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Isolation of antibacterial peptides from rabbit milk casein

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Introduction

Milk proteins have been considered for a long time for their nutritional value only, namely to supply the organism with essential amino acids. It is well established that proteolytical digestions of bovine and human caseins yield several bioactive peptide fragments, which are involved in the regulation of various physiological processes. Peptides derived from casein hydrolysates show immuno-modulatory and anti-inflammation effects, play important roles in passive immunity, are important for the growth and the development, inhibit platelet aggregation, and alter gastro-intestinal function. Therefore milk proteins are important factors not only in consideration of their nutritional value, but also for their intrinsic potentiality to be used as dietary supplements or in future as drugs [for reviews see 1, 2, 3].

Objectives

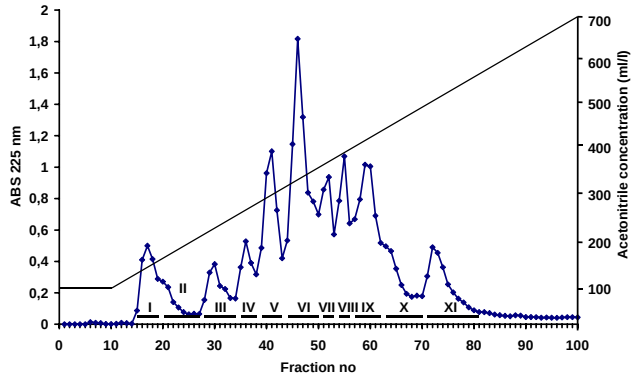
Caseins represent a heterogeneous class of milk proteins whose bactericidal properties have been poorly investigated. Some antibacterial activity has been ascertained for some peptides derived from the bovine casein only. The aim of our work was to search whether bactericidal peptides could be generated from the digestion of rabbit casein by endopeptidase present in the gastrointestinal tract. We expect from one side to learn more about the physiological function of casein and on the other side to explore the possibility to obtain antimicrobial compounds from food proteins.

Isolation of peptides

whole rabbit casein

enzyme *(trypsin)*

incubation at 37°C → ***RP-FPLC***



Collection of fractions: Fr. 1, 2, 3, 4, 5, 6, 7, 8, ... 100 (200)

pooling fractions belonging to the different peaks

Pool I, II, III, IV, V, ... → ***FREEZE-DRYING***

FREEZE-DRYING → ***antibacterial assay***

antibacterial assay → Isolated, antibacterial peptide

Isolated, antibacterial peptide → **sequencing**

sequencing → ***peptide synthesis*** → purification → ***RP-FPLC*** → ***antibacterial assay***

antibacterial assay → Pools with antibacterial acivity → ***RP-FPLC***

RP-FPLC → **Collection of fractions:** Fr. 1, 2, 3, 4, 5, 6, 7, 8, ... 100 (200)

1. step (column 120-10)

Pool I		Pool II		Pool III		Pool IV		Pool V		Pool VI		Pool VII		Pool VIII		Pool IX		Pool X		Pool XI	
E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	-	E.coli	0,5
B.subtilis	0.81	B.subtilis	1.77	B.subtilis	0.40	B.subtilis	0.03	B.subtilis	1.12	B.subtilis	-	B.subtilis	-	B.subtilis	-	B.subtilis	-	B.subtilis	1.42	B.subtilis	>3.0
St. lentus	0.17	St. lentus	0.08	St. lentus	-	St. lentus	-	St. lentus	0.37	St. lentus	0.49	St. lentus	-	St. lentus	0.04	St. lentus	0.04	St. lentus	0.95	St. lentus	0.10

2. step (column 100-5)

Pool 1		Pool 2	
B.subtilis	0.62	B.subtilis	0.75
St. lentus	-	St. lentus	-

2. step (120-10 oszlop)

Pool 1	
B.subtilis	0.44
St. lentus	0.07

2. step (column 120-10)

Pool 1

2. step (column 120-10)

Pool 1		Pool 2	
B.subtilis	0.15		
St. lentus	-		

Mixed by a mistake

2. step (column 120-10)

Pool 1		Pool 2		Pool 3		Pool 4	
B.subtilis	0.18	B.subtilis	0.04	B.subtilis	-	B.subtilis	0.12
St. lentus	0.06	St. lentus	-	St. lentus	0.08	St. lentus	-

2. step (column 120-10)

Pool 1		Pool 2		Pool 3	
B.subtilis	0.44	B.subtilis	0.23	B.subtilis	0.60
St. lentus	0.35	St. lentus	0.25	St. lentus	0.12

3. step (column 100-5)

Pool 1	
B.subtilis	-

3. step (column 120-10)

Pool1		Pool 2		Pool 3		Pool 4	
St. lentus	1.00	St. lentus	0.46	St. lentus	0.31	B.subtilis	0.56
						St. lentus	0.23

3. step (column 100-5)

Pool 1	

3. step (column 100-5)

Pool1	
B.subtilis	2.12
St. lentus	-

sequenced!
TP-3 (ILPFIQSLFPFAER)

4. step (column 100-5)

Pool 1	
St. lentus	0.46

sequenced!
TP-1 (HVEQLLR)

4. step (column 100-5)

Pool 1		Pool 2	
St. lentus	-	St. lentus	-

4. step (column 100-5)

Pool 1		Pool 2	
St. lentus	0.13	St. lentus	0.09

4. step (column 100-5)

Pool 1

5. step (column 100-5)

Pool 1	
St. lentus	0.11

sequenced!
TP-2 (FHLGHLK)

5. step (column 100-5)

Pool 1		Pool 2	
St. lentus	0.11	St. lentus	0.09

6. step (column HD)

Pool 1		Pool 2	
St. lentus	0.06	St. lentus	-

3. step (column 100-5)

Pool1		Pool 2	
B.subtilis	-	B.subtilis	0.14
St. lentus	-	St. lentus	-

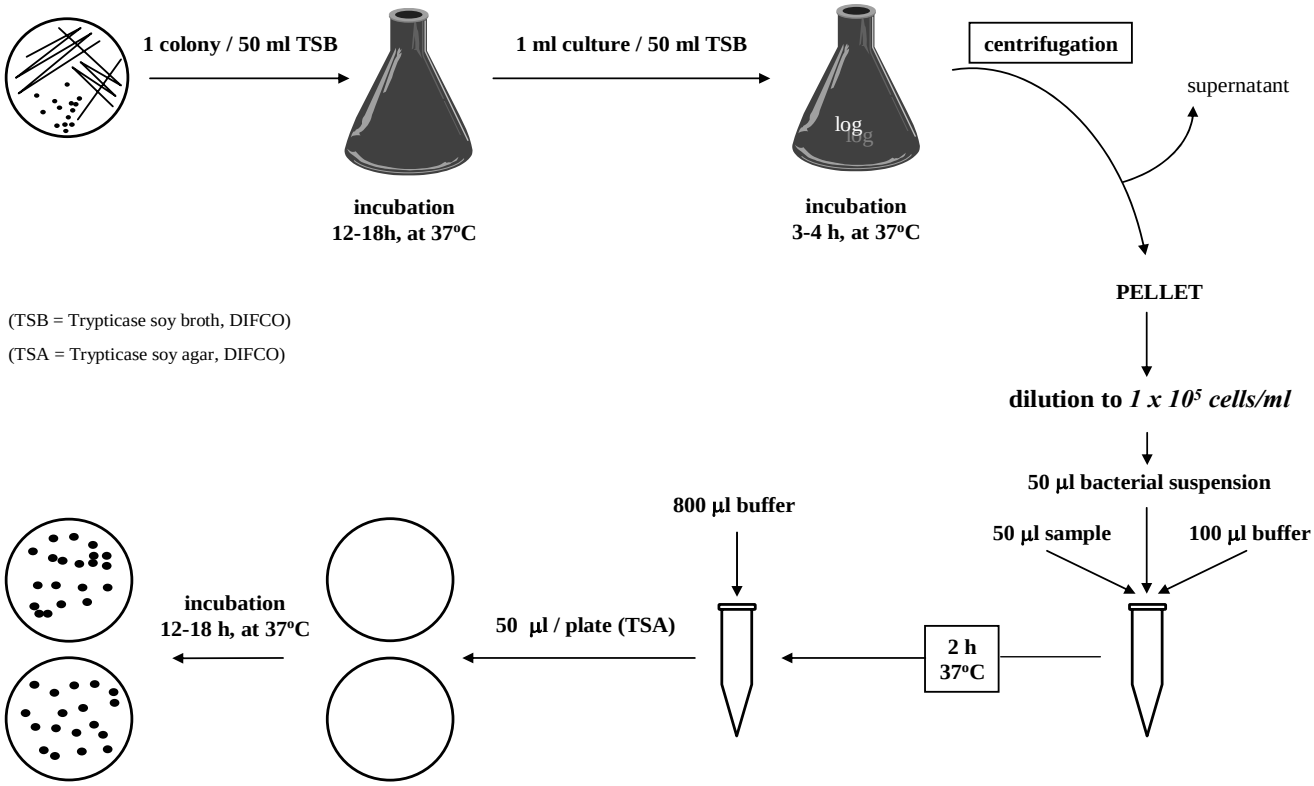
3. step (column 100-5)

Pool 1	Pool 2	Pool 3

Chromatographic separation

Chromatographic columns: Nucleosil 120-10 C18, Nucleosil 100-5 C18 (Macherey-Nagel)

Antibacterial assay



(TSB = Trypticase soy broth, DIFCO)

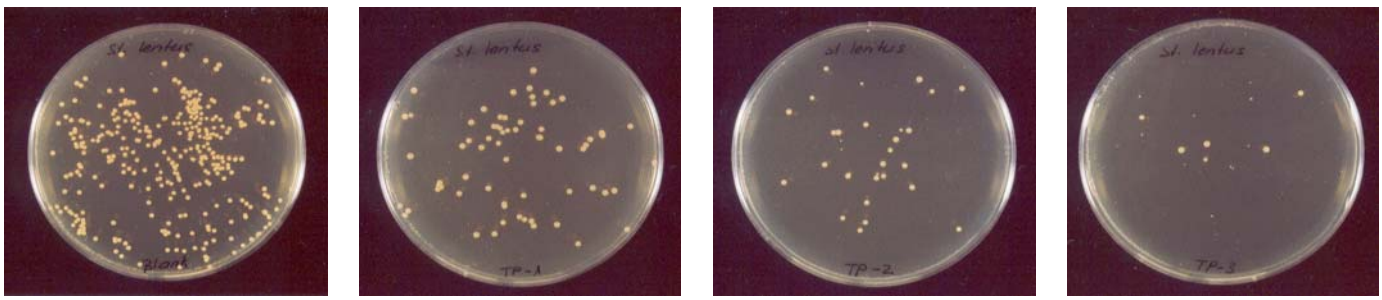
(TSA = Trypticase soy agar, DIFCO)

Calculation of antibacterial activity

$$\log (N_0 / N_1)$$

N_0 = number of control colonies

N_1 = number of colonies grown in the presence of antibacterial agent



Bacteria	Strain	Type
Escherichia coli	ATCC 25922	Gram-neg.
Bacillus subtilis	BGA	Gram-pos.
Staphylococcus lentus	WS	Gram-pos.
Klebsiella pneumonieae	ATCC 13883	Gram-neg.
Serratia marcescens	ATCC 8100	Gram-neg.
Streptococcus zooepidemicus	WS	Gram-pos.

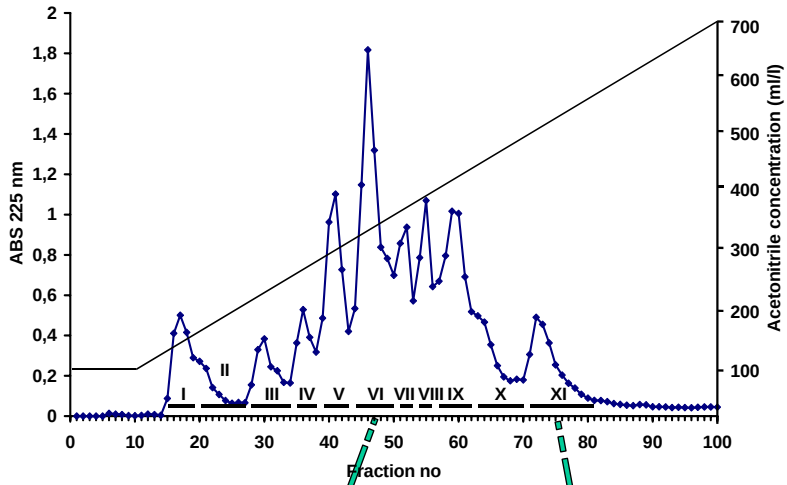
Testing the pools

Testing synthetic peptides

Results

	Esch. coli	*B. subtilis*	*Staph. lentus*
Pool I	**0**	**0.81 ± 0.37**	**0.17 ± 0.02**
Pool II	**0**	**1.77 ± 0.26**	**0.08 ± 0.02**
Pool III	**0**	**0.40 ± 0.03**	**0**
Pool IV	**0**	**0.03 ± 0.03**	**0**
Pool V	**0**	**1.12 ± 0.12**	**0.37 ± 0.07**
Pool VI	**0**	**0**	**0.49 ± 0.13**
Pool VII	**0**	**0**	**0**
Pool VIII	**0**	**0**	**0.04 ± 0.02**
Pool IX	**0**	**0**	**0.04 ± 0.02**
Pool X	**0**	**1.42 ± 0.09**	**0.95 ± 0.10**
Pool XI	**0.50 ± 0.30**	**>3.00 ± 0.00**	**0.10 ± 0.02**

Trypsin digestion



Further purification of pools VI and XI

HVEQLLR
(beta-casein)

FHLGHLK
(alpha-S1-casein)

ILPFIQSLFPFAER
(beta-casein)

Rabbit casein-derived antibacterial peptides

Rabbit alfa-S1-casein

```
        10         20         30         40         50
         |          | TP-2     |          |          |
MKLLILTCLV ATALARHKFH LGHLKLTQEQ PESSEQEILK ERKLLRFVQT

        60         70         80         90        100
         |          |          |          |          |
VPLELREEYV NELNRQRELL REKENEEIKG TRNEVTEEHV LADRETEASI

       110        120        130        140        150
         |          |          |          |          |
SSSSEEIVPS STKQKYVPRE DLAYQPYVQQ QLLRMKERYO IQEREPMRVV

       160        170        180        190        200
         |          |          |          |          |
NQELAQLYLQ PFEQPYQLDA YLPAPWYYTP EVMQYVLSPL FYDLVTPSAF

       210   215
         |     |
ESAEKTDVIP EWLKN
```

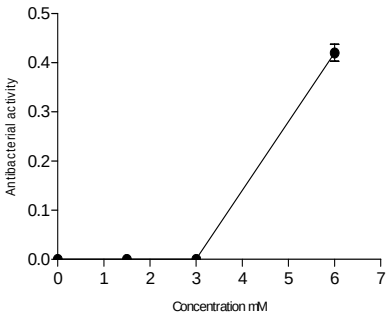
Rabbit beta-casein

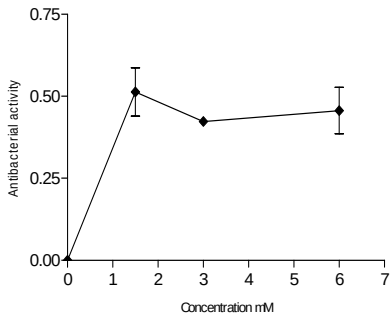
```
        10         20         30         40         50
         |          |          |          |          |
MKVLILACLV ALALAREKEQ LSVPTEAVGS VSSSEEITHI NKQKLETIKH

        60         70         80         90        100
 TP-1    |          | TP-3     |          |          |
VEQLLREEKL QDKILPFIQS LFPFAERIPY PTLPQNILNL AQLDMLLPLL

       110        120        130        140        150
         |          |          |          |          |
QPEIMEDPKA KETIIPKHKL MPFLKSPKTV PFVDSQILNL REMKNQHLLL

       160        170        180        190        200
         |          |          |          |          |
PQLLPFMHQV FQPFPQTPIP YPQALLSLPQ SKFMPIVPQV VPYPQRDMPI

       210        220      228
         |          |        |
QALQLFQELL FPTHQGYPVV QPIAPVNV
```

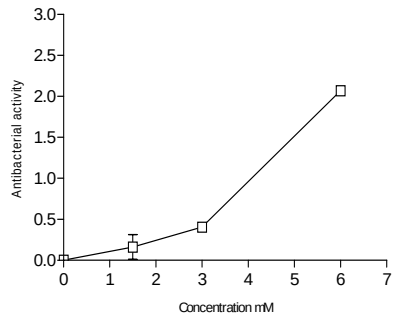
Peptide	*sequence*	*casein*	*position*	*pI*
TP-1	**HVEQLLR**	**β-CN**	**50-56**	**6,5**
TP-2	**FHLGHLK**	**α_{S1}-CN**	**19-25**	**8,7**
TP-3	**ILPFIQSLFPFAER**	**β-CN**	**64-77**	**6,0**

Antibacterial activity of synthetic peptides

B. subtilis





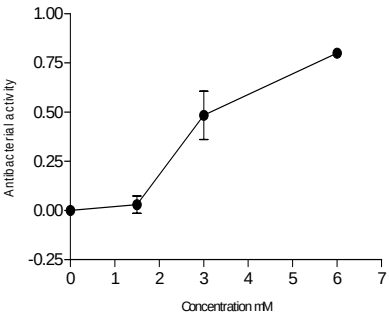


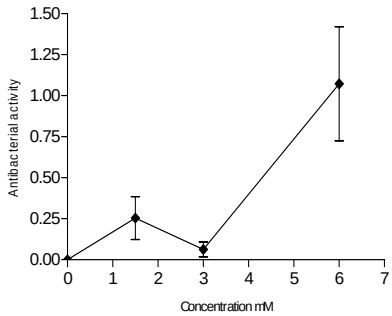
HVEQLLR
(*TP-1*)

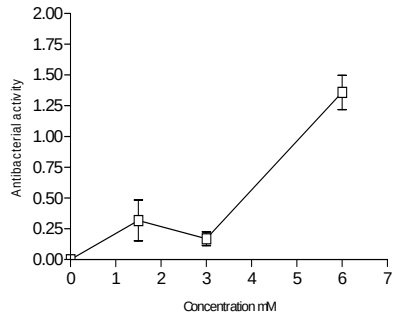
FHLGHLK
(*TP-2*)

ILPFIQSLFPFAER
(*TP-3*)

Staph. lentus







Whole rabbit casein was digested with trypsin and the arising peptide fragments were separated by reversed-phase FPLC. According to their amino acid sequence the identified peptides were synthetized and the antibacterial activity of the synthetic peptides tested against six Gram positive and Gram negative bacterial strains.

★ ***Trypsin digestion:***

- **4 pools strongly, 1 pool moderately inhibited the growth of B. subtilis**
- **2 pools moderately inhibited the growth of Staph. lentus**

★ ***Pools with antibacterial activity were further purified and the amino acid sequence of the isolated peptides was determined:***

- **2 antibacterial peptides were identified from pool VI**
  HVEQLLR (beta-casein, residues 50-56, pI 6.5) and
  FHLGHLK (alpha-S1-casein, residues 19-25, pI 8.7)
- **1 antibacterial peptide was identified from pool XI**

  ILPFIQSLFPFAER (beta-casein, residues 64-77, pI 6.0).

★ ***Synthetic peptides:***

- **Active only against the Gram-positive bacteria *B. subtilis* and *Staph. lentus*, but not against *Strep. zooepidemicus*. Not active against Gram-negative bacteria *Esch. coli, Kl. pneumoniae*, and *Ser. marcescens*.**

Acknowledgements

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References

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