Come funziona il test k

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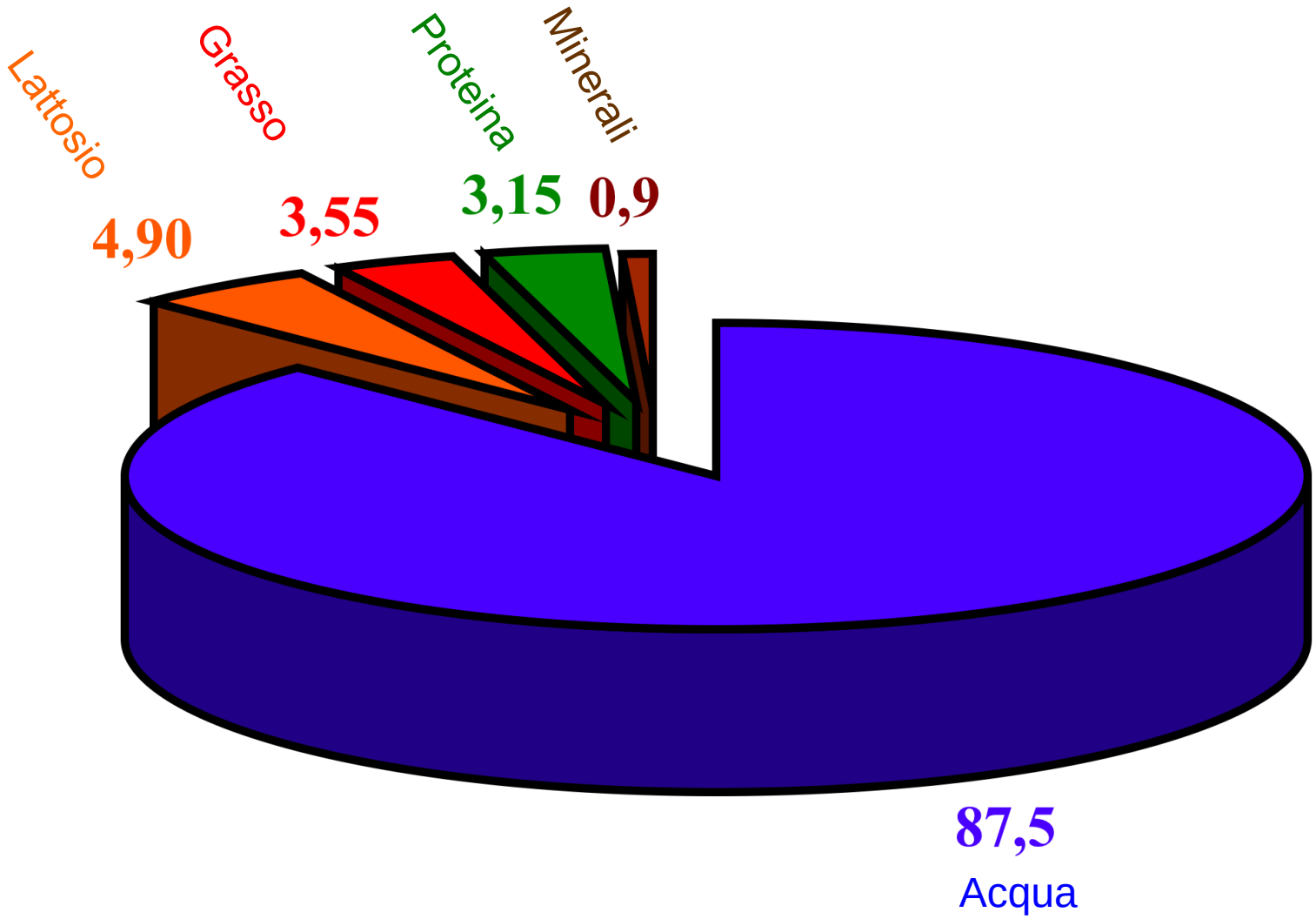
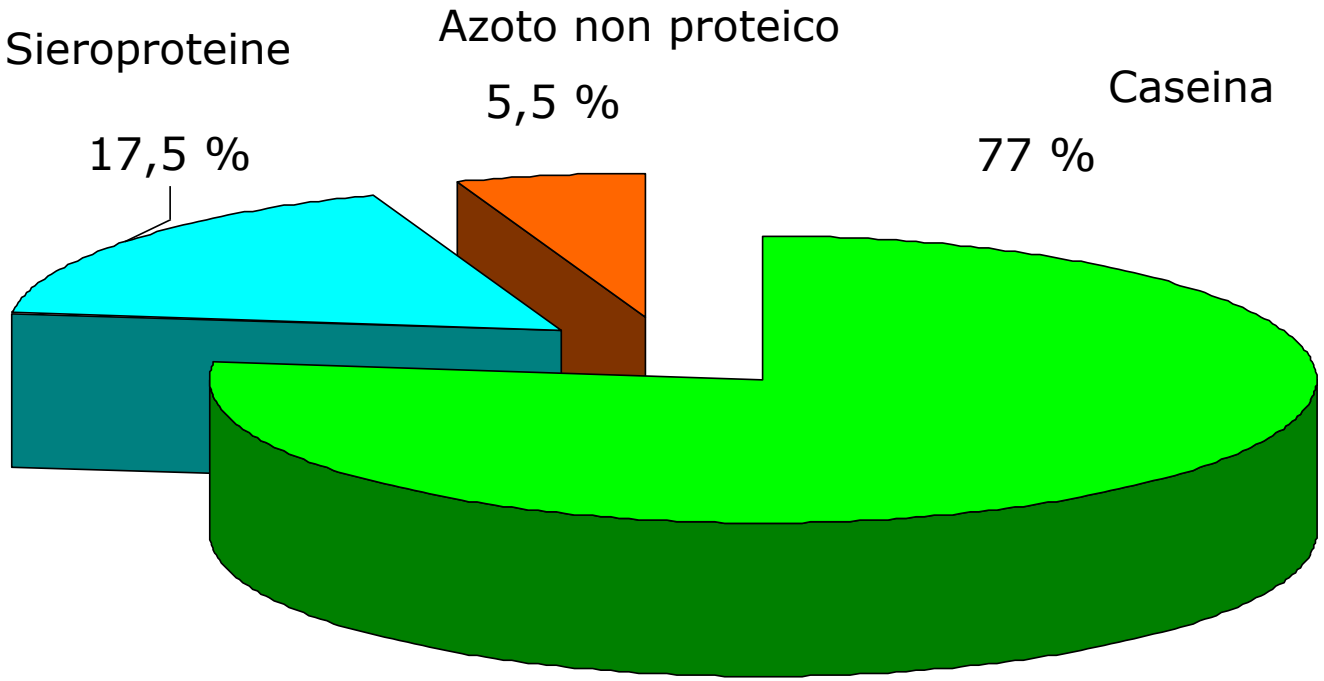
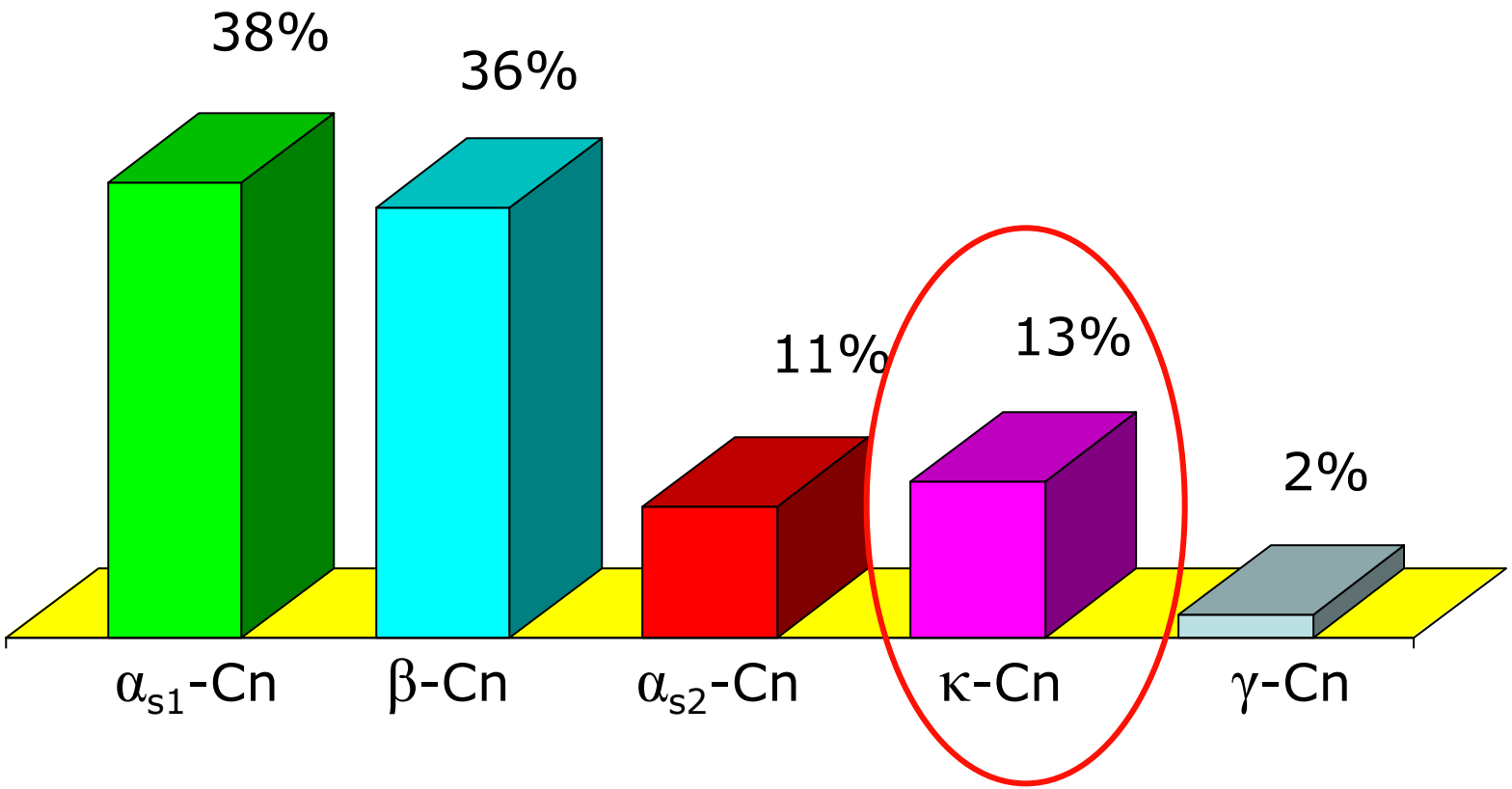
COMPOSIZIONE CHIMICA DEL LATTE



Le frazioni azotate del latte



Le caseine

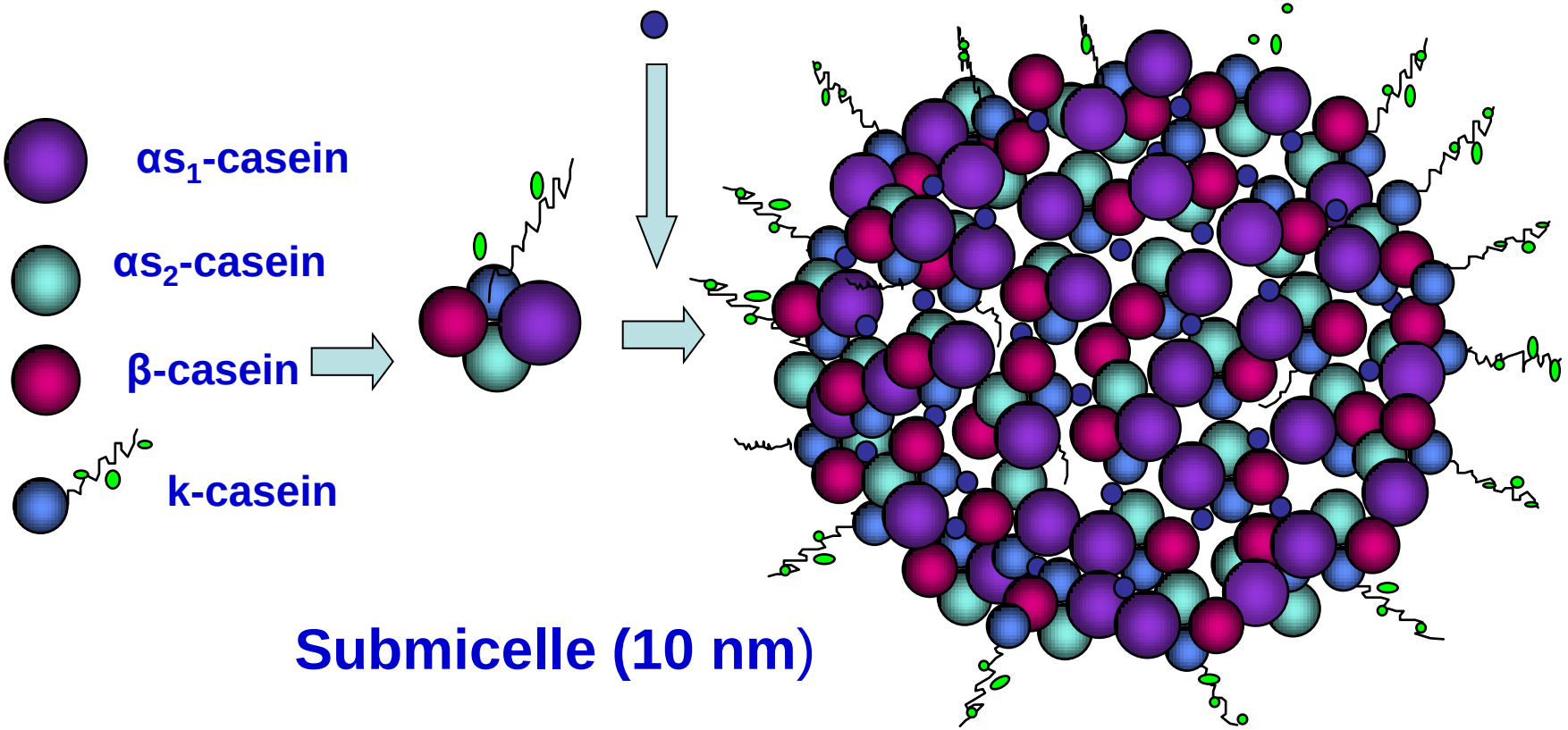


Caratteristiche chimiche e biochimiche k-caseina

- 169 amminoacidi
- Residui glicosilati nella porzione carbossi-terminale (da 0 a 4: isoforme)
- Insensibile all'azione precipitante degli ioni calcio
- rottura legame peptidico 105-106 ad opera della chimosina determina la coagulazione presamica, o enzimatica, del latte

(from Martin P, 1999, modified)

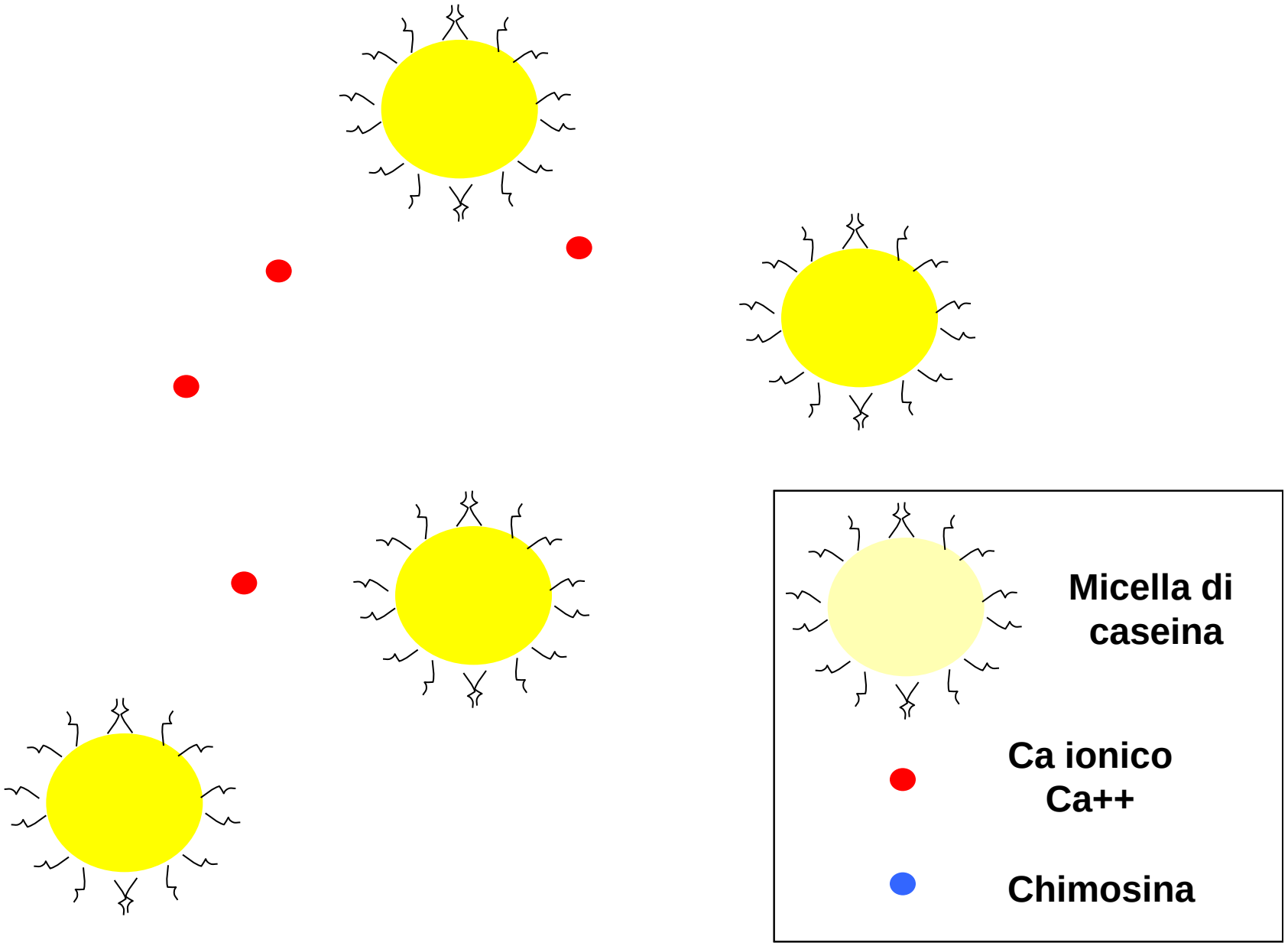
Fosfato di calcio colloidale $Ca_9(PO_4)_6$



Submicelle (10 nm)

Micella di caseina (20-300 nm)

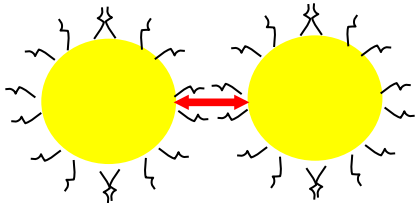
Stabilità delle micelle di caseina



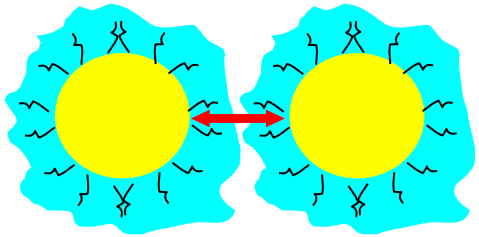
Basi molecolari della stabilità delle micelle di caseina

1 -Repulsione elettrostatica: le code della k-caseina sono cariche negativamente

2 –Ingombro sterico (o spaziale) delle code di k-caseina

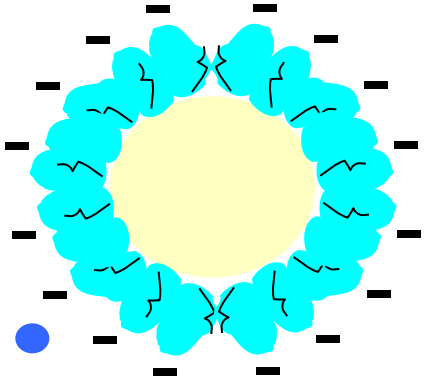


3 –Sfera di solvatazione (acqua) associata alle code di k-caseina



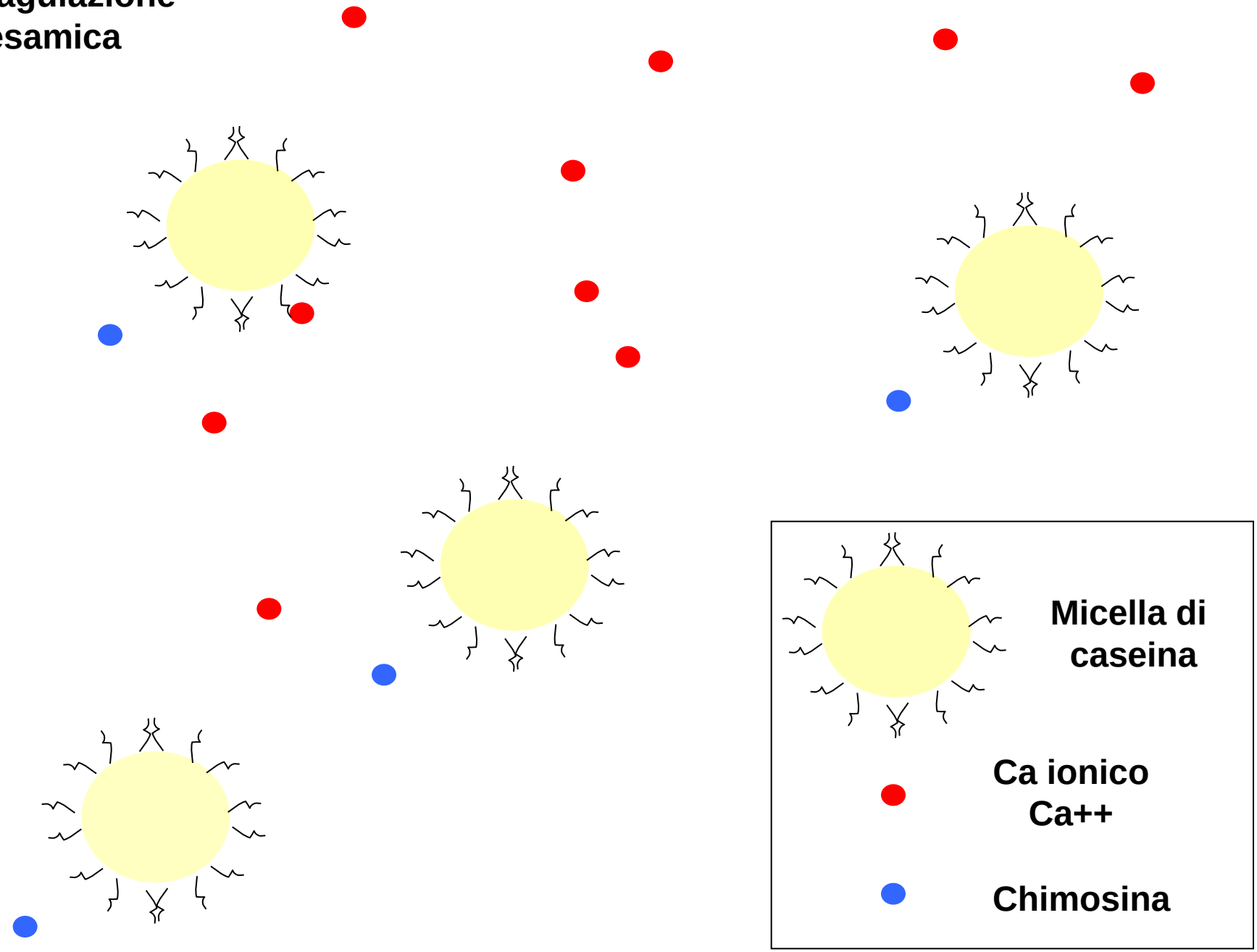
Le tre forze sono presenti simultaneamente

La chimosina rompe il legame 105-106 della k-caseina liberando nel siero la porzione carbossiterminale (106-169), denominata GlicoMacroPeptide (GMP)

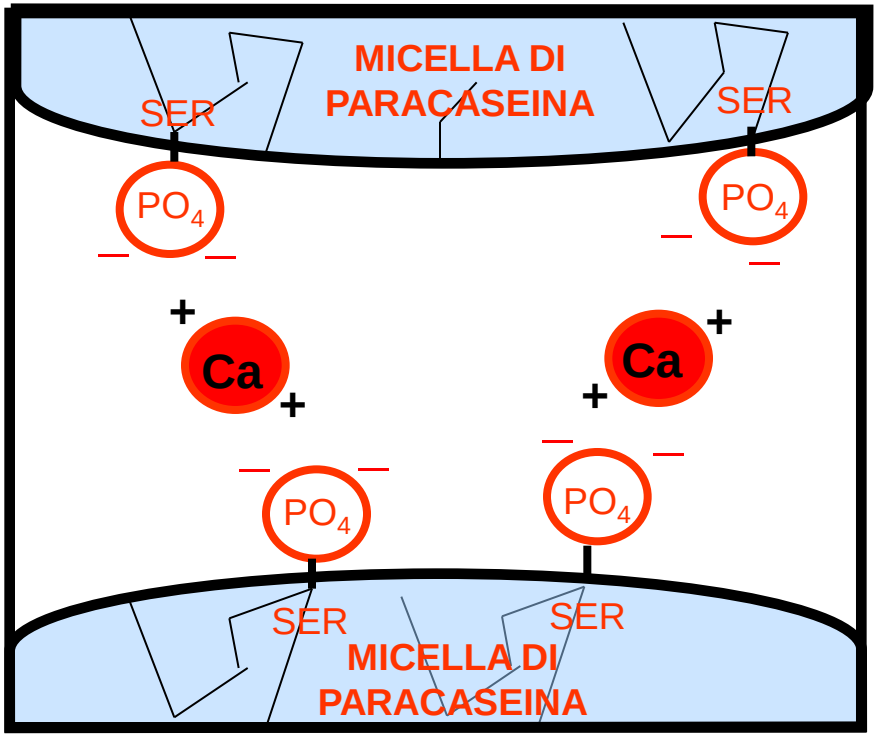


Il venir meno di tutte le forze responsabili della stabilità del latte determina l'aggregazione delle micelle di paracaseina (micella senza GMP), vale a dire la coagulazione presamica del latte

Coagulazione presamica



Coagulazione presamica



LEGAMI INTER-MICELLARI
Il calcio ionico interagisce, attraverso legami di natura secondaria, con i residui di fosfoserina della micelle di paracaseina non impegnati a legare il fosfato di calcio colloidale (**legami inter-submicellare**)

Polimorfismo genetico

Con questo termine si intende il fatto che ogni proteina del latte presenta due o più forme geneticamente determinate da alleli autosomici e codominanti.

Varianti genetiche k-CN

		10	97	104	135	136	148	155
k-CN	A	*Arg*	*Arg*	*Ser*	*Thr*	*Thr*	*Asp*	*Ser*
(169)	B					Ile	Ala	
	C		His					
	E							Gly
	F^1						Val	
	F^2	His						
	G^1		Cys		Ile			
	G^2						Ala	
	H					Ile		
	I			Ala				
	J					Ile	Ala	Arg

Evidenza scientifica:

la presenza di k-caseina B è associata a migliori caratteristiche tecnologiche del latte (coagulazione latte e resa in formaggio)

Idea:

determinare il contenuto di k-cn B nel latte di massa

Come:

test immuno-enzimatico

Requisito:

Anticorpo monoclonale

Specifico per la k-caseina B

NON REATTIVO verso k-caseina A e le altre frazioni proteiche del latte

Produzione dell’anticorpo monoclonale

HuCAL® Antibody Technology

Libreria di anticorpi sintetica
Non vengono utilizzati animali

Selezione dell'anticorpo monoclonale

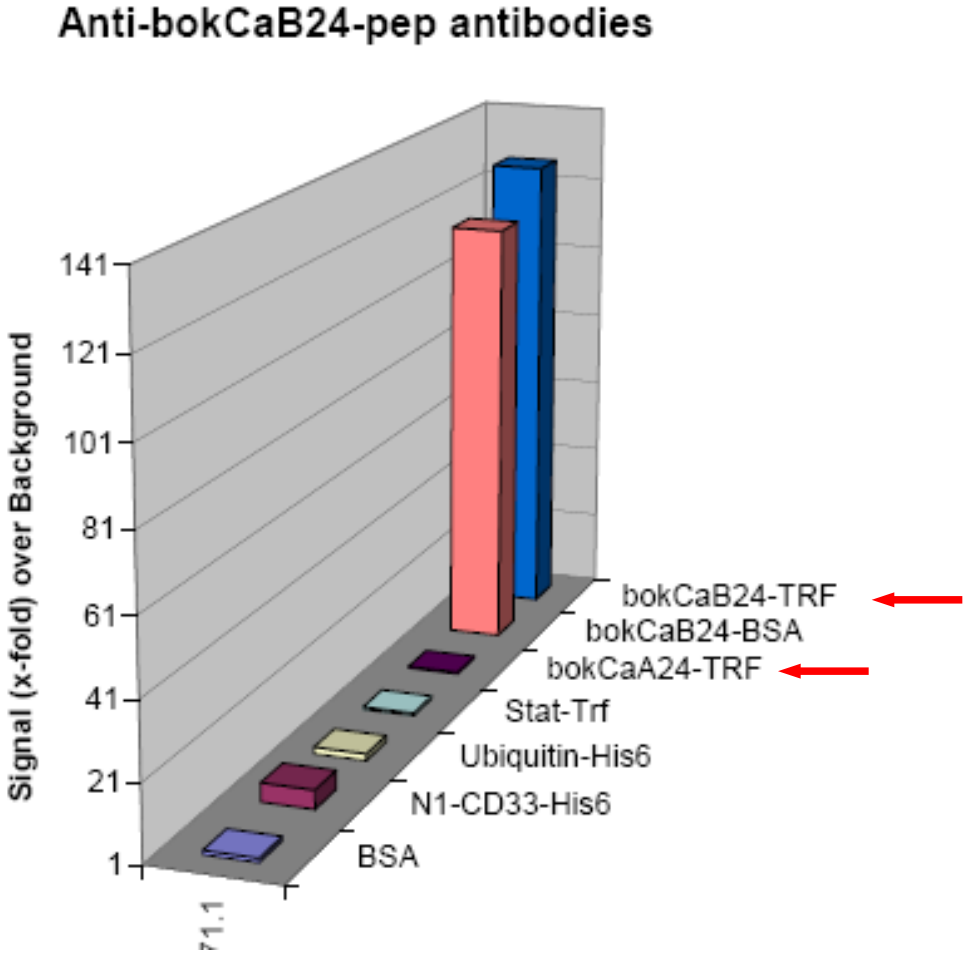
- Sintesi di oligopeptide corrispondenti alla sequenza 131-153 della k-caseina B
- Sintesi di un analogo oligopeptide per la k-caseina A

```
k-casein B:-21   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39
k-casein A:-21   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39

k-casein B: 40   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHP 99
k-casein A: 40   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHP 99
                                                      136          148
k-casein B: 100 HPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTVATLEASPEVIESPPEI 159
k-casein A: 100 HPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEI 159

k-casein B: 160 NTVQVTSTAV 169
k-casein A: 160 NTVQVTSTAV 169
```

bokCaB24-TRF TSTPTTEAVESTVATLEDSPEVI-**TRF**

bokCaA24-TRF TSTPTIEAVESTVATLEASPEVI-**TRF**

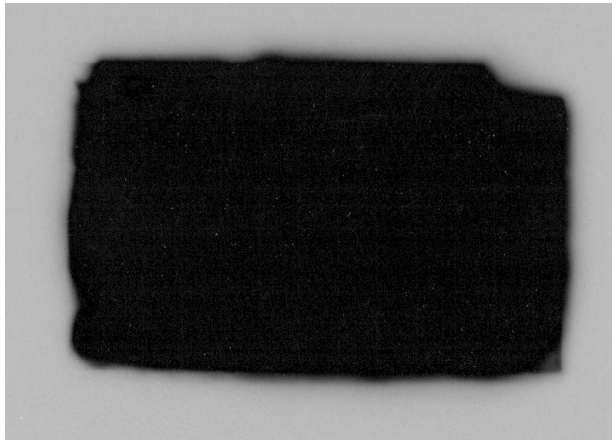
Selezione dell'anticorpo monoclonale



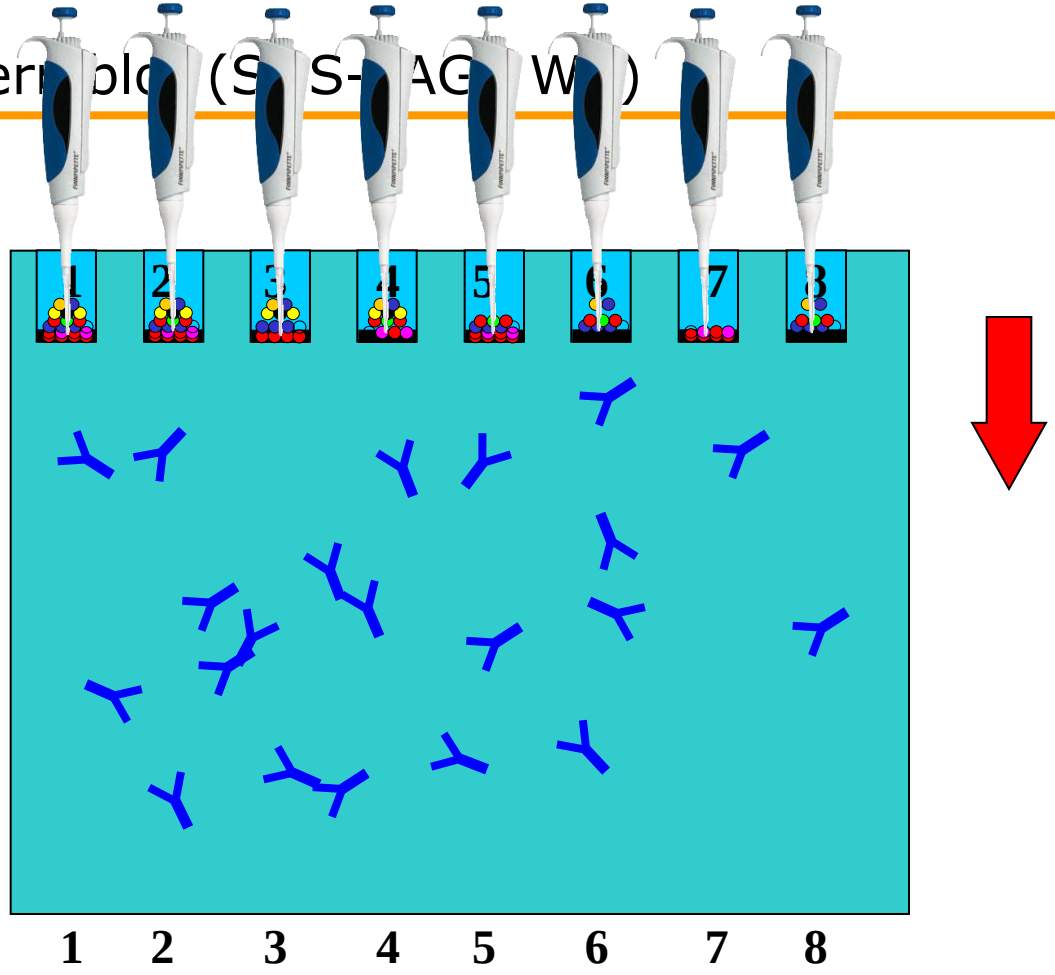
Caratterizzazione dell’anticorpo monoclonale

•Verifica della reattività dell’anticorpo monoclonale su campioni di latte individuale di vacche genotipizzate al locus per la k-caseina

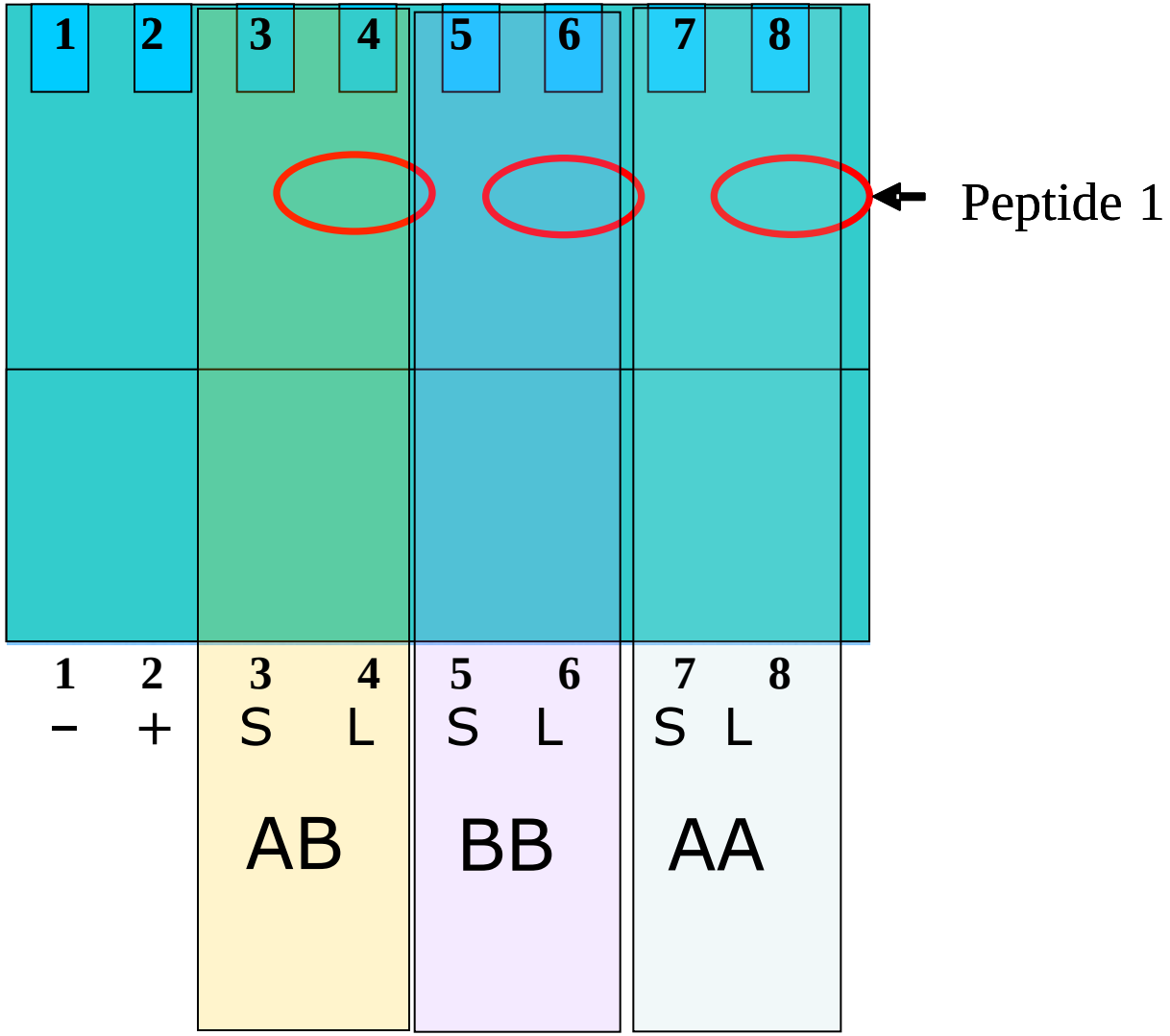
•Metodo impiegato: Western blot

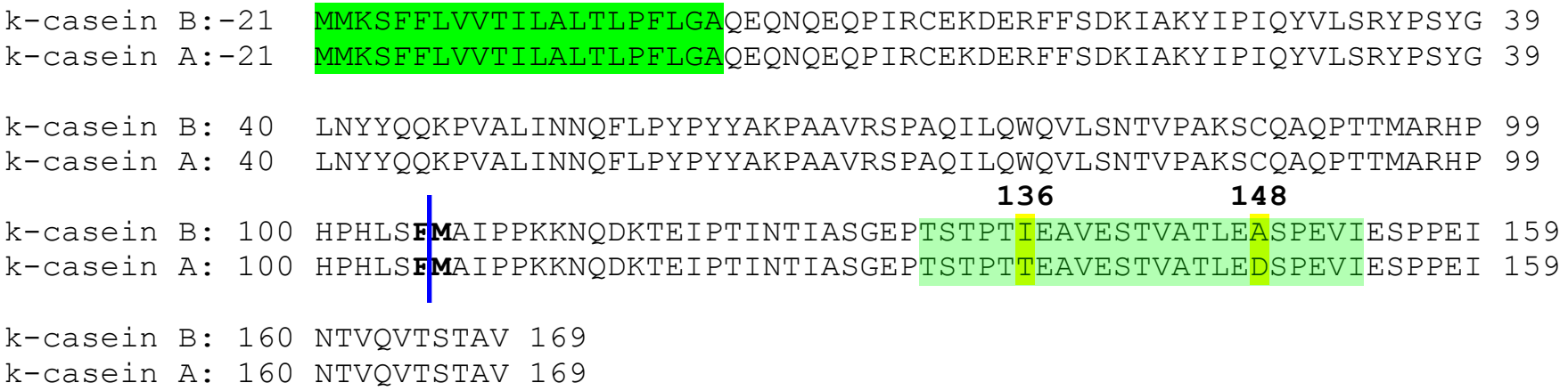


Western blot (SDS-PAGE Western)



Western blot (SDS-PAGE WB) latte e siero presamico




k-casein B:-21 MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39
k-casein A:-21 MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39
k-casein B: 40 LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHP 99
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k-casein B: 160 NTVQVTSTAV 169
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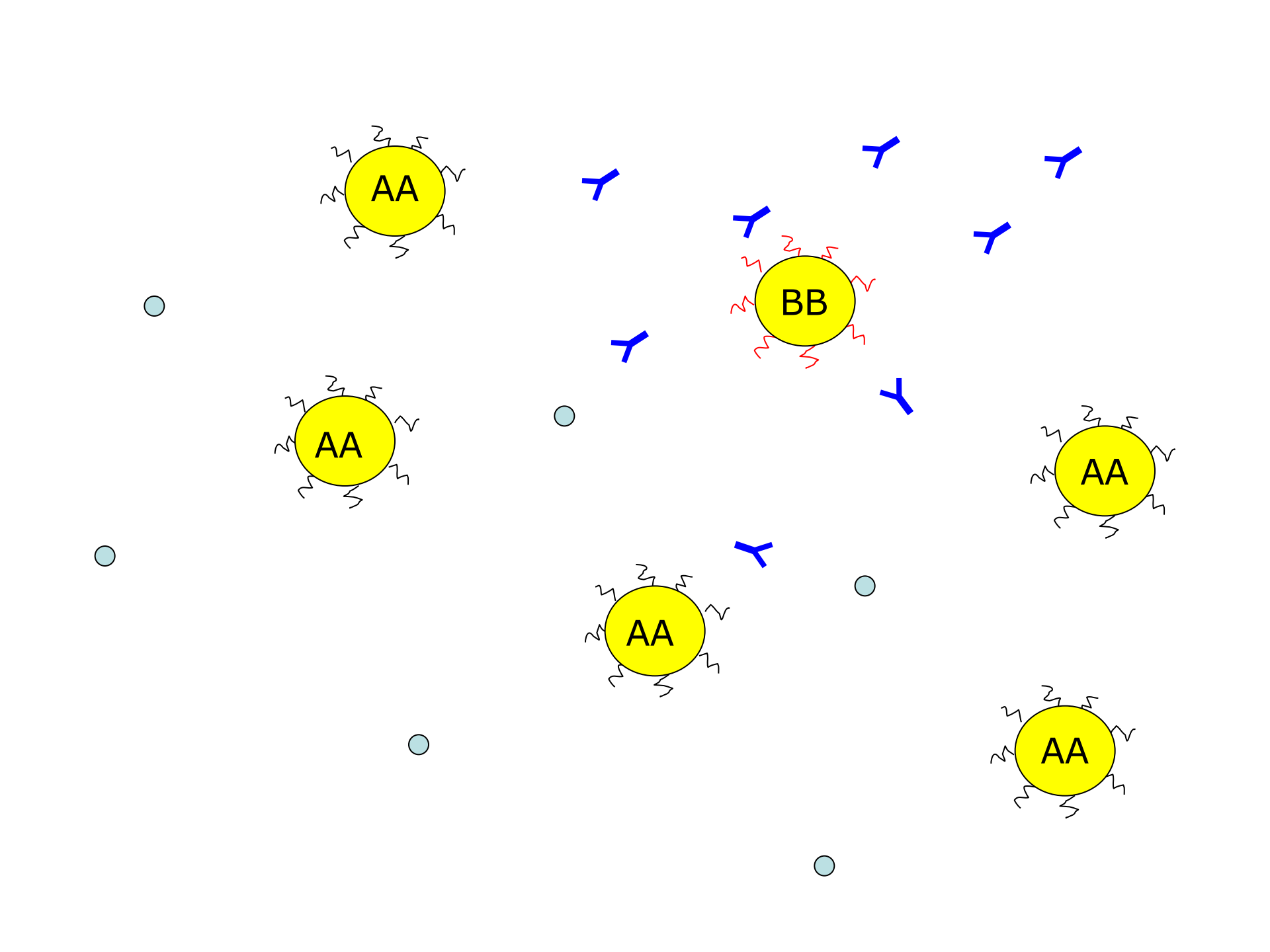
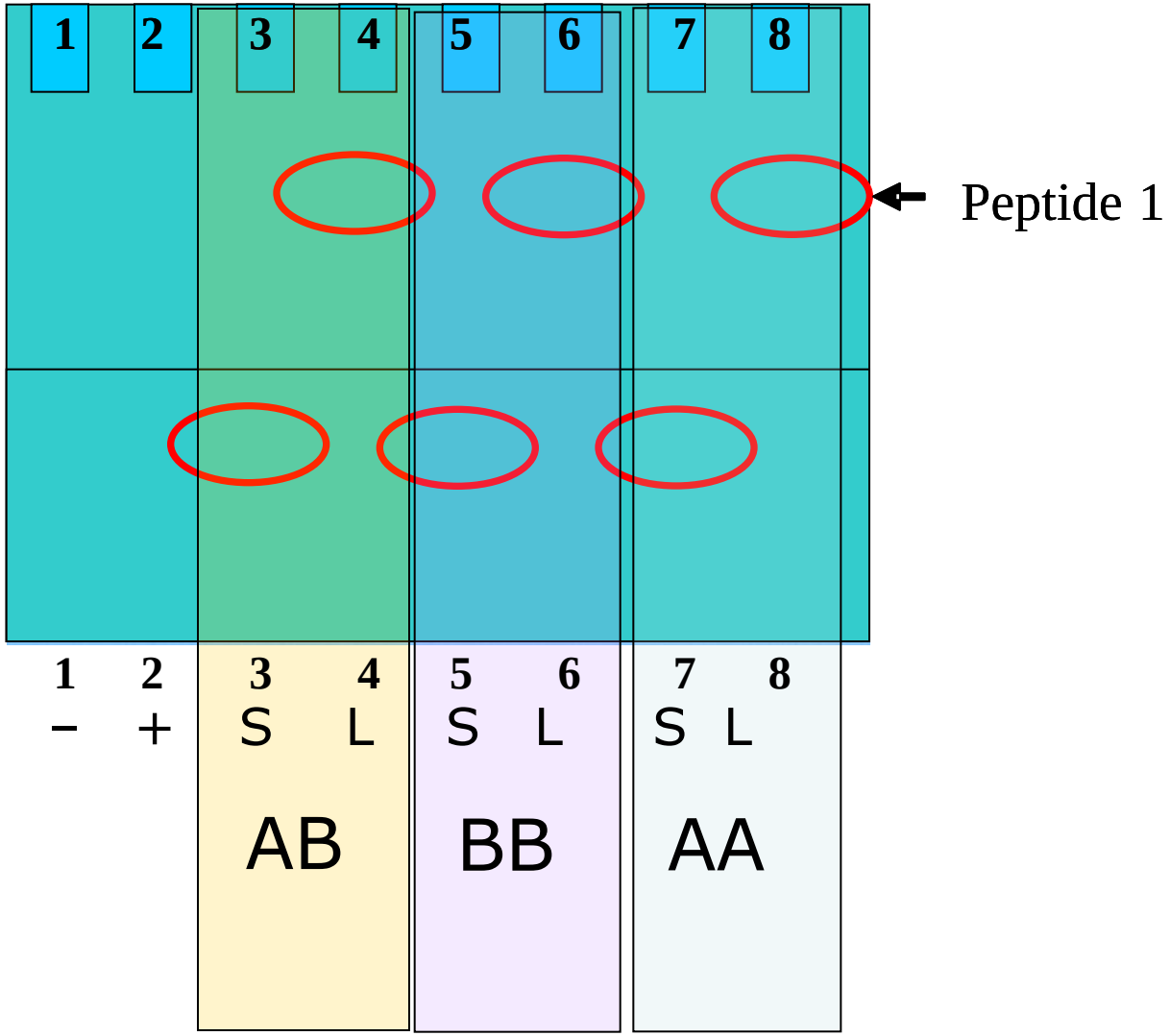

AA
BB
AA
AA
AA
AA

Western blot (SDS-PAGE WB) latte e siero presamico



Western blot (SDS-PAGE WB) latte k-caseina BB e caglio

Peptide 1

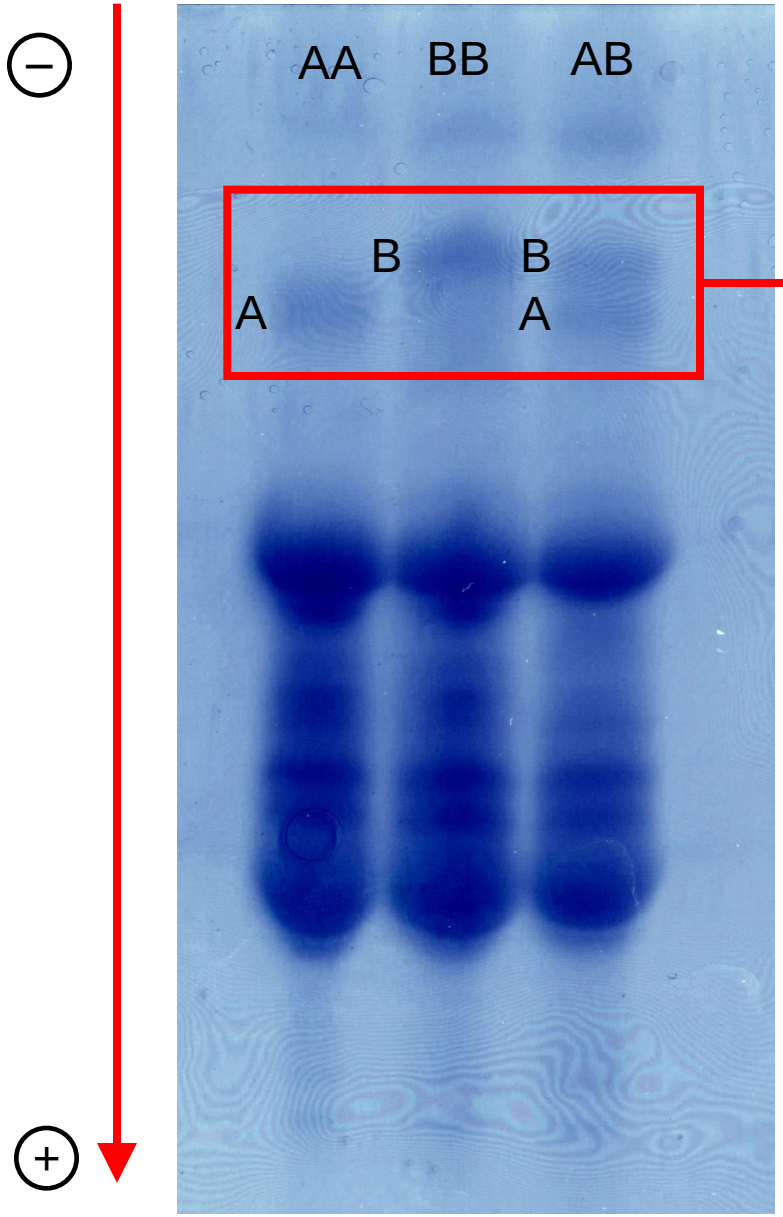
Peptide 2

Non specific peptides

1 2 3 4 5

0 10 20 30 >30 min.

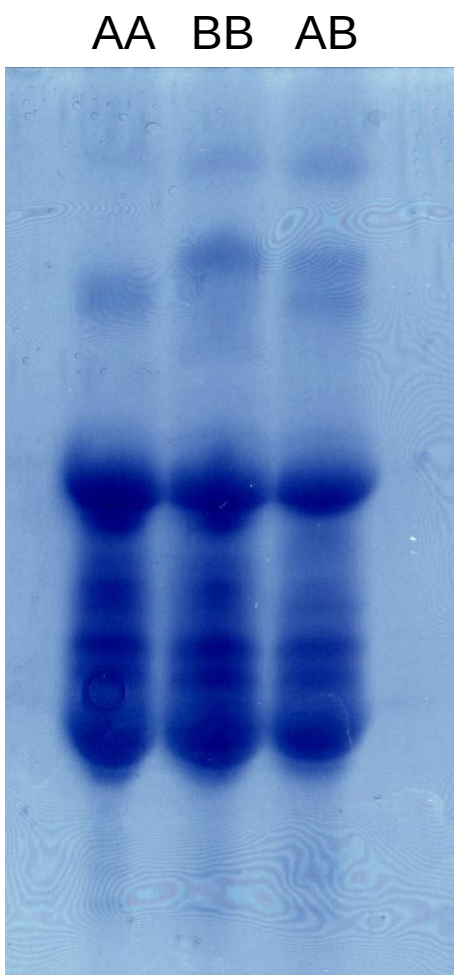
Western blot

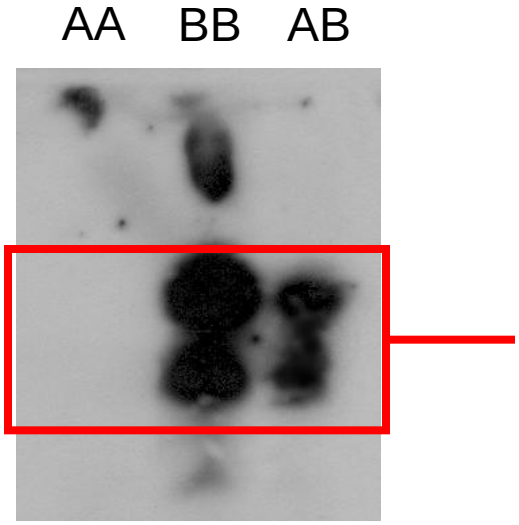


Urea-PAGE

Milk proteins are separated according to net charge and molecular weight.
In this case, it is possible to separate the B from the A allele.

Western blot





Urea-PAGE

A positive reaction of the monoclonal antibody was found only in BB and AB milks. In this case the band was a “smear” because of k-cn with different degree of glycosilation (k-cn isoforms) were separated in Urea-PAGE.
The smear band was in the migration zone of k-casein

Conclusioni

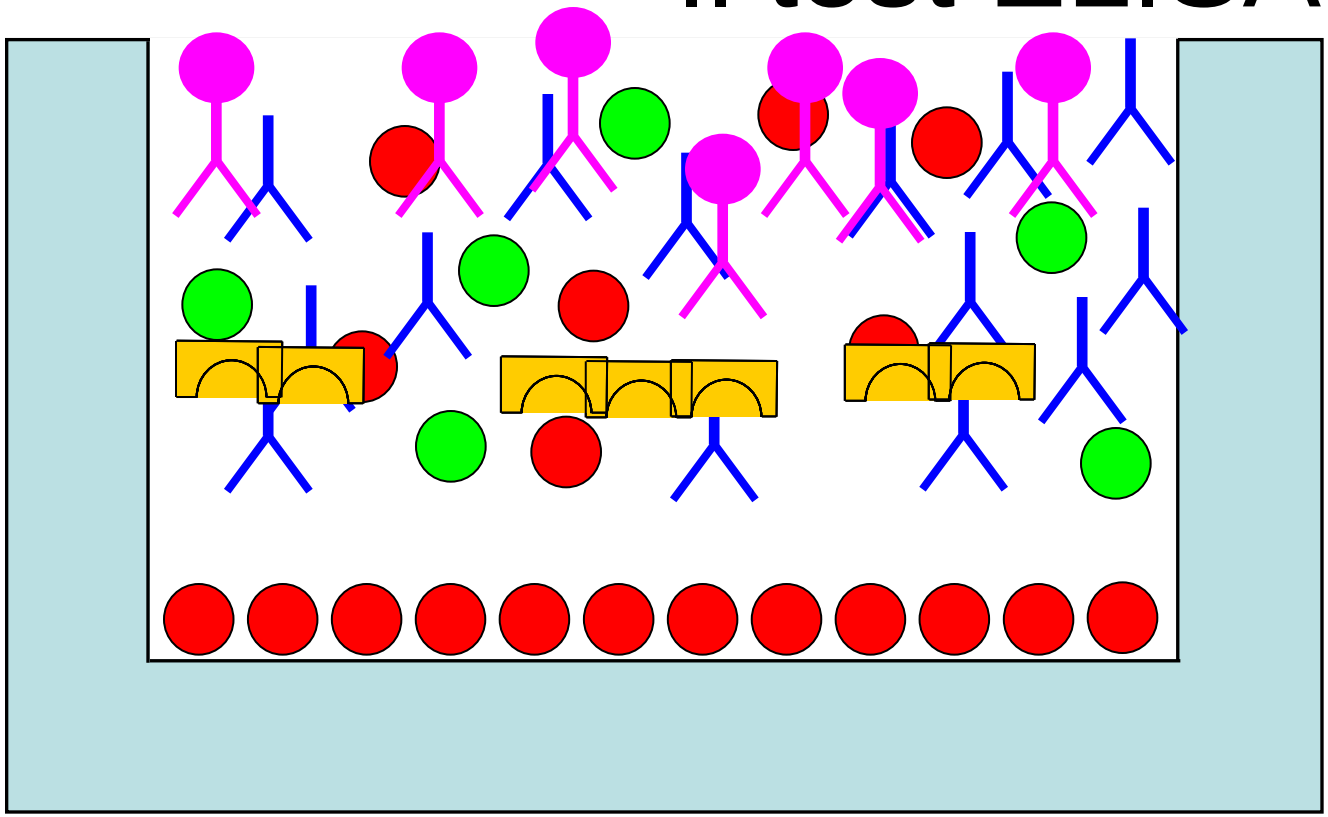
L’anticorpo riconosce in maniera specifica la variante B della k-caseina del latte di vacca

Non reagisce con la variante A della k-caseina

Non riconosce altri antigeni nel latte di vacca

Riconosce tutte le isoforme della k-caseina B

Il test ELISA



k-casein B

k-casein A

Antibody k-casein B

Secondary Antibody

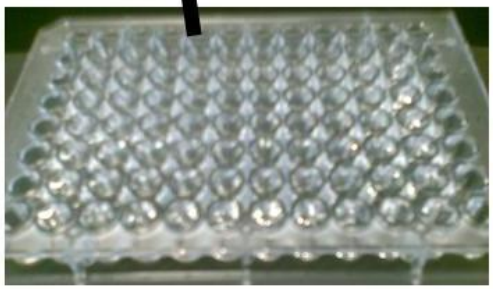
Il pozzetto è foderato di k-caseina B

Caricamento campione

Aggiunta quantità nota di anticorpo specifico per la k-caseina B

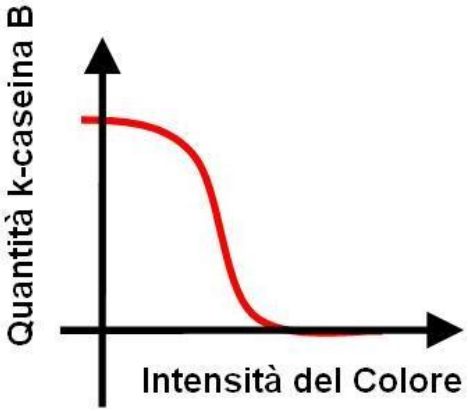
L'anticorpo reagisce con tutta la k-caseina B del campione e la parte in eccesso si lega alla k-caseina del pozzetto

Coloro l'anticorpo in eccesso che si è legato alla base del pozzetto

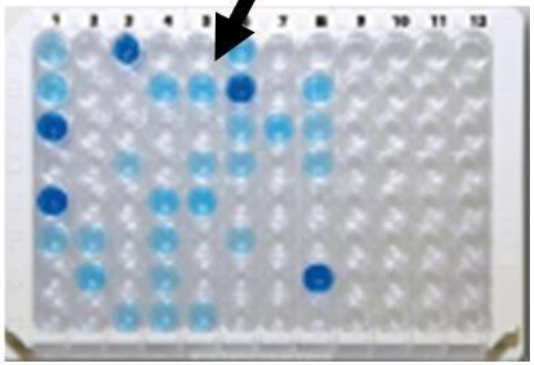


k-caseina B

k-caseina A



Maggiore è l'intensità del colore inferiore è il contenuto di k-caseina B nel campione



Anticorpo k-caseina B

Anticorpo secondario

Produzione e caratterizzazione dell'anticorpo monoclonale

- To synthesize an oligopeptide spanning region 157-169 of k-casein B variant.
- To synthesize an oligopeptide spanning region 157-169 of k-casein A variant (negative control).
- Make a monoclonal antibodies against the oligopeptide of the k-casein B variant.
- Verify that monoclonal antibodies does not react with the negative control (k-casein A oligopeptide).
- Verify that monoclonal antibodies react with the native k-casein B in the milk.
- Verify that monoclonal antibodies does not react with the native k-casein A in the milk.
- Verify that monoclonal antibodies react with the native k-casein B in the milk also in the presence of the native k-casein A (milk from cows with genotype CSN3 AB).

```
k-casein B:-21  MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39
k-casein A:-21  MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYG 39

k-casein B: 40  LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHP 99
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                                                      136         148
k-casein B: 100 HPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTVATLEASPEVIESPPEI 159
k-casein A: 100 HPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEI 159

k-casein B: 160 NTVQVTSTAV 169
k-casein A: 160 NTVQVTSTAV 169
```

Working steps

bokCaB24-TRF

TSTPTTEAVESTVATLEDSPEVI-**TRF**

bokCaA24-TRF

TSTPTIEAVESTVATLEASPEVI-**TRF**

Working steps

- To synthesize an oligopeptide spanning region 157-169 of k-casein B variant.
- To synthesize an oligopeptide spanning region 157-169 of k-casein A variant (negative control).
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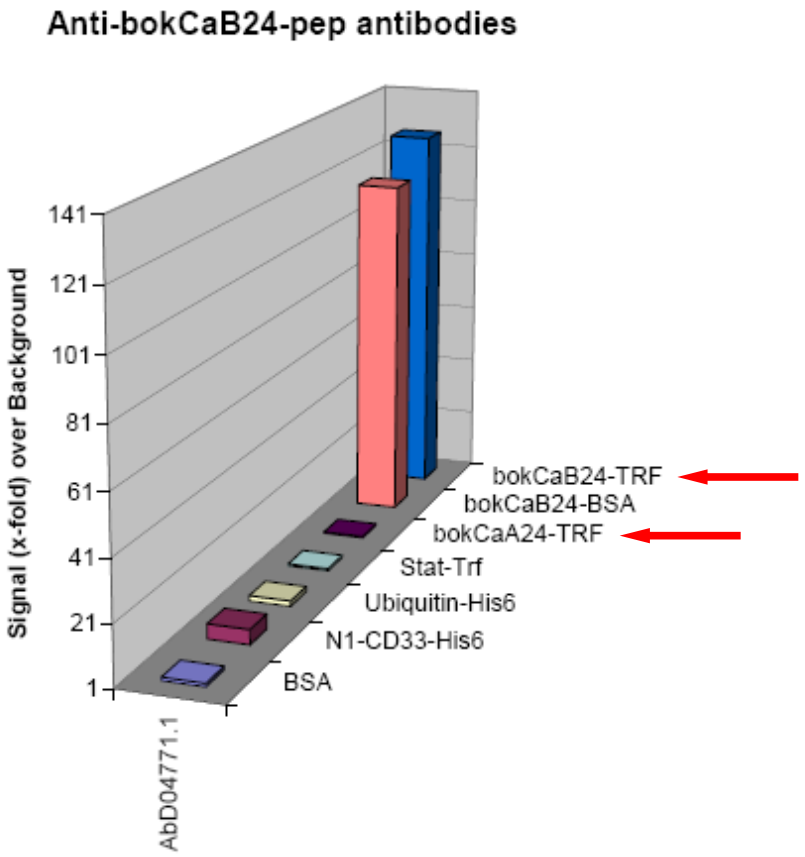
Working steps

ELISA test



Working steps

- To synthesize an oligopeptide spanning region 157-169 of k-casein B variant.
- To synthesize an oligopeptide spanning region 157-169 of k-casein A variant (negative control).
- Make a monoclonal antibodies against the oligopeptide of the k-casein B variant.
- Verify that monoclonal antibodies does not react with the negative control (k-casein A oligopeptide).
- Verify that monoclonal antibodies **react** with the native **k-casein B** in the **milk**.
- Verify that monoclonal antibodies **does not react** with the native **k-casein A** in the **milk**.
- Verify that monoclonal antibodies **react** with the native **k-casein B** in the **milk** also **in the presence of the native k-casein A** (milk from cows with genotype CSN3 AB).

Working steps

At this point, we need a method to CHARACTERIZE the reactivity of the monoclonal antibody against k-casein B IN MILK

HOWEVER

Besides k-casein, MILK is a complex matrix, mainly composed of 7 protein types (3 caseins and 4 whey proteins) and many degradation (proteolysis) products (peptides, gamma caseins, proteoso-peptones, *etc*.)

CONSEQUENTLY a further question arise:

WHAT IS THE IMMUNOLOGICAL BEHAVIOUR OF THE MONOCLONAL ANTIBODY TOWARD OTHER PROTEINS/PEPTIDES IN MILK?

Working steps

The method selected was:
Western Blot

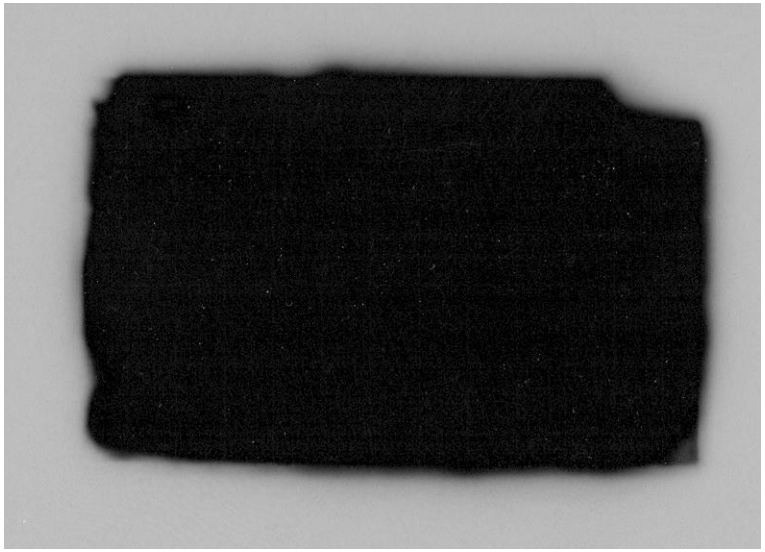
- SEPARATION OF MILK PROTEINS BY GEL ELECTROPHORESIS (SDS-PAGE, UREA-PAGE)

- Transfer of the gel to PVDF membrane (an hydrophobic membrane)

- Primary hybridazation with the monoclonal antibody (antikcnB)

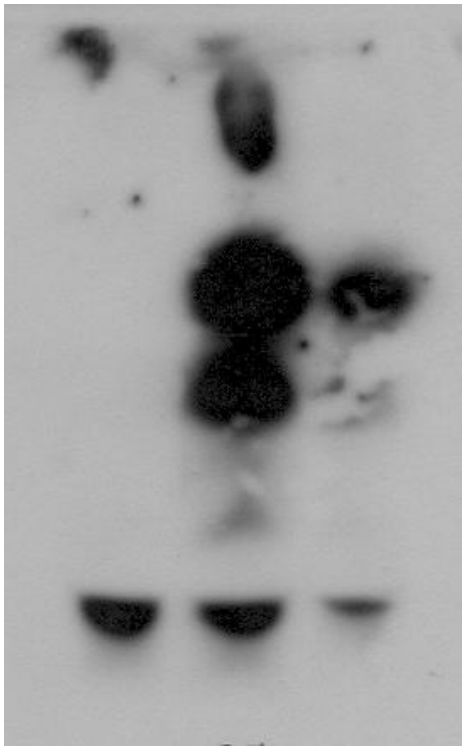
- Detection antigen-antibody reaction with a secondary antibody (anti-antikcnB)

The first WB
19 Dec 2006

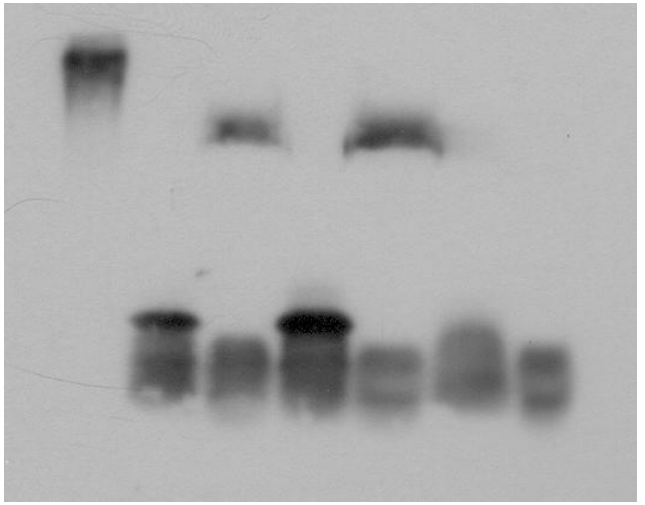


The last WB
8 March 2007

Urea-PAGE



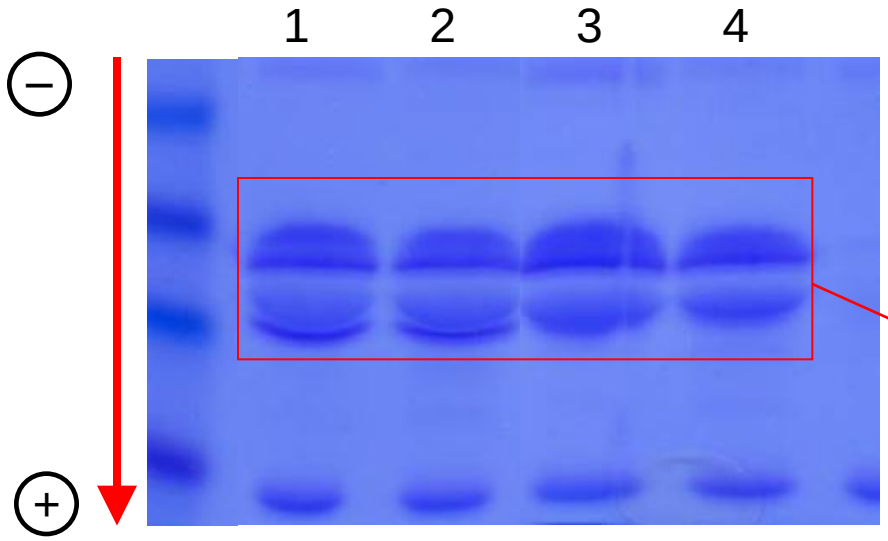
SDS-PAGE



Western blot

- Separation of milk proteins by gel electrophoresis (SDS-PAGE, Urea-PAGE)

SDS-PAGE



1 Milk AA
2 Milk BB
4 Milk AA + rennet
5 Milk BB + rennet

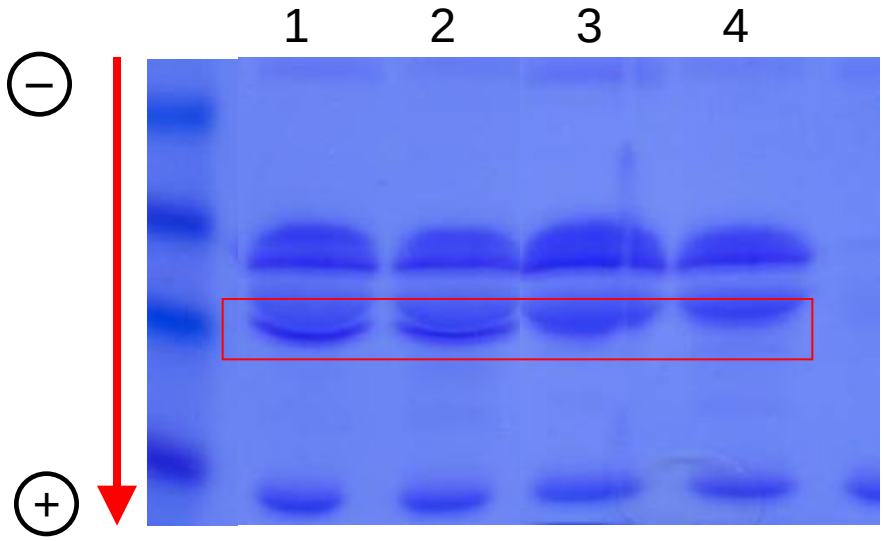
CASEINS

Milk proteins are separated according to their molecular weight

Western blot

- Separation of milk proteins by gel electrophoresis (SDS-PAGE, Urea-PAGE)

SDS-PAGE

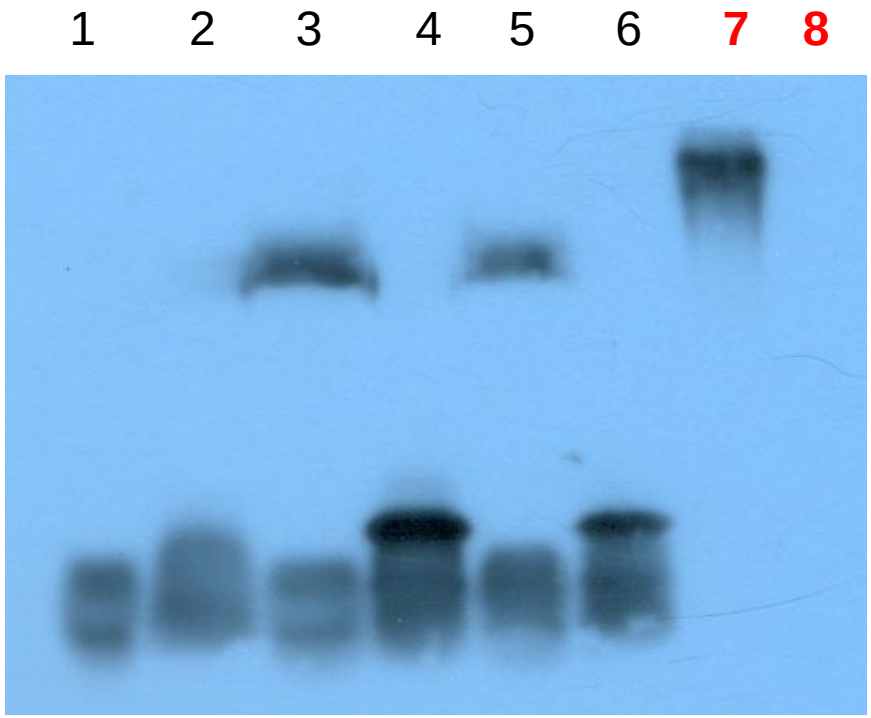


1 Milk AA
2 Milk BB
4 Milk AA + rennet
5 Milk BB + rennet

A band disappeared in the presence of the rennet in milk: k-casein

Western blot

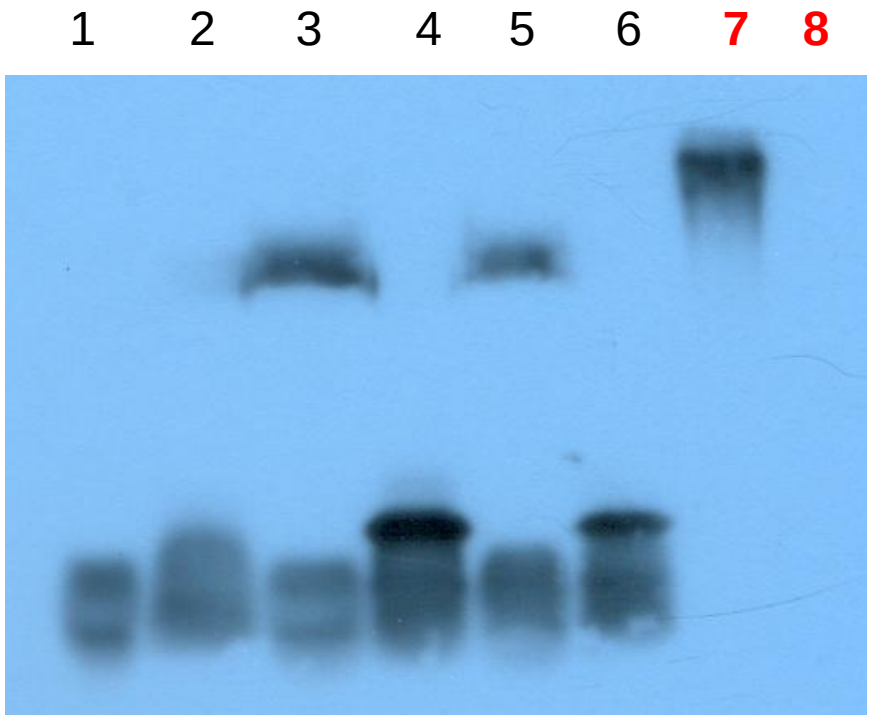
The SDS gel was transferred to PVDF membrane (blotting) and then hybridized with the monoclonal antibody. Here's the result:



Bands are milk proteins recognized by the monoclonal antibody

Western blot

The SDS gel was transferred to PVDF membrane (blotting) and then hybridized with the monoclonal antibody. Here's the result:



1 Milk AA

2 Rennet-whey AA

3 Milk BB

4 Rennet-whey BB

5 Milk AB

6 Rennet-whey AB

7 24aakB-TRF positive ctrl

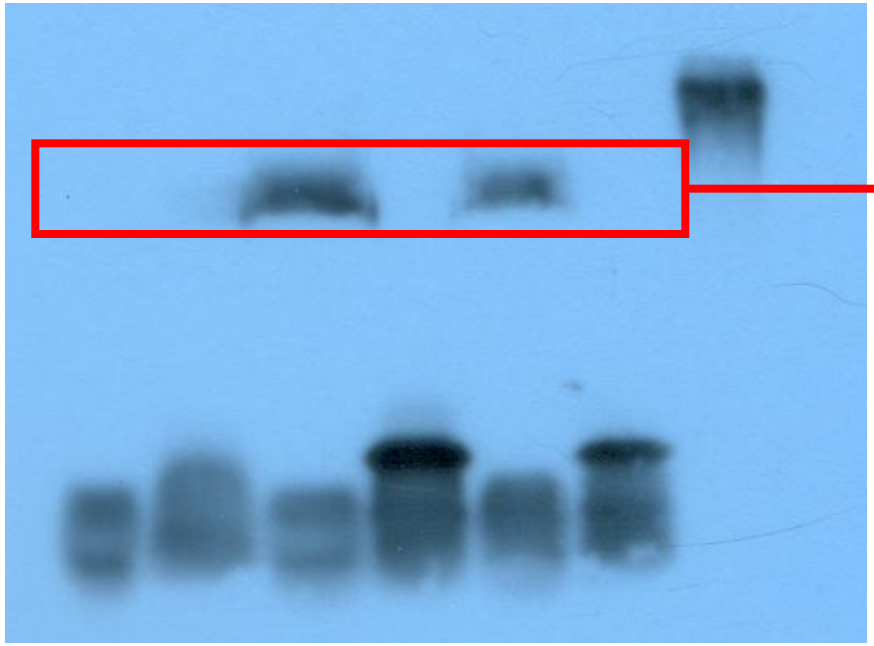
8 24aakA-TRF negative ctrl

The controls work! Now we can "read" other lanes

Western blot

- Reaction with monoclonal antibody

SDS-PAGE



M W M W M W

AA BB AB

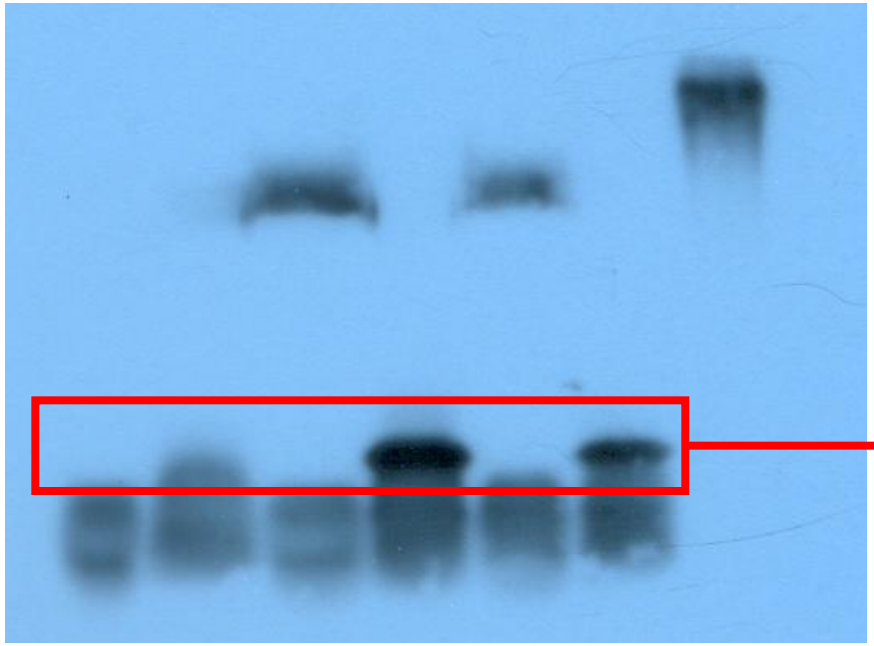
K-casein migration zone (169 aa)

A positive reaction was found in milk (M) from cows BB and AB, while no-reaction was found in AA milk and in rennet-whey of all milks

Western blot

- Reaction with monoclonal antibody

SDS-PAGE

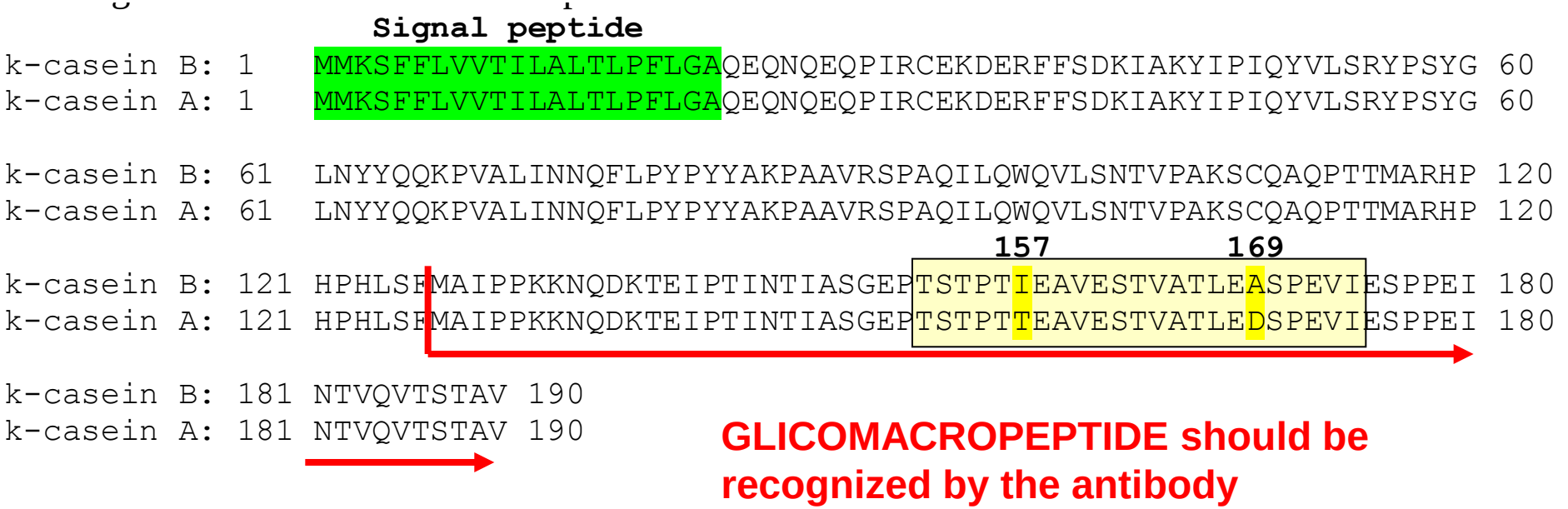


M W M W M W

AA BB AB

A band appear only in rennet-whey (W) of BB and AB milks: Glycomacropeptide from k-cn B (65 aa)?

Working steps

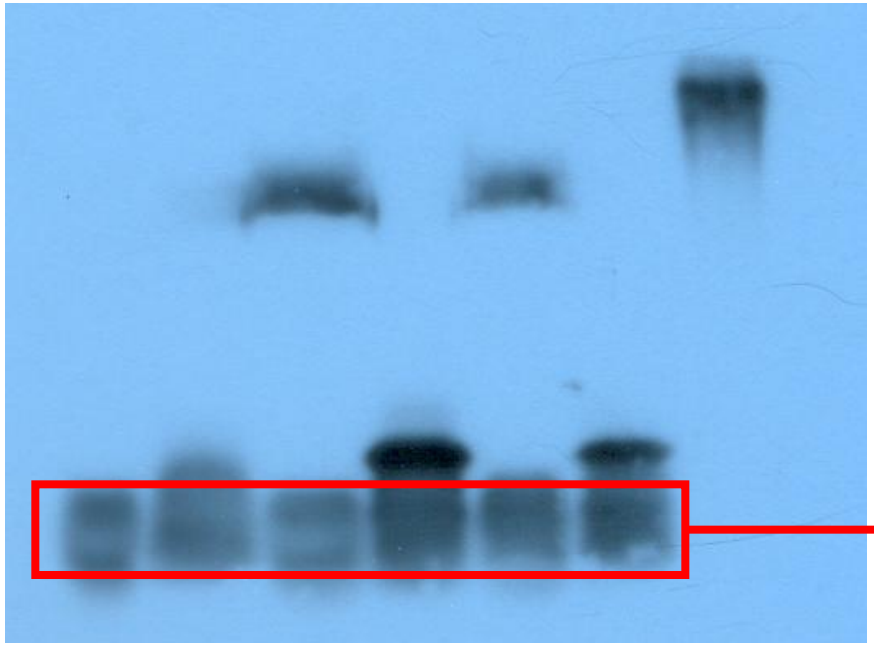


GLICOMACROPEPTIDE should be recognized by the antibody

Western blot

- Reaction with monoclonal antibody

SDS-PAGE



M W M W M W

AA BB AB

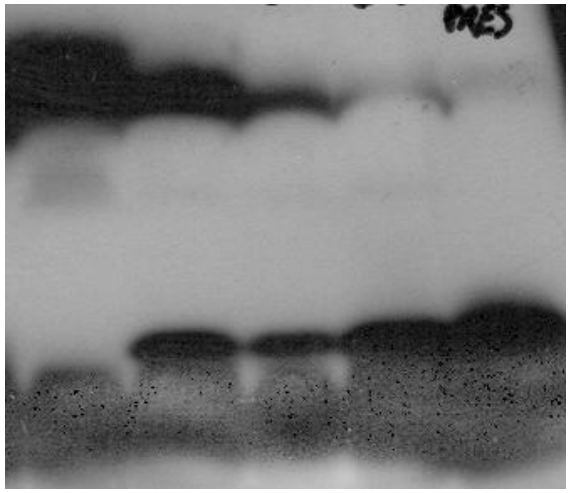
A non-specific reaction was found in all milks and rennet-whey (small size peptides?)

Western blot

- Reaction with monoclonal antibody

SDS-PAGE

1 2 3 4 5



1 BB Milk + rennet, 0 min

2 BB Milk + rennet, 10 min

3 BB Milk + rennet, 20 min

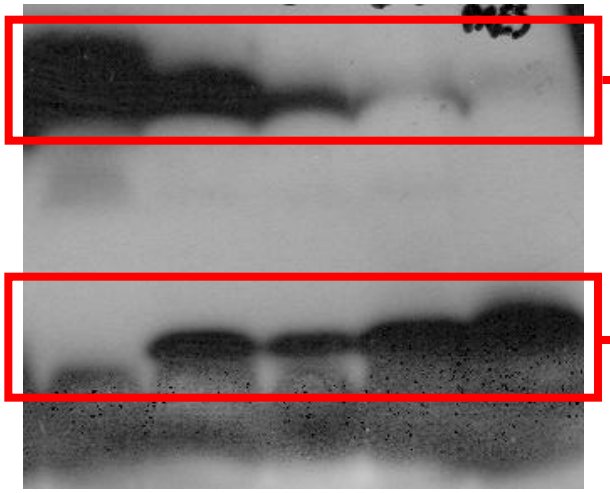
4 BB Milk + rennet, 30 min

5 BB Rennet-Whey

Western blot

- Reaction with monoclonal antibody

SDS-PAGE



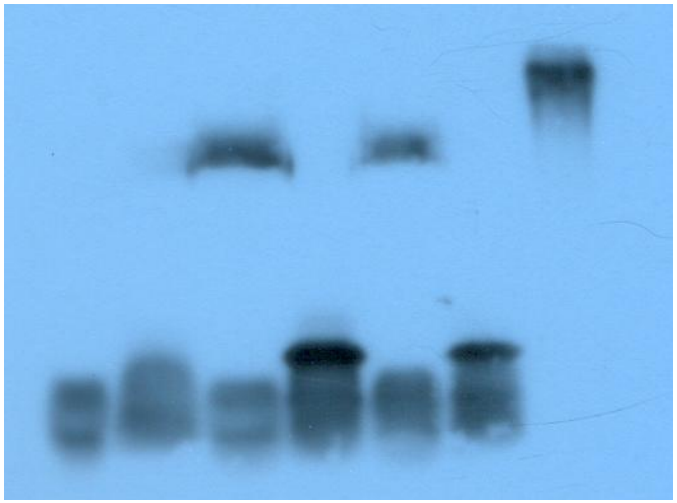
The band in the k-casein migration zone disappeared as time with rennet increase

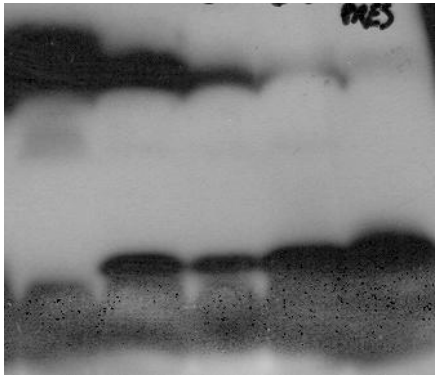
while the band corresponding to glicomacropeptide appear after 10 min and increase of intensity as time increase

Western blot

- Reaction with monoclonal antibody

SDS-PAGE



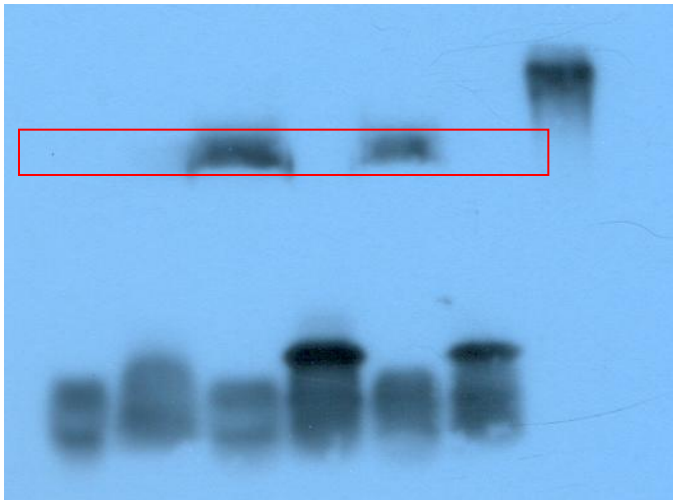


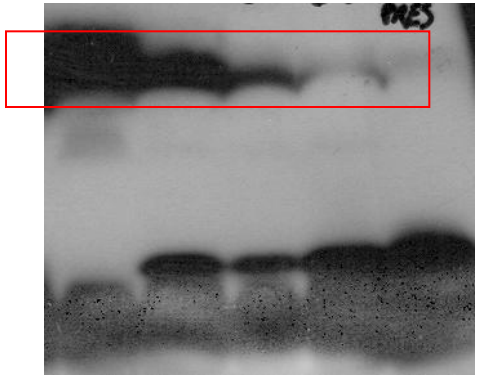
On the basis of these results:
- The antibody is specific toward the allele B of k-casein in milk with respect to A allele
- It reacts with glicomacropeptide of the B allele as well
- It also recognize some unidentified small pepetides in a non-specific manner

Western blot

- Reaction with monoclonal antibody

SDS-PAGE





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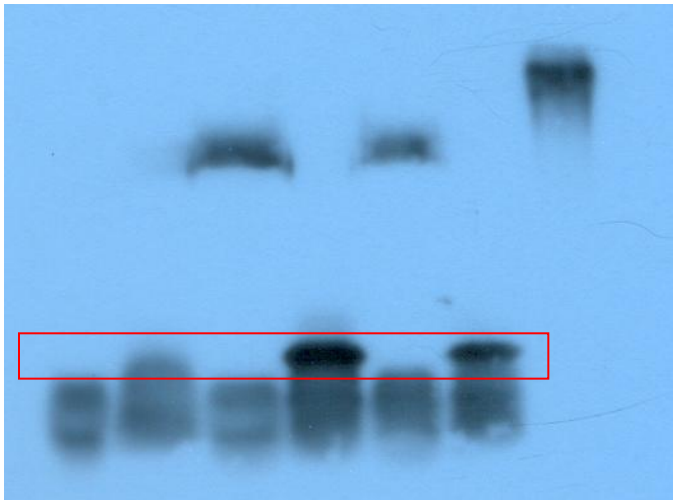
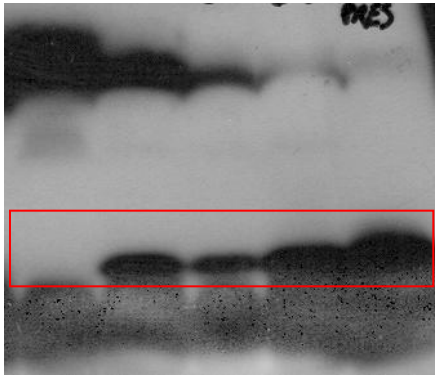
Western blot

- Reaction with monoclonal antibody

SDS-PAGE





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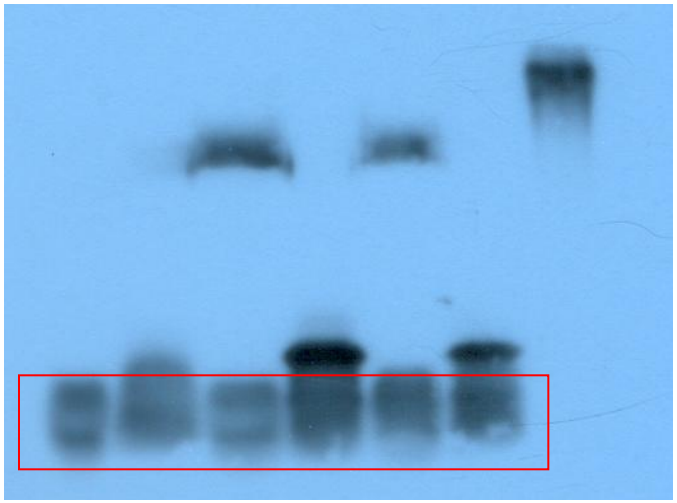
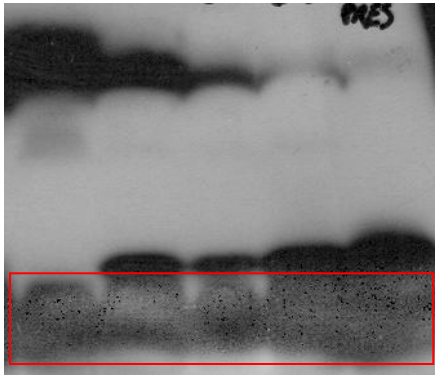
Western blot

- Reaction with monoclonal antibody

SDS-PAGE





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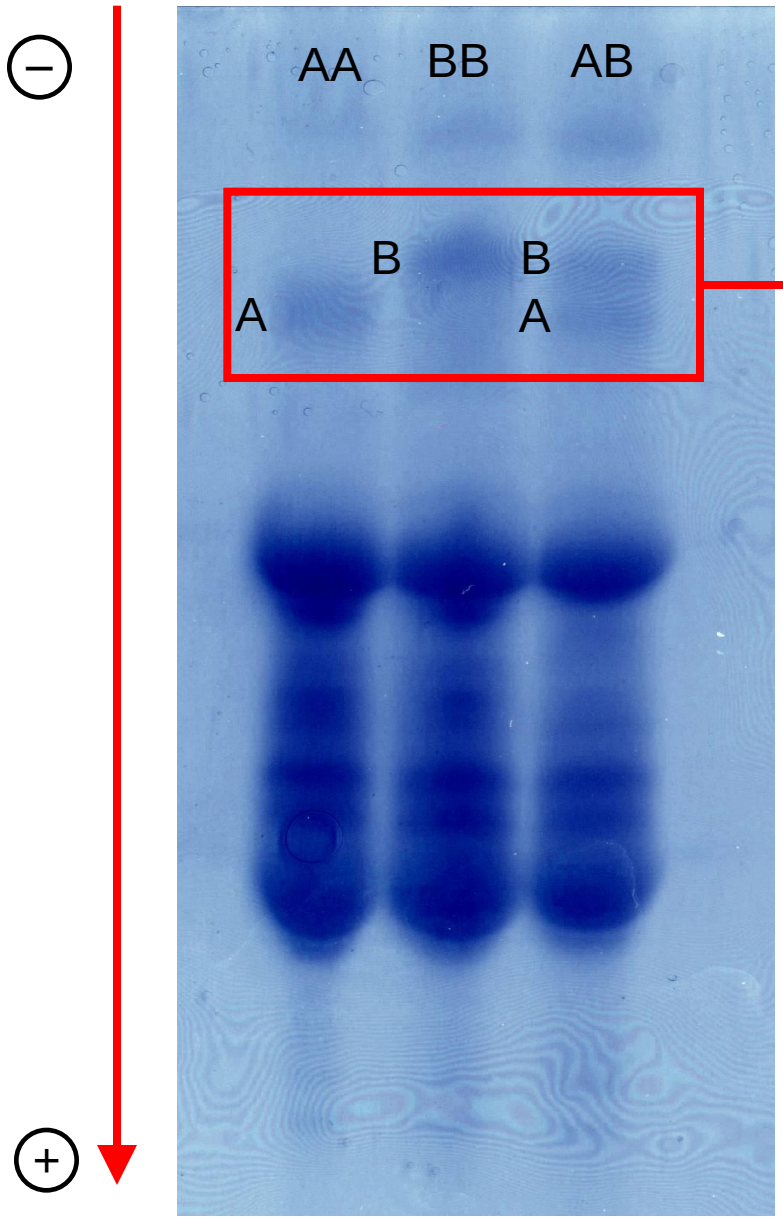
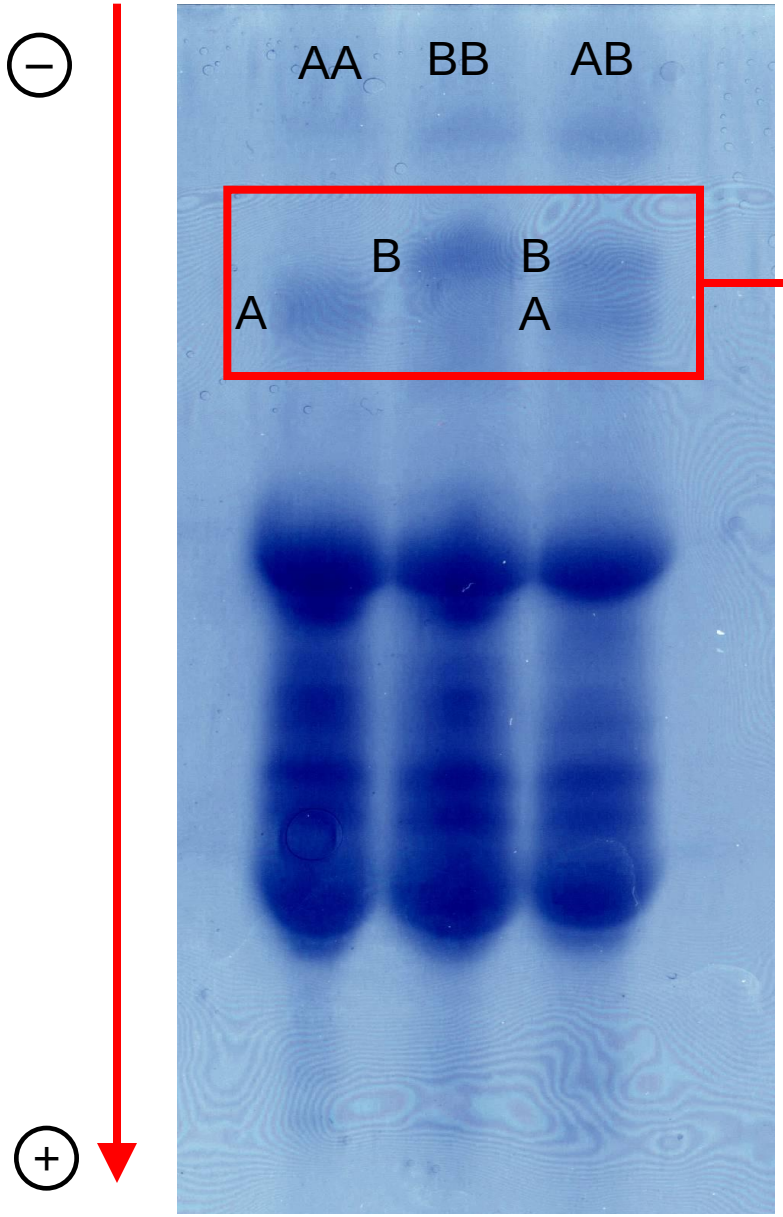
Western blot



Urea-PAGE

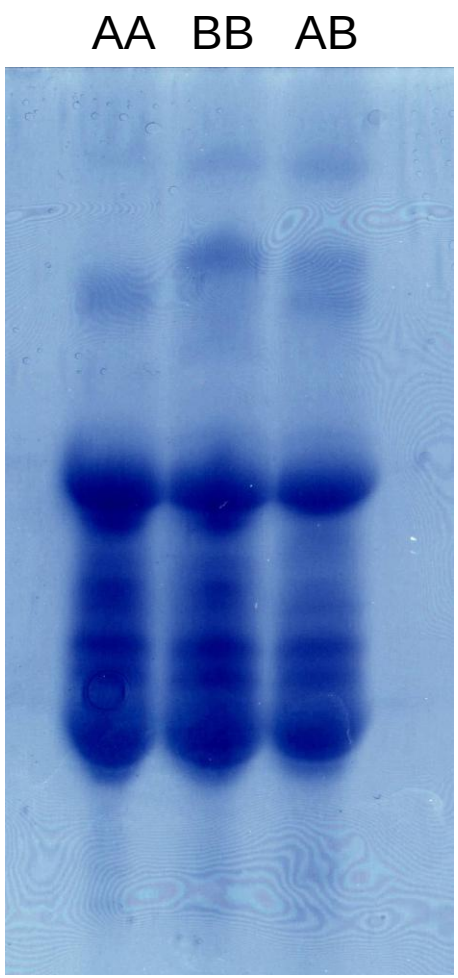
Western blot

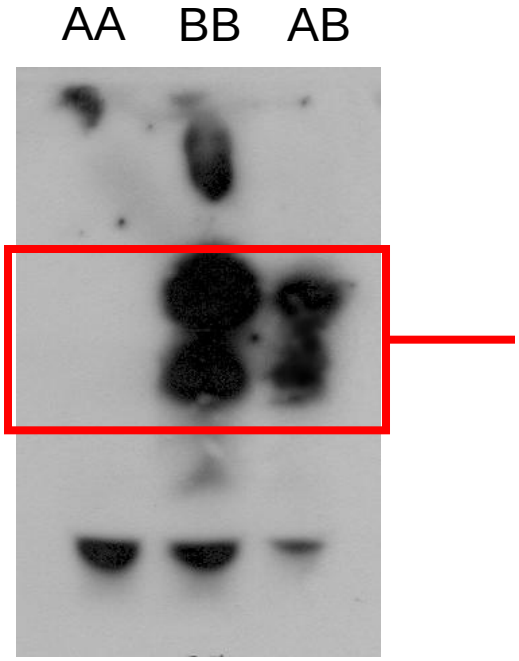


Urea-PAGE

Milk proteins are separated according to net charge and molecular weight.
In this case, it is possible to separate the B from the A allele.

Western blot



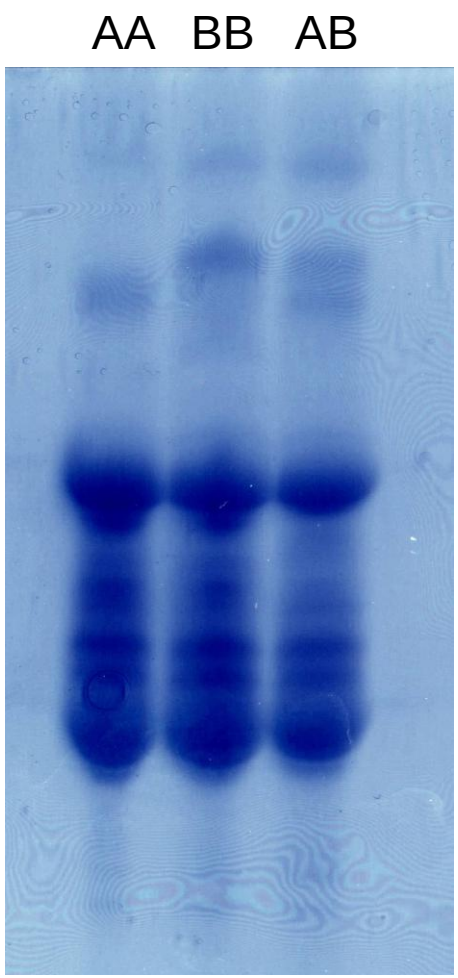


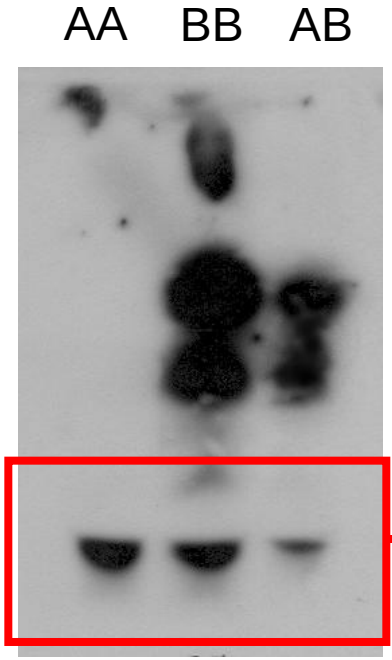
Urea-PAGE

A positive reaction of the monoclonal antibody was found only in BB and AB milks. In this case the band was a “smear” because of k-cn with different degree of glycosilation (k-cn isoforms) were separated in Urea-PAGE.
The smear band was in the migration zone of k-casein

Western blot

Urea-PAGE



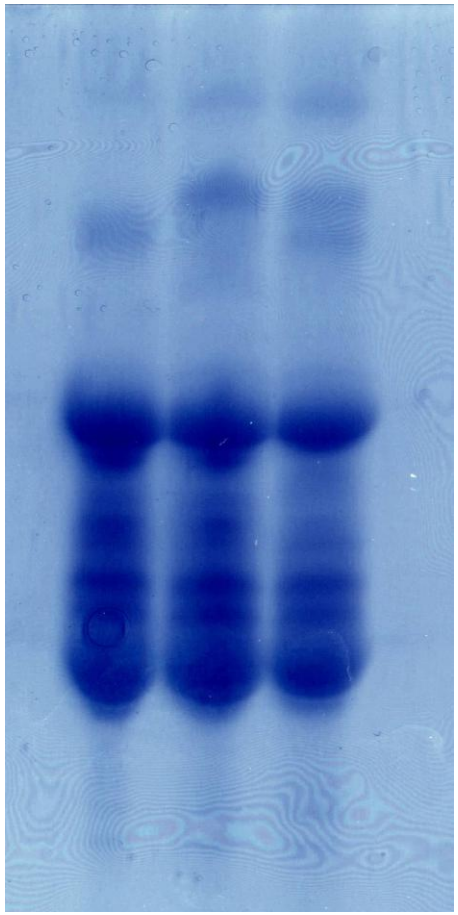


Also in this case, a non-specific reaction was detectable in all milks. However, according the correspondent position in the gel, the band seems in the migration zone of the beta-lg.

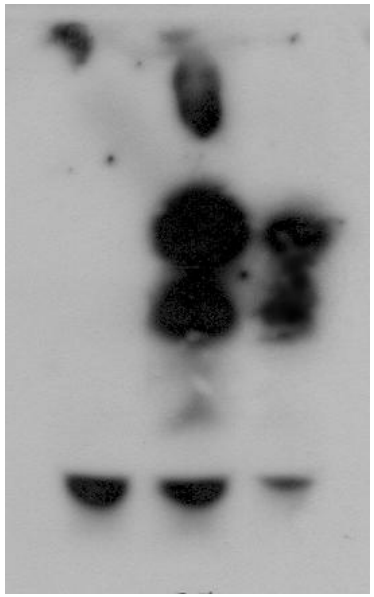
Western blot

Urea-PAGE

AA BB AB



AA BB AB

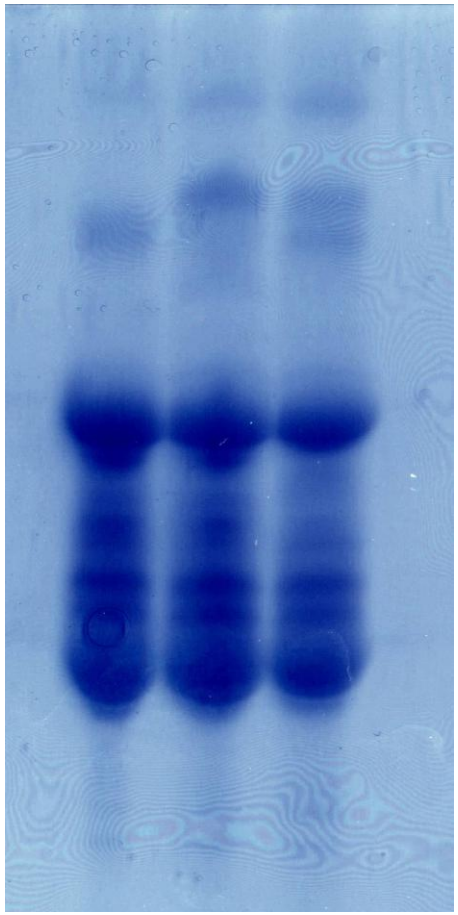


Further informations:
- The antibody recognize all the isoforms of k-casein
- The non-specific reaction seems to be related to beta-lg

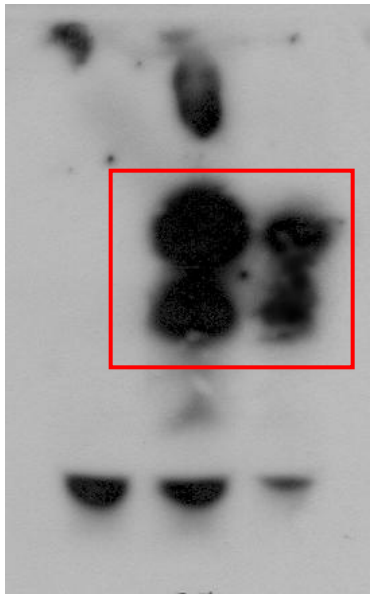
Western blot

Urea-PAGE

AA BB AB



AA BB AB



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Western blot

Urea-PAGE

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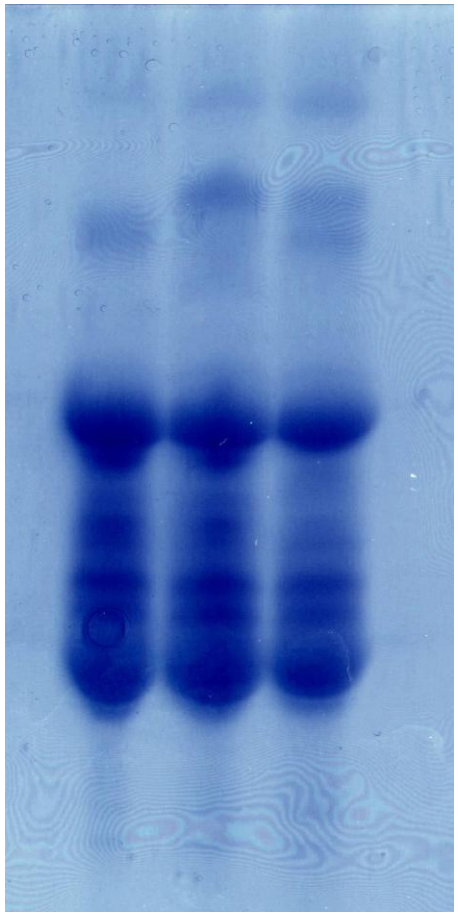
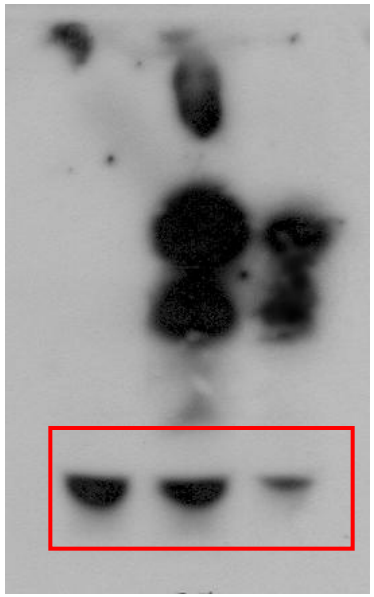


AA BB AB



Further results:

- The antibody recognize all the isoforms of k-casein
- The non-specific reaction seems to be related to beta-lg

Conclusion

The monoclonal antibody fits all the requirements reported in the project

The allele specificity of the monoclonal antibody is very promising in terms of set-up an ELISA quick test

As the antibody recognize the glicomacropeptide, it will be possible to work on the rennet whey in order to eliminate the interference/noise of caseins

However an non-specific reaction was detected in milk and its incidence on the final signal in ELISA most be carefully evaluated

Current works

Bulk milk samples

Other variants of k-casein

ELISA test