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Immunology

# High-pressure-mediated dissociation of immune complexes demonstrated in model systems

Charles Y. Cheung, David J. Green, Gerald J. Litt, and James A. Laugharn, Jr.*

**The use of pressure to disrupt immune complexes was demonstrated in two model systems: prostate-specific antigen (PSA) and anti-PSA antibody; and epiglycanin, a mucin glycoprotein, and an antibody specific to that protein. Dissociation of the anti-PSA antibody from the immobilized PSA antigen was observed when pressures of 415 MPa and 550 MPa (1 MPa ~144 psi) were applied at room temperature (~21 °C). Application of pressures ranging from 140 MPa to 550 MPa resulted in dissociation of antibody from epiglycanin. In both cases, the rebinding of dissociated antibody to immobilized antigen indicated that the effect of high pressure on the binding of the immune complexes was reversible. These findings suggest that application of high hydrostatic pressure has the potential to be used to significantly improve the sensitivity and specificity of clinical assays.**

Accurate quantitative analysis of biological samples is hampered when endogenous sample components bind to the analyte being measured. Examples of "binder pairs" are ubiquitous in biological systems and include DNA binding proteins binding to DNA, cell receptors binding to ligands, antigens binding to antibodies, proteases binding to protease inhibitors, and proteins binding to cofactors. Although significant advances have been made in the development of binding assay technology during recent years, in particular in the development of nonradiometric methods for the detection of analytes of clinical interest, there have been relatively few improvements in the development of techniques to prevent assay perturbation caused by endogenous sample components that bind to the analyte being measured. For example, immunoassays for thyroxine, estradiol, cortisol, and testosterone are perturbed by the binding of various serum globulins *[1]*; vitamin $B_{12}$ assays by the binding of transcobalamin *[2]*; and immunoassays for prostate-specific antigen (PSA) by the binding of $\alpha_1$-antichymotrypsin *[3]* and by $\alpha_2$-macroglobulin *[4]*.[1] Although binding assays for PSA can be adapted to detect PSA complexed with $\alpha_1$-antichymotrypsin, the detection of PSA complexed with $\alpha_2$-macroglobulin still remains problematic *[4, 5]*.

Sample treatment techniques such as solvent extraction *[6]*, boiling in the presence of stabilizers *[7]*, protein precipitation *[8]*, the addition of competitive inhibitors *[9]*, and measurement of "free" vs "bound" analyte *[10]* have been developed over the years to minimize the effect of interference by such endogenous binders. Other techniques include neutralization of the interfering agents, as well as the addition of disruptive substances such as detergents *[11]*. The binding of endogenous antibodies to antigens can cause grossly inaccurate quantification, or, in extreme cases, false negatives *[12, 13]*. In addition, sample treatment techniques such as solvent extraction, boiling, and protein precipitation usually cannot be used to eliminate the interference caused by endogenous antibodies binding to proteins because of the relative lability of the protein antigens being quantified. Although there has been limited success with the use of low pH to dissociate antigen:antibody complexes, the relatively high (and variable) amounts and the high binding affinities of the host-derived antibodies usually results in far less than complete dissociation. Most importantly, the completeness of the separation varies from sample to sample. Accurate quantification of the antigen is therefore usually quite problematic. For example, the problem of endogenous antibody interference is particularly acute in HIV-1 antigen assays where accurate quantification of the virus is only possible in the very early stages of infection *[14, 15]*.

High hydrostatic pressure is a powerful tool for studying the structure and function of proteins *[16, 17]*. Most proteins are denatured at high pressure because of irreversible changes in their secondary and tertiary structure *[18]*. The secondary and tertiary nature of these proteins are reversibly affected at pressures below the denaturation pressure *[18]*. Whereas some commercial applica-

BioSeq, Inc., 25 Olympia Ave., Unit F, Woburn, MA 01801-6307.
*Author for correspondence. Fax 617-932-8705; e-mail jlaugharn@bioseq.com.

[1] Nonstandard abbreviations: PSA, prostate-specific antigen; PBS-T, PBS-Tween 20; HRP, horseradish peroxidase; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); and EPGN, epiglycanin.

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tions of high hydrostatic pressure in the field of biotechnology have been reported or proposed *[19, 20]*, including its use for immunodesorption *[21, 22*, Dreir and Laugharn, manuscript in preparation*]*, the use of pressure to control and (or) modulate biomolecular interactions in sample preparation applications has received little attention to date. In this report, we describe the use of high hydrostatic pressure to dissociate immune complexes containing both IgG and IgM antibodies. These findings suggest that high-pressure-mediated dissociation of endogenous immune complexes has the potential to be applied commercially to improving the specificity of binding assays of analytes of clinical interest.

## Materials and Methods

A solid-phase ELISA for detecting antibodies to PSA was developed in-house. Polystyrene microtiter plates (Hi-Bind, Corning/Costar) were coated overnight at 4 °C with 0.1 mL of PSA (Sigma Chemicals) at concentrations ranging from 625 to 1265 $\mu$g/L in PBS, pH 7.4. Unreacted sites were blocked for 1 h with SuperBlock in PBS (Pierce Chemical). An anti-PSA mouse monoclonal antibody (DRG International) was then bound to the immobilized antigen by incubating the antigen-coated microwells with 0.1 mL of anti-PSA antibody (78–312 $\mu$g/L) in PBS/SuperBlock, pH 7.4, overnight at 4 °C. The wells were then washed five times with PBS-0.5 mL/L Tween 20 (PBS-T). To determine the amount of anti-PSA antibody bound to immobilized PSA, wells were reacted with goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (HRP) (Pierce Chemical). After five more washes in PBS-T, 100 $\mu$L of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), a colorimetric substrate for HRP, was then added, and the plates were read at 405 nm after a 1-h incubation.

A solid-phase ELISA for detecting a mouse monoclonal IgM antibody (AE3) to epiglycanin (EPGN), a mucin glycoprotein, was developed in-house. Polystyrene microtiter plates were coated with 0.1 mL of EPGN at 100 $\mu$g/L overnight at 4 °C in PBS, pH 7.4. Unreacted sites were blocked for 1 h with PBS/SuperBlock. The mouse monoclonal antibody was then bound to the immobilized antigen by incubating microwells with 0.1 mL of 100 $\mu$g/L antibody in PBS/SuperBlock overnight at 4 °C. After washing five times with PBS-T, the bound antibody was incubated with goat anti-mouse conjugated to HRP. After five more washes in PBS-T, 100 $\mu$L of ABTS was then added, and the plates were read at 405 nm after a 1-h incubation.

To determine the effect of pressure on the binding of the antibody to antigen, 0.1 mL of PBS/Superblock was added to microwells in which the antibody had been bound to immobilized antigen. Microwells were then overlaid with silicon oil (Sigma) to provide an immiscible phase by which pressure could be applied to the aqueous sample. The microwells were then inserted into a custom-designed high-pressure chamber *[23]* attached to a manually operated pressure apparatus (High Pressure Equipment Co.). High pressure was then applied for 30 min to the microwells containing PSA:anti-PSA, whereas pressure was applied for 20 min to those containing EPGN:AE3. Dissociated antibody was collected into the PBS medium. Control samples were overlaid with oil and held at ambient conditions during the application of high pressure to the test sample. After high pressure had been applied for the desired time, the test solutions were immediately transferred to a second set of microwells containing immobilized antigen to determine the amount of recoverable dissociated antibody. The amount of dissociated antibody from both pressurized and control microwells was measured with the ELISA described above. All experiments involving AE3 and EPGN were performed at ambient temperature (~21 °C), whereas temperatures ranged from ambient to 40 °C in experiments involving PSA and anti-PSA.

## Results and Discussion

Tables 1–4 show the experiments portraying typical results. In Tables 1 and 2, a decrease in the absorbance of a pressurized sample well is consistent with pressure-induced dissociation of antibody from antigen. Dissociation of antibody from antigen was further supported by observing increases in the absorbance values from the solutions removed from pressurized wells compared with those from solutions from control wells. These latter data are shown in Tables 3 and 4.

Absorbances presented in Table 1 show that increases in both pressure and temperature result in larger quanti-

**Table 1. ELISA absorbance values of pressurized and unpressurized microwells containing PSA:anti-PSA after removal of solutions.**

| Temperature, °C | Pressure, MPa | Absorbance | [PSA], $\mu$g/L | [anti-PSA], $\mu$g/L |
|---|---|---|---|---|
| 21 | 0.1 | 0.860 | 125 | 78 |
| 21 | 415 | 0.699 | 125 | 78 |
| 21 | 0.1 | 1.556 | 1000 | 312 |
| 21 | 550 | 1.052 | 1000 | 312 |
| 40 | 0.1 | 1.457 | 1000 | 312 |
| 40 | 415 | 0.937 | 1000 | 312 |
| 40 | 0.1 | 1.458 | 62.5 | 312 |
| 40 | 550 | 0.733 | 62.5 | 312 |

**Table 2. ELISA absorbance values of pressurized and unpressurized microwells containing EPGN:AE3 after removal of solutions.**

| Pressure, MPa | Absorbance |
|---|---|
| 0.1 | 0.868 |
| 140 | 0.41 |
| 0.1 | 0.832 |
| 275 | 0.538 |
| 0.1 | 0.739 |
| 415 | 0.565 |
| 0.1 | 0.853 |
| 550 | 0.357 |

**Table 4. ELISA absorbance values of solutions removed from pressurized and unpressurized microwells containing EPGN:AE3.**

| Pressure, MPa | Absorbance |
|---|---|
| 0.1 | 0.134 |
| 140 | 0.413 |
| 0.1 | 0.218 |
| 275 | 0.489 |
| 0.1 | 0.223 |
| 415 | 0.139 |
| 0.1 | 0.206 |
| 550 | 0.215 |

ties of anti-PSA dissociating from immobilized PSA. Raising the temperature to 40 °C during application of pressure significantly enhanced dissociation of anti-PSA from PSA. For example, as shown in Table 1, the combination of 550 MPa (1 MPa ~ 144 psi) and 21 °C resulted in the absorbance of the pressurized well decreasing to 68% of the control (1.556 decreasing to 1.052). In contrast, the combination of 550 MPa and 40 °C resulted in a relative absorbance decrease to 50% of the control (1.458 decreasing to 0.733). The higher level of absorbance decrease in the latter case was consistent with a higher level of dissociation under those conditions.

Although the absorbance measurements from the wells containing the EPGN:AE3 complex did not portray the same trend with increasing pressure as the PSA:anti-PSA system, absorbances from pressurized wells containing EPGN:AE3 nevertheless were significantly lower than the control wells at all pressures tested. As shown in Table 2, absorbance reductions were in the range of 37% ± 15% for the pressurized wells compared with control wells from 140 MPa to 550 MPa.

Tables 3 and 4 show the results of the ELISA in which the solutions from the pressurized and control wells were assayed for dissociated antibodies for both systems. In Table 3, larger absorbance values for solutions from pressurized wells were obtained than from control wells, indicating a greater recovery of anti-PSA antibody in these wells and confirming dissociation at high pressure. Also, the increased absorbances for solutions from pressurized wells at higher pressures and temperatures agree with the trends in absorbance measurements portrayed in Table 1. As shown in Table 4, antibodies that had been dissociated at 140 MPa and 275 MPa were able to rebind to immobilized EPGN. In contrast, antibodies that had been dissociated at 415 MPa and 550 MPa did not rebind to the solid phase. A possible explanation for this behavior at higher pressure may be due to the use of an IgM antibody in this system. IgM antibodies may be less stable than monomeric IgG antibodies, as the IgM antibodies may denature at higher pressures by dissociating into individual subunits. Thus, the recovery of antibody at 140 MPa and 275 MPa indicated that dissociation had indeed occurred at these conditions, whereas other mechanisms were responsible for the lower absorbances at 415 MPa and 550 MPa seen in Table 2.

Quantitative measurements to correlate an absorbance change with an approximate degree of dissociation were obtained by constructing calibration curves, in which different amounts of anti-PSA antibody were reacted with immobilized PSA. As presented in Tables 1 and 3, experiments were conducted at different concentrations of PSA and anti-PSA. As shown in the example calibration curve depicted in Fig. 1, with an initial antibody concentration of 78 $\mu$g/L (see the data point A at $4.8 \times 10^{-10}$ mol/L), a decrease in absorbance of approximately 0.250 in a pressurized well indicated that the pressure had caused dissociation of approximately 50% of the bound antibody (see the data point B at $2.4 \times 10^{-10}$ mol/L). Applying this analytical procedure in conjunction with appropriate calibration curves, PSA:anti-PSA dissociation was estimated,

**Table 3. ELISA absorbance values of solutions removed from pressurized and unpressurized microwells containing PSA:anti-PSA.**

| Temperature, °C | Pressure, MPa | Absorbance | [PSA], $\mu$g/L | [anti-PSA], $\mu$g/L |
|---|---|---|---|---|
| 21 | 0.1 | 0.051 | 125 | 78 |
| 21 | 415 | 0.154 | 125 | 78 |
| 21 | 0.1 | 0.135 | 1000 | 312 |
| 21 | 550 | 0.235 | 1000 | 312 |
| 40 | 0.1 | 0.113 | 1000 | 312 |
| 40 | 415 | 0.504 | 1000 | 312 |
| 40 | 0.1 | 0.248 | 62.5 | 312 |
| 40 | 550 | 0.629 | 62.5 | 312 |

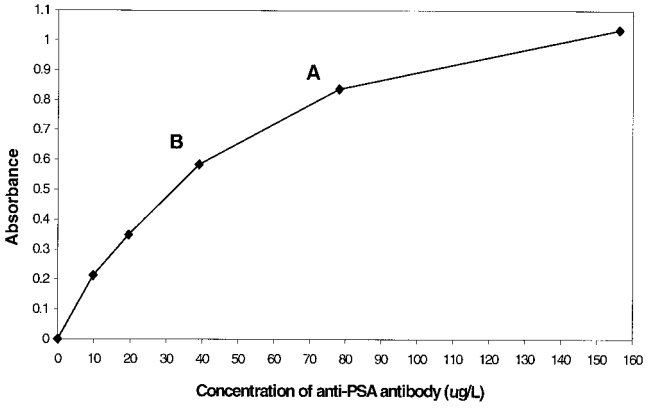


Fig. 1. Calibration curve for determining the degree of complexation between PSA and anti-PSA.

Note that a 0.250 drop in absorbance from point *A* to point *B* corresponds to a 50% drop in concentration of bound antibody.

for example, to be 20% at 415 MPa and 21 °C and 30% at 550 MPa and 21 °C. No calibration curves were constructed for the EPGN:AE3 system, and so all absorbance results remain qualitative.

The findings of this preliminary study indicate that the application of high hydrostatic pressure has the potential to be used to improve the clinical sensitivity and specificity of clinical assays. In an envisioned application, an endogenous "binder" that is "masking" an antigen from detection in an immunoassay would be dissociated by application of high pressure from the analyte of interest. Upon release of high pressure, the analyte would bind to an exogenously supplied binding reagent for subsequent detection in a conventional binding assay. Alternatively, if the interfering binder is more susceptible to pressure denaturation than the analyte of interest, the interfering binder could be selectively denatured. Success of this proposed procedure depends on the analyte being measurable after pressure treatment.

The potential for applying high hydrostatic pressure in conjunction with other sample treatment techniques to provide more precise control over dissociation is also indicated by this study. For example, a viable approach for heat-stable analytes might be to apply a combination of high pressure and increased temperature; high pressure to achieve dissociation of the interfering endogenous sample component and increased temperature to achieve denaturation of the interfering endogenous sample component. The effects of changing other variables such as pH, salt concentration, and denaturants in conjunction with pressure remain to be evaluated. A study of the effect of high pressure on the dissociation of PSA:$\alpha_1$-antichymotrypsin complexes would be of particular interest in such an evaluation.

In contrast to the results presented in this study, enhancement of binding between antigen and antibody was observed with HIV-1 p24 and anti-HIV-1 p24 *[23]*. The possibility of having enhancement of both association and dissociation of antigen to antibody at high pressure may be due to the varying responses of the interaction between antigen and antibody at different pressures and temperatures. Such behavior can be observed from pressure/temperature diagrams [24].

Overall, the use of high-pressure-mediated dissociation of biomolecular complexes has the potential to provide a fundamental new approach to the improvement of bioanalytical quantification. We anticipate that new high-pressure instrumentation will significantly improve the quantitative analysis of biological samples from technique improvements, by reducing assay times and allowing for higher clinical sensitivity and specificity in bioassays.

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