



RESEARCH NOTE

REVISED Protein-bound polyphenols create “ghost” band artifacts during chemiluminescence-based antigen detection [version 2; referees: 2 approved, 1 approved with reservations]

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Abstract

Antigen detection during Western blotting commonly utilizes a horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescent substrate. We utilized this technique to examine the impact of green tea-derived polyphenols on the binding of egg white protein-specific IgE antibodies from allergic human plasma to their cognate antigens. Our experiments unexpectedly showed that green tea-derived polyphenols, when stably complexed with egg white proteins, caused “ghost” band formation in the presence of horseradish peroxide. This study suggests that caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting using horseradish peroxidase and demonstrates that protein-bound polyphenols can be a source of “ghost” band artifacts on Western blots.

Open Peer Review

Referee Status:

	Invited Referees		
	1	2	3
REVISED			
version 2	report		report
published			
26 May 2017			
version 1			
published	report	report	
13 Mar 2017			

- Christopher P Mattison** , United States Department of Agriculture (USDA), USA
- R Hal Scofield** , Oklahoma Medical Research Foundation (OMRF), USA
Biji Kurien, University of Oklahoma Health Sciences Center, USA
- Jerónimo Carnés** , Laboratorios LETI, Spain

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Comments (0)

Corresponding author: Mary Ann Lila (imagemal@illinois.edu)

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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REVISED Amendments from Version 1

The authors have revised the manuscript based on referees' suggestions to create a new version that is more clear and streamlined. The following revisions were made:

1. Part of the introduction was revised to emphasize focus on HRP artifacts to coincide with the manuscript title
2. The term "hyperactivate/d" for HRP was removed and reworded, since there is no evidence for HRP hyperactivation
3. Authors' previous work containing "ghost" band artifacts was discussed
4. The NBT method was slightly reworded to increase clarity
5. The last paragraph of the immunoblotting method was slightly reworded to increase clarity
6. Additional proteins stained by NBT (Figure 1B) that were poorly stained by CBB were indicated with stars in the figure and text
7. The term "secondary HRP conjugated antibody" was rephrased to "biotinylated goat IgG anti-human IgE secondary antibody-NeutrAvidin HRP conjugate" for clarity
8. CBB staining of proteins on gels is now mentioned in [Supplementary Figure S1](#)

See referee reports

Introduction

Western blotting has been used extensively to identify and quantify relative amounts of specific proteins in complex mixtures. Proteins are identified using antigen-specific primary antibodies followed by various enzyme-coupled secondary antibodies. Commonly used conjugated enzymes are alkaline phosphatase and horseradish peroxidase (HRP)¹. HRP is more popular due to its stability and smaller size, which allows for conjugation of multiple HRP moieties per secondary antibody and increased sensitivity². Avidin-biotin systems can also be used to amplify reactivity and luminol-based enzyme substrates are commonly used to create a visible chemiluminescent signal.

We recently evaluated peanut protein-polyphenol aggregate particles for their binding capacity to peanut-specific plasma IgE from allergic patients using complementary assays, including chemiluminescence-based Western blotting³. Previous studies have shown "ghost" bands on some blots. In the present study, we demonstrate that protein-bound polyphenols can cause "ghost" band artifacts during chemiluminescence-based antigen detection. We investigated the binding of IgE antibodies to hen egg white proteins complexed with green tea-derived polyphenols. For detection on the blots, we used primary antibodies from allergic human plasma, secondary biotin-coupled goat anti-human IgE, avidin-HRP, and an enhanced luminol substrate. Results showed that HRP is required for "ghost" band formation. Caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

Methods

Materials

Precast mini TGX 4–20% polyacrylamide gels were purchased from BioRad (Hercules, CA, USA). Nitroblue tetrazolium and glycine were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). All other SDS-PAGE and immunoblotting reagents used are listed elsewhere³. Egg white protein (EWP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercially available organic dry green tea leaves (*Camellia sinensis* [L.] Kuntze) were provided by QTrade Teas & Herbs (Cerritos, CA, USA). Ground leaves were extracted and stored until further use as previously described¹. Extraction was performed for 2 h at 80°C.

Preparation of egg white protein-green tea polyphenol aggregate particles

The total phenolic content in the green tea extract was determined ($36.8 \text{ mg mL}^{-1} \pm 0.26 \text{ mg mL}^{-1}$, see [Table S1](#)) according to the 96-well microplate-adapted Folin-Ciocalteu method by Zhang *et al.*⁴ with modifications described by Herald *et al.*⁵. The amount of extract (mL) and protein powder (g) required to generate dry, stable protein-polyphenol aggregate particles containing 5, 10, 15, 30, or 40% polyphenols after complexation was added together and mixed under constant agitation for 15 min at room temperature. Mixtures were subsequently frozen at -20°C and freeze-dried (FreeZone12, Labconco, Kansas City, MO, USA) to form stable protein-polyphenol aggregate particles.

Nitroblue tetrazolium (NBT) staining to reveal polyphenols

Following transfer of proteins by electroblotting from unmodified EWP and aggregate particles to a polyvinylidene difluoride (PVDF) membrane, the membrane was briefly hydrated in 100% methanol. Subsequently, polyphenol-modified proteins were detected with NBT and glycinate as described by Hagerman [6; www.users.muohio.edu/hagermae/]. At alkaline pH, the catechol moiety of polyphenols catalyzes redox-cycling in the presence of glycinate, generating superoxide that reduces NBT to insoluble, visible formazan⁷.

SDS-PAGE and immunoblotting

Amounts of protein-polyphenol aggregate particles or unmodified EWP were normalized to provide 2 mg protein for SDS-PAGE. Samples were prepared in sample loading buffer containing 5% β -mercaptoethanol, resulting in 10 μg protein in 10 μL . Samples (10 μg protein/10 μL) were incubated for 5 min at 95°C, loaded onto a gel, run (40 min at 200 V), and then stained with Coomassie Brilliant Blue (CBB). The immunoblotting method used, including reagent sources, is described elsewhere³. The following minor modifications were made: Pooled human plasma (containing polyclonal antibodies, among them egg white-specific IgE) from 7 egg white-allergic individuals (PlasmaLab International, Everett, WA, USA; 1:80; v/v) was used to bind antigens on the membrane. EWP-specific IgE levels ranged from 15.4 to 100 kU L⁻¹ as determined via ImmunoCAP (Phadia, Uppsala, Sweden). Biotinylated polyclonal goat IgG anti-human IgE (Kirkegaard & Perry Laboratory, Inc., reference no. 01-10-04, Gaithersburg, MD, USA; 1:8,000; v/v)

and NeutrAvidin HRP conjugate (Thermo Scientific, Rockford, IL, USA; 1:20,000; v/v) were used to bind plasma antibodies.

In separate experiments, proteins in aggregate particles containing 15% polyphenols were blotted onto a PVDF membrane. The membrane was subsequently cut into strips and subjected to various combinations of immunoblotting reagents. Transferred proteins from unmodified EWP served as controls. The proteins from unmodified EWP were subjected to the full immunoblotting procedure.

Results and discussion

Protein distribution, NBT staining, and IgE binding capacity

The major EWPs ovotransferrin (76.6 kDa), ovalbumin (45 kDa) and lysozyme (14.3 kDa)⁸ from both aggregate particles and unmodified EWP were separated by SDS-PAGE and identified by staining with CBB (Figure 1A). An increase in molecular weight of ovotransferrin and ovalbumin, but not of lysozyme, was observed and this was polyphenol concentration dependent (Figure 1A). In fact, NBT staining indicated that ovalbumin and ovotransferrin, but not lysozyme were modified by polyphenols and the degree of staining was dependent on the concentration of polyphenol (Figure 1B). The staining also revealed several additional proteins poorly stained with CBB (indicated with stars), suggesting that the NBT staining of polyphenols more sensitively reveals the presence of protein than does CBB staining. As expected, control EWP did not react with NBT (Figure 1B). The finding that polyphenols remain bound to proteins following SDS-PAGE and membrane transfer suggests a strong, perhaps covalent association between the molecules.

As shown in Figure 1C, ovotransferrin, ovalbumin and lysozyme in unmodified EWP were recognized by antigen-specific IgE

antibodies from human plasma. However, for protein samples that contained polyphenols, ovotransferrin and ovalbumin as well as several of the proteins revealed by NBT but not CBB staining, appeared as white “ghost” bands (Figure 1C). Generally, “ghost” bands occur when the substrate is depleted quickly by the enzyme at that location and ceases to produce light². Commonly, this is a result of a high concentration of one or more of the components of the enzymatic reaction. However, in this case, the phenomenon was not observed for the EWP control sample (which did not contain polyphenols) and increased with increasing amount of polyphenols, suggesting that the polyphenols are triggering the excessive consumption of substrate and appearance of the “ghost” bands. The phenomenon was also observed with other aggregate particles including whey protein isolate-green tea polyphenol and whey protein isolate-blueberry polyphenol aggregate particles (see Figure S1) indicating that “ghosting” was not dependent on specific EWPs. “Ghost” bands also occurred on a few blots in our previously published work, however, this did not affect data interpretation^{3,9}. The same treatments were re-tested by fluorescence-based Western blotting and the data was consistent with that previously reported.

To further investigate the mechanism underlying “ghost” band formation on those blots, PVDF membrane-transferred unmodified and polyphenol-modified EWPs underwent treatment with a combination of different immunoblotting reagents. Results revealed that polyphenols promoted “ghost” band formation by interacting with HRP during HRP-substrate reactions (Figure 2). “Ghost” bands were only observed on membrane strips containing green tea polyphenols and HRP (Figure 2B, D, and G) and only HRP was required to produce “ghost” bands with polyphenol-modified EWPs (Figure 2G). No “ghost” bands were observed

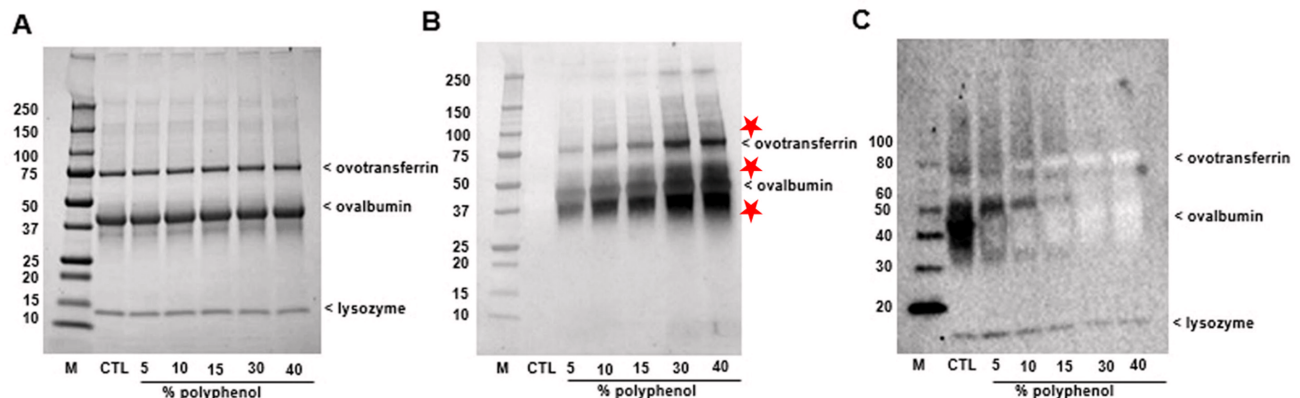


Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (A) SDS-PAGE of unmodified egg white protein (CTL) or egg white protein-polyphenol aggregate particles containing 5, 10, 15, 30, and 40% polyphenols and stained with CBB; (B) Staining of green tea polyphenol-bound egg white proteins by NBT, following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane; (C) corresponding Western blot. Pooled human plasma from 7 egg white-allergic individuals was used to bind antigens on the membrane. Egg white-specific IgE levels ranged from 15.4 to 100 kU L⁻¹ as determined via ImmunoCAP (Phadia, Uppsala, Sweden). Biotinylated goat IgG anti-human IgE was used as the secondary antibody and NeutrAvidin HRP conjugate and substrate were used for signal production. M: molecular weight marker (kDa); CTL: control (unmodified egg white protein). Approximate locations for egg white allergens are indicated. Gray scale was used for gels and membranes and contrast was optimized to improve visualization.

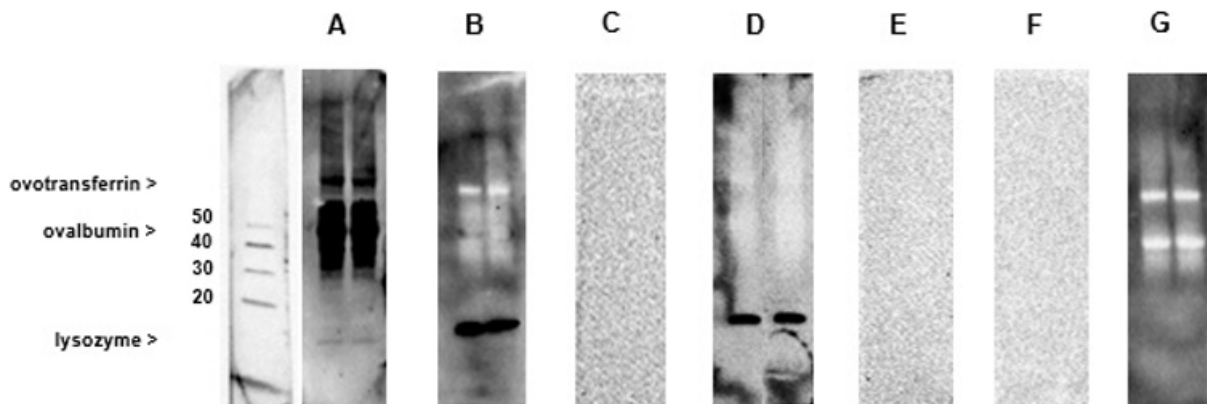


Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols. Western blot strips of (A) unmodified egg white proteins and (B-G) egg white protein-green tea polyphenol aggregate particles containing 15% total polyphenol content, after various immunoblotting treatments. (B) received all immunoblotting reagents after membrane blocking - primary antibody (pooled human plasma from 7 egg white allergic individuals with egg white-specific IgE levels ranging from 15.4 to 100 kU L⁻¹), biotinylated goat IgG anti-human IgE secondary antibody, NeutrAvidin HRP conjugate, and substrate; (C) the secondary antibody and NeutrAvidin HRP conjugate were omitted; (D) the primary antibody was omitted and (E) the primary and secondary antibody and NeutrAvidin HRP conjugate were omitted; (F) the primary antibody and NeutrAvidin HRP conjugate were omitted and (G) the primary antibody and secondary antibody were omitted. A molecular weight marker (kDa) is shown on the far left. Approximate locations for egg white allergens are indicated. Gray scale was used and contrast was optimized to improve visualization.

when substrate alone was added to a membrane containing polyphenol-bound proteins (Figure 2E). It should be noted that the light background in Figure 2C, E, and F is caused through a different mechanism than white “ghost” bands seen in B, D, and G. Since HRP is required for signal production, antibody-bound proteins on membranes not exposed to HRP (Figure 2C, E, and F) were not detected, hence, the membrane appeared blank when imaged (grey spotting is an imaging artifact). In contrast, on membranes that were treated with HRP and contained polyphenols (Figure 2B, D, and G), polyphenol-bound proteins appeared as white “ghost” bands due to depletion of locally available substrate and subsequent cessation of local light production. Interestingly, the lysozyme band was unaffected and apparently represents another artifact. This band did not require the presence of the primary antibody (Figure 2D), indicating it occurs due to a non-specific reaction between the biotinylated goat IgG anti-human IgE secondary antibody-NeutrAvidin HRP conjugate and the substrate. Further, the intensity of this band increased in the presence of polyphenols (Figure 2A, B and D), which seems contradictory since the NBT stain did not indicate polyphenols bound to lysozyme (Figure 1B). It is possible that in the presence of polyphenols, specific binding of primary and therefore secondary antibodies to proteins may be reduced resulting in excess free secondary antibodies to bind lysozyme (which did not contain bound polyphenols).

Based on this experiment, exact mechanisms of HRP promotion by polyphenols cannot be determined. It is possible, based on the fact that polyphenols are able to act as “bridges” between proteins¹⁰, that HRP non-specifically binds to protein-bound polyphenols at

high concentrations, therefore rapidly depleting substrate (luminol) in close proximity to the enzyme. Further, it is possible that protein-bound polyphenols are able to promote HRP activity, as has been observed similarly with digestive enzymes¹¹. In both cases, this could result in the cessation of light emittance (depletion of locally available luminol).

It is important to note that the observations made in this study applied to a specific set of protein samples, secondary antibody, enzyme and chemiluminescence substrate. Other types of conjugated or unconjugated secondary antibodies, enzymes (e.g. alkaline phosphatase), or substrates have not been evaluated. However, while proper Western blot experimental designs include appropriate controls such as evaluation of unmodified proteins or antibody-antigen specificity, no control for protein-bound polyphenols as shown above has been described to date. The present study highlights the importance of evaluating polyphenol effects on chemiluminescence-based antigen detection in order to prevent false interpretation of data and reveals a new source of “ghost” band artifacts.

Conclusion

We demonstrated that when attempting to evaluate IgE binding capacity of EWP-green tea polyphenol aggregate particles by enhanced chemiluminescence-based Western blotting, polyphenols which remained bound to egg white proteins after electrophoretic transfer to PVDF membrane created “ghost” bands in the presence of HRP. This study reveals protein-bound ligands as an unintended source of “ghost” band artifacts, and suggests that caution should be taken when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

Dataset 1. Raw data for Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity

<http://dx.doi.org/10.5256/f1000research.10622.d152366>

(Full legend and table are in the file).

Dataset 2. Raw data for Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols

<http://dx.doi.org/10.5256/f1000research.10622.d152367>

(Full legend and table are in the file).

Dataset 3. Raw data for Supplementary figure S1. Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity

<http://dx.doi.org/10.5256/f1000research.10622.d162634>

(Full legend and table are in the file).

Data availability

Dataset 1: Raw data for Figure 1. Protein distribution visualized by Coomassie Brilliant Blue staining (CBB), nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

DOI, [10.5256/f1000research.10622.d152366](https://doi.org/10.5256/f1000research.10622.d152366)¹²

Dataset 2: Raw data for Figure 2. Evaluation of horseradish peroxidase hyperactivation by polyphenols. (Full legend and table are in the file).

DOI, [10.5256/f1000research.10622.d152367](https://doi.org/10.5256/f1000research.10622.d152367)¹³

Dataset 3: Raw data for Supplementary Figure S1. Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

DOI, [10.5256/f1000research.10622.d162634](https://doi.org/10.5256/f1000research.10622.d162634)¹⁴

Author contributions

NJP carried out the research, contributed to experimental design and wrote a first draft of the paper. MAL served as corresponding author and contributed to the preparation of the manuscript. EAF contributed to the design of experiments and provided expertise in protein chemistry. SML helped design experiments, shared expertise in immunology and was involved in manuscript preparation. All authors were involved in manuscript revision and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

The authors declared that no grants were involved in supporting this work. The authors acknowledge the generous support for this project provided through the College of Agriculture and Life Sciences at NC State University.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We want to thank QTrade Teas & Herbs (Cerritos, CA, USA) for providing the green tea leaves.

Supplementary material

Figure S1: Protein distribution, nitroblue tetrazolium (NBT) staining, and IgE binding capacity. (Full legend and table are in the file).

[Click here to access the data.](#)

Table S1: Replicate measurements of green tea extract for total phenolic content. SD: standard deviation.

[Click here to access the data.](#)

References

- Kurien BT, Scofield RH: **Western blotting.** *Methods.* 2006; **38**(4): 283–293. [PubMed Abstract](#) | [Publisher Full Text](#)
- Alegria-Schaffer A, Lodge A, Vatterm K: **Performing and optimizing Western blots with an emphasis on chemiluminescent detection.** *Methods Enzymol.* 2009; **463**: 573–599. [PubMed Abstract](#) | [Publisher Full Text](#)
- Plundrich NJ, Kulis M, White BL, *et al.*: **Novel strategy to create hypoallergenic peanut protein-polyphenol edible matrices for oral immunotherapy.** *J Agric Food Chem.* 2014; **62**(29): 7010–7021. [PubMed Abstract](#) | [Publisher Full Text](#)
- Zhang Q, Zhang J, Shen J, *et al.*: **A simple 96-well microplate method for estimation of total polyphenol content in seaweeds.** *J Appl Phycol.* 2006; **18**(3): 445–450. [Publisher Full Text](#)

5. Herald TJ, Gadgil P, Tilley M: **High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour.** *J Sci Food Agric.* 2012; **92**(11): 2326–2331.
[PubMed Abstract](#) | [Publisher Full Text](#)
6. Hagerman AE: **Tannin Handbook.** Miami University, Oxford 45056. 2002.
[Reference Source](#)
7. Li CM, Zhang Y, Yang J, *et al.*: **The interaction of a polymeric persimmon proanthocyanidin fraction with Chinese cobra PLA₂ and BSA.** *Toxicon.* 2013; **67**: 71–79.
[PubMed Abstract](#) | [Publisher Full Text](#)
8. Stevens L: **Egg white proteins.** *Comp Biochem Physiol B.* 1991; **100**(1): 1–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Plundrich NJ, White BL, Dean LL, *et al.*: **Stability and immunogenicity of hypoallergenic peanut protein-polyphenol complexes during *in vitro* pepsin digestion.** *Food Funct.* 2015; **6**(7): 2145–2154.
[PubMed Abstract](#) | [Publisher Full Text](#)
10. Siebert KJ, Troukhanova NV, Lynn PY: **Nature of protein-polyphenol interactions.** *J Agric Food Chem.* 1996; **44**(1): 80–85.
[Publisher Full Text](#)
11. Tagliacucchi D, Verzelloni E, Conte A: **Effect of some phenolic compounds and beverages on pepsin activity during simulated gastric digestion.** *J Agric Food Chem.* 2005; **53**(22): 8706–8713.
[PubMed Abstract](#) | [Publisher Full Text](#)
12. Lila MA, Plundrich N, Foegeding E, *et al.*: **Dataset 1 in: Protein-bound polyphenols create “ghost” band artifacts during chemiluminescence-based antigen detection.** *F1000Research.* 2017.
[Data Source](#)
13. Lila MA, Plundrich N, Foegeding E, *et al.*: **Dataset 2 in: Protein-bound polyphenols create “ghost” band artifacts during chemiluminescence-based antigen detection.** *F1000Research.* 2017.
[Data Source](#)
14. Lila MA, Plundrich N, Foegeding E, *et al.*: **Dataset 3 in: Protein-bound polyphenols create “ghost” band artifacts during chemiluminescence-based antigen detection.** *F1000Research.* 2017.
[Data Source](#)

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Current Referee Status:



Version 2

Referee Report 03 July 2017

doi:10.5256/f1000research.12566.r23657



Jerónimo Carnés 

Research & Development Department, Laboratorios LETI, Madrid, Spain

General comments

The manuscript by Plundrich *et al.* describes that chemiluminescence Western blotting with horseradish peroxidase modifies the signal when protein-bound polyphenols are investigated by this methodology. However the authors demonstrate that this signal is an artifact of the methodology, named "ghost band artifact". According to these results the authors confirm that the interpretation of the results must be investigated in detail. This is a relevant study because this methodology is commonly used, independently of the proteins and extracts investigated and it is useful for the understanding of the reaction and the interpretation of the results. The highlights of the article are clearly described in the manuscript.

This reviewer considers that this findings are relevant when this methodology is going to be considered for the quantification of the proteins or when the objectives of these studies are to compare the concentration of different proteins in the same extract. In that case, the results obtained cannot be appropriate as a consequence of the different composition of the proteins (polyphenol aggregates particles). This topic should be discussed.

Another point to take into account is the concentration of polyphenol aggregates in the proteins. According to the SDS-PAGE shown in the article (Figure 1A and 1B) and Western blot (Figure 1C), the highest concentrations are influencing significantly the image, however, the percentage of polyphenolic components that the authors are adding does not seem realistic in a common extract. This topic should be clarified and discussed.

The authors should propose and include different alternatives with the objective to purify the natural extracts that contain these substances, by removing them, for example, using extensive dialysis or even treating the natural extracts. These studies could add value to the study.

Minor comments

The introduction section should be focused and introduce the application.

A reference should be add in paragraph 2. Previous studies have shown ghost bands... Please add what are these previous studies. Are they included in reference 3?

Figure 2. Please add KDa to indicate that 20, 30, 40 etc correspond to this unit

Methodology should be detailed

The authors should discuss their experience with other extracts. Are they comparable? Probably it would be better to move their experience with peanut to the discussion section.

The authors should propose different alternatives or at least include this topic in the discussion section.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 26 June 2017

doi:[10.5256/f1000research.12566.r23049](https://doi.org/10.5256/f1000research.12566.r23049)



Christopher P Mattison 

Southern Regional Research Center, FPSQ (Food Processing and Sensory Quality Research Unit) , ARS (Agricultural Research Service), United States Department of Agriculture (USDA), New Orleans, LA, USA

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 20 April 2017

doi:10.5256/f1000research.11446.r20891

**R Hal Scofield** ¹, **Biji Kurien** ²

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² University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

This study assessed the binding capacity of IgE antibodies to egg white protein (EWP)-green tea polyphenol complex by enhanced chemiluminescence-based Western blotting method. The authors of this study found polyphenols that remained bound to egg white proteins following electrophoretic transfer to a PVDF membrane hyperactivated HRP, leading to the formation of “ghost” bands. Based on the results of this study the authors suggest caution when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

While the article is of interest, this reviewer notes several concerns.

The authors should take into consideration the possibility that polyphenols bound to the protein prevents binding of primary antibody and thus could produce these artifacts. Will the effect go away if using lower amounts of protein in each well (e.g. 1, 2 or 5 µg/well; the study currently uses 10 µg/well)? The authors have studied various combinations of immunoblotting reagents, including exclusion of primary antibody or use of only HRP-avidin (using EWP with 15% polyphenol) to study the reason for the formation of these “ghost” bands. However, since there is the possibility of HRP-avidin interacting non-specifically with the antigen on the blot (in the absence of primary and secondary), the authors should try a system that does not involve the biotin-avidin system for increasing sensitivity of detection (just regular primary antibody, HRP secondary antibody and enhanced ECL detection). The authors should also try a non-chemiluminescence system to see if this problem could be reproduced (e.g. HRP with DAB detection).

There are few other issues-

Why is there a noticeable shift in lysozyme migration shown in Figure 1C if it does not bind polyphenols? Also, there is decreased detection of lysozyme in lanes with 30 and 40% polyphenols with the NBT system.

Were the gels of different composition? The protein migration pattern appears different in Figures 1A, 1B and 1C. The use of a different molecular weight marker in Figure 1C probably accentuates this observed effect. Actually 5 different molecular weight markers have been used in this work (10 to 250 kD; 6 to 98 kD; 20-220 kD; 20 to 100 kD and 20 to 50 kD)!

Figure S1A (and S1D), it is not clear how the proteins were stained? Were the proteins stained with Coomassie?

In experiments shown in Figure 1C, the authors show that the “ghost” band increases with increasing amounts of polyphenol bound to the proteins. However, Figure S1C shows that there is no “ghost” band in the lane with β-lactoglobulin bound to 40% polyphenol, which is contrary to the hypothesis put forward by the authors.

Do the authors have a reference to cite in support of the statement “Generally, “ghost” bands occur when the substrate is depleted quickly by the enzyme at that location and ceases to produce light”?

The authors should consider re-writing the following sentences-

“Following transfer of proteins by electroblotting from unmodified EWP and aggregate particles to a polyvinylidene difluoride (PVDF) membrane, the membrane was briefly hydrated in 100% methanol and polyphenol-modified proteins were detected with NBT and glycinate as described by Hagerman”

“Transferred proteins from unmodified EWP served as a control and underwent full immunoblotting procedure”

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 10 May 2017

Mary Ann Lila, North Carolina State University, USA

Comments to the Author:

“This study assessed the binding capacity of IgE antibodies to egg white protein (EWP)-green tea polyphenol complex by enhanced chemiluminescence-based Western blotting method. The authors of this study found polyphenols that remained bound to egg white proteins following electrophoretic transfer to a PVDF membrane hyperactivated HRP, leading to the formation of “ghost” bands. Based on the results of this study the authors suggest caution when evaluating polyphenol-bound proteins by enhanced chemiluminescence Western blotting.

While the article is of interest, this reviewer notes several concerns.”

****The authors should take into consideration the possibility that polyphenols bound to the protein prevents binding of primary antibody and thus could produce these artifacts. Will the effect go away if using lower amounts of protein in each well (e.g. 1, 2 or 5 µg/well; the study currently uses 10 µg/well)?***

Answer: Thank you. We have tested 5 µg/well for egg white protein-polyphenol complexes as well as for whey protein isolate-polyphenol complexes and observed “ghost” bands. We did not test even lower amounts of protein in this study. Our experiments have shown that “ghost” band formation appeared to be independent of primary antibody binding but dependent on the presence of HRP. However, it is possible that, in Figure 2B, the primary antibody was not able to bind but HRP, which was added as well, ultimately caused observed “ghost” bands. Figure 1B did not allow us to determine if the primary antibody bound to proteins that appeared as “ghost” bands or not.

****The authors have studied various combinations of immunoblotting reagents, including exclusion of primary antibody or use of only HRP-avidin (using EWP with 15% polyphenol) to study the reason for the formation of these “ghost” bands. However, since there is the possibility of HRP-avidin interacting non-specifically with the antigen on the blot (in the absence of primary and secondary), the authors should try a system that does not involve the biotin-avidin system for increasing sensitivity of detection (just regular primary antibody, HRP secondary antibody and enhanced ECL detection). The authors should also try a non-chemiluminescence system to see if this problem could be reproduced (e.g. HRP with DAB detection).***

Answer: Thank you. We agree with the referee and in fact we now have moved on to fluorescence based detection in our recent studies. Experiments have shown no artifacts using this system. We wanted to move away from chemiluminescence based detection systems all together.

There are few other issues-

****Why is there a noticeable shift in lysozyme migration shown in Figure 1C if it does not bind polyphenols? Also, there is decreased detection of lysozyme in lanes with 30 and 40% polyphenols with the NBT system.***

Answer: Thank you for your comment. Figure 1B (NBT stain) shows that lysozyme was not detected at all. Or more specifically, the NBT stain revealed that lysozyme was not bound by polyphenols. The upward shift seen in Figure 1C is likely an artifact from either running the gel or occurred during the protein transfer onto the PVDF membrane (gel could have been shifted/skewed a bit during “sandwich” preparation in the iBlot electroblotting system). It can be seen that all lanes in Figure 1C appear to be skewed. Figure 1A (SDS-PAGE) shows an even run of lysozyme.

****Were the gels of different composition? The protein migration pattern appears different in Figures 1A, 1B and 1C. The use of a different molecular weight marker in Figure 1C probably accentuates this observed effect. Actually 5 different molecular weight markers have been used in this work (10 to 250 kD; 6 to 98 kD; 20-220 kD; 20 to 100 kD and 20 to 50 kD)!***

Answer: Thanks. No, the same gels were used to create Figure 1 A, B and C (BioRad TGX mini

protean precast gels 4-20%). Yes, the 10 to 250 kDa marker (BioRad Precision Plus) was used for gels, the 6 to 98 kDa marker (Invitrogen SeeBlue2) was used for NBT blots since this was the available marker at the time of data collection, and a 20-220 kDa marker (Invitrogen Magic Mark XP, an IgG labeled marker) was used for Western blots. We did not use a 20 to 100 kDa nor a 20 to 50 kDa marker. Only visible marker bands are shown alongside the Western blots, hence the possible confusion.

****Figure S1A (and S1D), it is not clear how the proteins were stained? Were the proteins stained with Coomassie?***

Answer: Thanks. Yes, they were also stained with Coomassie Brilliant Blue and we have now added this information to the respective figure legend.

****In experiments shown in Figure 1C, the authors show that the “ghost” band increases with increasing amounts of polyphenol bound to the proteins. However, Figure S1C shows that there is no “ghost” band in the lane with β -lactoglobulin bound to 40% polyphenol, which is contrary to the hypothesis put forward by the authors.***

Answer: Thanks. The “ghost” band in Figure S1C is not very pronounced but can be seen for β -lactoglobulin.

****Do the authors have a reference to cite in support of the statement “Generally, “ghost” bands occur when the substrate is depleted quickly by the enzyme at that location and ceases to produce light”?***

Answer: Thank you. Yes, we have now added a reference.

****The authors should consider re-writing the following sentences-***

“Following transfer of proteins by electroblotting from unmodified EWP and aggregate particles to a polyvinylidene difluoride (PVDF) membrane, the membrane was briefly hydrated in 100% methanol and polyphenol-modified proteins were detected with NBT and glycinate as described by Hagerman”

Answer: Thanks. We have broken this rather long sentence into two, for greater clarity. It now reads: “Following transfer of proteins by electroblotting from unmodified EWP and aggregate particles to a polyvinylidene difluoride (PVDF) membrane, the membrane was briefly hydrated in 100% methanol. Subsequently, polyphenol-modified proteins were detected with NBT and glycinate as described by Hagerman”

“Transferred proteins from unmodified EWP served as a control and underwent full immunoblotting procedure”

Answer: Thanks. We have reworded for additional clarity. It now reads: “Transferred proteins from

unmodified EWP served as controls. The proteins from unmodified EWP were subjected to the full immunoblotting procedure”.

Competing Interests: No competing interests

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The findings of this manuscript should be published because the implications of the HRP findings on current and past research could be widespread. The authors should be congratulated for taking the time and effort to examine the artifacts they observed rather than just ignoring them and moving on. In its current form, however there are some important points that need to be addressed in the manuscript to ease reader comprehension and focus the research on one topic or more clearly describe the findings and implications of the 2 topics in the manuscript.

1. What is the focus of the manuscript, the study of the effect of polyphenols on egg, or the artifact(s) resulting from the use of HRP?
 - The title of the manuscript suggests the manuscript is focused to point out a potentially very serious and mis-leading artifact of using HRP for western blot signal generation, but the content of the text is mixed between pointing out the findings of the egg/tea polyphenol study findings and the HRP artifact
 - I would argue that the HRP artifact is the primary purpose of the paper (as suggested in the title) and more in-depth discussion of the findings/implications is needed
1. Please consider re-writing the second paragraph of the introduction to sharpen the focus of the manuscript to coincide with the title...rather than the focus of green tea polyphenols on egg allergens.
2. In the introduction some discussion and referencing of ‘ghost bands’ from past publications would be useful and possibly a discussion of the topic of reciprocity failure (if relevant here) in signal generation?
3. Would the ghost bands be expected to obscure ‘real’ bands nearby or migrating at the same pace?
4. Did the authors notice these artifacts in their own past publications on similar topics? Plundrich *et al* 2014¹. If so, this should be discussed and any discrepancies in their findings or conclusions that can be attributed to the HRP artifacts should be noted.
5. Findings using peanut allergens and tea (or other sources of) poly-phenols that lead to the same artifacts are important to point out.
6. Can the authors find a related published article/examples of other groups that may have suffered from the same artifact and mis-lead the authors of that research to put their findings in the context of other using the same reagents?
7. Could the authors please star/mark the bands that are considered “several additional proteins” that were detected with NBT staining but not CBB on Fig 1B?
8. Could the additional bands noted on Fig 1B represent oligomers/aggregates of the ovalbumin and ovotransferrin, and are these same bands present on the immunoblot?
9. Could you test directly the proposed interaction between HRP and green tea polyphenols observed by the blot in Fig 1C and Fig 2G?

10. Concerning the dark lysozyme band, this is a very important finding, but what evidence is there that this band is actually lysozyme? Are the authors aware of other examples of this non-specific artifacts with biotinylated 2ndary antibody-neutravidin-HRP complexes?
11. Consider changing the wording of the section; “due to a non-specific reaction between the secondary HRP-conjugated antibody” referring to the band in Fig 2D that I believe requires the secondary biotinylated antibody and the neutravidin-HRP conjugate.
12. In the conclusion the words “hyperactivated HRP” are mis-leading because there is no evidence of increased specific activity for the HRP so consider rewriting this sentence.

References

1. Plundrich NJ, Kulis M, White BL, Grace MH, Guo R, Burks AW, Davis JP, Lila MA: Novel strategy to create hypoallergenic peanut protein-polyphenol edible matrices for oral immunotherapy. *J Agric Food Chem.* 2014; **62** (29): 7010-21 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 May 2017

Mary Ann Lila, North Carolina State University, USA

Comments to the Author:

“The findings of this manuscript should be published because the implications of the HRP findings on current and past research could be widespread. The authors should be congratulated for taking the time and effort to examine the artifacts they observed rather than just ignoring them and moving on. In its current form, however there are some important points that need to be addressed in the manuscript to ease reader comprehension and focus the research on one topic or more clearly describe the findings and implications of the 2 topics in the manuscript. “

****What is the focus of the manuscript, the study of the effect of polyphenols on egg, or the artifact(s) resulting from the use of HRP?***

- ***The title of the manuscript suggests the manuscript is focused to point out a potentially very serious and mis-leading artifact of using HRP for western blot signal generation, but the content of the text is mixed between pointing out the findings of the egg/tea polyphenol study findings and the HRP artifact***
- ***I would argue that the HRP artifact is the primary purpose of the paper (as suggested in the title) and more in-depth discussion of the findings/implications is needed***

****Please consider re-writing the second paragraph of the introduction to sharpen the focus of the manuscript to coincide with the title...rather than the focus of green tea polyphenols on egg allergens.***

Answer: Thank you. We re-wrote the second paragraph of the introduction in consideration of these points, to emphasize that the HRP artifact is the primary purpose of sharing these research results.

****In the introduction some discussion and referencing of ‘ghost bands’ from past publications would be useful and possibly a discussion of the topic of reciprocity failure (if relevant here) in signal generation?***

Answer: Thanks. We have now included a sentence about previous studies.

****Would the ghost bands be expected to obscure ‘real’ bands nearby or migrating at the same pace?***

Answer: Thank you. Based on our observations, no. However, major proteins we investigated were well separated. We may not be able to exclude the possibility of “real” bands to be obscured by a (especially strong) “ghost” band close by and/or migrating at the same pace.

****Did the authors notice these artifacts in their own past publications on similar topics? Plundrich et al 2014¹. If so, this should be discussed and any discrepancies in their findings or conclusions that can be attributed to the HRP artifacts should be noted.***

Answer: Thank. Yes, this was observed in Figure 2 of the Plundrich et al. 2014 paper (soluble fraction, top of blot shows high molecular weight material that appeared as a “ghost” band/smear). It was also observed in the Plundrich et al. 2015 paper, Figure 3 B (peanut protein-cranberry polyphenol complex) above Ara h 2 in the digestive samples (appears that smeary lanes appeared somewhat as “ghost” bands. In both cases, however, this did not affect findings made and conclusions drawn. In addition, the same treatments were re-tested using a new protocol (fluorescence Western blotting) and the data was consistent with that previously reported. We now included a sentence about this in the discussion.

****Findings using peanut allergens and tea (or other sources of) polyphenols that lead to the same artifacts are important to point out.***

Answer: Thank you. Please see answer above.

****Can the authors find a related published article/examples of other groups that may have suffered from the same artifact and mislead the authors of that research to put their findings in the context of other using the same reagents?***

Answer: Thank you, this is a good question. At this time, we are not aware of any other studies that reported on similar artifacts such as those we found. After all, the detection method we used is one of many possible approaches.

****Could the authors please star/mark the bands that are considered “several additional proteins” that were detected with NBT staining but not CBB on Fig 1B?***

Answer: Thanks. We have now indicated those additional proteins and the respective sentence in the text slightly rephrased.

****Could the additional bands noted on Fig 1B represent oligomers/aggregates of the ovalbumin and ovotransferrin, and are these same bands present on the immunoblot?***

Answer: Thanks. The additional bands/smears observed are protein-polyphenol

complexes/aggregates that have been revealed by the NBT stain. Coomassie Brilliant Blue also stains proteins that are complexed with polyphenols (see smears in Figure 1 A), however, the NBT stain more sensitively stains proteins that have been modified by polyphenols. Those protein complexes are also present on the immunoblot, however, most of them appeared as “ghost” bands.

****Could you test directly the proposed interaction between HRP and green tea polyphenols observed by the blot in Fig 1C and Fig 2G?***

Answer: Thank you. This is a good question, and could be followed up on. We think green tea extract could directly be added to a PVDF membrane and similar experiments as in this study could be performed to test the direct effects between green tea polyphenols and HRP.

****Concerning the dark lysozyme band, this is a very important finding, but what evidence is there that this band is actually lysozyme? Are the authors aware of other examples of this non-specific artifacts with biotinylated 2ndary antibody-neutravidin-HRP complexes?***

Answer: Thanks. The tentative identification of lysozyme was based on literature. Lysozyme was the only protein found in the 15 kDa range (MW~14 kDa; Desert et al. J Agric. Food Chem., 2001, 49: 4553–4561). We are not aware of other examples of this non-specific binding. However, it is possible that the biotin-moiety of the secondary antibody was able to bind to lysozyme, as has previously been observed by Green et al. (Nature, 1968, 217: 254-256), although this group described weak interactions. It is also possible that the secondary antibody concentration used was high and resulted in non-specific binding to lysozyme when other reagents were omitted.

****Consider changing the wording of the section; “due to a non-specific reaction between the secondary HRP-conjugated antibody” referring to the band in Fig 2D that I believe requires the secondary biotinylated antibody and the neutravidin-HRP conjugate.***

Answer: Thanks. In fact, the “secondary HRP-conjugated antibody” refers to the “secondary biotinylated antibody that has been bound by neutravidin-HRP conjugate”. We have reworded the sentence to make it clear.

****In the conclusion the words “hyperactivated HRP” are misleading because there is no evidence of increased specific activity for the HRP so consider rewriting this sentence.***

Answer: Thank you. We agree and have reworded this sentence.

Competing Interests: No competing interests