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Antibacterial activity of casein-derived peptides isolated from rabbit (*Oryctolagus cuniculus*) milk

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Acid-precipitated rabbit 'whole casein' was digested by trypsin, chymotrypsin, pepsin, and clostripain to screen for possible peptides with antibacterial properties. The peptide fragments were separated by reversed-phase chromatography. The collected fractions were pooled and their antibacterial properties tested against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus lentus*. Three antibacterial peptide fragments derived from tryptic digestion of rabbit casein were isolated and identified. Their sequences were found as follows: HVEQLLR (residues 50–56 of β-casein), ILPFIQSLFPFAER (residues 64–77 of β-casein), and FHLGHLK (residues 19–25 of α_{s1}-casein). The three peptides were synthesized and found to exert antibacterial effect against Gram positive bacteria only. Proteolytic digestion of rabbit casein by chymotrypsin, pepsin and clostripain yielded several peptide fragments with antibacterial activity. Since antibiotic peptides can be released from casein during the digestion of milk proteins, our results suggest a possible antibacterial function of rabbit caseins. It is conceivable that antibacterial peptides can be generated by endopeptidases of the mammalian gastrointestinal tract possibly providing protection for new-born rabbits against aggression of micro-organisms.

Keywords: Antibacterial, bioactive peptide, casein, milk, rabbit.

Milk proteins have been long considered for their nutritional value only, namely to supply the organism with essential amino acids. Since the detection of the first opioid peptides (Brantl et al. 1979) derived from proteolytic digestion of casein *in vivo*, many bioactive peptides derived from casein have been investigated.

Bioactive peptides derived from casein have been reported to be involved in the regulation of various physiological processes like immunomodulation (Parker et al. 1984), opioid agonist (Jinsmaa & Yoshikawa, 1999), opioid antagonist (Chiba et al. 1989) and inhibition of platelets aggregation (Qian et al. 1995). Biologically active peptide sequences were obtained *in vitro* by proteolysis (Bellamy et al. 1992; Zucht et al. 1995; Recio & Visser, 1999) and *in vivo* by gastrointestinal digestion of milk proteins (Kuwata et al. 1998; Meisel & Bockelmann, 1999). Bioactive peptides with antibacterial properties have been discovered in animals and plants (Gennaro et al. 1989; Lee et al. 1989; Bevins & Zasloff, 1990; Lehrer et al. 1993; Boman, 1995; Cowan, 1999; Ganz & Lehrer, 1999). We have investigated the proteolytic digestion of aprotinin (Pellegrini et al. 1996), lysozyme (Pellegrini et al. 1997), α-lactalbumin (Pellegrini et al. 1999), and β-lactoglobulin (Pellegrini et al. 2001) and obtained peptide fragments with antibacterial properties. Casein, the main protein fraction of the milk, is a good source of bioactive peptides which exert several physiological functions, thus it is important to determine whether proteolytic digestion of casein *in vitro* would generate antibacterial peptides. We have previously attempted the proteolytic digestion of bovine κ-casein and searched for antibacterial peptides (not published). We observed that the bovine κ-casein fragments were only weakly antibacterial (unpublished).

Since caseins represent a heterogeneous class of milk proteins we decided to investigate the antibacterial properties of rabbit casein, which were not studied before. The aim of the present work was to find out whether antibacterial peptides could be generated through proteolytic digestion of rabbit casein by endopeptidases of the gastrointestinal tract. Here we will show that several antibacterial peptides can be generated from rabbit casein. Three such peptides generated by trypsin were sequenced and produced synthetically. The antibacterial properties of the synthetic peptides and of the fragments created by the proteolytic digestion of rabbit casein are discussed.

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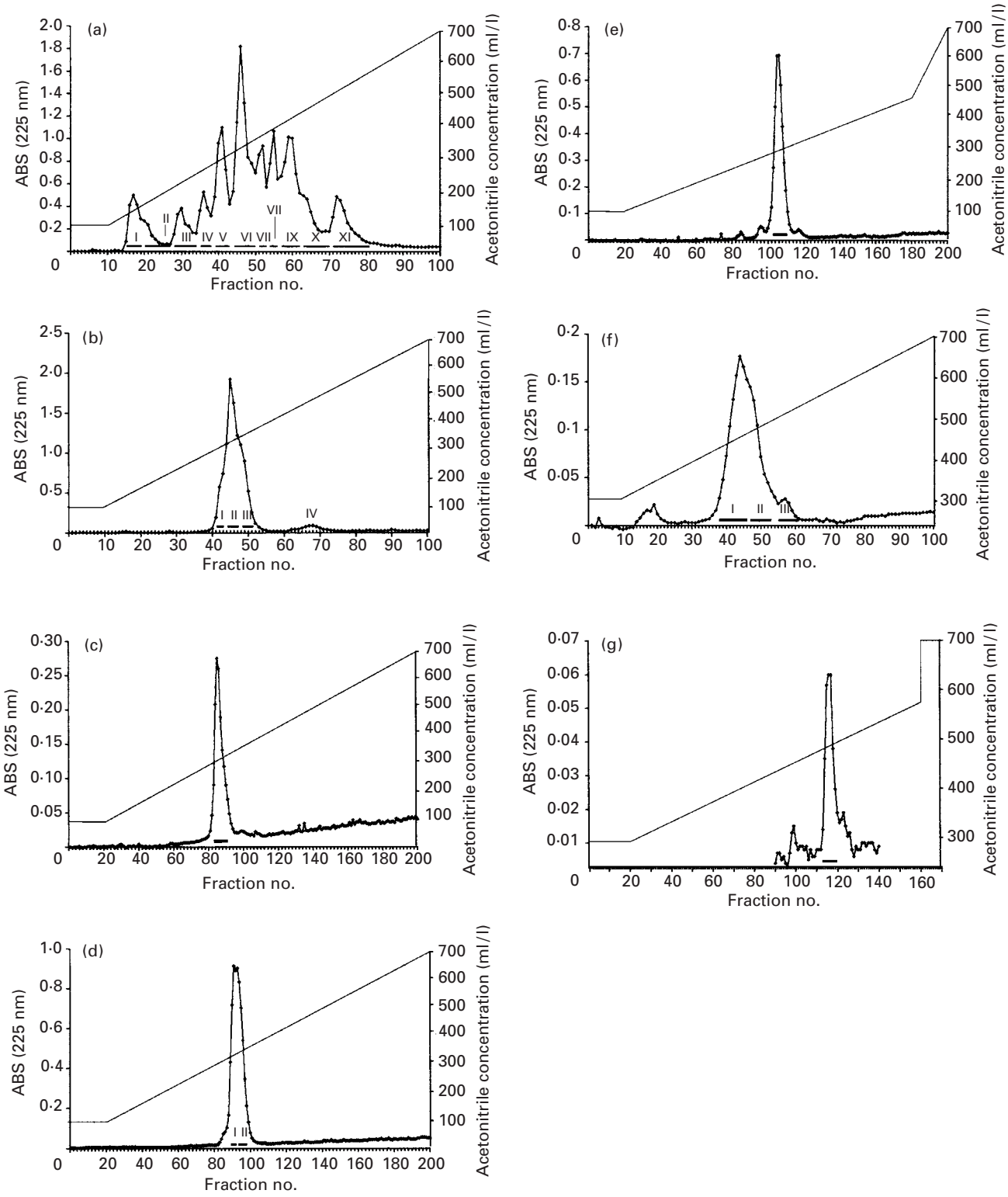

Fig. 1. RP-FPLC of the peptide fragments derived from rabbit casein digested with trypsin. (a) Elution diagram from RP-FPLC on Nucleosil 120-10 C18. Pool 1a-VI was moderately antibacterial against *Staph. lentus,* whereas pool 1a-XI exerted a strong antibacterial activity against *B. subtilis* (Table 1). (b) Further purification of pool 1a-VI (panel a) performed by RP-FPLC on Nucleosil 120-10 C18. (c) Purification of pool 1b-I (panel b). The material was resolved by RP-FPLC on Nucleosil 100-5 C18 column as a single peak. It was identified as the peptide HVEQLLR (RCDT1). (d) Pool 1b-II (panel b) was resolved by RP-FPLC on Nucleosil 100-5 C18 in two peaks. (e) Further purification of pool 1d-I (panel d) through Nucleosil 100-5 C18 column resulted in a single peak. The

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Materials and Methods

Materials

Reagents were obtained from the following sources: Trypsin (2 × crystallised from porcine pancreas) from Serva (Catalys, CH-8205 Wallisellen, Switzerland); chymotrypsin Type I-S (from bovine pancreas), clostripain and synthetic oxytocin from Sigma (Sigma AG, CH-6371 Stans/Lucern, Switzerland). Peptides HVEQLLR, FHLGHLK and ILPFIQSLFPFAER were synthesized at the Institute of Biochemistry of the Medicine Faculty, University of Lausanne (CH-1066 Epalinges, Switzerland) according to our order. Chemicals for sequence analysis were from Agilent Technologies (D-71034 Böblingen, Germany).

Nucleolsil 120-10 C18 and Nucleosil 100-5 C18 columns for reversed-phase chromatography were from Macherey-Nagel (Macherey-Nagel AG, CH-4702 Oensingen, Switzerland). Reagents for chromatography were of HPLC grade and purchased from Biosolve (5554 HA Valkenswaard, The Netherlands). *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, and *Serratia marcescens* ATCC 8100 were from Medical & Veterinary Supplies Limited (MK 182LR Buckingham, UK); *Staphylococcus lentus* and *Streptococcus zooepidemicus* (wild strains) were from the Institute of Bacteriology of the Veterinary Hospital Zurich (CH-8057 Zurich, Switzerland); *Bacillus subtilis* BGA was from Merck (Merck-Schweiz AG, CH-8953 Dietikon, Switzerland).

Rabbit (*Oryctolagus cuniculus*) milk was collected from New Zealand White rabbits at the Institute for Small Animal Research (H-2100 Gödöllő, Hungary).

Antibacterial assay

Antibacterial assays were essentially performed as described earlier (Pellegrini et al. 1997). Samples were mixed with bacteria harvested at logarithmic phase (ca. 10^5 cfu/ml) and incubated in 10 mM-sodium phosphate buffer, pH 7·4 containing 20 g trypticase soy broth (TSB)/l at 37 °C for 2 h. The bacterial suspension was diluted and plated on trypticase soy agar (TSA) for cfu evaluation. The assays were conducted in triplicate. The antibacterial activity was calculated as $\log(N_0/N_1)$, where N = cfu after 2 h incubation, grown without (N_0) and in the presence (N_1) of the tested sample material.

Sequence analysis of the casein fragments

Peptide sequence analysis was performed at the Peptide Sequence Analysis Centre, University of Zurich. The analysis was carried out on a Model G1005 A protein sequencer Agilent Technologies using version 3.0 chemistry according to the manufacturer's recommendations.

Purification of the synthetic polypeptides

The synthetic peptides FHLGHLK and HVEQLLR were dissolved in H_2O containing 100 ml acetonitrile and 0·5 ml TFA/l, whereas the dissolving solution used for the peptide ILPFIQSLFPFAER contained 300 ml acetonitrile/l. The peptides were purified by reversed phase chromatography (RP-FPLC) on a Nucleosil 100-5 C18 column using a linear gradient of acetonitrile from 100 ml/l (for the purification of the peptides FHLGHLK and HVEQLLR) or from 300 ml/l (for the peptide ILPFIQSLFPFAER) to 700 ml/l (all containing 0·5 ml TFA/l).

Preparation of rabbit whole casein

Rabbits were injected intramuscularly with 2 I.U. oxytocin and milk was collected after 5 min by hand. Whole casein was prepared by acid precipitation (pH 4·6). Mixed milk from 10 New Zealand white rabbits was diluted with an equal volume of distilled water, skimmed by centrifugation at 2500 ***g*** at room temperature for 20 min, and then acidified with 1 M-sodium acetate pH 4·6, and stirred for 10 min at room temperature. The pH of the solution was around 4·6 (detected by litmus paper). After centrifugation at 1500 ***g*** for 15 min the supernatant was discarded, the pellet (casein fraction) washed twice with distilled water and freeze-dried.

Digestion of rabbit casein with trypsin

Freeze-dried rabbit casein (1 g) and 20 mg trypsin were dissolved in 4 ml 0·2 M-triethanolamine (TRA) buffer containing 20 mM-$CaCl_2$ pH 7·8. The mixture was stirred at 37 °C for 24 h. The solution was acidified by adding 75 µl TFA (1000 ml/l) and centrifuged at 50 000 ***g*** for 20 min.

Separation of tryptic casein fragments by RP-FPLC

The supernatant of trypsin digested rabbit casein was loaded (100 µl per run) in 42 separate runs onto a Nucleosil 120-10 C18 column which was previously equilibrated with 100 ml acetonitrile/l H_2O containing 1 ml TFA/l. The column was eluted at a flow rate of 0·8 ml/min and fractions of 200 µl were collected. After 10 fractions a linear gradient from 100 to 700 ml acetonitrile/l containing 1 ml TFA/l was applied. The collected fractions were pooled according to the elution diagram (Fig. 1a). From each pool an aliquot of 150 ml/l was taken. Pools and

material eluted in the main peak was identified as the peptide FHLGHLK (RCDT2). (f) Reversed phase chromatography of the pool 1a-XI (panel a) by Nucleosil 120-10 C18 yielded three pools whose materials showed antibacterial activity. (g) Purification of pool 1f-III (panel f) by Nucleosil 100-5 C18 column. The material eluted in the main peak showed antibacterial activity. It was identified as the peptide ILPFIQSLFPFAER (RCDT3).

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aliquots were freeze-dried separately. The aliquots were then dissolved in 350 µl H_2O and assayed for antibacterial activity against *Esch. coli*, *B. subtilis*, and *Staph. lentus*.

RP-FPLC of pool 1a-VI from Nucleosil 120-10 C18

Pool 1a-VI (pool VI on Fig. 1 panel a) was dissolved in 1·0 ml H_2O containing 100 ml acetonitrile and 0·5 ml TFA/l and loaded in 10 separate runs onto the Nucleosil 120-10 C18 column again. The column was eluted at a flow rate of 0·8 ml/min and fractions of 200 µl were collected. After 10 fractions a linear gradient from 100 ml to 700 ml acetonitrile/l containing 0·5 ml TFA/l was applied. The peak fractions were pooled (Fig. 1b). From each pool an aliquot of 100 ml/l was taken. Pools and aliquots were freeze-dried separately. The aliquots were then dissolved in 350 µl H_2O and assayed for antibacterial activity against *Staph. lentus*.

Pool 1b-I (Fig. 1b) was dissolved in 1·0 ml H_2O containing 100 ml acetonitrile and 0·5 ml TFA/l and loaded in 10 separate runs onto a Nucleosil 100-5 C18 column which was equilibrated with acetonitrile (100 ml/l containing 0·5 ml TFA/l). The column was eluted at a flow rate of 0·3 ml/min. For every run 100 µl was loaded and fractions of 100 µl were collected. After 20 fractions a linear gradient (18 ml) from 100 ml to 700 ml acetonitrile/l was applied (Fig. 1c). The peak fractions were pooled, assayed for antibacterial activity and then sequenced.

Pool 1b-II from Nucleosil 120-10 C18 (Fig. 1b) was dissolved in 1·0 ml H_2O containing 100 ml acetonitrile and 0·5 ml TFA/l and loaded in 10 separate runs onto a Nucleosil 100-5 C18 column. The purification protocol for this step was the same as above. The elution diagram revealed two unresolved peaks. Fractions were pooled as reported in Fig. 1d. From each pool an aliquot of 100 ml/l was separately freeze-dried and taken for antibacterial assay. The antibacterial pool was dissolved in 1·0 ml H_2O containing 100 ml acetonitrile and 0·5 ml TFA/l. In five separate runs 200 µl were loaded onto a Nucleosil 100-5 C18 column. The column was eluted at a flow rate of 0·3 ml/min and fractions of 100 µl were collected. After 20 fractions a linear gradient (16 ml) from 100 ml to 450 ml acetonitrile/l was applied. Respective peak fractions were pooled according to the elution diagram (Fig. 1e), freeze-dried, assayed for antibacterial activity against *Staph. lentus* and then sequenced.

RP-FPLC of pool 1a-XI from Nucleosil 120-10 C18

Pool 1a-XI (Fig. 1a) was dissolved in 1·0 ml H_2O containing 300 ml acetonitrile and 0·5 ml TFA/l and loaded in 10 separate runs onto the Nucleosil 120-10 C18 column. The column was eluted at a flow rate of 0·8 ml/min and fractions of 200 µl were collected. After collecting 10 fractions a linear gradient from 300 ml to 700 ml acetonitrile/l was applied. Peak fractions were pooled according to the elution diagram (Fig. 1f). From each pool an aliquot of 100 ml/l was taken. Pools and aliquots were freeze-dried separately. The aliquots were dissolved in 0·25 ml distilled water then assayed for antibacterial activity against *B. subtilis* and *Staph. lentus*.

The material of pool 1f-III (Fig. 1f) was dissolved in 600 µl H_2O containing 300 ml acetonitrile and 0·5 ml TFA/l and loaded in 3 separate runs onto a Nucleosil 100-5 C18 and eluted at a flow rate of 0·3 ml/min. For every run 200 µl were loaded and fractions of 100 µl were collected. After collecting 20 fractions a linear gradient from 300 ml to 580 ml acetonitrile/l was applied (Fig. 1g). The peak fractions were pooled, freeze-dried, assayed for antibacterial activity against *B. subtilis* and *Staph. lentus* and then sequenced.

Digestion of rabbit casein with chymotrypsin

Freeze-dried rabbit casein (50 mg) was processed following the same procedure as described for the trypsin digestion of casein. Trypsin was replaced in the digestion mixture with 5 mg of chymotrypsin.

Separation of the chymotryptic casein fragments

The chymotryptic casein fragments were separated following the same protocol used to separate the tryptic fragments. Fractions were pooled according to the elution diagram (Fig. 2a), freeze-dried, then dissolved in 2·3 ml H_2O and assayed for antibacterial activity.

Digestion of rabbit casein with pepsin

Rabbit casein (50 µl) and pepsin (5 mg) were dispersed in 3·5 ml 0·1 M-sodium citrate buffer, pH 2·0. After stirring at 37 °C for 6 h the enzymic digestion of casein was stopped by incubating the solution at 80 °C for 45 min. The solution was centrifuged at 50 000 ***g*** for 20 min and the precipitated material discarded.

Separation of peptic casein fragments

The supernatant of the previous step was loaded onto a Nucleosil 120-10 C18 column, which was eluted following the same protocol as described for the tryptic digestion of casein. Fractions were pooled according to the elution diagram (Fig. 2b), freeze-dried, then dissolved in 2·3 ml H_2O and assayed for antibacterial activity.

Digestion of rabbit casein with clostripain

For activation, 200 U of clostripain were dissolved in 1 ml digestion buffer (50 mM-Tris-HCl buffer, pH 7·5, containing 10 mM-$CaCl_2$, 160 mM-NaCl and 2·5 mM-DTT), and incubated for 3 h at room temperature. The enzyme solution was then stored at 4 °C until use (not longer than 15 h).

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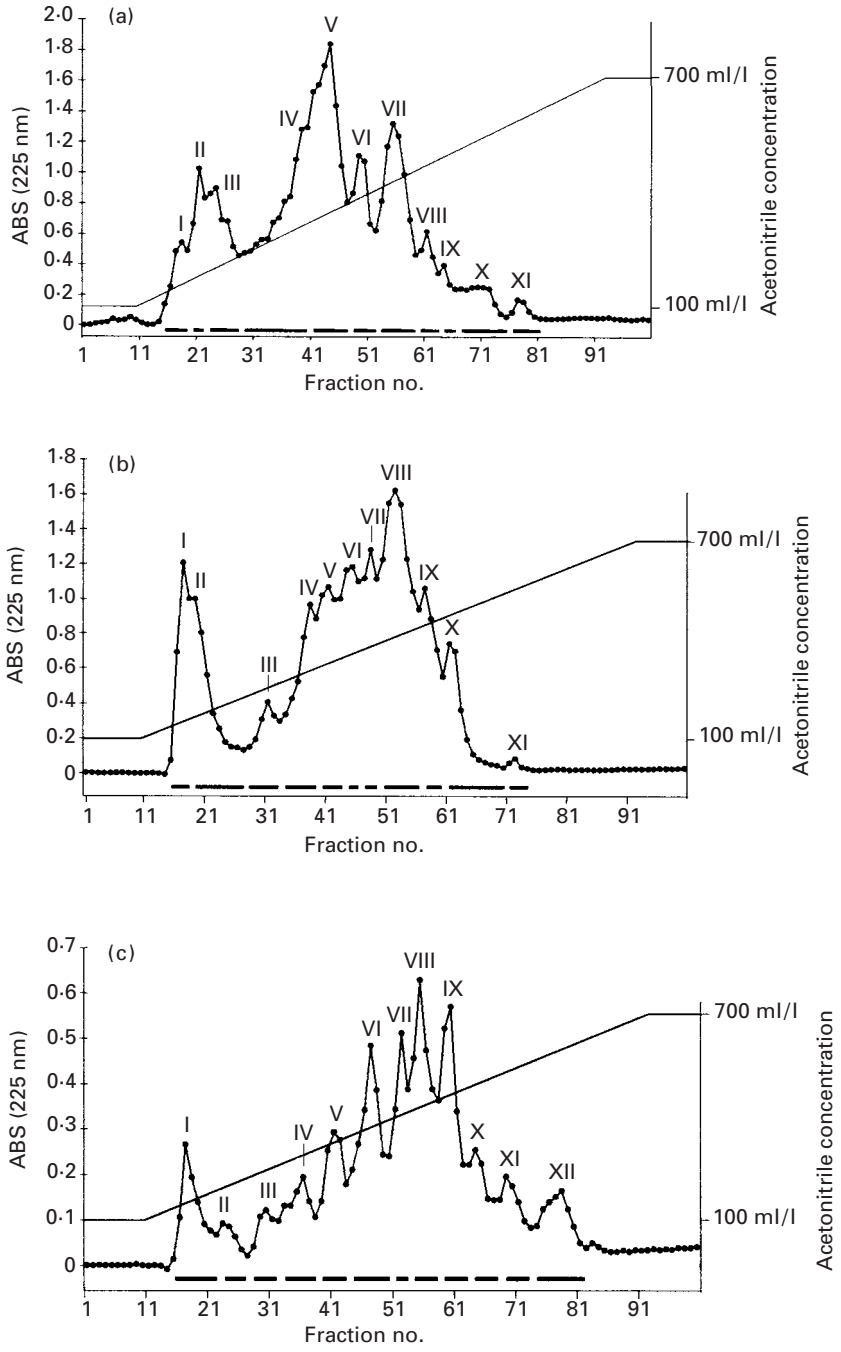

Fig. 2. Separation of antibacterial peptide fragments derived from the proteolytic digestion of rabbit casein by RP-FPLC on Nucleosil 120-10 C18 column. (a) Rabbit casein digested by chymotrypsin. Strong antibacterial activity was detected in pools 2a-I, 2a-II, 2a-III, 2a-IV, 2a-V, 2a-VI, 2a-VIII, 2a-X and 2a-XI (Table 2). (b) Rabbit casein fragments obtained from digestion by pepsin. Antibacterial activity was detected in pools 2b-I, 2b-II, 2b-III, 2b-IV, 2b-VII and 2b-VIII (Table 3). (c) Rabbit casein digested by clostripain. A very weak antibacterial activity was present in the materials eluted in the pools 2c-IX and 2c-XII (Table 4).

Rabbit casein (50 mg) was dissolved in 2·5 ml of the same digestion buffer, and added to the activated clostripain solution. This mixture was incubated at 37 °C for 6 h with stirring, then acidified by adding 525 µl 100 ml TFA/l and centrifuged at 50 000 ***g*** for 20 min.

Separation of Clostripain casein fragments

The supernatant of the previous step was loaded onto a Nucleosil 120-10 C18 column, which was eluted following the same protocol as described for the separation of the tryptic fragments. Fractions were pooled according to the

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Table 1. Antibacterial activity of the pools derived from RP-FPLC of trypsin digested rabbit casein

Values are means of three trials ±SD; lethal effect is indicated as >3·0

	Esch. coli	*B. subtilis*	*Staph. lentus*
Pool 1a-I	0	0·81±0·37	0·17±0·02
Pool 1a-II	0	1·77±0·26	0·08±0·02
Pool 1a-III	0	0·40±0·03	0
Pool 1a-IV	0	0·03±0·03	0
Pool 1a-V	0	1·12±0·12	0·37±0·07
Pool 1a-VI	0	0	0·49±0·13
Pool 1a-VII	0	0	0
Pool 1a-VIII	0	0	0·04±0·02
Pool 1a-IX	0	0	0·04±0·02
Pool 1a-X	0	1·42±0·09	0·95±0·10
Pool 1a-XI	0·50±0·30	>3·0	0·10±0·02

elution diagram (Fig. 2c) freeze-dried, then dissolved in 2·3 ml H_2O and assayed for antibacterial activity.

Results

Antibacterial activity of rabbit whole casein

Antibacterial activity of acid precipitated whole rabbit casein could be detected only at a concentration of 60 mg casein/ml. At this concentration the antibacterial activity against *Esch. coli* was 0·56±0·07. This concentration was lethal for *B. subtilis* and *Staph. lentus*, fully inhibiting the bacterial growth.

Digestion of rabbit casein by trypsin

Trypsin digestion of rabbit casein yielded several peptide fragments, which could be partially separated through RP-FPLC. The fractions eluted from the column were pooled as shown in Fig. 1a and assayed for antibacterial activity. Four pools (1a-II, 1a-V, 1a-X and 1a-XI) strongly inhibited the growth of *B. subtilis* and two pools (1a-I and 1a-III) inhibited this microorganism moderately (Table 1). *Staph. lentus* was moderately inhibited by pools 1a-V, 1a-VI and 1a-X. The growth of *Esch. coli* was not effected by any pool.

Pool 1a-VI was re-chromatographed. The elution diagram (Fig. 1b) consisted of a main peak with two shoulders and a small peak that eluted with higher retention time indicating that the material was not homogenous. Antibacterial activity against *Staph. lentus* was present in all of the four pools.

The elution diagram from the purification step of Pool 1b-I consisted of one peak (Fig. 1c). Antibacterial activity could be detected against *Staph. lentus*. This material was subjected to peptide sequence analysis and found to be HVEQLLR, which corresponds to sequence 50–56 of rabbit β-casein. This peptide was designated RCDT1 (rabbit casein digested by trypsin).

The elution diagram from the purification step of Pool 1b-II consisted of two unresolved peaks very tightly bound to each other (Fig. 1d). The fractions were pooled in two separate pools and assayed for antibacterial activity. Most of the antibacterial activity was present in pool 1d-I. Pool 1d-I was further purified on the same column using a different acetonitrile gradient. The elution diagram from this purification step consisted of a main peak and three small ones (Fig. 1e). Antibacterial activity was present in the main peak only. The material eluted with the main peak was subjected to peptide sequence analysis. The sequence was determined as FHLGHLK, which corresponds to the sequence of 19–25 of rabbit α_{S1}-casein. This peptide was called RCDT2.

Pool 1a-XI was further purified in two steps through RP-FPLC at first on Nucleosil 120-10 C18 and then on Nucleosil 100-5 C18. The elution diagram of the first step consisted of a main peak presenting a shoulder and a small peak eluted at a higher retention time (Fig. 1f). Fractions were pooled according to the elution diagram. Antibacterial activity was present in each of the three pools. Purification of pools 1f-I and 1f-II was attempted with several different RP-FPLC procedures using different columns and different elution gradients. The derived materials were always very heterogeneous and could not be subjected to amino acid sequence analysis. Pool 1f-III was then further purified on RP-FPLC on Nucleosil 100-5 C18. The elution diagram from this step consisted of a main peak and some contaminants eluted either with lower or with higher acetonitrile concentrations (Fig. 1g). The material eluted in the main peak showed antibacterial activity against *B. subtilis*. It was subjected to peptide sequence analysis and found to be ILPFIQSLFPFAER corresponding to the sequence 64–77 of rabbit β-casein. This peptide was named RCDT3.

Purification of pools 1a-I, 1a-II, 1a-III, 1a-V, and 1a-X (Fig. 1a), which showed antibacterial activity against *B. subtilis* (Table 1), was also attempted but peptides that were sufficiently pure to be subjected to amino acid sequence analysis were not obtained.

Antibacterial activity of the peptides RCDT1, RCDT2 and RCDT3

Following characterization, the peptides RCDT1, RCDT2 and RCDT3 were synthesized and purified. The antibacterial activity of the synthetic peptides was investigated at a concentration of 3·0 mM against Gram-positive and Gram-negative bacteria. The peptides were active against the Gram-positive bacteria only. The Gram-negative bacteria *Esch. coli, Kleb. pneumoniae, Serr. marcescens* were not susceptible to the action of RCDT1, RCDT2 and RCDT3. They inhibited only the Gram-positive *B. subtilis* and *Staph. lentus*. They were however ineffective against *Str. zooepidemicus*. The survival of *B. subtilis* and *Staph. lentus* was investigated at various concentrations of the peptides. The dose response curves of antibacterial activity

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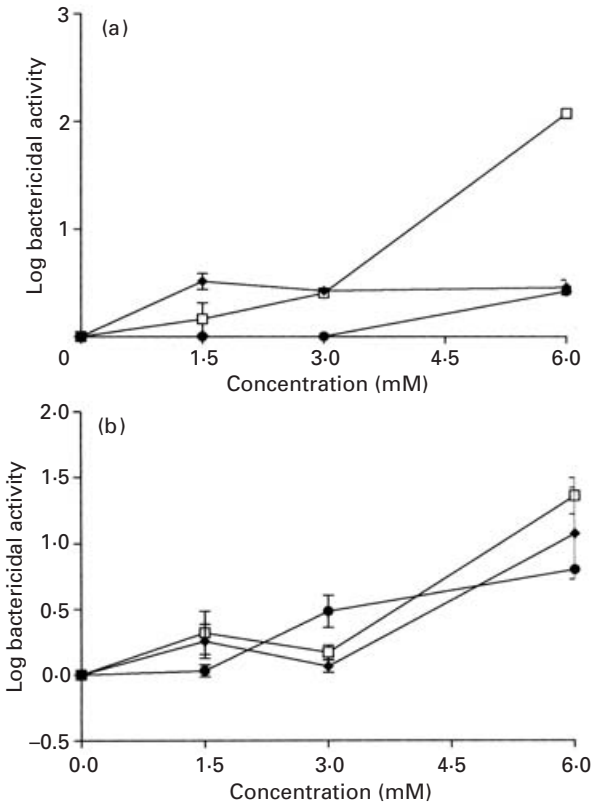

Fig. 3. Susceptibility of (a) *B. subtilis* and (b) *Staph. lentus* to the peptides RCDT1 (●), RCDT2 (◆) and RCDT3 (□). Bacterial suspension mixed with sample solution and buffered TSB was incubated at 37 °C for 2 h and then plated on TSA for CFU evaluation. Values are mean ± SD for $n=3$.

against *B. subtilis* are reported in Fig. 3a. It shows that the most active peptide against *B. subtilis* was RCDT3. At a dose of 6 mM the antibacterial activity was 2·07 ± 0·03 whereas that of RCDT1 and RCDT2 was 0·42 ± 0·02 and 0·46 ± 0·07, respectively. The titration curves of antibacterial activity against *Staph. lentus*, reported in Fig. 3b, showed a similar slope indicating, that under the assay conditions used, the peptides possess the same effectiveness in killing *Staph. lentus*. At the concentration of 6 mM the most antibacterial peptide was RCDT3 (1·36 ± 0·14) whereas the weakest was RCDT1 (0·80 ± 0·02).

Digestion of rabbit casein by chymotrypsin

Digestion of rabbit casein by chymotrypsin yielded several peptide fragments, which after a partial purification by RP-FPLC were grouped in eleven pools (Fig. 2a). Antibacterial assays of the pools showed that they were almost ineffective against *Esch. coli* and *Staph. lentus*. Strong antibacterial activity against *B. subtilis* was detected in pools 2a-I, 2a-II, 2a-III, 2a-IV, 2a-V, 2a-VI 2a-VIII, 2a-X and 2a-XI (Table 2). A moderate antibacterial activity was revealed in the pools 2a-VII and 2a-IX.

Table 2. Antibacterial activity of the pools derived from RP-FPLC of chymotrypsin digested rabbit casein

Values are means of three trials ± SD; lethal effect is indicated as >3·0

	Esch. coli	*B. subtilis*	*Staph. lentus*
Pool 2a-I	0	>3·0	0·09 ± 0·03
Pool 2a-II	0	2·02 ± 0·21	0·04 ± 0·01
Pool 2a-III	0	2·02 ± 0·21	0·07 ± 0·02
Pool 2a-IV	0	>3·0	0·04 ± 0·02
Pool 2a-V	0	1·43 ± 1·04	0·04 ± 0·02
Pool 2a-VI	0·06 ± 0·03	1·56 ± 0·92	0·13 ± 0·01
Pool 2a-VII	0·30 ± 0·02	0·95 ± 0·60	0
Pool 2a-VIII	0	2·06 ± 0·21	0
Pool 2a-IX	0	0·50 ± 0·02	0
Pool 2a-X	0	1·29 ± 0·11	0·08 ± 0·02
Pool 2a-XI	0	2·21 ± 0·06	0·08 ± 0·02

Digestion of rabbit casein by pepsin

RP-FPLC of pepsin digested rabbit casein resulted in the appearance of several peaks. The corresponding fractions were pooled as shown in Fig. 2b. Pool 2b-I and 2b-VIII were lethal (indicated as >3·0 in the tables) or strongly antibacterial against all the bacteria strains investigated (Table 3). Pool 2b-II had a lethal effect on *B. subtilis* and *Staph. lentus* but only weakly inhibited *Esch. coli*. Pool 2b-VII was lethal for *Staph. lentus* only, and pool 2b-III had a strong inhibitory effect on the growth of *B. subtilis*. The other pools affected the bacterial growth only poorly or not at all.

Digestion of rabbit casein by clostripain

Almost all of the pools derived from the digestion of rabbit casein with clostripain (Fig. 2c) did not show any significant antibacterial activity when assayed against *Esch. coli*, *B. subtilis* and *Staph. lentus*. A very weak antibacterial activity against *Staph. lentus* was present in the materials eluted in the pools 2c-IX and 2c-XII (Table 4).

Discussion

Proteolytic digestion of rabbit casein led to the generation of several peptide fragments possessing antibacterial properties. Casein itself exhibited antibacterial activity only when assayed at very high concentration. The high concentration required for antibacterial activity could be a peculiar characteristic of casein. However one cannot exclude that it could be due to some whey contaminant like lactoferritin present in a very small amount in the sample. In fact the rabbit casein fraction was assayed after the acid precipitation without further purification.

Not all the peptides produced from rabbit casein presented antibacterial properties. Pools 1a-IV, 1a-VII, 1a-VIII and 1a-IX derived from the tryptic digestion of casein were devoid of any antibacterial activity. Lack of antibacterial activity was also present in the pools 2b-X and 2b-XI

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Table 3. Antibacterial activity of the pools derived from RP-FPLC of pepsin digested rabbit casein

Values are means of three trials ±SD; lethal effect is indicated as >3·0

Pepsin	*Esch. coli*	*B. subtilis*	*Staph. lentus*
Pool 2b-I	>3·0	>3·0	>3·0
Pool 2b-II	0·17±0·09	>3·0	>3·0
Pool 2b-III	0	1·57±0·07	0·16±0·01
Pool 2b-IV	0	1·09±0·32	0·36±0·02
Pool 2b-V	0	0	0·20±0·02
Pool 2b-VI	0	0	0·51±0·04
Pool 2b-VII	0	0·38±0·04	>3·0
Pool 2b-VIII	2·02±0·34	>3·0	>3·0
Pool 2b-IX	0	0	0·16±0·01
Pool 2b-X	0	0	0
Pool 2b-XI	0	0	0·05±0·03

Table 4. Antibacterial activity of the pools derived from RP-FPLC of clostripain digested rabbit casein

Values are means of three trials ±SD

Clostripain	*Esch. coli*	*B. subtilis*	*Staph. lentus*
Pool 2c-I	0	0	0
Pool 2c-II	0	0	0
Pool 2c-III	0	0	0
Pool 2c-IV	0	0	0
Pool 2c-V	0	0	0
Pool 2c-VI	0	0	0
Pool 2c-VII	0	0	0
Pool 2c-VIII	0	0	0·04±0·02
Pool 2c-IX	0	0	0·12±0·05
Pool 2c-X	0	0	0
Pool 2c-XI	0	0	0
Pool 2c-XII	0	0·09±0·01	0·56±0·19

obtained from the peptic digestion of rabbit casein. Proteolytic digestion of rabbit casein by clostripain did not yield any peptide fragments with meaningful antibacterial activity. Clostripain, a protease present in Clostridium bacteria, which is a component of the intestinal microflora, was previously successfully used to yield antibacterial peptides from cathepsin G (Bangalore et al. 1990), lysozyme (Pellegrini et al. 1997) and aprotinin (Pellegrini et al. 1996). A peculiarity of clostripain is its ability to cut specifically the peptide bond after an Arg residue. In previous analogue systems it has been observed that the positively charged Arg located in the C-terminal side, was an important factor for antibacterial properties (Shafer et al. 1996; Pellegrini et al. 1997). Our results have shown that the presence of C-terminal Arg is not a sufficient condition for antibacterial activity but that other configurations are required for antibacterial activity.

The antibacterial activity of the generated peptides was mostly directed against Gram-positive bacteria species. Antibacterial activity against *Esch. coli*, a Gram-negative micro-organism, was only detected in two pools derived from the pepsin digestion of rabbit casein. In previous investigations on the antibacterial peptides obtained from the proteolytic digestion of aprotinin, α-lactalbumin and β-lactoglobulin (Pellegrini et al. 1996, 1999 & 2001) we observed a similar situation. The negatively charged antibacterial peptides were antibacterial for Gram-positive bacteria only. Interaction between negatively charged peptides and Gram-negative bacteria is hindered by the fact that in the membrane of these bacteria species negatively charged lipopolysaccharides are present as a major component of the cell membrane. The lack of antibacterial activity of some of the fragments generated by proteolytic digestion of rabbit casein against Gram-negative bacteria suggests they could be anionic peptides. In fact the theoretical pI of RCDT1 was 6·5 and that of RCDT3 was 6·0. However, despite its cationic character, RCDT2 (pI 8·7) was ineffective against the Gram-negative bacteria. This result suggests that as well as the charge other unknown physical-chemical properties are important factors for antibacterial activity against Gram-negative bacteria. We observed a similar event in one of our previous investigations on antibacterial peptides (Pellegrini et al. 2001). A cationic peptide generated from the digestion of β-lactoglobulin was effective only against Gram-positive bacteria. The peptides RCDT1-3 were less antibacterial than the peptides derived from the hydrolysis of α-lactalbumin and β-lactoglobulin. However, since casein is present in milk at a higher concentration than whey proteins, a larger amount of casein peptides will be released during the digestion of milk, which in turn will compensate for their weaker antibacterial action.

Sequence homology analysis of RCDT1-3 indicates that they do not share any homology with the sequences of known milk proteins present in other animal species.

Enzymatic hydrolysis of bovine (Jinsmaa & Yoshikawa, 1999), sheep (Qian et al. 1995), minipig (Meisel & Frister, 1989) and human caseins (Chabance et al. 1995) have been previously studied *in vitro* and *in vivo*, and the bioactive peptides generated were investigated in other terms than their antibacterial properties. Bioactive peptides derived from casein have been reported previously (Migliore-Samour & Jollès, 1988; Meisel, 1997) and showed to exert beneficial physiological effects which include immunostimulation (Parker et al. 1984), binding to opioid receptor (Jinsmaa & Yoshikawa, 1999), inhibition of angiotensin I-converting enzyme (Maruyama et al. 1985), and inhibition of platelets aggregation (Qian et al. 1995). In the present work it was shown that rabbit caseins (never previously investigated) contain several antibacterial domains, which can be released by endoproteases of the mammalian gastrointestinal tract. Antibacterial domains of proteins were previously isolated after proteolytic digestion of other proteins like cathepsin G (Bangalore et al. 1990), lactoferrin (Bellamy et al. 1992), aprotinin (Pellegrini et al. 1996), lysozyme (Pellegrini et al. 1997) and α-lactalbumin (Pellegrini et al. 1999). Our results extend the previous findings and provide further evidence that antibacterial

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peptides can be generated from food proteins by endopeptidases of the mammalian gastrointestinal tract.

The antibacterial pools derived from the hydrolysis of rabbit caseins were eluted with different retention times from the RP-FPLC column and only three antibacterial peptides could be isolated in an homogeneous form to be sequenced. Since each pool contains at least one antibacterial peptide the large number of antibacterial pools obtained illustrates the wide antibiotic arsenal of rabbit casein.

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