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Hydrolysis of Casein Micelles by AprX Enzym from *Pseudomonas fluorescens* F Induces their Destabilization

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ALIMENTATION
 AGRICULTURE
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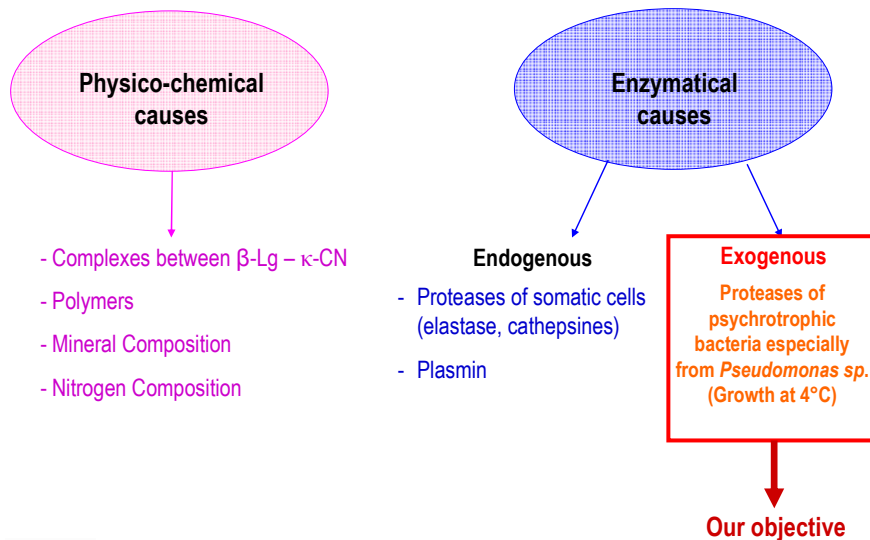
Context ↪ Destabilisation of UHT Milks During their Storage

- Phenomena described since several years
 - Destabilisation with presence of sediment
 - Destabilisation with the presence of gel
 - Destabilisation in the package or during heating by the consumer
- } Before the limit date of consumption



Necessity to understand this phenomena

Multiple Causes of UHT Milk Destabilisation



Physico-Chemical Characteristics of the Protease from *Pseudomonas*

- Extracellular protease secreted (*variability between strains*)
- Name of enzyme : **AprX** from F strain of *Pseudomonas*
- 464 amino acids (without signal peptide) and glycosylated
- Molecular mass : about 50 kDa
- Optimal activities between 5 and 8 for pH and 30 and 40°C for temperature
- Thermostable :
 - Absence of S-S bridges
 - Important content in small amino acids
 - Presence of Zn and Ca in the protein.
 - Variability in the thermostability between strains *Pseudomonas fluorescens*

Main Objective and Steps of the Study

Understand the destabilisation of UHT milk and propose a mechanism

1. Select a *Pseudomonas* strain able to destabilise UHT milk during storage
2. Purify and characterize its extracellular protease
3. Characterize the proteolytic action of enzyme in UHT milk and the consequences on the stability of casein micelles by different methods :
 - ✓ Visual observation
 - ✓ Size particle
 - ✓ Zeta potential
 - ✓ Quantitative and qualitative proteolysis

STEP 1 : Select one *Pseudomonas* strain destabilising UHT milk during storage

Study different strains of *Pseudomonas* : CNRZ 798, F, DSM 4358, LMG 6812, LMG 5825, LMG 1244, LMG 5167, CIP 6913 and CIP 7325.

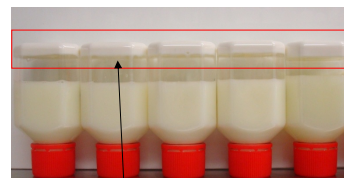
- Inoculation in raw skimmed milk previously microfiltered (removal of somatic cells)
- UHT treatment 140°C – 4 sec - sterile packaging - storage during 3 months

Observation of destabilisation ↪ 2 groups

No destabilisation of UHT milk



Destabilisation of UHT milk

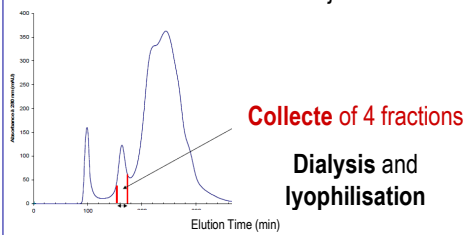


Selection of the F strain of *Pseudomonas fluorescens*

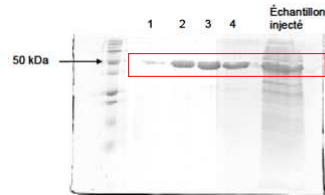
STEP 2 : Purify and characterize the extracellular protease from strain F

1. **Culture** during 72 h of *Pseudomonas* strain F in *ad hoc* complex media

2. Chromatographic purification from supernatant of culture (HiPrep Sephacryl 26/60 S-200 / 10 ml of injection



3. Characterization - Identification



- ↳ MW = 48 154 Da by mass spectrometry
- ↳ Identified as extracellular alkaline metalloprotease from *Pseudomonas fluorescens*

STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles

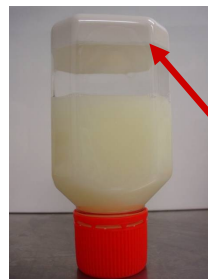
1. Addition of 0.2 mg of enzyme /l of raw skimmed milk previously microfiltered
2. UHT treatment 140°C, 4 sec followed by a sterile packaging

3. Physico-chemical analyses of milks (with and without enzyme) especially casein micelles during 3 months

Milk control
without enzyme



No destabilisation
after 90 days



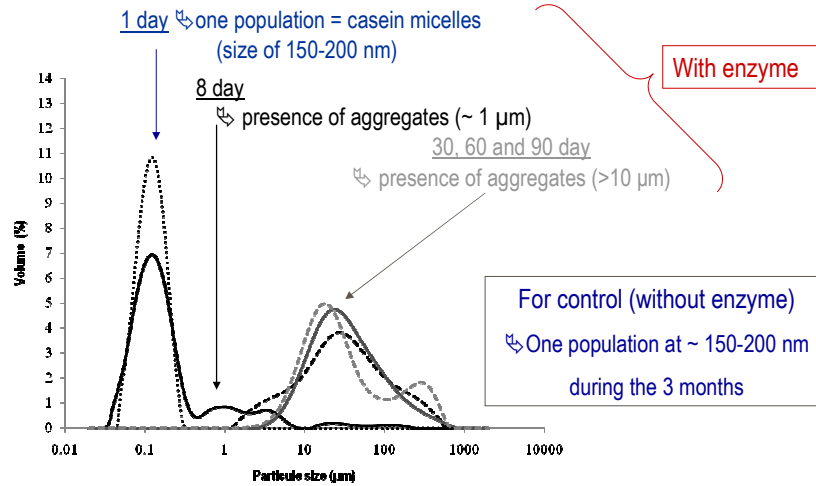
Milk with enzyme



Destabilisation
after 8 days and
more

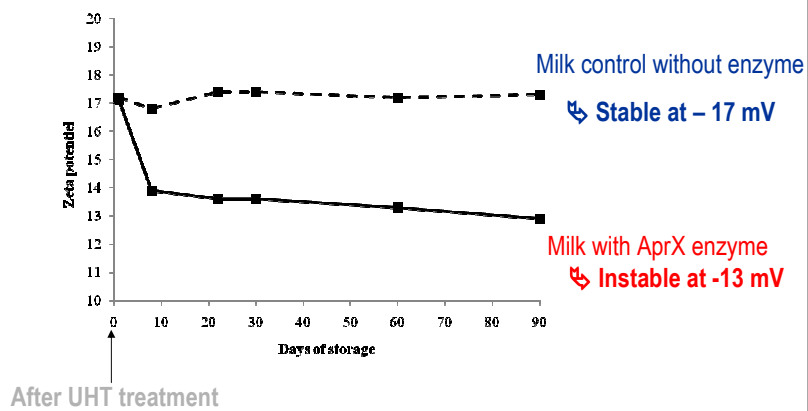
STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles

Particle size distribution by laser granulometry



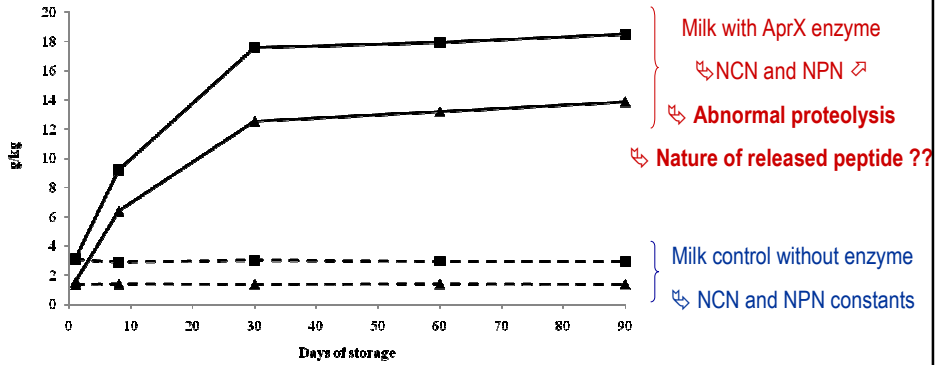
STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles

Zeta Potential (mV) : Electrical charge of casein micelles



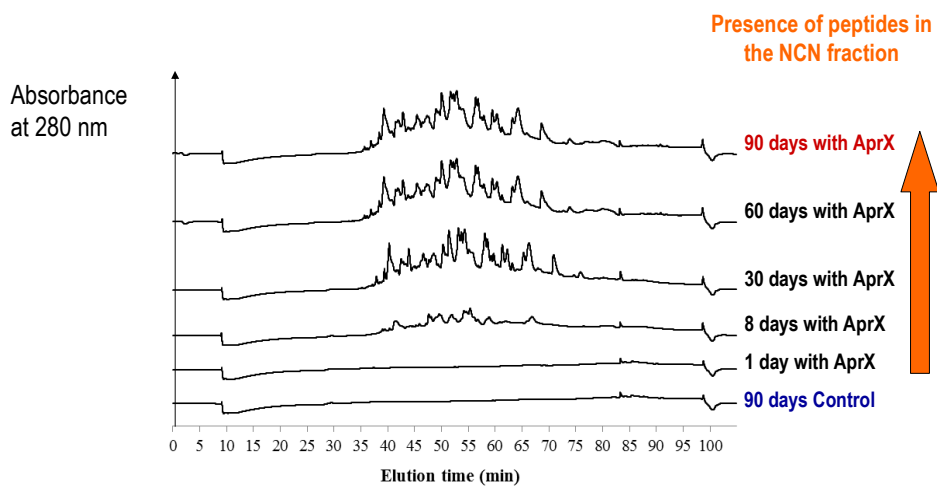
STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles

Non Casein Nitrogen (NCN) and Non Protein Nitrogen (NPN) contents
 ↳ indicators of proteolysis

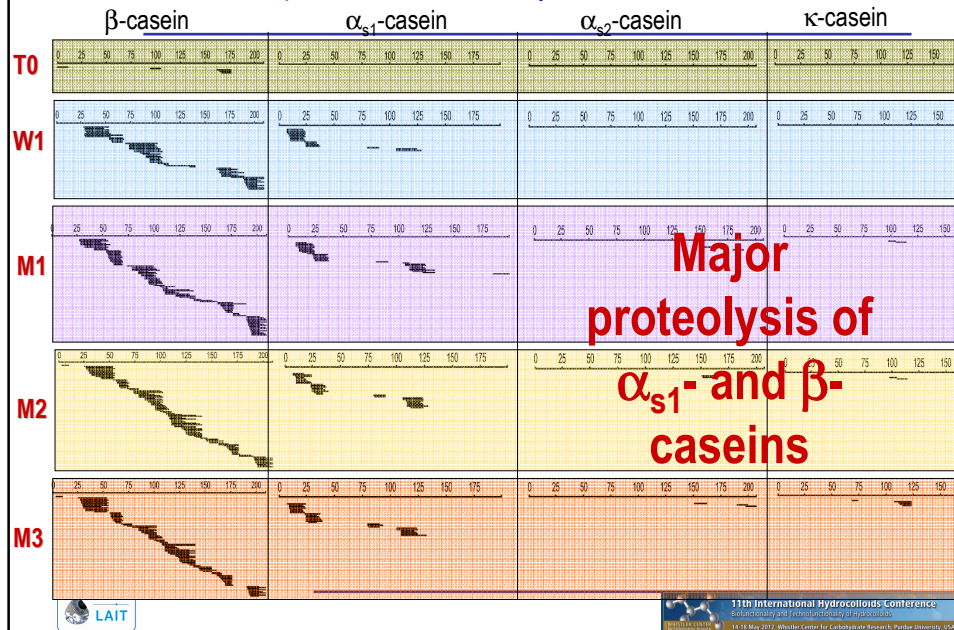


STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles

RP-HPLC of the Non Casein Nitrogen (NCN) fraction as a function of time storage



STEP 3 : Characterize the proteolytic action in UHT milk and the consequences on the stability of casein micelles



Summary of Proteolysis Characteristics

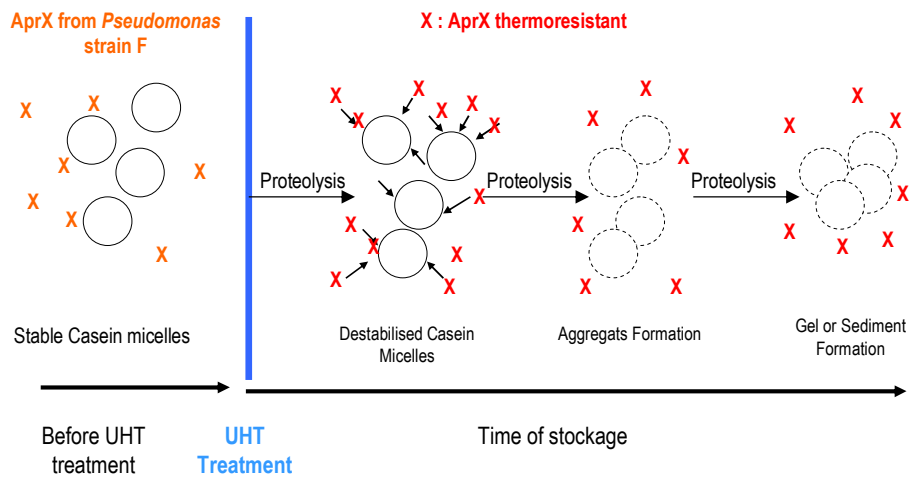
- | Thermoresistance of AprX (activity detected after UHT treatment)
- | Large specificity of the enzyme with preferential proteolysis of β and α_{s1} -caseins (identification of released peptides by mass spectrometry) \rightarrow No specific peptidic marker

Proteolysis \Rightarrow Destabilisation of casein micelles :

- > Sedimentation
- > Formation of aggregates $> 1 \mu\text{m}$ in size
- > \searrow in their zeta potentials

AprX Enzyme from *Pseudomonas fluorescens* F induces proteolysis of casein micelles and their destabilizations

Implication of AprX in the Mechanism of UHT Milk Destabilisation



Questions to Resolve & Perspectives

- ▮ All the strain of *Pseudomonas* are destabilizing (probably NO) ?
And Why ?
- ▮ How avoid destabilisation of UHT milk ?
- ▮ How predict a potential destabilisation of UHT milk during storage ?
 - > Global test
 - Phosphate test
 - Alcohol test
 - > Specific tests
 - Numeration of the psychrotrophic flora
 - Detection of AprX enzyme in raw milk (immunoenzymology,...)

Thanks for your attention

