ENGINEERING OF DAIRY PROTEINS AND THE MODULATION OF THEIR STRUCTURES -

MICELLISATION AND IMMUNO REACTIVITIES OF DIMERIC BETA CASEINS.

Thomas Haertlé

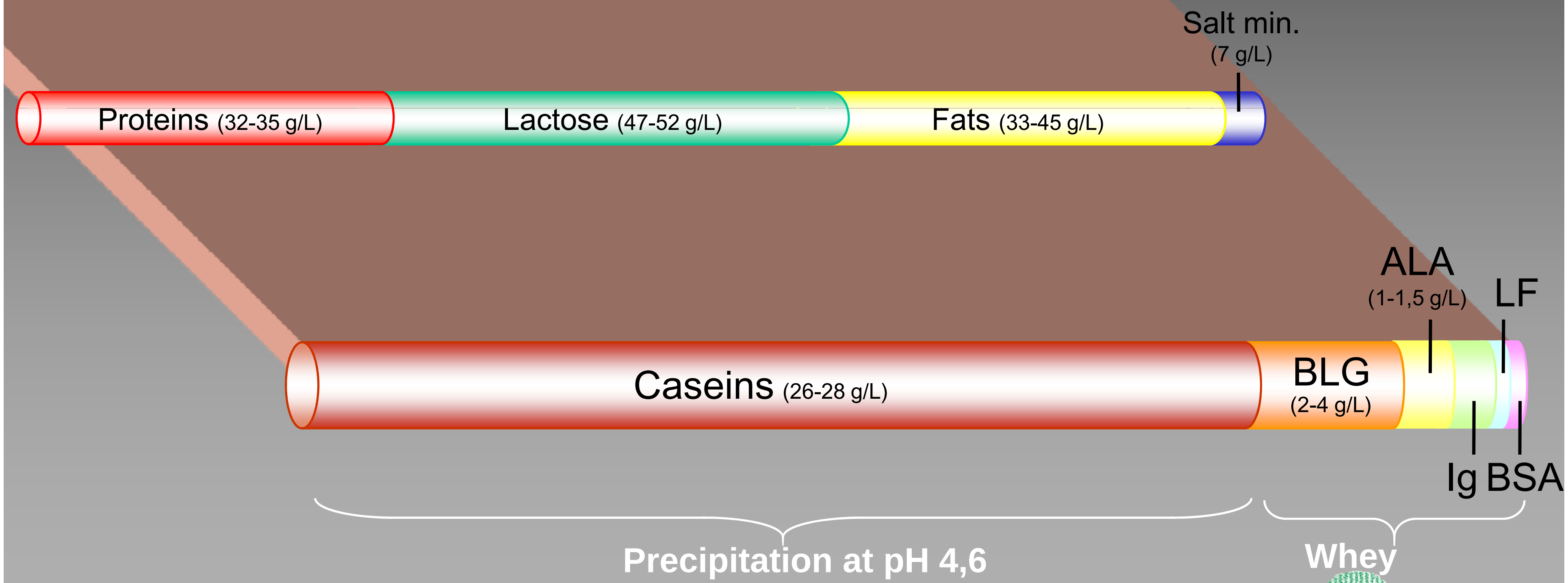
Institut National de la Recherche Agronomique, Nantes, France

Importance of milk

- Essential food of newborn (nutrition, immune protection, enzymes).
- Cow, goat and sheep in Western countries.
  Mare, water buffalo, camel, yak…in Eastern countries.
- Consumed without or after transformation (cheeses, fermented milks).
- Of great importance for food industries of each human society.

Average composition of cow milk

- Synthetisised by secretory epithelial cells (synthesis of components or their capture from blood)
- 12-13 % of dry matter (120-130 g/L)

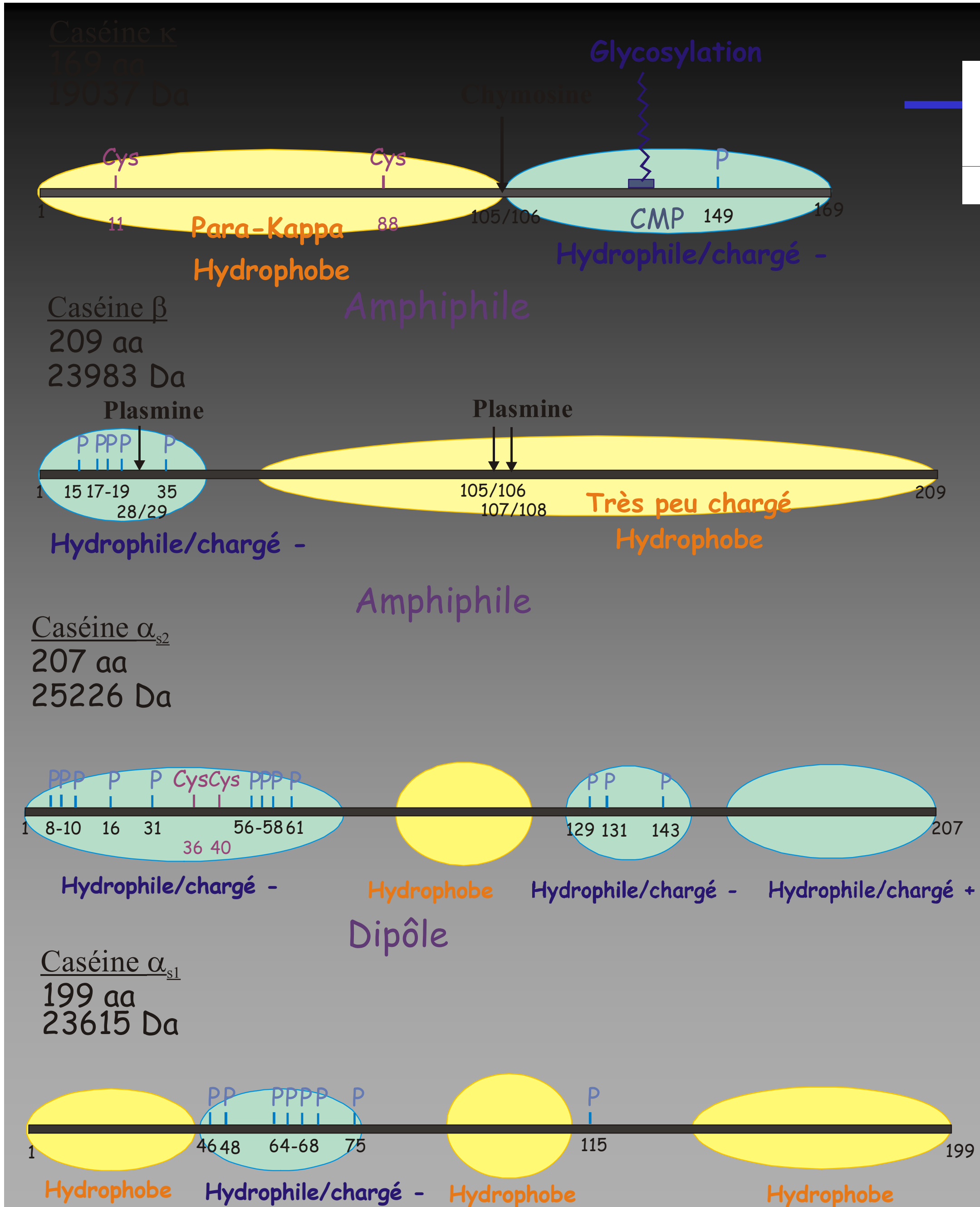


Bovine caseins

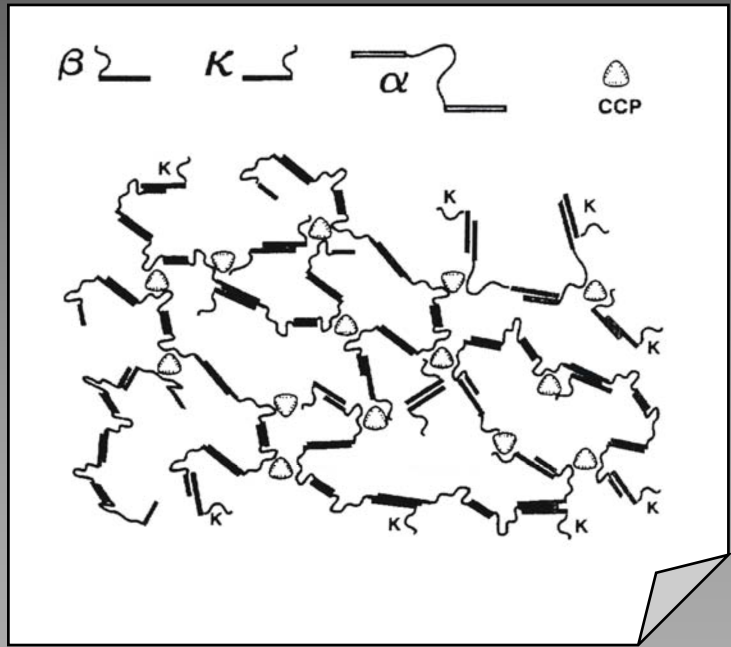
Casein	αS1	αS2	β	κ
AA	199	207	209	169
MM (kDa)	23,6	25,25	24	19
Variants	5	3	6	6
Cys	0	2	0	2
S-S Bridges	0	0	0	0
Ser-P	8	11	5	2
Glycosylation	No	No	No	Yes
Major ep.	6	4	6	8
Minor ep.	3	6	3	0

- Unknown three-dimensional structures
- Unknown functions (nutritional, only ?)
- Quite often allergenic.

Physico-chemical properties of caseins



Casein micellisation according to Holt (1998)



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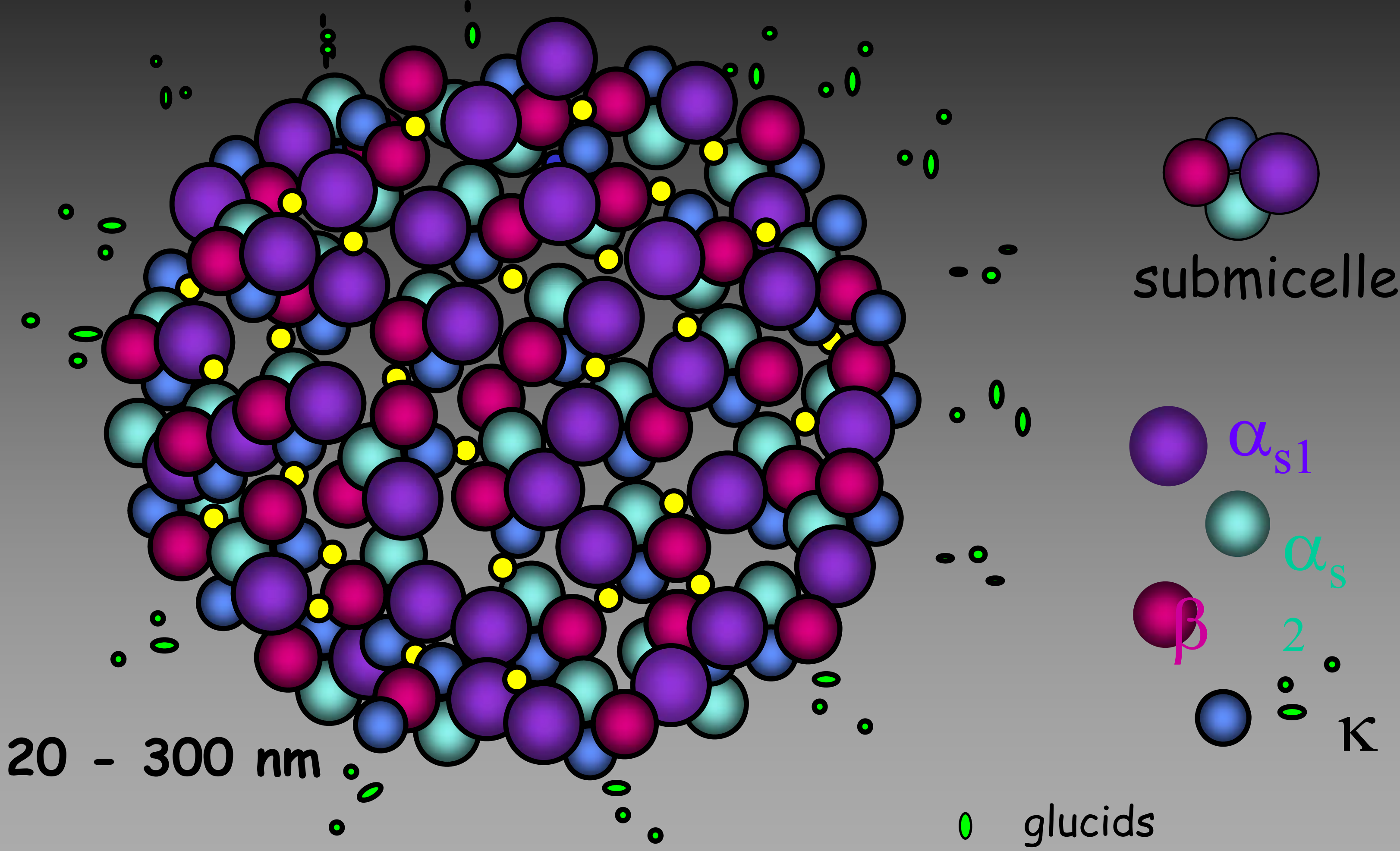
Casein micelle



CCP Colloidal Calcium Phosphate

(Schmidt, 1982 ; Walstra, 1984)

β casein

P P P P P

MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
PIQAFLLYQE PVLGPVRGPF PIIV

❖ Phosphorylation ➔ calcium fixation (binding of other caseins by phosphocalcic bridges)

❖ Hydrophilic N-terminus (several negative charges)

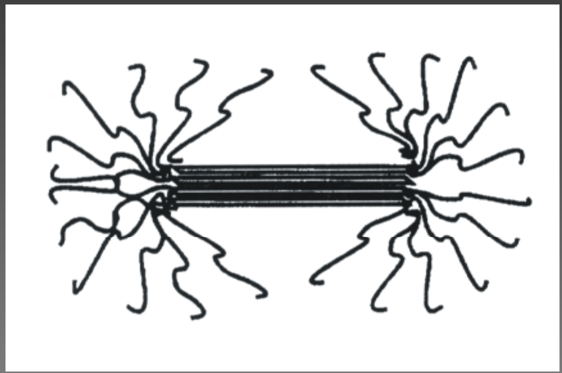
❖ Long hydrophobe C-terminal sequence

➢ AMPHIPHILIC PROTEIN

➔ structures polyproline II

❖Numerous prolines (35) ➔ scarce classic secondary structures (β sheets, α helices …)

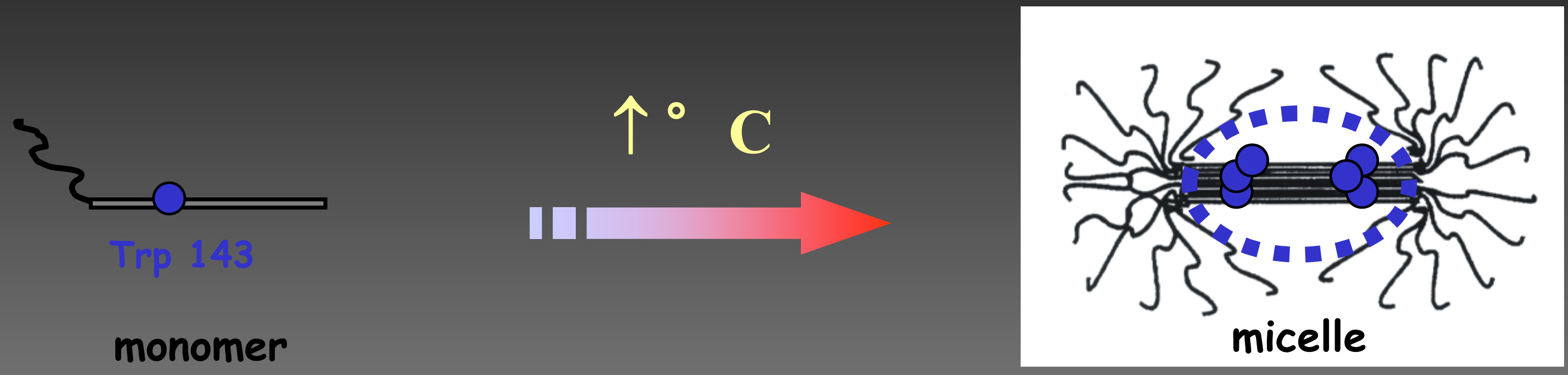
Self-association of β casein

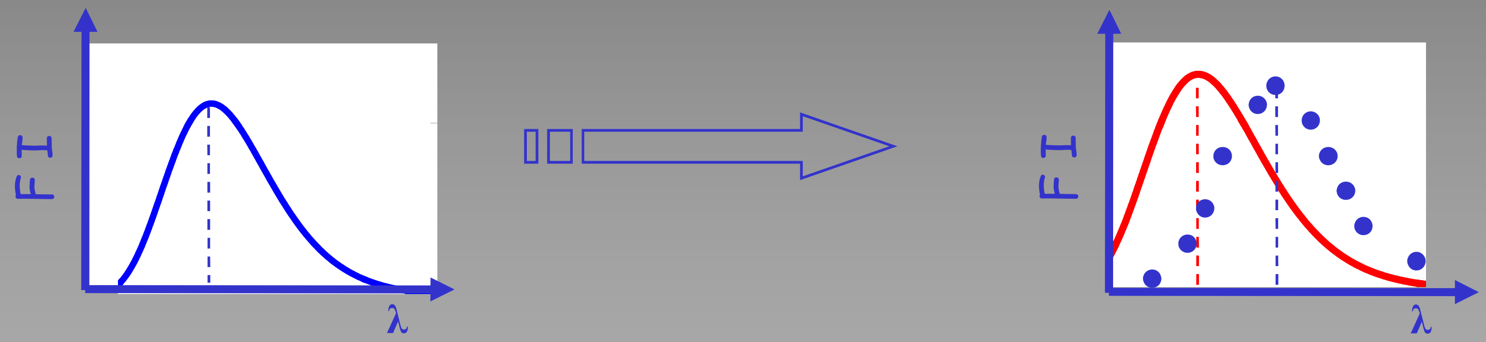


- Micellar aggregates
- Temperature and concentration dependent

T (°C)	N	R_h (nm)	Methods	References
14	20	10		Andrews *et al.*, 1979
	41–50		LS	Payens *et al.*,1963
	30		Sedimentation	Farrell *et al.*, 2001
	38–40	15	SANS–LS	Thurn *et al.*, 1987
	51	15.4	SAXS	Kajiwara *et al.*, 1988
	48		LS	Sood *et al.*, 1990
	47	12	SANS	Sood *et al.*, 1997
70	70		SANS	Leclerc *et al.*, 1998
4.5–70	5–73	13.5	SANS	Leclerc *et al.*, 1997

Intrinsic Fluorescence of tryptophan (1)





λ_{max} Trp = 341 nm

λ_{max} Trp = 336 nm

Enginering of β casein

Recombinant β casein, wild type, expressed in *E. coli* : WT

Recombinant β casein, mutated, expressed in *E. coli*: MU

```
    MRELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ
TEDELQDKIH PFAQTQSLVY PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP
EVMGVSKVKE AMAPKHKEMP FPKYPVEPFT ESQSLTLTDV ENLHLPLPLL
QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY PQRDMPIQAF
LLYQEPVLGP VRGPFPIIV
```

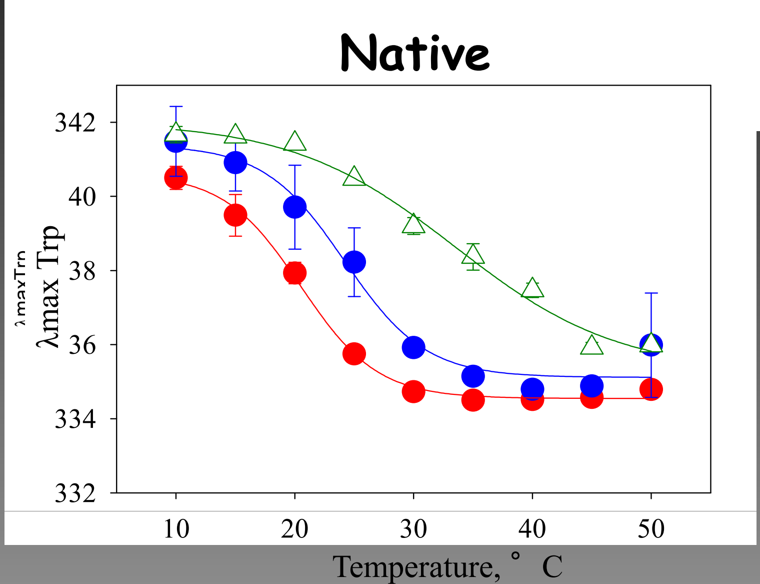
❖ Sequence containing three glutamates well conserved in ***Ruminants***

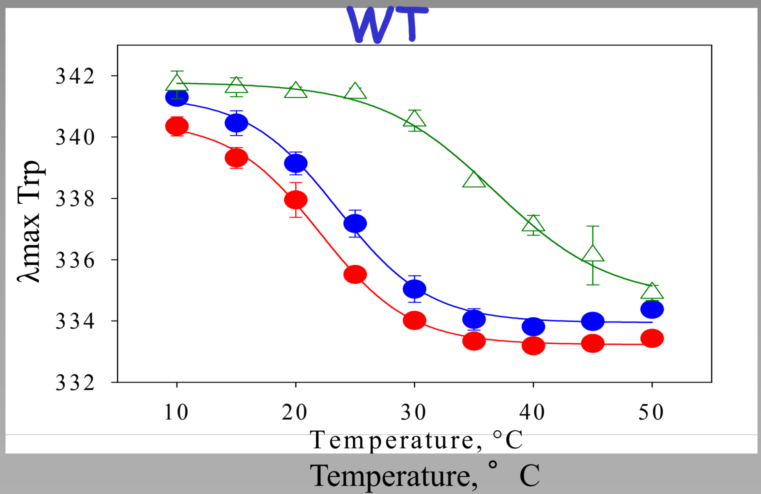
❖ Addition of three negative charges: change of hydrophilic/hydrophobic ratio

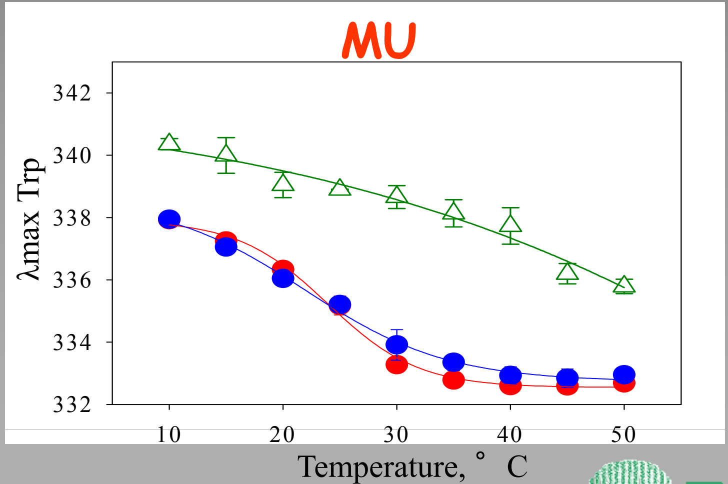
Intrinsic Fluorescence of Tryptophan (1)

Same conditions as for DLS.

- 1 mg/mL
- 0,4 mg/mL
- 0,2mg/mL







Beta casein native

$^{+}_{3}$HNeRELEELNVPGEIVE**S**L**SSS**EE**S**ITRINKKIEKFQ**S**EEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS
LPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQ
SWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
COO^{-}

Beta casein simple mutant **C4**

$^{+}_{3}$HNeMGRELE**C**LNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTP
VVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSL
SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV COO^{-}

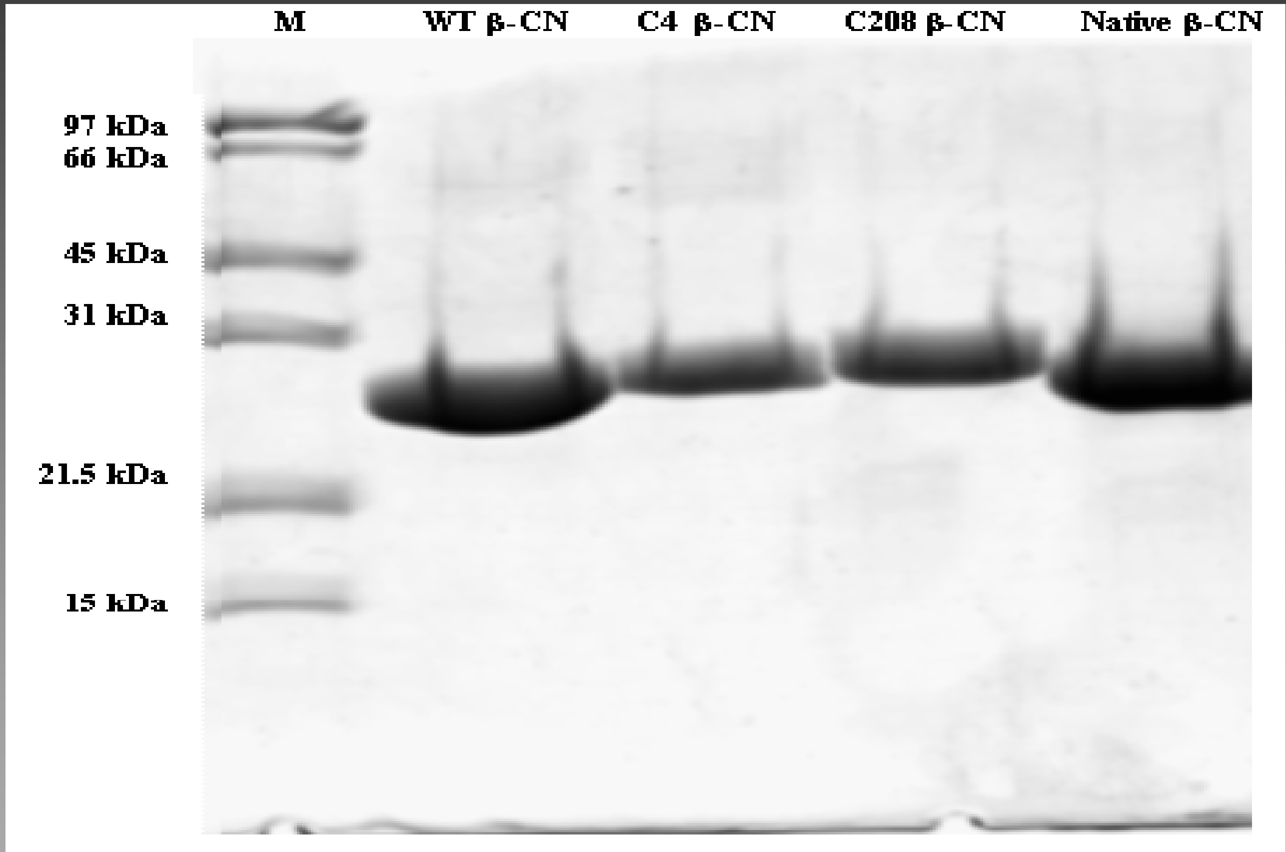
Beta casein simple mutant **C**208

$^{+}_{3}$HNeMGRELEKLNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPV
VVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLS
QSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPI**C**V COO^{-}

Beta casein double mutant **C**4 - 208

$^{+}_{3}$HNeMGRELE**C**LNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTP
VVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSL
SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPI**C**V COO^{-}

SDS-PAGE of bovine β casein and mutants.



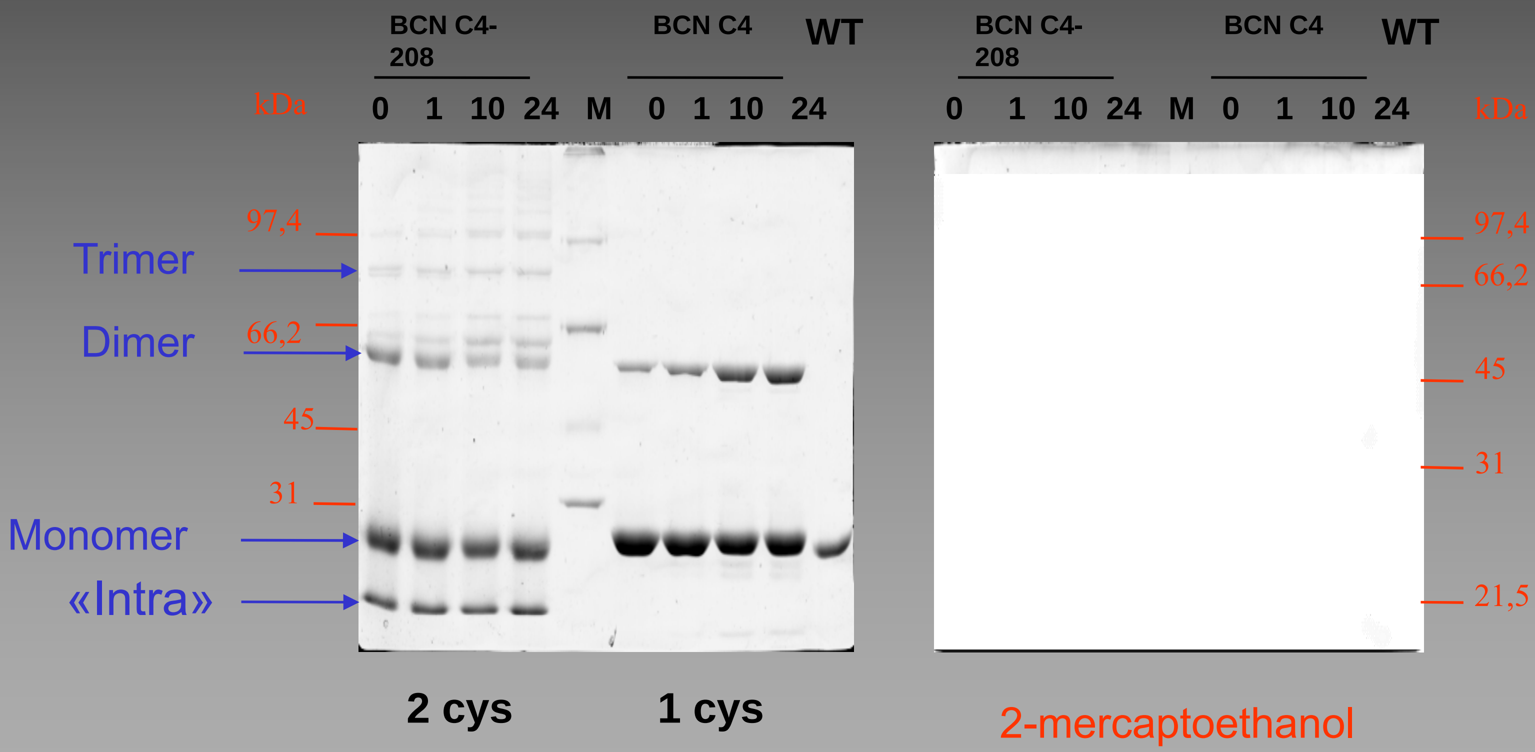
Experimental condition: SDS-PAGE (12.5 % gel), under reducing conditions.

Coomasie blue staining.

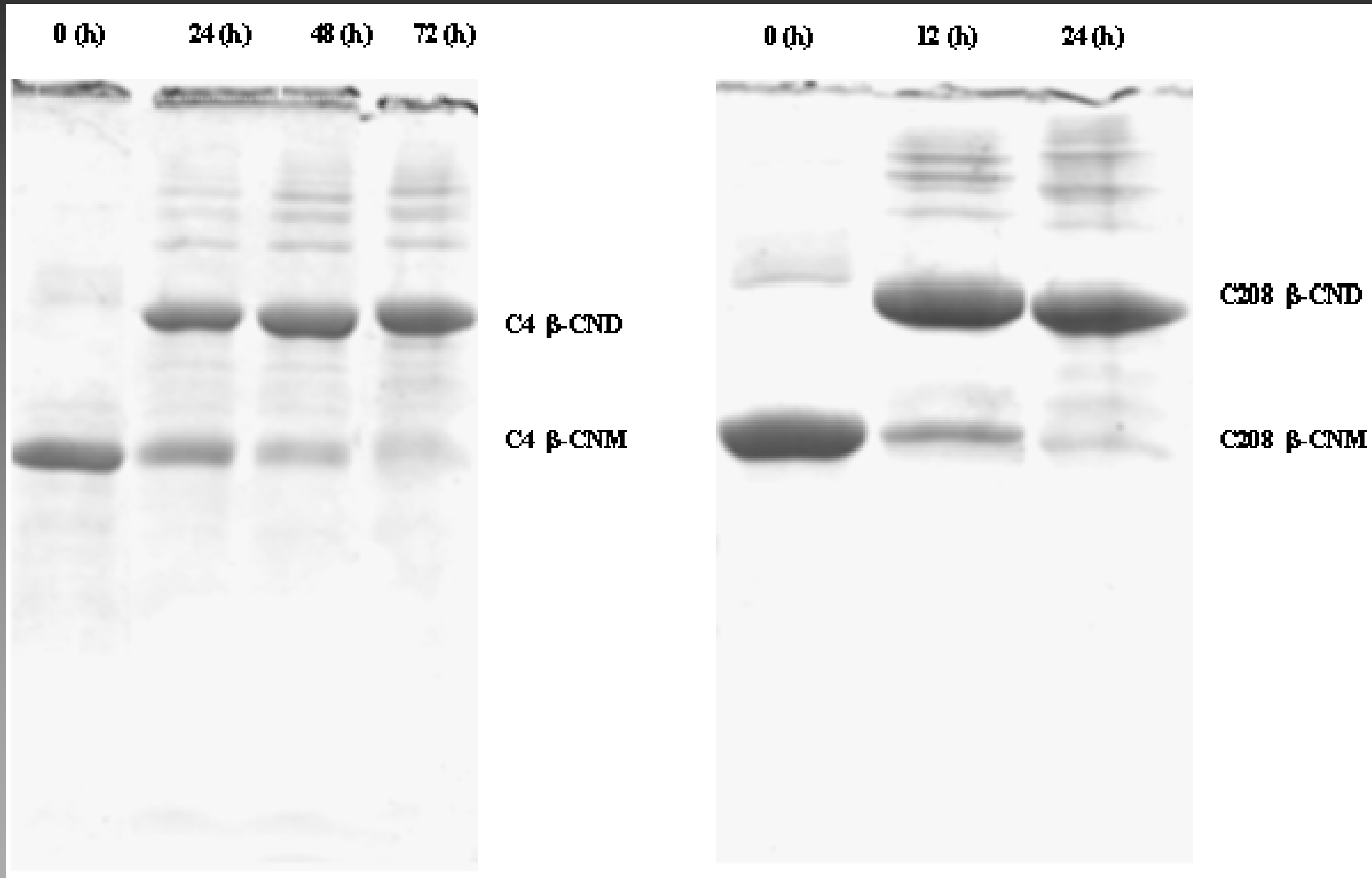
Oxydation

24 h at 25° C pH 6,5

SDS-PAGE



SDS-PAGE of the dimerizing β casein mutants



Condition: Tris buffer 50 mM, pH 8.2 including 80 mM NaCl and H_2O_2, 37 ° C, stirred, sterile.

- Reasons for quicker dimerization kinetics of C208 ß-CNs

✂ ☑ Greater mobility of C-terminal segment
✂ ☑ Easier access of SH function
✂ ☑ Hydrophobic interactions

- **The dimerization sites in C6- and C-208 ß-CNs**

- **RELECSH(4)NVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDEL QDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGV**
- **SKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMH**
- **QPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDM PIQAFLLYQ**
- **EPVLGPVRGPF PIIVCSH (208)**

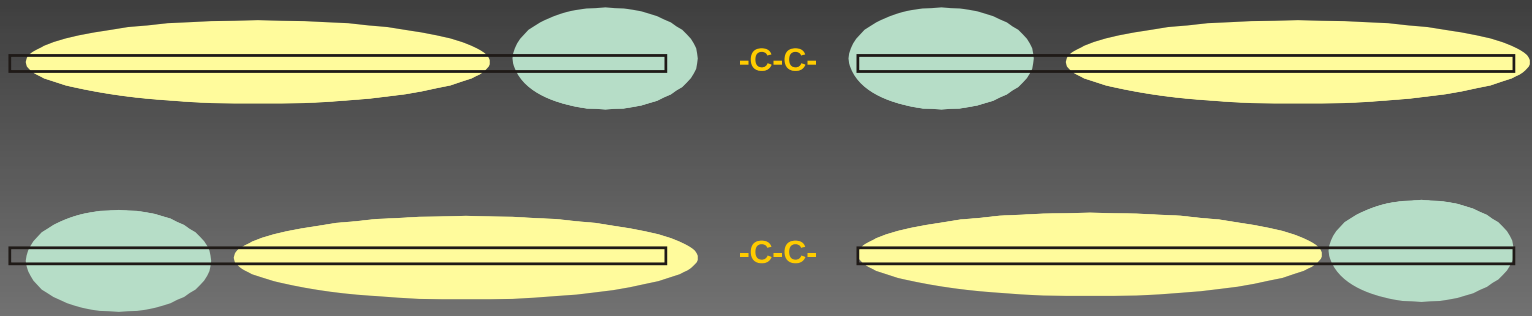
Hydrophilic-Hydrophilic patterns of different β CNs

Native β CN has distinct arrangement of hydrophilic (ψ) -hydrophobic(φ) fragments (ψ- φ).

The dimeric β CNs are in some sense symmetrical

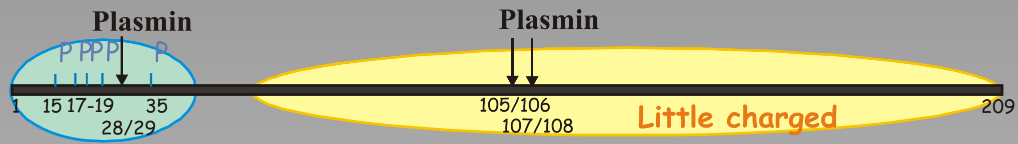
C208 β CND (ψ - φ ~ φ - ψ)
C4 β CND (φ - ψ ~ ψ - φ)

"Palindromic"



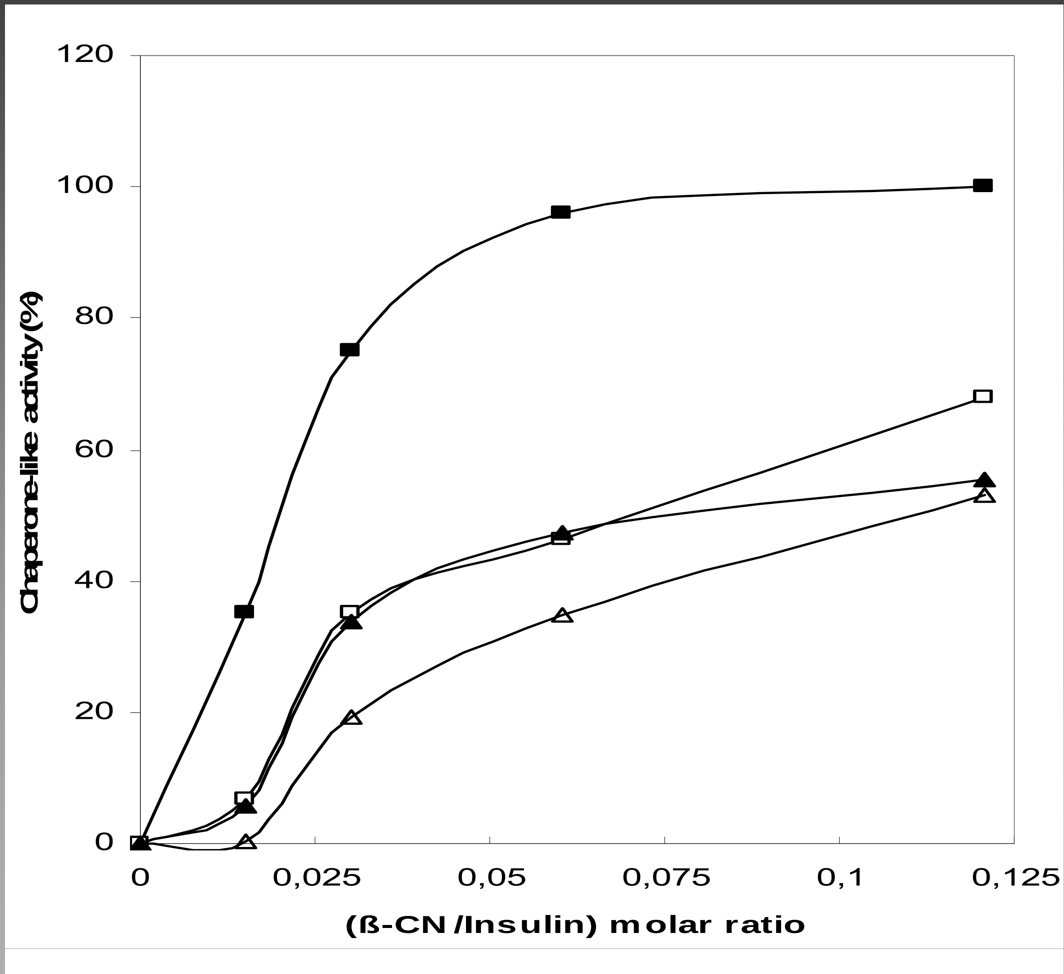
Casein β

23983 Da



Amphiphilic

Chaperone-like activities of native and mutant monomeric β-CN. Target protein - insulin



☑ Chemically-induced aggregation of insulin performed in the présence of 20 mM DTT at 40 ° C.

☑ Mutant β-CNs exhibit considerably smaller chaperone-activities than that of native β-CN.

Native β-CN (■), Wild type β-CN (□), C4 β-CN (▲) and C208 β-CN (Δ)

Importance of phosphoryl residues for chaperone acivities of ß-CNs.

☑ The hydrophilic segment of chaperones plays an essential role increasing solubilities of target proteins.

☑ The expressed in *E. coli* recombinant ß-CNs are not phosphorylated what decreases their amphiphilicity.

MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDEL
QDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPK
HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSL
SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

☑ Smaller polarity of hydrophilic domain is the reason for poorer chaperone activities of the mutant ß-CNs.

Consequence of Cys incorporation on chaperone-like activities of ß-CN.

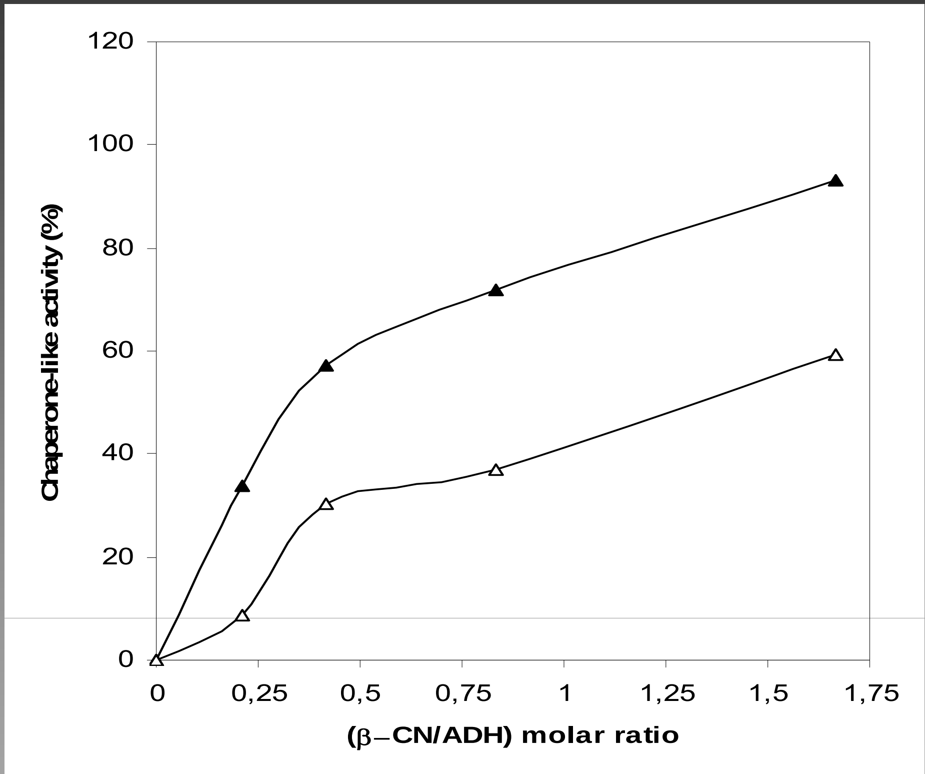
☑ Absence of cysteinyl residues, is one of the common features among different chaperone families.

☑ The C4 ß-CN and C208 ß-CN have cysteinyl residue in position 4 and 208, respectively.

☑ The ß-CNs containing cysteine showed almost similar chaperone-like activities as WT ß-CN.

☑ Thus incorporation of cysteine has no significant consequence in chaperone-like activity of monomeric ß-CN.

Chaperone-like activities of the dimeric β-CN. Target protein-ADH.



C208 β-CND (▲): Hydrophobic core, Polar ends.

C4 β-CND (Δ): Polar core, Hydrophobic ends.

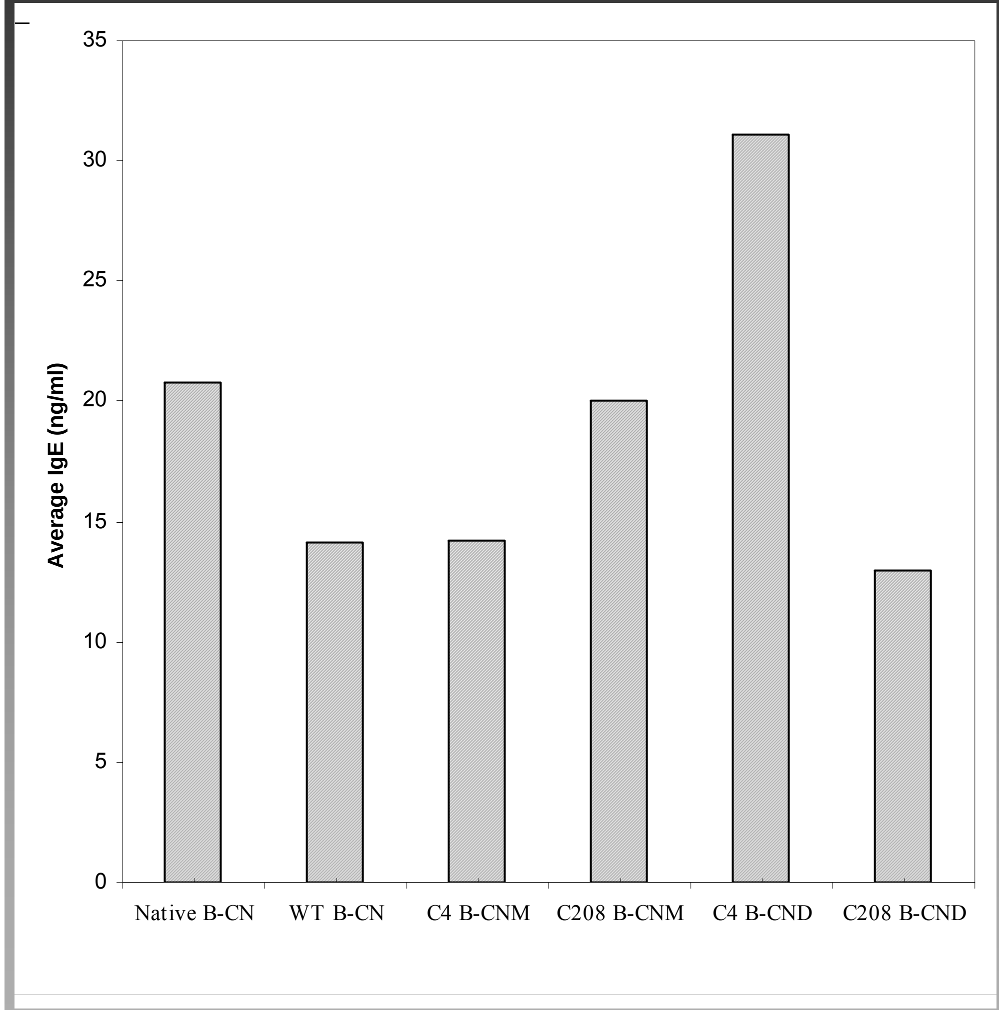
☑ Due to combination of flexibility and amphiphilicity, ß-CN acts as a surfactant molecule in solution.

☑ The dimeric β-CNs resembles somehow bis (gemini) - surfactants.

☑ C208 β-CND (ψ - ϕ ~ ϕ - ψ) has greater chaperone activity than C6 β-CND (ψ - ϕ ~ ϕ - ψ).

☑ C208 β-CND also shows greater chaperone activity than mutant β-CNs in monomeric state.

The IgE-binding (immuno reactivity) of different molecular forms of β-CN.



Sera from patients with milk allergies were used to determine immuno reactivities of different molecular forms of β-CN.

Averaged results of 16 out of 37 tested sera showing significantly greater IgE-responses to different forms of β-CN

Summary

- ß-CN and its mutants undergo temperature-induced concentration-dependent micellisation.
- Polar phosphate residues and the N-terminal hydrophilic domain are important functional elements enhancing the chaperone-like activities of native β-CN.
- Dimerization of C208 β-CN with two distal hydrophilic domains improved considerably its chaperone-like activity in comparison with its monomeric form and with C4 β-CND.
- N-terminal hydrophilic domain plays significant role as important functional elements enhancing the chaperone-like activity of native β-CN
- Exposure of hydrophobic domains increases immuno reactivity in 208 β-CN dimer






Our INRA team

Isabelle Bronnec
Jean-Marc Chobert
Yvan Choiset
Michèle Dalgalarrondo
Hanitra Rabesona
Thomas Haertlé

Aynur Akhmadova – PhD student
Shady El Gaish – PhD student
Tatyana Konnova – PhD student
Asghar Taheri Kafrani – PhD student
Iuliya Schutskaya – PhD student
Reza Yousefi – post doctoral

Collaborations

- Prof Dr V.I. Muronetz, Faculty of Bioengineering, Lomonosov University, Moscow, Russia
- Prof Dr Yu. D. Zuev, Institute of Biochemistry and Biophysics, RAS, Kazan, Russia.
- Prof Dr A. A. Moosavi-Movahedi, Institute of Biochemistry and Biophysics, Tehran University, IR Iran.