Imperial Journal of Interdisciplinary Research (IJIR)
Vol-2, Issue-6, 2016
ISSN: 2454-1362, http://www.onlinejournal.in

Determination of the Proteins of Salmonella Enteritidis Involved in Colonization of Chicken Ceca 2-Analysis of the Degradation of *in vivo* Proteins Preparation of *S. Enteritidis*

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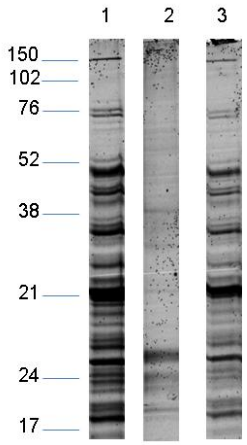
Abstract : *To overcome the problem of the degradation of in vivo proteins of S. Enteritidis harvested from the chicken gut and when the tri reagent kit was used for protein extraction, a very good extraction was obtained from in vitro proteins compared to reduced numbers of bands in the in vivo preparation visualized on SDS-PAGE. This revealed that this method of protein extraction did not improve degradation of in vivo Salmonella proteins. When the effect of washing was investigated, it was found that washing once, twice or three times did not affect the increase of bands in in vivo proteins. Also boiling had no effect as the in vivo proteins still appeared to be degraded indicating that heat sensitive proteases are not responsible for the in vivo protein degradation. Furthermore, using NP-40 as lysis buffer had no effect on the number of protein bands visible from in vivo preparation as no bands were detected on SDS-PAGE. Regarding the effect of mixing in vitro grown bacteria with non-infected cecal contents more than thirty bands were seen in the untreated invitro protein compared to only four bands seen in in vitro preparation from the in vivo protein treated with chicken cecal contents. This suggests that the contents of the gut adversely affect the quality of the protein profile and is likely to be responsible for the degradation.*

Key words: *S. Enteritidis*, caecal content, *in vivo*, *in vitro* protein, degradation, protease inhibitors, NP-40 lysis buffer, Tri-reagent kit.

Introduction

The previous section of this study revealed that the whole-cell protein profile of *S. Enteritidis* harvested from the chicken intestine was very different to that of bacteria cultured *in vitro* in nutrient broth. As visualised on SDS-PAGE, the *in vivo* protein extract had a lower protein content and a reduced number of visible protein bands; after protein extraction by sonication, and despite the fact that the amount of protein loaded was the same for both *in vivo* and *in vitro* extracts, very few protein bands from the *in vivo* bacteria were detectable on SDS-PAGE. Compared with more than thirty bands for the *in vitro* protein extract, only 2 to 4 bands could be detected for the *in vivo* extract, suggesting that most are degraded and disappeared off the gel. Therefore, it seemed important to attempt to overcome the issue of the degradation to be able to make a good comparison between the *in vitro* and *in vivo* protein profile of *S. Enteritidis*. For that reason various techniques have been used including Tri-reagent kit, additional washing prior to cell lysis, boiling of the *in vivo* preparation, using a different lysis buffer, and mixing *in vitro* culture with non-infected caecal contents,



‘’Figure 1. This figure is a Coomassie Blue Stained 1-D SDS-PAGE of whole Cell Sonicated Protein Preparations Prepared from S. Enteritidis Cultured either in Nutrient Broth
(Lanes 1 and 3) or Harvested from Chicken Ceca (lane 2), showing a Large Difference Between *in vitro* Profile with More Than Thirty Bands and only Few *in vivo* Bands.”

Experimental Plan

Several experiments were carried out in an attempt to inactivate the potential proteases and to investigate the issue of the potential degradation using different techniques including the use of a different lysis buffer, a different extraction technique, heating the *in vivo* sample to heat-inactivate any proteases, adding an additional washing step for the *in vivo* preparation and also mixing *in vitro* culture with uninfected caecal contents, with use of bacterial and mammalian protease inhibitor cocktail from sigma . All these experiments involved loading the same amount of *in vivo* and *in vitro* proteins for SDS-PAGE and staining the gel with Imperial Protein Staining from Per bio Science (cat no 24617) and scanning it by a Bio-Rad GS800 densitometer scanner**.**

Materials and Methods

Using of Tri-reagent Kit for Protein Extraction

Tri-reagent kit from Sigma (Cat T9424), which is a quick and convenient reagent for use in the simultaneous isolation of RNA, DNA and protein, from various biological materials, including samples of human, animal, plant, yeast, viral and bacterial source, was used for the proteins extractions from *S. Enteritidis* harvested from the chicken gut as well as *in vitro* grown bacteria in NB instead of sonication method according to the manufactures protocol.

Effect of Additional Washing

A single wash might not be sufficient and perhaps proteases present from the gut were inadequately removed so further washing steps have been used to investigate the effect of different washings on protein extraction. To do that the same additional washing steps were performed after diluting the caecal contents in PBS and prior to sonication.

Effect of Heating

The aim of this section was to consider the consequence of heating on the *in vivo* preparation, and inhibit any heat sensitive proteases in caecal material that might be involved in the process of degradation. To achieve that, cecal contents that were infected with *S. Enteritidis* harvested from chicken gut were diluted in PBS and boiled for 10 minutes prior to sonication.

Using NP-40 as Lysis Buffer Rather Than PBS

One additional option was to change the lysis buffer, using NP-40 instead of PBS, to asses if it would afford a more efficacious lysis of cells and solubilisation of proteins. NP-40 is a non-ionic polyoxyethylene surfactant that is frequently used as a component of cell lysis buffers or other solutions intended to extract and solubilise proteins. Proteins were extracted by sonication using the same protocol described in previous sections substituting the PBS with the NP-40 buffer.

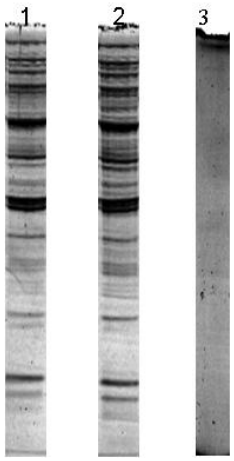
Mixing *in vitro* Grown Bacteria with non-Infected Cecal Contents

Unfortunately, after unsuccessful attempts to reduce the activity of suspected proteolytic degradation using the approaches described above we wanted to determine whether the proteolytic activity was associated with the chicken gut contents itself. To do this 2.5 ml of *S. Enteritidis* grown in nutrient broth culture were mixed with the same volume of uninfected chicken cecal contents then the proteins were extracted from this mixture.

Results

Using of Tri-reagent

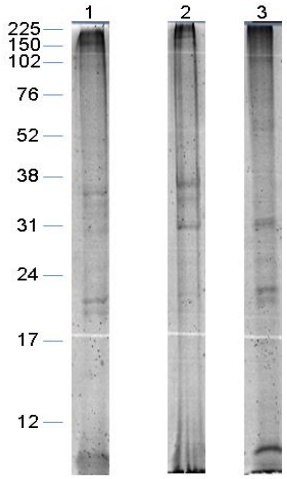
To overcome the problem of the degradation of *in vivo* proteins of *S. Enteritidis* harvested from the chicken gut, the Tri-reagent kit from Sigma (Cat T9424), a standard reagent for nucleic acid and protein preparation was used according to the manufacturer's instructions. This kit was used at first for extraction of *in vitro* proteins and extracted proteins were used to run SDS-PAGE followed by Coomassie blue staining which demonstrated that a very good extraction was obtained from an *in vitro* preparation as most of the *in vitro* protein band (more than thirty) were detected; while when Tri-reagent kit was employed for proteins extraction from *in vivo* grown bacteria in chicken, as caecal contents were harvested directly into Tri-reagent, the protein profiles obtained were very similar with reduced numbers of bands in the *in vivo* preparation, since the majority of the *in vivo* protein bands still could not be visualized on SDS-PAGE as shown in Figure 2. This revealed that the use of this method to extract the proteins from *in vivo* grown bacteria in chicken did not improve degradation of *in vivo Salmonella* proteins.



"|Figure 2**.** Coomassie Blue Stained Gel of *E*nteritidis Protein Preparation Extracted Using Tri-reagent kit, Showing that the Use of this Technique for Protein Extraction had no Effect on Increasing Protein Bands which were Visible for *in vivo* Preparation (lane 3),compared with *in vitro* proteins (lane 1 and 2)".

Effect of Additional Washing

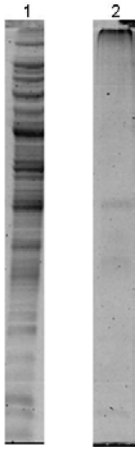
As mentioned in materials and methods section above, *S. Enteritidis* proteins were extracted from infected chicken cecal contents by sonication using different washing times and after measuring the proteins concentration the same protein amount from each preparation were loaded in SDS-PAGE and the result as illustrated in Figure 1 indicated that only few bands (2 – 5) of *in vivo* protein could be detected compared with the majority of *in vitro* bands (more than thirty), could be clearly visualized in a Coomassie blue stained gel.



"|Figure 3. Coomassie Blue -Stained Gel of *E*nteritidis *in vivo* Protein Preparation, Washed Three Times in PBS Before Sonication (lane one), washed twice (lane 2) and once (lane 3); which Indicated that Additional Washes had no Effect on Increasing the Number of Visible *in vivo* Protein Bands".

Effect of Heating

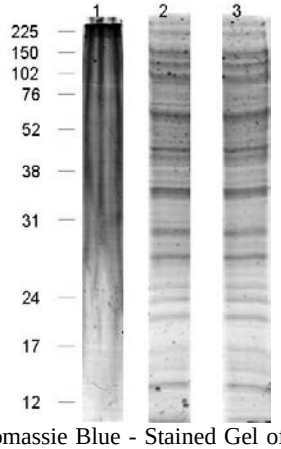
As illustrated in Figure 4 below the result showed that boiling had no effect as the *in vivo* preparation still appeared to be degraded and most of the bands could not be distinguished; compared with the *in vitro S. Enteritidis* proteins only about 5 bands were seen out of more than thirty bands of *in vitro* proteins which were detected



"Figure 4. Coomassie Blue- Stained Gel of Boiled *S. Enteritidis* Sonicated Proteins *in vitro* Preparation (Lane 1), and *in vivo* Preparation (Lane 2), Suggesting that Heat - Sensitive Proteases are not Responsible for the degradation of the *in vivo* Proteins".

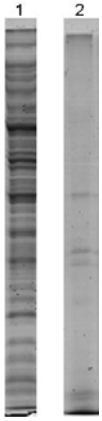
Using of NP-40 as a Lysis Buffer

.Figure 5 showed that using NP-40 also had no effect on the number of protein bands visible from the *in vivo* preparation. This preparation was poorly stained. However, in comparison with the more than thirty bands seen in the *in vitro* preparation none at all was visible from the *in vivo* preparation, despite of loading same protein amount.



"Figure 5. Coomassie Blue - Stained Gel of *S. Enteritidis* Proteins Showing that Using NP-40 as Lysis Buffer had no Effect on the Number of Protein Bands Visible from the *in vivo* Preparation (Lane 1), Compared With More Than Thirty Bands Seen in the *in vitro* Preparation Lanes 2 and 3) Nothing at all was Visible from the *in vivo* Preparation."

Mixing *in vitro* Grown Bacteria with Non-Infected Cecal Contents



One hypothesis was that, if proteolytic digestion is occurring with the *in vivo* samples, then this is the result of proteolytic activity arising from the chicken intestine. In comparison with the more than thirty bands visible in the untreated *in vitro* protein preparation, only four were visible in the preparation from the *in vitro* protein treated with chicken caecal contents as illustrated in Figure 6. These data do suggest that the content of the gut adversely affects the quality of the protein profile and is likely to be responsible for the degradation.

"Figure 6: Coomassie Blue Stained Gel Showing that only 4 Bands were Visible in the Preparation from the *in vitro Enteritidis* Protein Treated with Uninfected Chicken Cecal Contents (Lane 2), in Comparison with the More Than 30 Bands Visible in the Untreated *in vitro* Protein Preparation (Lane 1)."

Discussion

Different techniques were used to defeat the issue of degradation of the *in vivo S.* Enteritidis proteins loading same protein amount into SDS-PAGE to be able to make a logical comparison between *in vitro* and *in vivo* protein profiles.

When protein extracted from *in vivo* cultured *S. Enteritidis* in chickens were compared with that of *in vitro* grown bacteria in NB, fewer bands were detectable in the *in vivo* preparations. Although the levels of bacterial gene expression in chicken gut are in general known to be lower than *in vitro* (Barrow et al, unpublished results), the concentrations of the proteins obtained from both *in vivo* and *in vitro* preparations were very similar (data not presented). It seemed likely that enzymic degradation of the *S. Enteritidis in vivo* protein preparation was taking place during preparation as a consequence of contact with the contents of the chicken cecal component. When cecal contents were harvested directly into Tri-reagent, the protein profiles obtained were very similar with reduced numbers of bands in the *in vivo* preparation. Other approaches used to attempt to improve the number of protein bands obtained included (i) using an increased number of washes of bacterial suspension before sonication (ii) using NP-40 as lysis buffer (Berndt et al, 2007), (iii) heating the bacterial suspension at 100^{o}C for 10 minutes and (iv) adding bacterial and mammalian protease inhibitors (Sigma) to the bacterial suspension prior to sonication. None of these had any effect on the poor quality of the bacterial protein sample obtained from the ceca in comparison with the *in vitro* preparation. It seemed likely that heat-resistant avian proteases were responsible as when an *in vitro* protein preparation was mixed with cecal contents from uninfected birds in equal volumes for 10 minutes followed by sonication, the *in vitro* protein preparation, which normally produced many (more than thirty) clear bands, more closely resembled the *in vivo* preparation with a very small number of indistinct bands proving that contact between the *in vitro* protein preparation and the caecal contents of the chicken resulted in degradation of the bacterial protein. Unfortunately, the target have not been achieved, as the results of this investigation confirmed that the proteins of *S. Enteritidis* harvested from chicken ceca are still most likely to be degraded compared with the protein of *in vitro* grown bacteria in nutrient broth; this is compatible with the result previously obtained by P. Barrow who carried out a similar experiment comparing the *in vitro* and *in vivo* proteins profile of *S. Typhimurium* in chickens, (Paul Barrow unpublished data and personal communication). These results may not also be concurred with the results obtained by David O'Connor and others when they compared the proteome of *in vivo* and *in vitro* grown *S.* Typhimurium and stated that more proteins are down-regulated *in vivo* than up-regulated (David O'Connor et al 2006). What they have got might be because of the degradation of the *in vivo* proteins as most of *the latters* were not represented for the reason of degradation. Moreover, the degree of up-regulation is generally less than the degree of down-regulation *in vivo*. Thus, protein synthesis seems to be lower *in vivo*, as perhaps would be expected in an anaerobic environment.

This might also give further explanation for the results obtained during this work, as disappearance of the majority of the *in vivo* proteins bands from SDS-PAGE could be caused by poor protein synthesis under anaerobic condition (in chicken) and not degraded as have been mentioned at some point of this study. However,, further work focusing on identifying the heat-resistant proteases present in the chicken gut can be carried out to overcome the issue of degradation and to know which proteases are present in our sample (caecal

contents) can be performed. This may include use of ProteSEEKER^{TM} kit from GBiosciences which is designed to identify the specific type of proteases present in a cell or tissue lysate.

In our case the general protease inhibitor cocktails might be inefficient resulting in degradation of S Enteritidis *in vivo* proteins as shown in gel electrophoresis compared with the *in vitro* proteins. To overcome this, two choices can be utilized : one is to increase the amount of general protease inhibitor cocktails used, which is very cost ineffective, the second option is to identify the elevated protease and then supplement our extraction or lysis buffer with extra protease inhibitor specific for that protease; this technique was not performed because of time limitation and expense.

Conclusion

To investigate the problem of the degradation of in vivo proteins of *Salmonella Enteritidis* harvested from the chicken ceca various attempts were used .

The study revealed that the degradation observed in this *in vivo* protein preparation obtained from chicken ceca is due to the effect of the contents of the gut which adversely affect the protein profile.

References

[1] Berndt, A., Wilhelm, A., Jugert, C., Pieper, J., Sachste, K., & Methner, U., 2007. "Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness". *Infect Immun*, 75, 2007, pp 5993-6007.

[2] David O'Connor et al, Proteomics, 2006