellulose membranes and air-dried for at least 15 min. Then, the membrane was blocked using 5 % (w/v) BSA in wash buffer 1: 150 mM sodium acetate, 10 mM EDTA, 100 mM NaCl, 0.5% (v/v) Tween 20, pH 4.5, for 1 h. Then, the membranes were rinsed with wash buffer 1. After rinsing, rabbit polyclonal anti-myoglobin IgG half-antibody reduction mixture was diluted 1/100 in 0.5 % (w/v) BSA wash buffer 1 or wash buffer 1 were added. The membranes were incubated for 1 h. Non-specifically bound antibody was rinsed twice with wash buffer 1 and twice with wash buffer 2: 100 mM phosphate buffered saline, 0.5% (v/v) Tween 20. Afterwards, 1 mg/mL goat anti-rabbit antibody-horseradish peroxidase conjugate at a dilution of 1/10000, was added and incubated for 1 h. Then, the membranes were rinsed with wash buffer 2 twice and with 100 mM PBS twice. Enhanced chemiluminescent substrate was used for developing the signal (Thermo Scientific, USA).

3. Results and discussion

Sheep polyclonal anti-digoxin IgG and rabbit polyclonal anti-*E.coli* IgG were subjected to reduction to half-antibody fragments with a milder reductant 2-MEA and a stronger reductant TCEP (Figure 1). Partial reduction of these bonds might yield different products (Table 1). Possible reduction products were based on the results presented in studies examining class IgG antibody reduction and reoxidation stating that these processes undergo in certain patterns and no independent disulphide bond formation (for example, between the sulfhydryl

groups of the light chains) might occur (Sears, 1975). The molecular weight of the half-antibody fragment (HL) should be ~75 kDa.

Table 1. Expected antibody reduction products and their approximate molecular weights. H – heavy chain, L – light chain.

Reduction product	Molecular weight, kDa
H_2L_2	~150
H_2L	~125
H_2	~100
HL	~75
H	~50
L	~25

The initial reduction conditions were chosen according to a previous protocol (Hermanson 2008) which states that at 1-10 mg/mL IgG, good reduction occurs with 50 mM–100 mM 2-MEA with 10 mM EDTA; and that varying absolute concentration of IgG still yields acceptable results. For a stronger reductant, a 3-fold molar excess of TCEP over the amount of IgG is sufficient. The pH of the reduction reaction can vary from pH 6.0 to 8.0 with pH 8.0 being optimal.

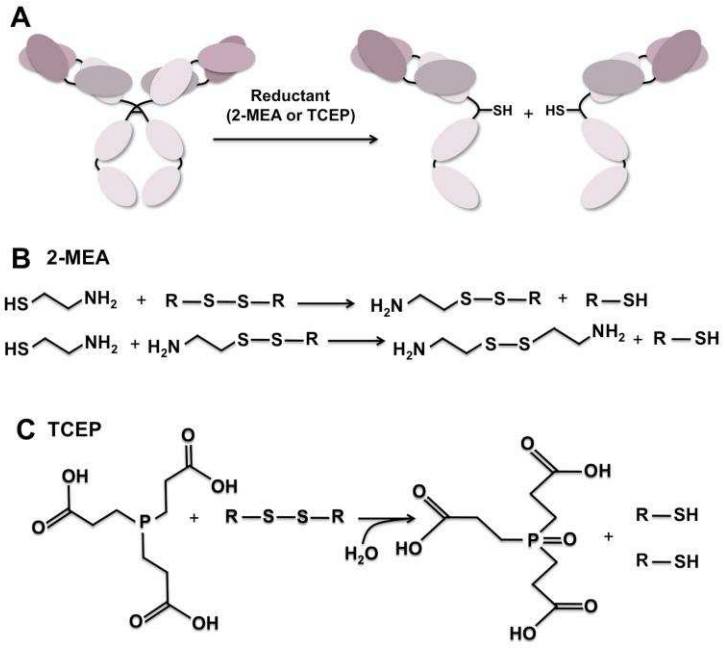

Figure 1. Schematic of IgG class antibody reduction to half-antibody fragments (A) and reduction mechanisms using 2-mercaptoethylamine (B) and (tris(2-carboxyethyl)phosphine) (C).

Following these guidelines, 5 mg/mL (~33.3 μM) preparations of sheep anti-digoxin IgG and rabbit anti-*E.coli* IgG, reduction mixtures with 53 mM 2-MEA and 100 μM TCEP in 100 mM PBS with 10 mM EDTA, pH 7.4 were prepared and the reduction products were subjected to SDS-PAGE analysis (Figure 2).

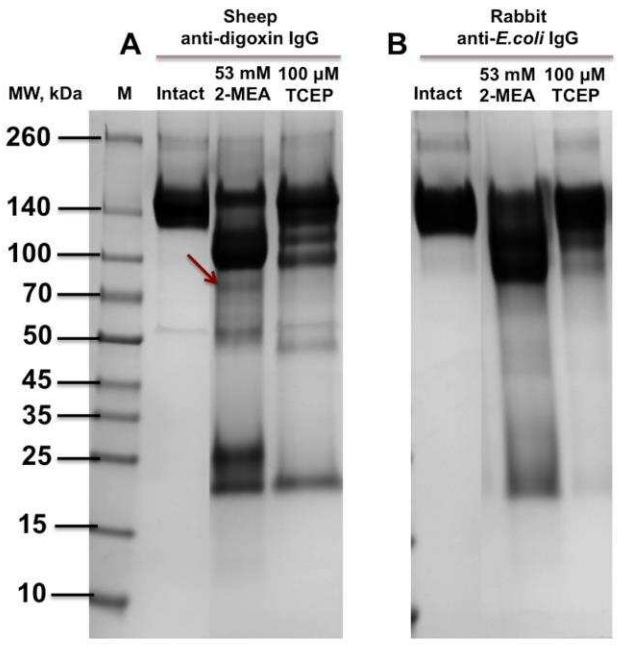

Figure 2. SDS-PAGE analysis of sheep polyclonal anti-digoxin IgG (A) and rabbit polyclonal anti-*E.coli* IgG (B) reduction products obtained using 2-mercaptoethanolamine (2-MEA) and (tris(2-carboxyethyl)phosphine) (TCEP). The arrow indicates the band corresponding to the HL fragments.

Reduction at these conditions resulted in a mixed population of antibody fragments. In the case of sheep anti-digoxin IgG reduction with 2-MEA (Figure 2A), a band corresponding to the HL fragment can be observed but its yield is quite low as opposed to bands of other antibody fragments. If this preparation was further to be used for bioconjugation or purification, their efficiency would be impaired due to low concentration of the half-antibody fragments and interference from other reduction products. In the case of TCEP reduction, the reaction resulted in a mixture of fragments with molecular weights around ~100 kDa and higher. No band corresponding to the half-antibody fragment could be observed. Rabbit anti-*E.coli* IgG reduction (Figure 2B) resulted in a similar distribution of the products with both reductants. However, in the case of rabbit anti-*E.coli* IgG reduction the molecular weights of the IgG fragments were difficult to identify, which implied that more fragments or fragment aggregates were obtained. Additional bands that were observed in the gels corresponded to the intermediates of reduction or reoxidation reactions (Table 1).

In some cases faster-migrating shoulders of the bands were observed, which were treated as one with their corresponding bands.

There could be two ways in which HL fragment yields could be improved. One of them could be to increase the concentration of the reductants. Another could be to prevent reoxidation of the antibody fragments.

When comparing the reduction conditions of earlier studies (Palmer et al. 1963) and currently available protocols, it was noted that the reduction was previously performed at pH 5.0. First, pH lower than the pI value of IgG molecules should result in electrostatic repulsion disrupting hydrophobic, or electrostatic interactions. Second, it has been reported that in acidic media (pH 2.0-5.0), sulfhydryl groups are equally resistant to oxygen both in the presence and absence of variable metal ions in the solutions (Bagiyan et al. 2003). The influence of pH on the antibody reduction yield was investigated using increased reductant concentrations in more acidic media using 2-MEA for reduction (Figure 3). pH 4.5 was used as it results in only limited changes in the conformation and stability of the antibody molecules (Ejima et al. 2007, Latypov et al. 2012), and still falls into the pH interval that strongly suppresses the reoxidation.

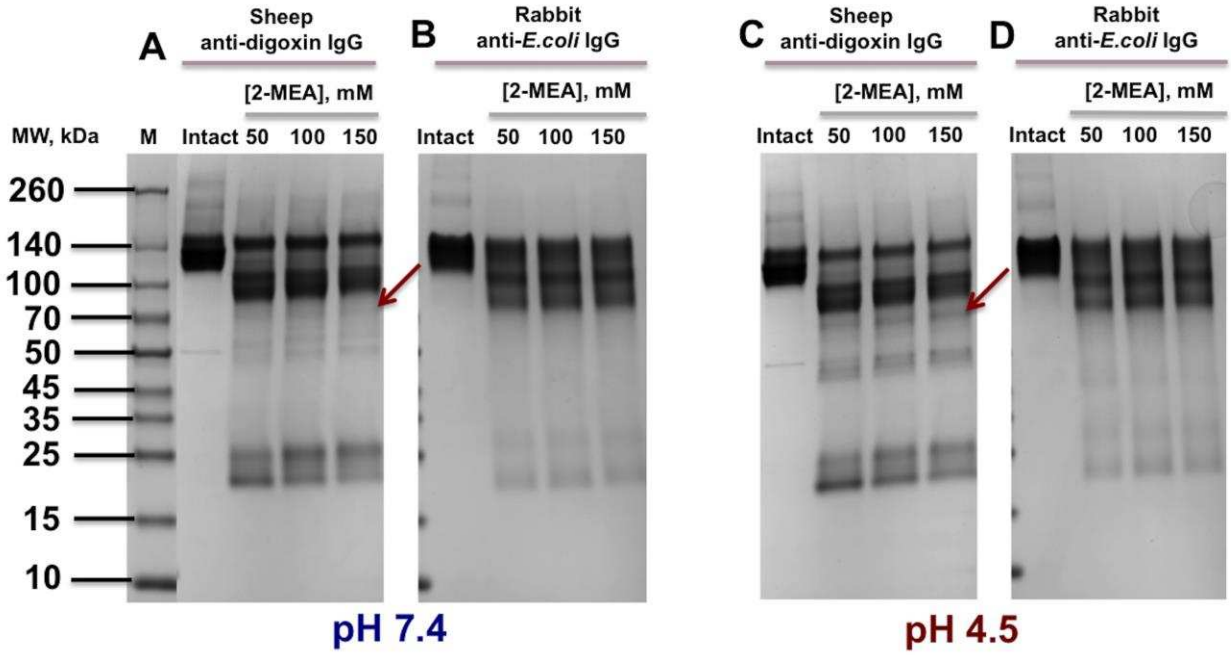

Figure 3. SDS-PAGE analysis of sheep polyclonal anti-digoxin IgG (A, C) and rabbit polyclonal anti-*E.coli* IgG (B, D) reduction products obtained using 2-mercaptoethanolamine

(2-MEA) at pH 7.4 and 4.5. The arrows indicate the bands corresponding to the HL fragments.

In the case of sheep anti-digoxin IgG reduction (Figure 3 A), no increase in the intensity of the HL band was observed with increased 2-MEA concentrations at pH 7.4. However, at pH 4.5 (Figure 3C) more defined HL bands were observed. The intensity of these bands increased with the increased 2-MEA concentrations. These results confirmed that the insufficient yield of the HL fragment was caused by reoxidation of the sulfhydryl groups. Consequently, pH 4.5 was used for all further experiments.

Interestingly, no differences in rabbit antibody reduction were observed despite the increased 2-MEA concentrations and a decreased pH value (Figure 3 B,D). In order to investigate whether a higher yield of sheep anti-digoxin and rabbit anti-*E.coli* HL fragments could be obtained, 2-MEA concentrations were increased up to 1 M (the upper limit of 2-MEA solubility). In addition, whether the reduction products of rabbit anti-*E.coli* IgG were characteristic to the particular antibody preparation used in the experiment or if they could be replicated using another type of rabbit IgG was investigated. To this end, rabbit polyclonal anti-myoglobin IgG was subjected to the same reduction conditions (Figure 4).

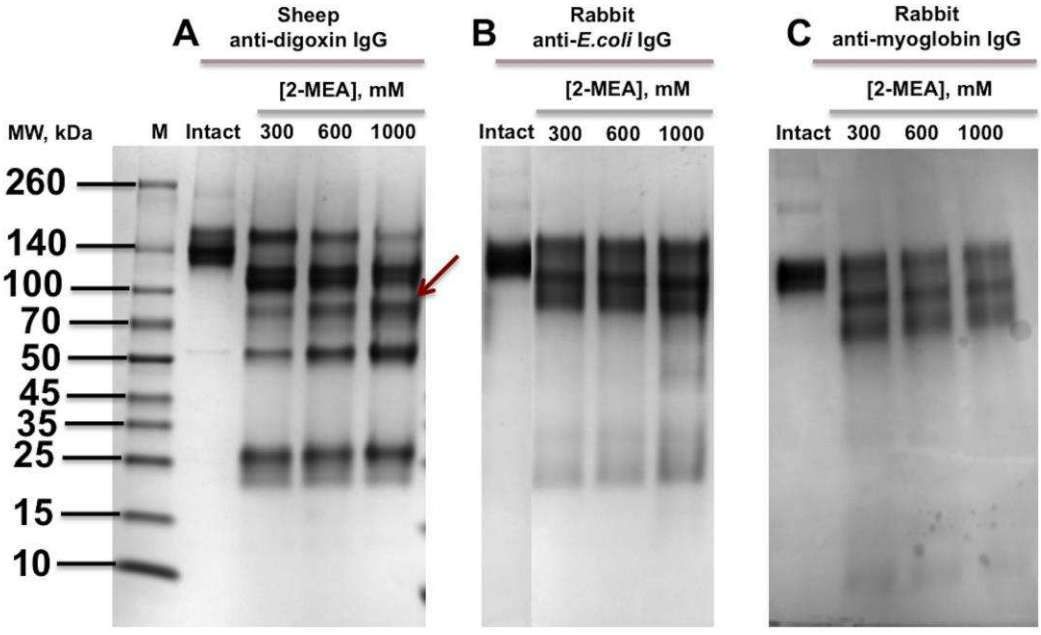

Figure 4. SDS-PAGE analysis of sheep polyclonal anti-digoxin IgG (A), rabbit polyclonal anti-*E.coli* IgG (B) and rabbit anti-myoglobin IgG (C) reduction products obtained using 2-

mercaptoethanolamine (2-MEA) at pH 4.5. The arrows indicate the bands corresponding to the HL fragments.

In the case of sheep anti-digoxin antibodies (Figure 4A), the HL fragment yield increased with increased 2-MEA concentrations. Nevertheless, although after reducing sheep anti-digoxin IgG with 1M 2-MEA the highest HL fragment band intensity was obtained, fairly high amounts of other reduction products could also be observed in the gel. It is likely that the yield of these products could be diminished by using higher reductant concentrations. However, solutions of higher 2-MEA concentrations could not be prepared.

After reducing rabbit anti-*E.coli* IgG with 1 M 2-MEA (Figure 4 B), the HL band could be observed, which was not present at lower 2-MEA concentrations. In the case of rabbit anti-myoglobin IgG reduction products (Figure 4 C), a very similar component distribution in the gel was observed. However, even with the highest 2-MEA concentration no HL fragment band was obtained.

In order to further optimize the production of rabbit IgG HL fragments, it was decided to return to using a stronger reductant TCEP. In addition, it was examined whether this reductant yielded a different distribution of fragments between sheep and rabbit antibodies. Three TCEP concentrations (5 mM, 25 mM, 125 mM) were used (Figure 5).

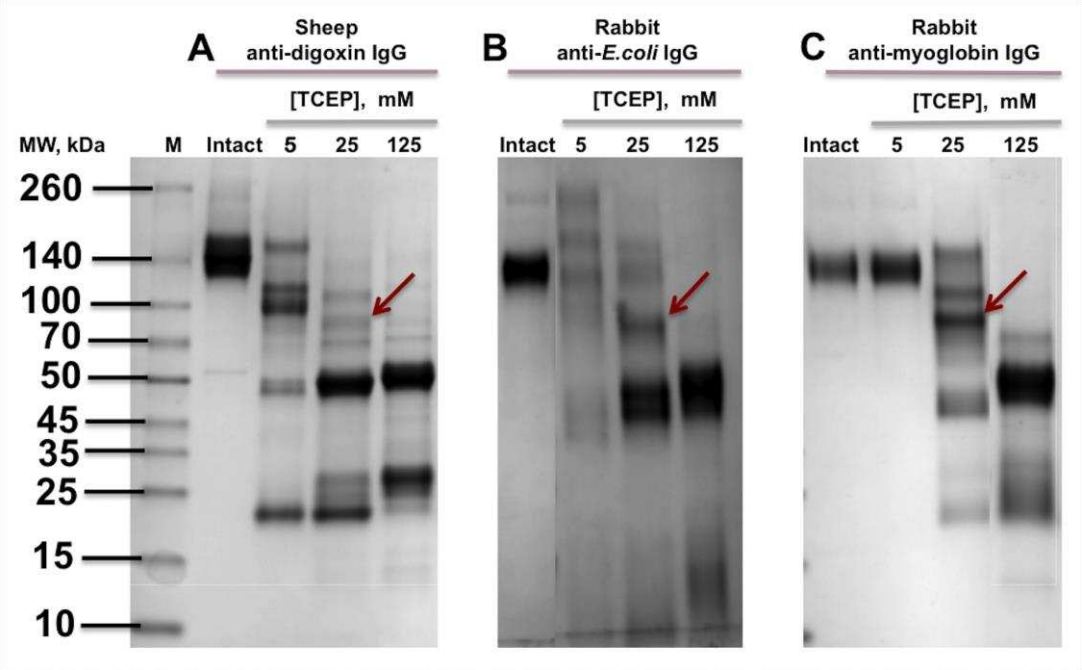

Figure 5. SDS-PAGE analysis of sheep polyclonal anti-digoxin IgG, rabbit polyclonal anti-*E.coli*, anti-myoglobin IgG reduction products obtained using (tris(2-carboxyethyl)phosphine) (TCEP) at pH 4.5. The arrows indicate the bands corresponding to the HL fragments.

The most intensive HL band of sheep anti-digoxin IgG was observed after reduction using 25 mM TCEP (Figure 5 A). However, the yields of other antibody fragments (H and L) were much higher. The same tendency was observed after rabbit IgG anti-*E.coli* and anti-myoglobin reduction (Figure 5 B,C). The major difference from sheep IgG was that the yield of rabbit HL fragments was higher as well as the ratio of HL to other antibody fragments.

Reduction data using 2-MEA and TCEP suggest that sheep antibodies are more prone to reduction than rabbit antibodies. Preferential cleavage in the hinge region could be obtained using a milder reductant and the reduction reaction progressed further using a stronger reductant. Apparently, in the case of rabbit IgG reduction, reduction to H and L and reoxidation occur faster and result in a mixed population of different components. This might be influenced by the fact that there are two subclasses in sheep IgG with different amino acid sequences in the hinge region and only one IgG subclass in rabbit (Clarkson et al. 1993).

Rabbit anti-myoglobin IgG were used to evaluate the activity of the HL fragments. The highest yield of the HL fragments was obtained using these antibodies and the reduced preparation had a smaller amount of higher molecular weight antibody fragments in comparison to sheep anti-digoxin IgG reduction. This is important because the higher molecular weight components could have antigen-binding sites and might interfere with the evaluation of the activity of HL fragments. In order to obtain the highest HL fragment yield and to minimize the yield of other antibody fragments, rabbit anti-myoglobin reduction was optimized using a range of TCEP concentrations (Figure 6).

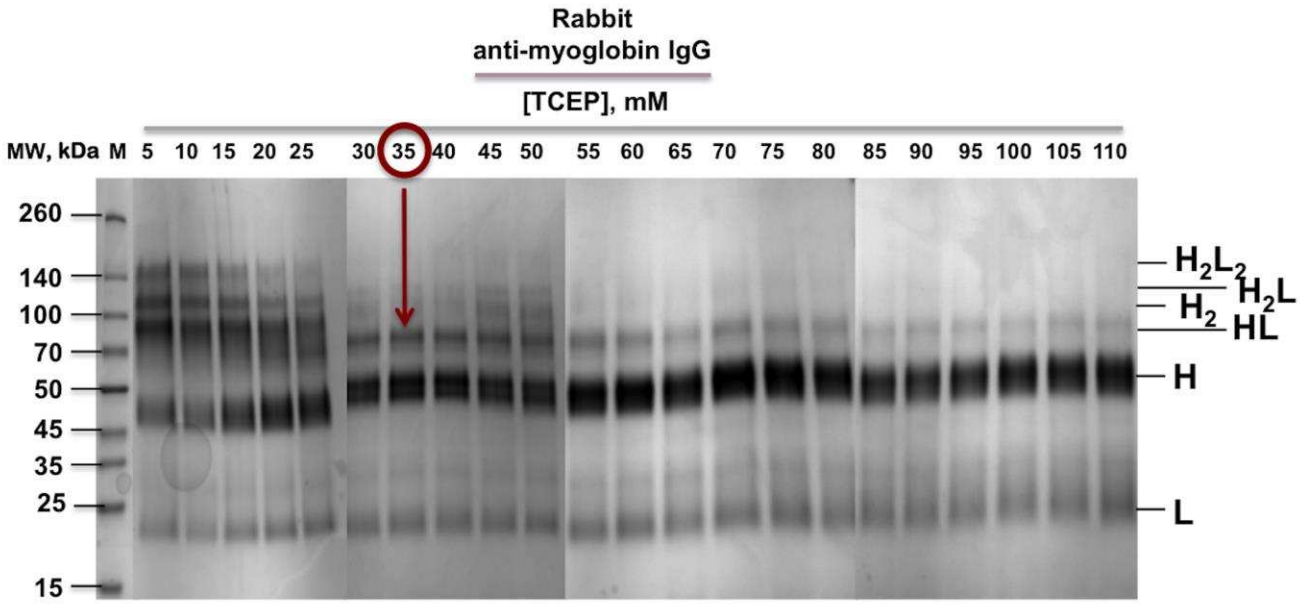

Figure 6. SDS-PAGE analysis of rabbit polyclonal anti-myoglobin IgG reduction products obtained using a range of (tris(2-carboxyethyl)phosphine) (TCEP) concentrations at pH 4.5. The arrow indicates the band corresponding to the HL fragments.

Figure 6 illustrates the course of the reduction reaction. First, when 5 mM–15 mM TCEP is used for reduction, bands identified as H_2L_2, H_2L, H_2, H and L components could be observed in the gel. The best results (the highest HL band intensity *vs.* other band intensities) were obtained when reducing with 35 mM TCEP at pH 4.5. These reduction conditions were used for further experiments.

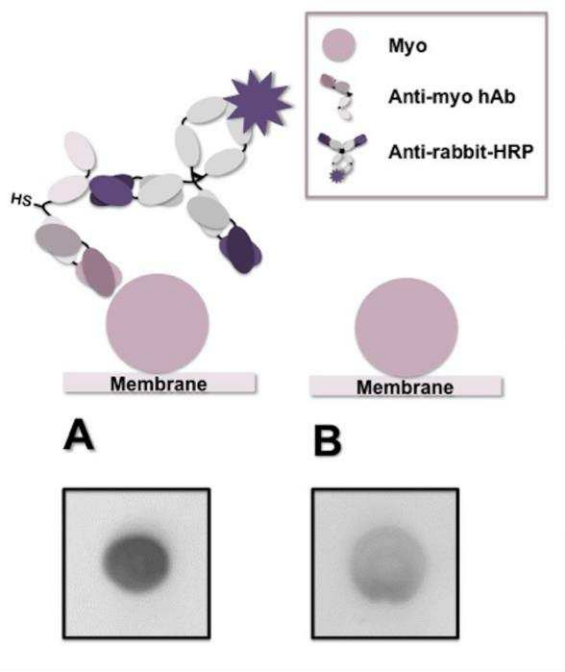

Figure 7. A dot blot immunoassay of the rabbit polyclonal anti-myoglobin IgG reduction products (anti-myo hAb) using 35 mM (tris(2-carboxyethyl)phosphine) at pH 4.5. A – myoglobin was adsorbed on a nitrocellulose membrane, anti-myo hAb was used instead of primary antibody and anti-rabbit IgG conjugated with horseradish peroxidase (anti-rabbit-HRP) was used as secondary antibody; B – for negative control myoglobin was adsorbed on a nitrocellulose membrane, no primary antibody was used and anti-rabbit-HRP was used as secondary antibody.

Finally, HL fragments obtained after reduction with 35 mM TCEP were analyzed by a dot blot immunoassay (Figure 7). The reduction mixture containing HL fragments was incubated with myoglobin adsorbed on the nitrocellulose membrane. Afterwards, it was incubated with goat anti-rabbit antibody-horseradish peroxidase conjugate and imaged with enhanced chemiluminescent substrate. Reduction products containing HL fragments showed good reactivity to their myoglobin proving that HL fragments retained their activity and could eventually be employed for biosensor, drug delivery or other biomodification applications.

4. Conclusions

This study has illustrated the optimization of half-antibody production, which could be critical to their continued use in biosensor manufacture and other applications. Results here show how a significantly higher yield of half-antibody fragments can be obtained by careful manipulation of factors including antibody host, pH, reductant and reductant concentration. The presented findings put emphasis on the importance of carefully evaluating the preparation of half-antibody fragments. In this study the yield of half-antibody fragments could be significantly increased by preventing reoxidation of sulfhydryl groups. Moreover, it is important to take into account that large discrepancies in the reduction products of IgG obtained from different hosts might be observed. In addition, in some cases reduction mixtures containing half-antibody fragments might well contain fragments of higher molecular weights having undamaged antigen-binding sites. Non-homogeneous populations of reduced antibody fragments have been reported in a study examining antibody conjugation to fluorophores (Shrestha et al. 2012). Limiting these fragments might prevent interference with half-antibody fragments in surface modification applications, reducing unexpected characteristics of the developed systems (e.g., larger size of the modified nanostructures) and increasing consistency. Reducing yields of other reduction products might also simplify the purification of half-antibody fragments. Therefore, in order to fully exploit the advantages of this antibody immobilization technique, it has to be tailored to particular antibodies with a focus on fragment recombination, host species and suitable reductants. During the course of the reduction reaction characteristic mixtures of reduced antibody fragments are produced. As illustrated here, knowing what antibody fragments are obtained at a starting point can facilitate the optimization of the reduction and to obtain the desired fragments more quickly and efficiently.

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