Phosphorylation of breast-milk $\alpha_{s1}$-casein induced conformational changes and abolished TLR4-agonisticity as well as formation of fibril structure

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Phosphorylation of breast-milk $\alpha_{s1}$-casein induced conformational changes and abolished TLR4-agonisticity as well as formation of fibril structure.
Abstract: Breast-milk α_{s1}-casein is a Toll-like receptor (TLR4) agonist which induced proinflammatory cytokine secretion. Phosphorylated α_{s1}-casein (P-α_{s1}-casein) is non-agonistic. The objective of this study was to analyze structural characteristics underlying these observations. Recombinant α_{s1}-casein was shown to exist in two conformations, an α-helical TLR4-agonistic conformation and a non-agonistic conformation with lower α helical and higher random coil content. TLR4-agonistic α_{s1}-casein conformation was found at a pH-range between 7.4 and 2. α_{s1}-Casein bound itself (KD-value: 2 µM) formed large aggregates (between Ø 73 nm [pH7] and Ø 826.2 nm [pH2]). Using Thioflavin T assay and atomic force microscopy showed that α_{s1}-casein adopted fibril-like structure. P-α_{s1}-casein was observed in a less α helical conformation, not inducing IL-8 secretion. P-α_{s1}-casein bound itself stronger (KD-value: 0.5 µM) than α_{s1}-casein and did not form fibrils. In conclusion, TLR4-agonistic and non-agonistic conformations of α_{s1}-casein could be differentiated. It was demonstrated that human caseins are able to adopt fibril structure. These kind of structures are often disease related. We postulate, that phosphorylation could be a switch of two conformations regulating immunomodulatory effects of human α_{s1}-casein especially in immune system development.

Keywords: Breast milk; human α_{s1}-casein; TLR4 agonist; fibril structure, CK2.
Human $\alpha_{s1}$-casein

Expressed in:
- Breast- and prostate cancer
- Synovia of patients (arthritis)
- breast milk (functional food)
  transport of molecules, minerals
  induces life long IgG response

- $\alpha_{s1}$-casein bound TLR4-receptors
- In vitro phosphorylated $\alpha_{s1}$-casein
  did not bind TLR4-receptors

Is there a structure-function relationship for $\alpha_{s1}$-casein activating TLR4?

Phosphorylation of $\alpha_{s1}$-casein abolished this.
**In silico predicted structure and in vitro analysis α_{S1}-casein**

- **(intrinsically) disordered**
  - flexible structure, known phosphorylation site

- **ordered**
  - conserved structure, less accessible

**N-Terminus**
- S41
- S33
- R16

**C-Terminus**
- W185

- **partial α-helical structure**
  1. ratio (222 nm/208 nm) of 0.74
  2. maxima at 1661 nm

- **high intensity for used concentration (CD)**
  - **result of multimerization?**

- **maxima at 1625 nm (β-sheet?)**

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α₅₁-casein binds itself?

(intrinsically) disordered flexible structure, known phosphorylation site

ordered conserved structure, less accessable

K98

N-Terminus

R16

S33

S41

S89

C-Terminus

W185

+DTT (mM)

+SDS (%) 0,2 2 10 100

<table>
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<tr>
<th>kDa</th>
<th>170</th>
<th>70</th>
<th>55</th>
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<th>35</th>
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<th>25</th>
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 oligomere
 trimer
 dimer
 monomer

95 °C

Microsale Thermophoresis

- Homomers
- „K_D-value: 2.2 µM“
- Diameter of particels: 73.4 nm (PI: 0.6)

α₅₁-casein binds to itsself
Correlation of α-helical structure and effects via TLR4

IL-8 secretion via TLR4
- RT, pH7: yes
- 95 °C: no
- Phosphorylation: no
- pH2: yes

- αS1-casein had higher α-helical content at RT (pH7 and pH2) than phosphorylated and heated one
Difference in binding of $\alpha_{S1}$-casein to itself

- Phosphorylation could be a mechanism to control multimerization
- Unphosphorylated: slower, structured
- Phosphorylated: faster, unstructured

**SPR**

- Phosphorylated: 0.5 µM
  - Higher affinity, faster binding to itself
- Unphosphorylated: 2 µM

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β-Sheet content and multimerization hint that $\alpha_{S1}$-casein could form fibril structures

- Unphosphorylated $\alpha_{S1}$-casein formed fibrils (shown by Thioflavin T Assay and AFM)
- Phosphorylated $\alpha_{S1}$-casein did not form fibrils, but aggregates.
Conclusions

- $\alpha_{s1}$-casein was shown to have two conformations, an $\alpha$-helical TLR4-agonistic and a non-agonistic conformation with lower $\alpha$-helical content.
- Phosphorylation of $\alpha_{s1}$-casein as well as incubation at 80 °C led to the non-agonistic conformation.
- $\beta$-Sheets and aggregation allowed us to identify fibril-like structures of specifically for $\alpha_{s1}$-casein by ThT-assay and AFM.
- phosphorylation could be a switch between two conformations of $\alpha_{s1}$-casein regulating immunomodulatory processes of the immune system.
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