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The Extracellular P_I-Type Proteinase of *Lactococcus lactis* Hydrolyzes β-Casein into More than One Hundred Different Oligopeptides

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The peptides released from β-casein by the action of P_I-type proteinase (PrtP) from *Lactococcus lactis* subsp. *cremoris* Wg2 have been identified by on-line coupling of liquid chromatography to mass spectrometry. After 24 h of incubation of β-casein with purified PrtP, a stable mixture of peptides was obtained. The trifluoroacetic acid-soluble peptides of this β-casein hydrolysate were fractionated by high-performance liquid chromatography and introduced into the liquid chromatography-ion spray mass spectrometry interface. Multiply charged ions were generated from trifluoroacetic acid-soluble peptides under low nozzle voltage conditions, yielding the MH^+ mass of each eluted peptide. All peptides corresponding to each of the MH^+ calculated masses were determined. In those cases in which different peptides were possible, further identification was achieved by collision-induced dissociation under higher nozzle voltage conditions. Hydrolysis of β-casein by PrtP was observed to proceed much further than reported previously. More than 40% of the peptide bonds are cleaved by PrtP, resulting in the formation of more than 100 different oligopeptides. With the exception of Phe, significant release of amino acids or di- and tripeptides could not be observed. Interestingly, one-fifth of the identified oligopeptides are small enough to be taken up by the oligopeptide transport system. Uptake of these peptides could supply *L. lactis* with all amino acids, including the essential ones, indicating that growth of *L. lactis* might be possible on peptides released from β-casein by proteinase only.

Lactococci have very limited capacities of synthesizing amino acids and therefore must utilize exogenous nitrogen sources for optimal growth. The amino acid requirement is strain dependent, but Glu or Gln, Ile, Leu, His, Met, and Val are essential for the growth of most *Lactococcus lactis* strains (6). The addition of several other amino acids was found to be growth stimulatory (34, 25). The concentrations of essential amino acids in milk are very low, especially those of Ile and Leu (less than 1 mg/liter) (27). Moreover, the concentrations of other free amino acids are too low to explain the growth of *L. lactis* to the cell densities normally reached in coagulated milk (27, 44). Thus, for optimal growth in milk, lactococci depend on the utilization of milk proteins, such as caseins. Casein hydrolysis by lactococci is mediated by a complex proteolytic system which includes a cell envelope-located proteinase (P_I- or P_{III}-type proteinase [PrtP]) and several peptidases (for recent reviews, see references 31, 32, and 42).

According to proposed models, PrtP is involved in the first step of casein degradation (32, 39). The action of purified PrtP on β-casein has been studied extensively (28, 29, 33, 46, 47). After separation of the proteolytic products by reverse-phase high-performance liquid chromatography (HPLC), the different peptides are collected, purified when needed, and identified by biochemical methods (i.e., amino acid composition, determination of the N- and C-terminal amino acids, and sequencing of the peptide, etc.). In some cases, additional information is obtained by mass spectrometrical (MS) analysis of the purified peptide. From these studies, the formation of 39 different peptides released from β-casein by PrtP has been reported. Only very few of these reported peptides are small enough to be taken up by the peptide transport systems of *L. lactis* (17, 38, 45). The peptidases have therefore been postulated to be present extracellularly in order to explain the growth of *L. lactis* on media containing casein as the only source of amino acids. However, all the peptidases of *L. lactis* studied to date are located intracellularly, as judged by immunological techniques (41), and leader sequence motifs are not present in the proteins (31). If indeed peptidases are not present extracellularly, growth of *L. lactis* on milk can be explained only if hydrolysis of casein by PrtP proceeds much further than previously reported. We therefore decided to reanalyze in detail the PrtP-generated degradation products of β-casein using the very sensitive method of ion spray MS (43). The use of on-line liquid chromatography (LC)-MS makes it possible to determine the molecular weight of each peptide, even when HPLC column fractions contain more than one peptide (7, 36). The results demonstrate that β-casein is indeed hydrolyzed more extensively than reported previously. A large range of peptides are formed, many of which are small enough to be taken up by the oligopeptide transport system of *L. lactis* (17, 45).

MATERIALS AND METHODS

Organism and culture conditions. *L. lactis* subsp. *lactis* MG611 was obtained from A. J. Haandrikman (Department of Genetics, University of Groningen, The Netherlands). This strain, a derivative of *L. lactis* subsp. *lactis* MG1363, carries the plasmid-encoded proteinase genes *prtP* and *prtM* of *L. lactis* subsp. *cremoris* Wg2 on the chromosome and contains approximately eight stably integrated proteinase gene copies (23). The organism was maintained routinely on M17 broth containing 0.5% glucose (wt/vol) and 5 μg of erythromycin per ml. *L. lactis* MG611 was grown overnight in M17 broth containing 1% glucose (wt/vol) and 5 μg of erythromycin per ml at 29°C and a controlled pH of 6.0.

Proteinase purification. The proteinase from *L. lactis* subsp. *lactis* MG611 was isolated by the procedures described previously (1, 18). Cells of a 12-liter culture (A_{660} of ~1.8) were washed twice in 50 mM Tris-HCl (pH 8) containing 30 mM

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$CaCl_2$. The proteolytic activity was released from the washed cells by incubation in 600 ml of 50 mM Tris-HCl (pH 8), containing 15 mM Na-EDTA (releasing buffer) at 30°C. After 30 min of incubation, the cells were removed by centrifugation, and $CaCl_2$ (30 mM, final concentration) was added to the proteinase-containing supernatant. A second release procedure was performed with 200 ml of releasing buffer. All subsequent steps were performed at 4°C in 50 mM Tris-HCl (pH 8) plus 30 mM $CaCl_2$. Anion-exchange chromatography was carried out as follows: 200 ml of proteinase-containing supernatant was filtered through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.) and loaded onto a Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden). After the column was washed with 200 ml of 50 mM Tris-HCl (pH 8) plus 30 mM $CaCl_2$, the proteinase activity was eluted with a linear NaCl gradient (0 to 0.35 M). The active fractions (see below) from four successive runs were pooled and concentrated by using a 10,000-Da-pore-size ultrafiltration membrane (YM10; Amicon Corp., Danvers, Mass.). The pH of the concentrated PrtP solution was adjusted to 6.0 prior to storage at −80°C. A further gel filtration control step was carried out on PrtP samples, by using a Hi-Load 16/60 Superdex 200 column (Pharmacia) and 50 mM Tris-HCl (pH 8)–30 mM $CaCl_2$–0.20 M NaCl as the eluant. Proteinase activity was measured by using the chromogenic peptide methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitro-anilide (Chromogenix, Mölndal, Sweden) as the substrate (9). Protein concentrations were determined as described previously, with bovine serum albumin as the standard (24).

Peptidase activity. The PrtP solution was tested for the presence of peptidase activities. Fifty microliters of a 2 μM PrtP solution was incubated for 2 h at 37°C with substrates (2 mM) specific for different peptidases (i.e., Lys-para-nitroanilide (pNA), Glu-pNA, Gly-Pro-pNA, Leu-Gly-Gly, bradykinin, and Met-enkephalin). Peptidase activity was checked either by determining the A_{405} in the case of chromogenic substrates or by HPLC analysis in the case of nonchromogenic substrates.

β-Casein purification. Whole casein was obtained by acidic precipitation of defatted milk obtained from a cow selected for producing a homozygote β-casein (40). β-casein (A_2 variant) was purified essentially as described previously (13). Total caseins (60 mg) were resuspended in 6 ml of 5 mM Tris-HCl (pH 8) containing 4.5 M urea and 3 mM dithiothreitol, filtered through a 0.45-μm-pore-size filter (Millipore Corp.), and loaded onto a Mono Q HR 10/10 column (Pharmacia). After washing of the column with 50 ml of 5 mM Tris-HCl (pH 8) containing 4.5 M urea, β-casein was eluted with a linear NaCl gradient (0 to 0.31 M) at 40°C. The β-casein-containing fractions from 10 runs were pooled, dialyzed at 4°C against Milli Q water (Millipore Corp.), and freeze-dried in a SpeedVac concentrator (Savant Instruments Inc., Farmingdale, Colo.).

Hydrolysis of casein. Hydrolysis of β-casein A_2 (7 mg/ml) was done in 50 mM Tris-HCl (pH 6.5) containing 20 mM $CaCl_2$, for 24 h at 25°C. The pH value corresponds to growth conditions in milk at which the proteinase has to become functional in order to allow the lactococci to grow to high optical densities. Moreover, these experimental conditions did not result in a significant inhibition of PrtP activity, whereas autoproteolysis of the enzyme was drastically reduced (18). An enzyme/substrate concentration ratio of about $1.7 \cdot 10^{-4}$ was used. The reaction was stopped by diluting the enzyme-substrate mixture into sample buffer (see below) and subsequent boiling for 3 min in the case of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by adding trifluoroacetic acid (TFA; 1%, final concentration) in the case of HPLC analysis. After centrifugation to remove the TFA-insoluble peptides, the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore) and concentrated by freeze-drying.

SDS-PAGE and Western blotting. SDS-PAGE was performed as described previously (21), with a Mini-PROTEAN II vertical electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.). The acrylamide content of the running gel was either 8% (wt/vol) for enzyme extracts or 15% for casein hydrolysates. The protein samples were diluted 1:4 in sample buffer (10% SDS, 30% glycerol, 0.1% bromphenol blue, 10% β-mercaptoethanol in 50 mM Tris-HCl [pH 6.8]), heated for 3 min at 100°C, and applied to the gels. Migration occurred at room temperature in 25 mM Tris-HCl (pH 8.3) containing 0.1% SDS and 0.19 M glycine. After electrophoresis, casein hydrolysate-containing gels were stained with Coomassie brilliant blue. Enzyme extract-containing gels were either silver stained (48) or used for Western blotting (immunoblotting). The molecular masses of the protein bands were estimated by using high- and low-molecular-mass range SDS-PAGE standards (Bio-Rad) for 8 and 15% acrylamide gels, respectively. Western blotting was performed as described previously (20).

HPLC analysis. The HPLC study was performed by using a Waters (Milford, Mass.) 717 automatic sampler, a Waters 625 LC gradient system, and a Waters 486 tunable absorbance detector. The TFA-soluble peptides were separated on a reverse-phase HPLC column (250 by 4.6 mm, Hi-Pore 318; Bio-Rad). Solvent A was 0.11% TFA (vol/vol) in Milli Q water, and peptides were eluted at 30°C by using a linear gradient (0 to 80%) of solvent B (0.1% TFA, 60% [vol/vol] acetonitrile in Milli Q water) within 40 min. The flow rate was 1 ml/min, and peptides were detected by UV absorption at 214 nm.

LC-MS analysis. LC-MS analyses were performed essentially as described previously (43), with a Kratos Spectroflow 450 gradient controller, two Kratos Spectroflow 400 pumps, and a Kratos Spectroflow 757 UV detector (Applied Biosystems, Forest City, Calif.) as the HPLC unit, which was connected to a Nermag R 30-10 triple quadrupole MS. Peptides obtained from β-casein hydrolysis were loaded with a 100-μl loop in a Rheodyne (Cocati, Calif.) 7125 injector. Column and elution conditions were the same as those described above. By flow splitting of the column eluate, approximately 1% of the sample was introduced into the LC-ion spray MS interface (5). The MS had a custom-built prototype atmospheric pressure ionization source (4) and was used in the positive ion mode. Different conditions were selected as follows: to generate multiply charged ions without fragmentation, a low nozzle voltage (70 V) was used; to generate fragment ions by collision-induced dissociation from peptides, higher nozzle voltages (170, 270, and 370 V) were used (2, 3, 15). Full-scan mass spectra were recorded at mass-to-charge ratios (m/z) of from 50 to 1,999. To correlate mass spectral data to the peptide sequence, the computer program MacProMass (version 1.05) was used (22).

Free amino acid determination. The large peptides of the β-casein hydrolysate were precipitated by 5-sulfosalicylic acid (3% final concentration) (12). After centrifugation to remove the 5-sulfosalicylic acid-insoluble peptides, the supernatant was diluted with an equal volume of 100 mM lithium chloride–68.5 mM sodium citrate (pH 2.2) and filtered through a 0.45-μm-pore-size filter (Millipore). The free amino acid content of the filtrate was determined with an LC 5000 amino acid autoanalyzer (Biotronik, Munich, Germany).

Purification of peptides. For complementary experiments, some of the peptides obtained from β-casein hydrolysis were purified, by using two different solvent systems. The first system is described above. Eluted fractions were collected and concentrated by freeze-drying. The coeluted peptides were separated on the same Hi-Pore 318 (Bio-Rad) reverse-phase HPLC column with a second solvent system. Solvents A and B were 10 mM potassium phosphate and 10 mM potassium phosphate plus acetonitrile (40 and 60%, respectively) (pH 7.2), respectively. Elution and detection conditions were identical to those described above. Purified peptides were collected and freeze-dried.

Hydrolysis of purified peptides by proteinase. The purified peptides were dissolved in Milli Q water, in order to obtain an identical HPLC peak response for each of them. The peptide solution (50 μl) was mixed with 10 μl of 50 mM Tris-HCl (pH 6.5)–50 μl of 2 mM methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-pNA–10 μl of a 2 μM PrtP solution. After 2 h of incubation at 30°C, the reaction was stopped by adding TFA (1%, final concentration) to the reaction mixture. The results of hydrolysis were analyzed by reverse-phase HPLC, by using the TFA-acetonitrile solvent system previously described.

RESULTS

Purification of proteinase. The cell envelope-associated proteinase PrtP was released from *L. lactis* MG611 by incubation in a calcium-free buffer. The proteinase was purified by anion-exchange chromatography and gel filtration. SDS-PAGE of the active fractions showed one main band, with an apparent molecular mass of 164,000 Da, which is in good agreement with values reported previously (19). Several other bands with lower intensities were visible by silver staining. All these proteins reacted with a mixture of monoclonal antibodies raised against PrtP (data not shown) (20). The molecular masses of the minor bands ranged from 150,000 to 58,000 Da and could be identified as autoproteolytic products (18, 19). The PrtP-containing fractions did not have any measurable peptidase activity (data not shown). The specific activity of the proteinase was 4.32 μmol/min/mg of protein, as determined by the rate of *p*-nitroanilide release from methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-pNA under saturating conditions. The purification factor was 77, and the yield was approximately 25%.

Hydrolysis of casein. In the absence of the proteinase, no degradation of β-casein was observed. The time course of β-casein hydrolysis by purified PrtP was followed by SDS-PAGE (Fig. 1). In the initial phase of degradation, four main protein bands were formed, with maximal intensities after 6 h of hydrolysis. Determination of the molecular masses of these degradation products was not possible because of the anomalous behavior of β-casein on SDS-PAGE (8). These four proteins could be precipitated by TFA (data not shown) and thus were large hydrolyzed fragments of β-casein. After 24 h of incubation, almost all β-casein was digested and three of the four initially observed proteins were no longer detectable on SDS-PAGE. The residual PrtP activity was decreased to about 0.1% of the initial activity. The amount of low-molecular-mass products increased with incubation time. One peptide with a

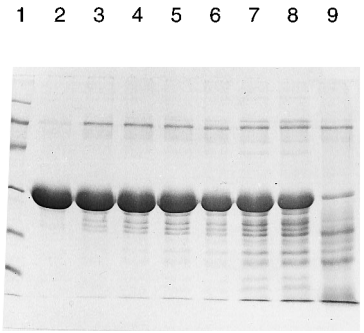

FIG. 1. SDS-PAGE showing hydrolysis of β-casein by purified proteinase of *L. lactis* subsp. *cremoris* Wg2. Lanes: 1, standard proteins; 2 to 9, after 0, 0.5, 1, 1.5, 2, 4, 6, and 24 h of incubation at 25°C, respectively. The standard proteins (molecular weights given in parentheses) were phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). Hydrolysis was performed at 25°C and pH 6.5. The enzyme/substrate concentration ratio was 1.7×10^{-4}. The amount of β-casein applied to the gel was approximately 14 μg.

molecular mass similar to that of bovine serum albumin increased with incubation time.

The HPLC separation profile of the TFA-soluble peptides of the PrtP hydrolysate of β-casein after 24 h of incubation is shown in Fig. 2. No TFA-soluble peptides were detected when PrtP was incubated without β-casein, suggesting that the autoproteolytic products of PrtP were all TFA insoluble. Thus, all the degradation products detected in the chromatogram were the result of hydrolysis of β-casein by PrtP. In the earliest stages of degradation (up to 3 h of incubation), six main protein peaks were detected (retention times of 16.6, 19.9, 22, 23, 33.1, and 34.1 min, respectively), accounting for more than 75% of the total area of the chromatogram. During incubation, these six proteins were partially degraded further, and after 24 h of hydrolysis, HPLC indicated the formation of a large number of peptides in relatively low amounts.

Identification of peptides. Peptides have been identified by on-line coupling of HPLC to MS. The first analysis was performed at a nozzle voltage of 70 V. Under these conditions, multiply charged ions were generated. An example is shown in Fig. 3a. The peptide that eluted at a retention time of 16.6 min was detected as a single ion (*m/z* of 802) and as a doubly charged ion (*m/z* of 402), yielding an MH^+ mass of 802. In the

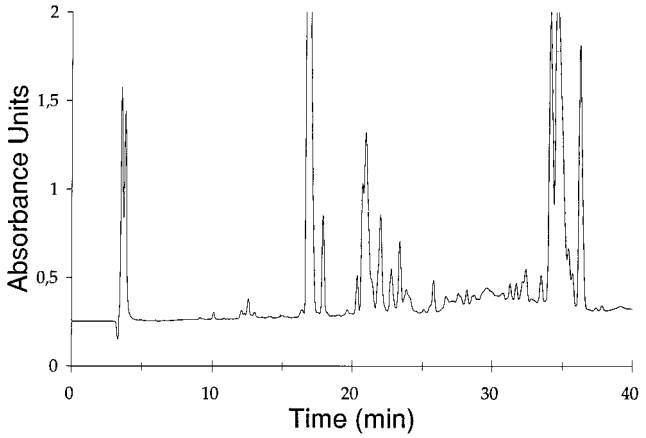

FIG. 2. Reverse-phase HPLC profile of TFA-soluble peptides from β-casein hydrolyzed for 24 h at 25°C by purified proteinase of *L. lactis* subsp. *cremoris* Wg2.

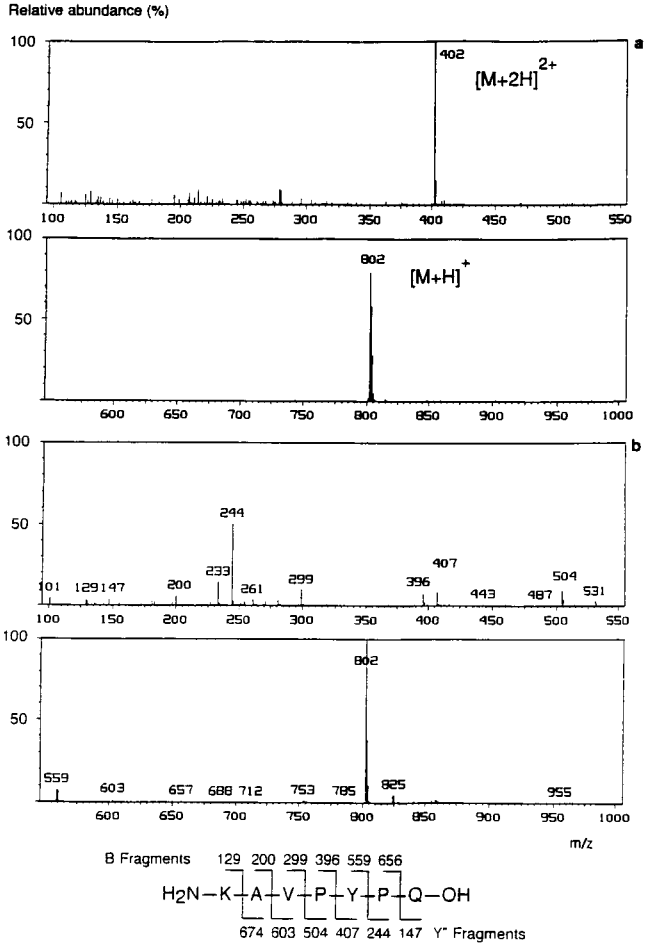

FIG. 3. Mass spectra of peak eluting at retention time of 16.6 min. (a) Without fragmentation (nozzle voltage, 70 V); (b) with fragmentation by collision-induced dissociation (nozzle voltage, 270 V). Masses of the fragment ions from collision-induced dissociation are indicated on the bottom of the figure. B fragments are from the N-terminal part; Y fragments are from the C-terminal part of the peptide.

amino acid sequence of β-casein, seven peptides with an MH^+ mass of 802 ± 1 could be found: the peptide from residues 133 to 139 (hereafter referred to as 133-139) (LHLPLPL), 134-140 (HLPLPLL), 140-145 (LQSWMH), 174-180 (PQKAVPY), 175-181 (QKAVPYP), 176-182 (KAVPYPQ), and 186-192 (PIQAFLL). Discrimination between these seven possibilities was not possible by HPLC, since peptides with identical amino acid compositions have identical HPLC indices (for example, the three peptides 174-180, 175-181, and 176-182 have an HPLC index of 29.50). To overcome these problems, other sets of LC-MS injections were performed, with higher nozzle voltages. Under these conditions, peptides (R_n-CH-CO-NH-CH-R_{n+1}) are easily fragmented, because of the cleavage of the peptide bond (B-Y″ dissociation type), generating [R_n-CH-C≡O^+] N-terminal fragments and [NH_2-CH-R_{n+1}]H^+ C-terminal fragments (35). This ion fragmentation is shown for the peptide eluting at a retention time of 16.6 min (Fig. 3b). Several ion fragments were formed (MH^+ masses of 129, 147, 200, 244, 299, 396, 407, 504, 559, and 656). A comparison of the masses of the ion fragments with the amino acid sequences of the seven possible peptides allowed discrimination between the different possibilities. With these procedures, almost all the

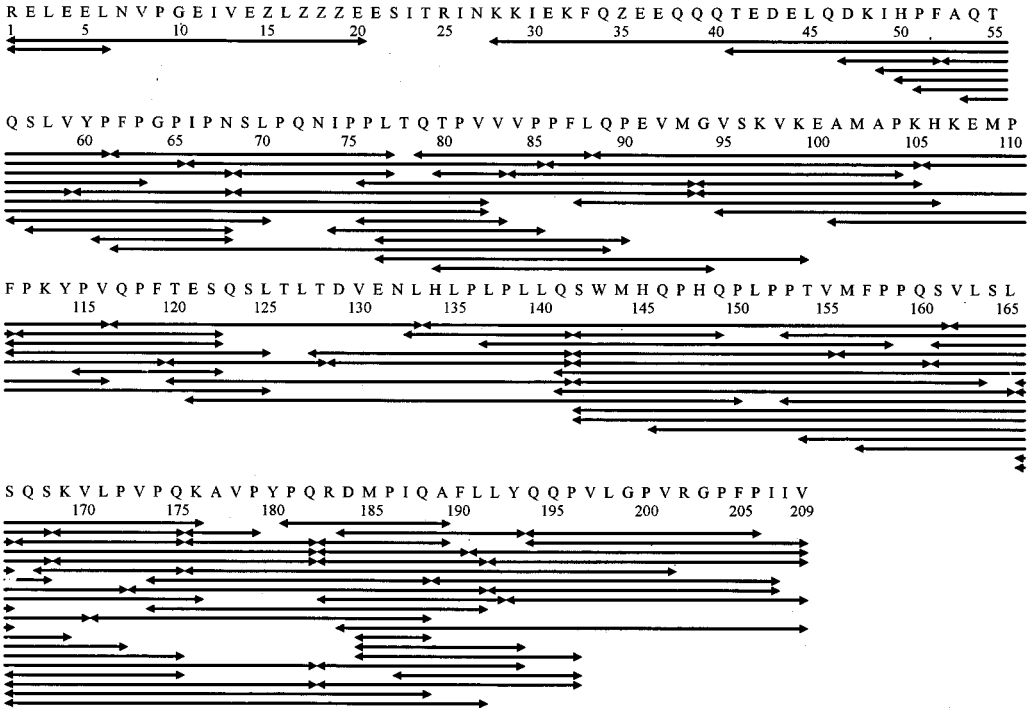

FIG. 4. Localization of identified peptides released by the action of purified PrtP on β-casein (A_2 genetic variant).

peptides in the PrtP hydrolysate of β-casein obtained after 24 h of incubation were identified (Fig. 4). Over 100 different β-casein-derived peptides were found in the PrtP hydrolysate. The identification of only a few peptides was not possible. One peptide (retention time of 21.5 min) yielded ion fragments (MH^+ masses of 116, 129, 292, 356, 550, and 980) which could originate from two different peptides (72-81 and 113-121). Since the HPLC indices of these peptides were too close (35.10 and 43.80, respectively), further identification could not be done. One peptide, eluting at 19.1 min, could not be identified as no peptide sequence with an MH^+ mass of 5,509 could be found in the amino acid sequence of β-casein. Finally, four peptides could not be identified because their MH^+ masses were too high (5,696, 4,996, 5,283, and 5,193, respectively), yielding ion fragments out of the recorded *m/z* range. These peptides were presumably very large β-casein fragments, which were not completely removed by the TFA precipitation. The peptide eluting at a retention time of 17.5 min had an MH^+ mass of 803. In the amino acid sequence of β-casein, seven peptides with an MH^+ mass of 803 ± 1 are found (127-133, 132-138, 133-139, 134-140, 174-180, 175-181, and 176-182). Several ion fragments of the peptide were detected (MH^+ masses of 559, 505, 408, 396, 299, and 245), all of which could have been derived from one of the seven peptides. Three fragments were obtained from the N-terminal part of the peptide 176-182 (MH^+ masses of 559, 396, and 299 [Fig. 3b]), but fragments of the complementary C-terminal parts (i.e., MH^+ masses of 244, 407, and 504, respectively) could not be detected. The masses of the detected ion fragments were systematically 1 mass unit higher than expected (i.e., MH^+ masses of 245, 408, and 505, respectively). The explanation for these observations has to be that the peptide contains a C-terminal glutamate instead of a glutamine (molecular masses of 147.1 and 146.1, respectively) and that the sequence of the peptide is KAVPYPE instead of KAVPYPQ. A KAVPYPE peptide is not found in the amino acid sequence of β-casein. It could have been derived from the initial KAVPYPQ sequence by chemical modification (deamination) under acidic conditions (1% TFA). Such a chemical deamination of glutamine has been observed previously (49). About 50% of the peptides in the β-casein hydrolysate were composed of 8 to 16 amino acids (Fig. 5). However, 18 peptides containing eight or fewer residues have been identified. No di- or tripeptide could be detected in the β-casein hydrolysate. Amino acid analysis showed the presence of a trace of free phenylalanine, but other amino acids could not be detected (data not shown).

Extent of hydrolysis. After 24 h of incubation, the PrtP activity had dropped to 0.1% because of autoproteolysis of the enzyme (18). To check whether the peptides obtained by PrtP hydrolysis of β-casein can be hydrolyzed further by PrtP, 13 peptides were purified and subjected to degradation by newly added PrtP (i.e., peptides 164-168, 176-182, 94-105, 120-128, 169-175, 167-175, 166-175, 69-77, 183-192, 183-193, 194-209, 193-209, and 62-77). Although all peptides contained one or more potential cleavage sites, none of them was hydrolyzed. In order to exclude the possibility that the added PrtP was inactive or severely inhibited by the peptides, the chromogenic peptide methoxy-succinyl-L-arginyl-L-prolyl-L-tyrosine-pNA was added to the reaction mixture. After 2 h of incubation, more than 90% of the chromogenic peptide was hydrolyzed in the presence or absence of purified peptides in the incubation mixes. Again, the quantity of each purified peptide did not change significantly during incubation with PrtP, and no new peaks could be detected in the chromatograms, indicating that further hydrolysis of the peptides in the PrtP hydrolysate obtained from β-casein after 24 h did not occur. The peptide mixture obtained from β-casein after 24 h of incubation with PrtP

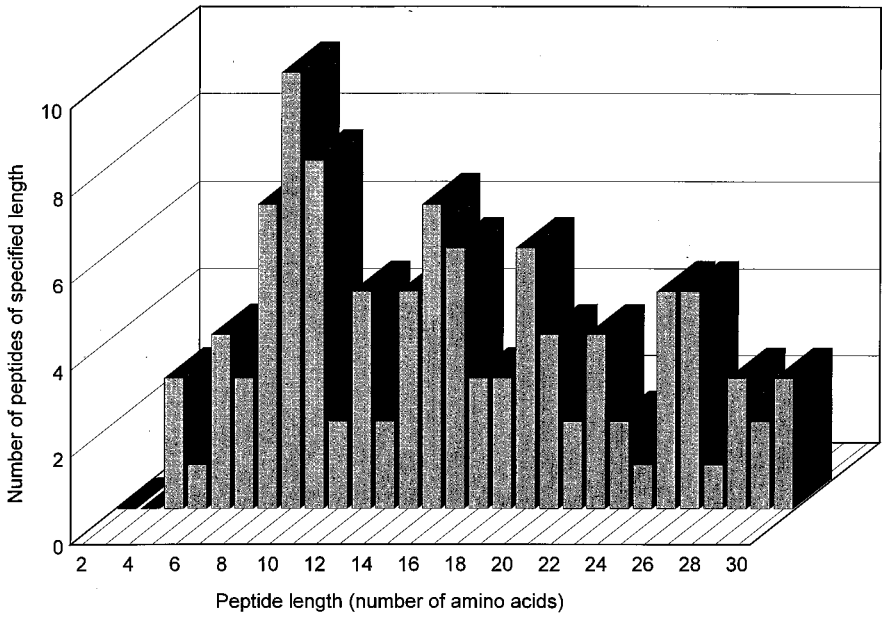

FIG. 5. Size distribution of peptides released by the action of purified proteinase from *L. lactis* subsp. *cremoris* Wg2 on β-casein.

should therefore be considered as an end product of the proteolysis reaction.

DISCUSSION

The peptides released from β-casein by the action of purified PrtP from *L. lactis* subsp. *cremoris* Wg2 (P_I type) have been identified by on-line coupling of HPLC to MS. This analysis proved to be very powerful and allowed the identification of more than 100 different peptides in the hydrolysate. This is considerably more than the 39 peptides which have been found previously in different analyses of the β-casein PrtP hydrolysate (42). The results extend considerably the observations regarding the hydrolysis patterns reported for PrtPs. The C-terminal part and the 60 to 105 region of the β-casein molecule appeared to be the areas most susceptible to hydrolysis by PrtP. More than 50% of the peptides identified originate from the C-terminal part (residues 142 to 209), whereas about half of the remaining peptides are derived from the 60 to 105 region (Fig. 4). The N terminus of the molecule is very poorly hydrolyzed by PrtP. In total, 91 different cleavage sites have been detected; they constitute 44% of all peptide bonds present in β-casein, showing that PrtP has a very broad substrate specificity. Since no detectable amounts of di- and tripeptides and only traces of Phe are formed, one can conclude that hydrolysis of peptides smaller than eight residues is a very rare event, if it occurs at all.

The importance of particular amino acids in the flanking regions of the cleavage sites was analyzed as described previously (30). The positions of the amino acids at the N- and C-terminal sides of the cleavage site are indicated as PX and P′X, respectively, in which X represents the distance from the cleavage site in number of residues (designation according to reference 37). The number of times a cleavage occurs when a particular amino acid is present at position PX was divided by the total number of cleavage sites, and this cleavage frequency was divided by the frequency of occurrence of this amino acid in β-casein. Positive effects on cleavage were found in position P6 (Asp, Arg, and His), P4 (Asp, Ala, Phe, and Tyr), and P′1 (Asp, Thr, Ala, and Tyr). When Ala was present in position P4 or Tyr was present in position P1, the corresponding peptide bonds were always hydrolyzed. In contrast, when Asn or Arg was in position P′2, no hydrolysis of the corresponding peptidic bond was found. A strong negative effect was also noticed with Phe in P6 and Glu in P1. A similar analysis was performed on the three different classes of amino acids (i.e., hydrophobic, hydrophilic, and neutral). Hydrophobic residues at position P2 had a clear positive effect on cleavage, whereas hydrophilic residues at the same position had a negative effect. Moreover, the presence of hydrophobic residues at positions P4, P3, and P′2, or that of neutral amino acids at position P4, resulted in a lower frequency of hydrolysis.

Eleven peptide bonds (Asn-68–Ser-69, Gln-141–Ser-142, Leu-163–Ser-164, Leu-165–Ser-166, Ser-166–Gln-167, Ser-168–Lys-169, Gln-175–Lys-176, Gln-182–Arg-183, Gln-188–Ala-189, Leu-191–Leu-192, and Tyr-193–Gln-194) are preferentially cleaved. These preferential cleavage sites yield six peptides that are produced during the initial phase of degradation. Interestingly, the proposed three-dimensional molecular model of β-casein A_2 (11) indicates that these preferential cleavage sites are on the outside of the molecule. The positive influence of prolyl residues in the close environment (positions P2, P4, and P6) of these early accessible cleavage sites and that of serine in position P′1 has been confirmed (30). These cleavages may result in a modification of the conformation of the substrate and the exposure of new sites for hydrolysis.

From the results presented here, it is clear that not all possible cleavage sites are hydrolyzed. For example, bond Ser-168–Lys-169 is not cleaved, because otherwise the peptide 168-175 would not have been detected. For that reason, it is not possible to define precise rules about the specificity of the proteinase based on β-casein degradation experiments alone. The observation that not all β-casein molecules are hydrolyzed in the same way is most likely due to the complexity of the structure of β-casein. It may explain why some complementary fragments of the β-casein molecule are not found (especially in the N-terminal region of the molecule) and why it is not possible to reconstruct the whole β-casein molecule only from the

identified TFA-soluble peptides. In conclusion, hydrolysis of β-casein is a complex process, in which several parameters are involved, i.e., the specificity of the proteinase, the complex structure of the substrate, the possible refolding of the substrate after cleavage, and the aggregation of β-casein molecules.

Growth of lactococci in milk is strongly dependent on β-casein degradation (10). Only traces of free Phe were produced, and the release of essential free amino acids could not be detected upon hydrolysis of β-casein by purified proteinase from *L. lactis*. To serve as a source of amino acids for *L. lactis*, the peptides released from β-casein by PrtP have to be either translocated across the cytoplasmic membrane or hydrolyzed further by extracellular peptidases. To date, no solid biochemical and/or genetical evidence for an extracellular location of lactococcal peptidases is available (26, 41). The peptidases therefore do not appear to play an essential role in the initial phase of β-casein utilization. Transport into the cells of peptides released from β-casein by PrtP prior to their hydrolysis by intracellular peptidases seems a more likely sequence of events.

Two different peptide transport systems have been identified in lactococci, i.e., a di-tripeptide and an oligopeptide transport system (17, 38). No di-tripeptides have been detected in the present study, although at least two were expected (Ile-208–Val-209 and Ile-207–Val-209). The 18 oligopeptides of from 4 to 8 amino acid residues are possible substrates for the oligopeptide transport system (16, 17, 45). All amino acids found in β-casein, including the essential ones, are present in these 18 peptides with the exception of phosphoserine. Thus, the oligopeptide transport system can provide lactococci with all amino acids, including the essential amino acids Glu or Gln, His, Val, Ile, Met, and Leu (16). This arrangement also explains why the oligopeptide transport system is essential for growth of *L. lactis* on milk and β-casein (16, 45). Most other peptides released from β-casein by PrtP are very likely too long to be transported into the cells. During growth of lactococci in milk, these peptides will remain outside the cells, without being used (unless they are cleaved by peptidases released upon lysis of the cells). This is in full agreement with the inability of a proteinase-lacking strain of lactococci to grow in milk in which a parental proteolytic strain has been cultured previously (14). In summary, the present analysis of β-casein hydrolysis by PrtP allows us to explain the utilization of β-casein by lactococci despite the absence of extracellularly located peptidases. In addition to a better understanding of the proteolytic pathway of *L. lactis* as a whole, we have gained additional information on the specificity of P_I-type proteins.

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