

Production of Human S100A8 and S100A9 Proteins for Characterization of Their Roles as Danger Signals

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Original title: Expression and Purification of Recombinant Human S100A8 and S100A9 for Characterization of Their Roles as Danger Signals

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S100A8 and S100A9 are two proteins that have become the focus of many current researches since they seem to play a central role as danger signals in many human diseases, activating various cell signalling pathways via receptors. The initial events during binding of the proteins to their receptors, however, have yet to be clarified. It is therefore of interest to produce S100A8 and S100A9 in order to characterize their interactions with especially TLR4 (Toll-Like Receptor 4) and RAGE (Receptor for Advanced Glycation Endproducts). This study proposed a second approach to produce human S100A8 and confirmed binding activities of the proteins using in vitro characterization with Surface Plasmon Resonance and with NF- κ B Luciferase Reporter Assay.

Introduction

The direct involvement of S100 calcium-binding proteins A8 (S100A8) and A9 (S100A9) to numerous human diseases have been discovered more than 20 years ago, where increased levels of both proteins have been found in inflammation and various cancers. Both S100A8 and S100A9 are expressed mainly by phagocytes, and are abundant in the cytosol. *In vivo*, they can form both homo- and heterodimers, with the latter being the most predominately occurring form with higher stability [1]. When phagocytes are activated by stress responses, both proteins are up-regulated and secreted to the extracellular compartment by a non-classical secretory pathway [2]. Increased levels of S100A8 and S100A9 as homo- and

heterocomplexes become crucial danger signals during inflammatory processes in infection, autoimmunity and cancer. The complexes are recognized by sentinel cells (danger-sensing cells) by acting as endogenous activators of TLR4 [3][4] and RAGE [5], leading to amplified inflammatory reactions and activation of various cell-signalling pathways. One important signalling pathway that is activated is the NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B cells) signalling pathway. The initial events during binding of the proteins to their receptors, however, have yet to be clarified. Hence, it is of great interest to produce these proteins and study their binding activities to especially TLR4 and RAGE in order to further define the molecular mechanisms.

Method and Results

Production of S100A8 and S100A9

To be able to study the binding activities of human S100A8 and S100A9 to TLR4 and RAGE, the S100 proteins had to be produced. Both S100A8 and S100A9 were expressed as recombinant proteins in its wild-type form (wt-rhS100A8 and wt-rhS100A9) and eventually wt-rhS100A8/S100A9 heterocomplexes were formed of the two. A second approach was also to produce S100A8 fused to a His₆-tag (His₆-rhS100A8) and compare this with the wt-rhS100A8 in terms of expression levels, purification and product yield, in order to find the better approach. The His₆-rhS100A8 showed to be more superior in all three aspects, requiring only one-step purification whereas the wt-rhS100A8 could not be purified without major protein loss, yielding no products [6]. In addition, the wt-rhS100A8 were extremely unstable compared to the fused protein, and were fragmented whenever exposed to air. A suggestion is that, due to the fact that wt-rhS100A8 is extremely prone to oxidation, it becomes fragmented.

An ambition was also to cleave off the His₆-tag with enzymatic cleavage and study the protein with the uncleaved protein, but since the chosen methods resulted in unsuccessful cleavage, a suggestion is to find other approaches and attempt further trials.

Qualitative analyses of the produced proteins were performed prior to characterization, using Western blot for identity analysis, SDS-PAGE for purity analysis and LC-MS

(Liquid Chromatography-Mass Spectrometry) to determine exact masses. The produced proteins could be identified with monoclonal antibodies (mAb) that specifically recognize human S100A8 and S100A9. The purity was >90-95% and the exact masses were close to the theoretical mass, differentiating with only 1-2 Da.

In vitro Characterization

The *in vitro* characterizations of the produced S100A8 and S100A9 were performed to study their binding activities to TLR4-MD2 complex and RAGE. Two different systems were used; one with Surface Plasmon Resonance (SPR) studies and one with NF- κ B Luciferase Reporter Assay.

The SPR studies were performed on a Biacore instrument that utilizes chips immobilized with TLR4-MD2, RAGE and mAbs specifically recognizing human S100A8 and human S100A9. This study demonstrated that His₆-rhS100A8 exhibited high binding activities to TLR4-MD2 and also RAGE, which has previously not been confirmed (see *Figure 1A and 1B*) [6]. In addition, it was also recognized by the mAb, which suggests that the identity of the protein is correct (see *Figure 1C*). The wt-rhS100A8/S100A9, on the other hand, showed no or very low signals compared to the homocomplex S100A9 both over TLR4-MD2 and RAGE (data not shown). This suggests that the heterocomplex have much lower binding activities than the homocomplexes.

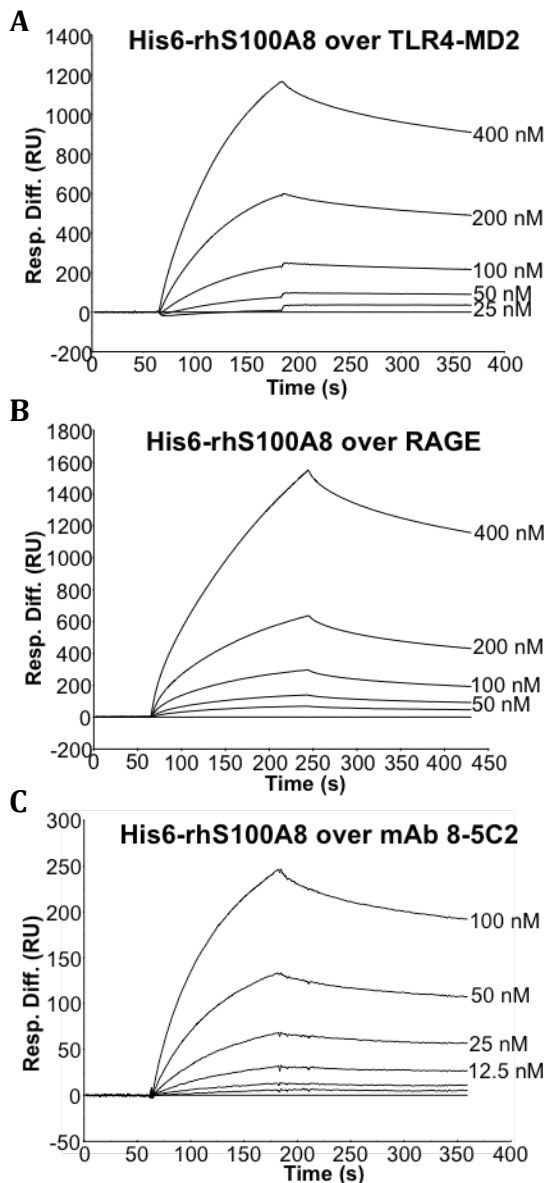


Figure 1. Bindings of His₆-rhS100A8 in Biacore SPR over immobilized (A) human TLR4-MD2 (B) human RAGE and (C) monoclonal antibody (anti-human S100A8). Responses are measured as Response Units (RU) over time.

The NF- κ B Luciferase reporter assay is a cell-based *in vitro* system that was used to measure the binding activities of S100A8, S100A9 and S100A8/S100A9 to TLR4-MD2 as activation of the NF- κ B cell-signalling pathway. The assay demonstrated binding activities of the endotoxin-reduced wt-rhS100A8/S100A9 heterocomplex to TLR4-MD2 (see

Figure 2) [6] as well as with His₆-rhS100A8 (data not shown), although the data over His₆-rhS100A8 is highly questionable due to the likelihood of endotoxin influences (experiments with endotoxin-reduced proteins are strictly necessary since endotoxins are also able to activate the NF- κ B cell-signalling pathway, resulting in extra false responses).

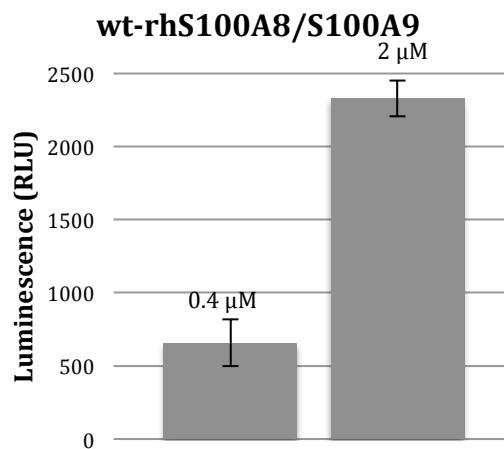


Figure 2. Measured Relative Light Units (RLU) by NF- κ B Luciferase reporter assay as a result of incubation of rhS100A8/S100A9. Data have been adjusted from a blank signalling response of 760 RLU.

Conclusion and Future Prospects

For the production of human recombinant S100A8, the recommendation is to continue expressing the protein fused to a His₆-tag as this has shown to be more superior in terms of expression levels, purification and protein stability compared to the wild-type form. The attempts to cleave off the tag was however unsuccessful with the chosen method. The recommendation is to find other methods as it would be interesting to know if there are any effects of the His₆-tag to S100A8,

which in its wild-type form is known to be unstable and extremely prone to oxidation [7].

The *in vitro* characterization of His₆-S100A8 and wt-rhS100A8/S100A9 confirmed: i) binding activities of His₆-S100A8 to TLR4-MD2 and RAGE with SPR studies and ii) cell-based binding activities of wt-rhS100A8/S100A9 to TLR4 with NF-κB Luciferase reporter assay.

The project also confirmed that S100A8 and S100A9 have high binding activities as homocomplexes, but when S100A8 and S100A9 exist as heterocomplex, the binding activities seem to be drastically decreased.

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Abbreviations

LC-MS	Liquid-chromatography mass-spectrometry
mAb	Monoclonal antibody
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
RAGE	Receptor for advanced endoproteins
rh	Recombinant human
RLU	Relative light unit
RU	Response unit
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TLR4	Toll-like receptor 4
wt	Wild-type

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