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# Purification and Characterization of an Endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2

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**An endopeptidase has been purified to homogeneity from a crude cell extract of *Lactococcus lactis* subsp. *cremoris* Wg2 by a procedure that includes diethyl-aminoethane-Sephacel chromatography, phenyl-Sepharose chromatography, hydroxylapatite chromatography, and fast protein liquid chromatography over an anion-exchange column and a hydrophobic-interaction column. Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a molecular mass of the purified enzyme of 70,000 Da. The endopeptidase can degrade several oligopeptides into various tetra-, tri-, and dipeptides. The endopeptidase has no aminopeptidase, carboxypeptidase, dipeptidase, or tripeptidase activity. It is optimally active at pH 6.0 to 6.5 and in the temperature range of 30 to 38°C. The enzyme is inactivated by the chemical agents 1,10-phenanthroline, ethylenedinitrilotetraacetate, β-mercaptoethanol, and phenylmethylsulfonyl fluoride and is inhibited by $Cu^{2+}$ and $Zn^{2+}$. The ethylenedinitrilotetraacetate- or 1,10-phenanthroline-treated enzyme can be reactivated by $Co^{2+}$. Immunoblotting with specific antibodies raised against the purified endopeptidase indicated that the enzyme is also present in other *Lactococcus* spp., as well as in *Lactobacillus* spp. and *Streptococcus salivarius* subsp. *thermophilus*.**

The breakdown of casein into peptides and amino acids and the uptake of small peptides and amino acids are vital activities for lactic acid bacteria growing in milk (20, 31). The activities of this proteolytic system also contribute to the development of flavor during the ripening of cheese (30, 33). Therefore, proteolytic systems of lactic acid bacteria have been studied extensively (6, 15, 18, 21, 39, 40, 43). The first step in proteolysis of casein is catalyzed by cell wall-bound proteinases (7, 10, 27, 46). The proteinase of *Lactococcus lactis* hydrolyzes β-casein into several oligopeptides of five or more amino acids (25, 26, 44, 45). Subsequently, peptidases can degrade these peptides to smaller peptides and amino acids. Several peptidases of lactic acid bacteria have been isolated and characterized. Endopeptidases (48, 49) hydrolyze large casein fragments into smaller peptides which can then be degraded by several aminopeptidases (1, 8, 13, 29, 38). Specific X-prolyl-dipeptidylaminopeptidases (2, 14, 22, 50) degrade the proline-rich peptides while di- and tripeptidases (3, 11, 41) and prolidases (12) release free amino acids, which finally complete the degradation of casein.

Amino acid and peptide uptake systems play a key role in the proteolytic system (16, 32, 35, 37). Recent studies in *L. lactis* subsp. *lactis* showed that a dipeptide-tripeptide uptake system is vital for the growth of *L. lactis* on casein (34, 36). This and other observations indicate that some peptidases perform their hydrolytic action outside the cytoplasmic membrane and provide peptides for these uptake systems, while other peptidases are active inside the cytoplasm. To date, three peptidases have been isolated from *L. lactis* subsp. *cremoris* Wg2 and biochemically characterized: a dipeptidase (41), a tripeptidase (3), and an aminopeptidase (38). These three enzymes are all exopeptidases and show no endopeptidaselike activity.

Obviously, such endopeptidase activity is essential for complete degradation of casein. Our aim was to search for such activity. As expected, such activity was found, and this paper reports the purification and characterization of an endopeptidase from *L. lactis* subsp. *cremoris* Wg2. This new peptidase can hydrolyze large β-casein peptides into smaller fragments. This enzyme appears to be distinctly different from other reported endopeptidases (48, 49).

## MATERIALS AND METHODS

**Organisms and preparation of cell extract.** *L. lactis* subsp. *cremoris* Wg2 was obtained from the Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. *Lactococcus lactis* subsp. *cremoris* H61 was obtained from the National Institute of Animal Industry, Ibaraki, Japan; *L. lactis* subsp. *cremoris* P8-2-47 was obtained from the Institut für Mikrobiologie, Bundesanstalt für Milchforschung, Kiel, Germany. *L. lactis* subsp. *cremoris* AM2, also called CNRZ 380, was obtained from the culture collection of the Centre de Recherches de Jouy-en-Josas, Jouy-en-Josas, France, and was stored at −18°C in litmus milk.

The organisms were routinely maintained in 10% (wt/vol) sterile reconstituted skim milk containing 0.1% (wt/vol) tryptone (Difco Laboratories, Detroit, Mich.) and stored at −20°C. *L. lactis* subsp. *cremoris* Wg2 was grown on MRS broth (4) at a controlled pH of 6.3 in a 5-liter fermentor. Cells were harvested at an optical density at 660 nm of 1.9 by centrifugation at 7,000 × *g* for 15 min at 4°C, washed in 400 ml of 50 mM potassium phosphate (KPi) (pH 6.0), and resuspended in 50 ml of 20 mM KPi (pH 6.0). Cells were disrupted with a French press (Aminco, Silver Spring, Md.) at 4°C. Cell extracts were obtained by centrifugation of the disrupted cells for 20 min at 17,500 × *g* at 4°C.

**Enzyme assays.** Hydrolysis of peptides was detected by thin-layer chromatography (TLC). Endopeptidase activity was assayed as follows: the reaction mixture containing 1.25 mM metenkaphalin (tyrosyl-glycyl-glycyl-phenylalanyl-methionine) in 20 mM 2-amino-2-(hydroxymethyl)-1,3-propandiol–HCl (Tris-HCl) at pH 7.0 was incubated with an appropriate amount of enzyme for 15 min at 30°C. Samples (50 μl)

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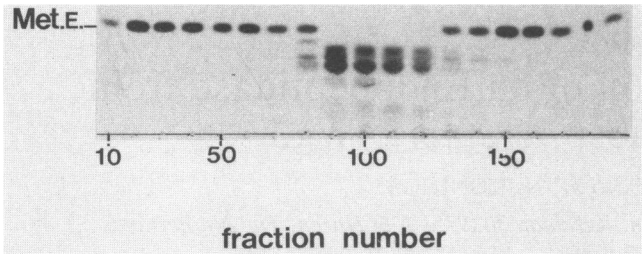


FIG. 1. TLC of metenkaphalin hydrolysis by fractions 10 to 180. Fractions were obtained by DEAE-Sephacel column chromatography of a crude extract of *L. lactis* subsp. *cremoris* Wg2. Metenkaphalin-hydrolyzing activities were detected in fractions 90 to 120. Met.E., metenkaphalin.

were taken, and the reaction was stopped by adding 10 μl of 30% acetic acid and cooling the mixture to 4°C. The mixture (10 μl) was then spotted onto a precoated 0.25-cm-thick silica gel 60 plate (Merck, Darmstadt, Germany), and TLC was performed as described by Tan and Konings (38). In some cases, 0.05% fluorescamine in 99% acetone was used to stain the gels. Peptides and amino acids became visible in UV light.

Endopeptidase activity was determined by incubating an appropriate amount of endopeptidase with 1.25 mM metenkaphalin in 20 mM Tris-HCl (pH 7.0) at 30°C. Samples (50 μl each) were taken every 60 s, and the reaction was stopped by adding 10 μl of 30% acetic acid and cooling the mixture to 4°C. TLC was performed as described above. The time point at which complete hydrolysis was observed was determined. The time needed for complete hydrolysis decreased linearly with the protein concentration. On the assumption that hydrolysis proceeded linearly in time, the activity was calculated and expressed in micromoles of metenkaphalin hydrolyzed per minute per milligram of protein at 30°C and pH 7. β-Casein peptides were provided by DMV Campina, Veghel, The Netherlands.

**DEAE-Sephacel column chromatography.** A DEAE-Sephacel column (1.6 by 20 cm) was equilibrated with 20 mM KPi (pH 6.0) containing 0.1 M NaCl. Cell extract (400 ml), obtained from *L. lactis* subsp. *cremoris* Wg2, was diluted with 20 mM KPi to the same ionic strength as the buffer used for equilibrating the DEAE-Sephacel column and was subsequently applied to the column. Ionic strength was measured with a radiometer (Radiometer, Copenhagen, Denmark). After the column was washed with 2 volumes of equilibration buffer, the enzyme was eluted (54 ml/h) with a linear gradient of 0.1 to 0.4 M NaCl in 20 mM KPi. The highest metenkaphalin-hydrolyzing activities were found in fractions 90 to 120 (Fig. 1). The fractions were combined.

**Phenyl-Sepharose chromatography.** A phenyl-Sepharose (CL-4B) column (1.8 by 8.5 cm) was equilibrated with 20 mM Tris-HCl (pH 6.0) containing 4 M NaCl. The combined enzyme fractions of the DEAE-Sephacel chromatography (186 ml) were brought to the same osmolarity as the equilibration buffer by the addition of NaCl. Subsequently, the enzyme solution was applied to the column. After the column was washed with 2 volumes of equilibration buffer, the enzyme was eluted (30 ml/h) with a linear gradient of 4 to 0 M NaCl in 20 mM Tris-HCl (pH 6.0). Fractions (7.5 ml each) were collected and tested for endopeptidase activity. The highest metenkaphalin-hydrolyzing activities were found with 0.9 to 0.6 M NaCl.

**Hydroxylapatite chromatography.** The enzyme fractions obtained by phenyl-Sepharose chromatography were combined (90 ml) and subsequently applied to a hydroxylapatite column (1.5 by 6.3 cm) that had been preequilibrated with 0.01 M Na-phosphate (NaPi) (pH 6.0). The column was washed with 2 volumes of the same buffer, and the enzyme was eluted with a linear gradient of NaPi from 0.01 to 0.4 M at pH 6.0. The flow rate was 40 ml/h. The highest metenkaphalin-hydrolyzing activities were found in the fractions with 0.21 to 0.25 M NaPi.

**Second anion-exchange chromatography.** For fast protein liquid chromatography (FPLC), a Mono Q HR 5/5 column was used (Pharmacia, Uppsala, Sweden), which was preequilibrated with 20 mM Tris-HCl (pH 6.0) containing 0.1 M NaCl. The combined fractions of hydroxylapatite chromatography (75 ml) were applied to the column. The enzyme was eluted with a linear gradient from 0.1 to 0.3 M NaCl in 20 mM Tris-HCl (pH 6.0) at a flow rate of 1 ml/min. The metenkaphalin-hydrolyzing activities were found in the fractions with 0.23 to 0.27 M NaCl.

**Second hydrophobic-interaction chromatography.** FPLC with a phenyl-Superose HR 5/5 column (Pharmacia) was done after preequilibration of the column with 20 mM Tris-HCl (pH 6.0) containing 4 M NaCl. With the addition of NaCl, the combined active fractions eluted from the Mono Q column were brought to the same osmolarity as the equilibration buffer and subsequently applied to the phenyl-Superose HR 5/5 column. The enzyme was eluted with a linear gradient from 4 to 0 M NaCl in 20 mM Tris-HCl (pH 6.0) at a flow rate of 0.5 ml/min. The enzyme eluted as a single peak at 1.6 M NaCl.

**HPLC.** High-performance liquid chromatography (HPLC) was performed as described by Bosman et al. (3). Purified endopeptidase (100 μl containing 25 μg of protein) was injected on a Ultropac TSK G3000 SW gel filtration column (7.5 by 600 mm; Pharmacia LKB Biotechnology). The molecular weight of the protein was estimated by using the low-molecular-weight protein standards (Pharmacia LKB Biotechnology). The single peak was tested for metenkaphalin hydrolysis.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (19). The protein samples were mixed 4:1 with sample buffer (0.05 M Tris-HCl [pH 6.8], 10% SDS, 22% glycerol, 10% β-mercaptoethanol, 0.18% bromphenol blue) and applied to the gels. The molecular weights of the protein bands were estimated by using the low-molecular-weight SDS-PAGE standards (Bio-Rad Laboratories, Richmond, Calif.). The gels were stained either with Coomassie brilliant blue or with silver by the method of Wray et al. (47).

**Isoelectric focusing.** Isoelectric focusing on slab gels was performed with Phast System (Pharmacia) and a ready-to-use 5% polyacrylamide gel containing Pharmalyte 3-9 (Phastgel; Pharmacia). Determination of the isoelectric point was carried out with the reference proteins from the broad-pI-range calibration kit (Pharmacia). Gels were automatically stained by the Phast System with Coomassie brilliant blue.

**Temperature and pH dependence of endopeptidase activity.** The effect of temperature on the endopeptidase activity was measured in the range of 4 to 60°C. The enzyme mixture was equilibrated for 5 min at the temperatures tested before the addition of metenkaphalin. The effect of the pH was determined in the range from pH 4 to 10 by using a buffer consisting of 20 mM (each) malic acid, 2-(*N*-morpholino) ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and boric acid adjusted to the appropriate pH values.

**Effect of divalent cations and chemical agents on enzyme activity.** Purified enzyme (99 μg) was preincubated for 30 min at 4°C with EDTA (1 mM) in Tris-HCl (pH 6.0). After dialysis for 24 h against the same buffer, the enzyme solution was incubated with several concentrations of divalent cations for 30 min at 4°C before metenkaphalin was added to start the reaction. The reaction was stopped and the reaction products were analyzed as described above. An appropriate amount of enzyme was incubated with chemical agents (1 mM) for 30 min at 4°C, and the effect on the enzyme activity was studied.

**Antiendopeptidase serum.** The antiserum to the enzyme was raised in rabbits after immunization by the following protocol. Samples (100 μl) of purified enzyme (0.5 mg of protein per ml) were injected with complete Freund adjuvant. After 1 month, a booster injection of 50 μg of purified enzyme was given. The rabbits were bled 7 and 14 days after each booster injection. Serum was prepared from blood by centrifugation at 10,000 × *g* for 10 min.

**Immunoblotting.** After PAGE of samples containing the crude extracts of different *L. lactis* subspecies, *Lactobacillus delbrueckii* subsp. *bulgaricus* var. *diacetylactis*, and *Streptococcus salivarius* subsp. *thermophilus* strains or purified endopeptidase, the proteins separated on the gel were transferred to polyvinylidene difluoride sheets with a semidry electroblotter (Ancos, Copenhagen, Denmark) by the technique of Kyhse-Andersen (17). The polyvinylidene difluoride membrane was saturated with 1% skim milk and then incubated with rabbit antiendopeptidase serum diluted 1/7,000. Subsequently, the membrane was incubated with alkaline phosphatase-conjugated goat-rabbit immunoglobin serum (Sigma Chemical Co., St. Louis, Mo.) diluted 1/1,000. After being washed with TBST (10 mM Tris-HCl [pH 8.0] containing 150 mM NaCl and 0.05% Tween 20), the enzyme-antibody complex was visualized by incubating the membrane in an alkaline carbonate buffer (pH 9.8) containing 1% (vol/vol) Nitro Blue Tetrazolium (30 mg/ml) in 70% dimethylformamide and 1% (vol/vol) 5-bromo-4-chloro-3-indolylphosphate (BCIP) (15 mg/ml) in 100% dimethylformamide.

**CIE.** Crossed immunoelectrophoresis (CIE) of the purified enzyme (80 μg of protein per ml) and cell extract of *L. lactis* subsp. *cremoris* Wg2 (0.32 mg of protein per ml) was carried out as described previously (5, 42) against antibodies (0.5 mg of protein per ml) raised against the cell extract of *L. lactis* subsp. *cremoris* Wg2 (9) and against antibodies (17.8 mg of protein per ml) raised against the purified endopeptidase. The gels were run at 30 V/cm for 90 min in the first dimension and at 40 V/cm for 18 to 24 h in the second dimension. The gels were stained with Coomassie brilliant blue to show the precipitate.

**Casein hydrolysis.** An appropriate amount of purified enzyme was added to a mixture of α-, β-, and κ-casein (1.5 mg/ml) in Tris-HCl (pH 7). After overnight incubation at 30°C, the samples were boiled for 5 min in sample buffer as described above. Proteins were separated by electrophoresis on a 12.5% polyacrylamide–SDS gel.

**Amino acid analysis.** Amino acid composition analysis were carried out by Eurosequence b.v. (Groningen, The Netherlands), as described previously (3, 24, 28, 38).

**Protein determination.** Protein concentrations were determined by the method of Lowry et al. (23), with bovine serum albumin as the standard.

**Chemicals.** All chemicals were of reagent grade and were obtained from commercial sources.

TABLE 1. Purification of an endopeptidase from *L. lactis* subsp. *cremoris* Wg2

| Purification step | Total amt (mg) of protein | Total activity[a] | Yield (%) | Activity[b] | Purification (fold) |
|---|---|---|---|---|---|
| Cell extract | 6,640 | 278.9 | 100 | 0.042 | 1 |
| DEAE-Sephacel | 902.1 | 387.9 | 139 | 0.430 | 10 |
| Phenyl-Sepharose | 102.6 | 112.4 | 40.3 | 1.096 | 26 |
| Hydrolxylapatite | 38.5 | 96.3 | 34.5 | 2.500 | 60 |
| Mono Q | 2.4 | 48.5 | 17.4 | 20.208 | 481 |
| Phenyl-Superose | 1.7 | 48.4 | 17.4 | 28.470 | 678 |

[a] Total activity is expressed as micromoles of metenkaphalin hydrolyzed per minute.

[b] Activity is expressed as micromoles of metenkaphalin hydrolyzed per milligram of protein per minute.

## RESULTS

**Enzyme purification.** The enzyme purification procedures (see Materials and Methods) are summarized in Table 1. A total yield of the purification procedure of 17.4% and a purification factor of 678 was estimated. After the last purification step, a single peak was eluted. Hydrolysis of metenkaphalin completely into amino acids requires several peptidase activities (Fig. 1). The tripeptide tyrosyl-glycyl-glycine was not further hydrolyzed with the enzyme fraction obtained from the hydroxylapatite column, indicating that tripeptidase activity and aminopeptidase activity had been removed. However, dipeptidase activity was still present, as indicated by the hydrolysis of Phe-Met. The fractions obtained from the Mono Q column hydrolyzed metenkaphalin into Tyr-Gly-Gly and Phe-Met, indicating that only endopeptidase activity was retained (Fig. 2, lanes a and d). However, SDS-PAGE showed that the fractions were still contaminated with other proteins (Fig. 3A, lane e). These contaminations were removed in the last purification step with a phenyl-Superose column. After every purification step, activity increased (Table 1).

**Molecular mass and isoelectric point.** The molecular mass of the enzyme was estimated to be 70,000 Da by HPLC with a TSK G3000 SW column (results not shown) and by SDS-PAGE on a 10% polyacrylamide gel (Fig. 3A). On SDS-PAGE, the purified enzyme gave one band with silver staining as well as with Coomassie brilliant blue staining.

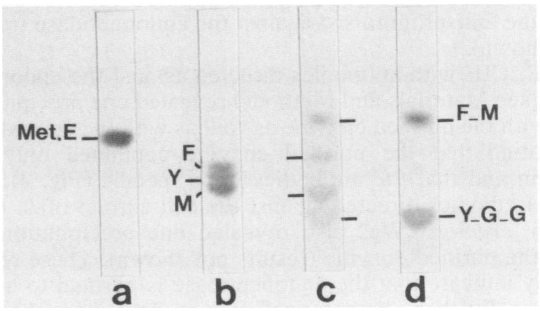


FIG. 2. TLC of metenkaphalin before and after hydrolysis with peptidase-containing fractions. Fractions of metenkaphalin before (a) and after incubation with a fraction obtained after DEAE-Sephacel chromatography (b), with a fraction obtained after hydroxylapatite chromatography (c), with a fraction obtained after Mono Q chromatography (d). Abbreviations: Met.E, metenkaphalin; F, phenylalanine; Y, tyrosine; M, methionine; F-M, phenylalanyl-methionine; Y-G-G, tyrosyl-glycyl-glycine.

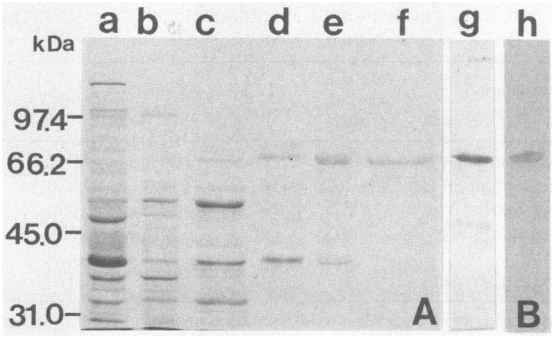


FIG. 3. (A) SDS-PAGE analysis of the protein-containing fractions obtained in the different purification steps of the endopeptidase. Electrophoresis was performed on a SDS-polyacrylamide gel (10% polyacrylamide) with low-molecular-size protein standards (see Materials and Methods; also data not shown). Crude cell extract (22 μg of protein) before chromatography (lane a), after DEAE-Sephacel column chromatography (14 μg of protein) (lane b), after phenyl-Sepharose column chromatography (11 μg of protein) (lane c), after hydroxylapatite column chromatography (10 μg of protein) (lane d), after Mono Q (FPLC) column chromatography (5 μg of protein) (lane e), and after phenyl-Superose HR 5/5 (FPLC) column chromatography (2 μg of protein) (lane f). (B) Immunoblots of cell extract (20 μg of protein) incubated with polyclonal antibodies against the purified endopeptidase (lane g) and of the purified enzyme (2 μg of protein) incubated with polyclonal antibodies against the purified endopeptidase (lane h).

Treatment of the purified enzyme with β-mercaptoethanol after boiling for 5 min or under native conditions yielded the same electrophoretic migration, indicating that the enzyme consists of a single polypeptide.

The pI of the enzyme was estimated to be 4.3 by isoelectric focusing (data not shown).

**Immunoblotting.** Figure 3B shows the results of immunoblotting with the purified enzyme (lane g) and with cell extract from strain Wg2 (lane h). These results confirm the purity of the enzyme. A very small cross-reaction between the antiserum and one protein band (ca. 45 kDa) in the crude extract could be observed. Immunoblotting with cell extracts of *L. lactis* subsp. *cremoris* H61, P8-2-47, E8, AM2, SK11, and HP, *L. lactis* subsp. *lactis* ML1 and ML3, *Streptococcus thermophilus* A147, and *Lactobacillus bulgaricus* B131 also revealed a 70-kDa band after incubation with the antiserum raised against the endopeptidase (results not shown).

**CIE.** CIE with antibodies directed against the endopeptidase (see Materials and Methods) revealed one precipitation line with the purified enzyme as well as with the cell extract, indicating that the purified enzyme contained only one protein and that the antibodies were specific (Fig. 4). CIE with antibodies directed against the cell extract of *L. lactis* subsp. *cremoris* Wg2 also revealed one precipitation line with the purified enzyme (results not shown). These results clearly indicate that the endopeptidase is purified to homogeneity. Further characterization was done with the pure endopeptidase.

**Temperature and pH dependence of endopeptidase activity.** The effects of temperature and pH on the endopeptidase activity were measured. The optimum temperature for metenkaphalin-hydrolyzing activity was found to be between 30 and 38°C and the optimum pH for hydrolyzing metenkaphalin appeared to be between pH 6.0 and 6.5. No hydrolysis of metenkaphalin was detected below pH 4.5 and above pH 10.0.

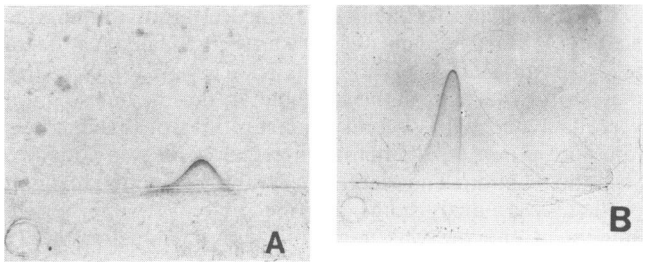


FIG. 4. CIE of the purified endopeptidase against antibodies raised against whole cells of *L. lactis* subsp. *cremoris* Wg2 (A) and of cell extract of *L. lactis* subsp. *cremoris* Wg2 against polyclonal antibodies raised against the purified endopeptidase (B).

**Substrate specificity.** Hydrolysis of various peptides by the endopeptidase is shown in Table 2. The products of hydro-

TABLE 2. Substrate specificity of an endopeptidase from *L. lactis* subsp. *cremoris* Wg2[a]

| Substrate | Activity[b] |
|---|---|
| Lys-pNA[c] | − |
| Gly-Pro-pNA[c] | − |
| Ala-Pro-pNA[c] | − |
| Benzoyl-Gly-Pro | − |
| Benzoyl-Gly-Lys | − |
| Carbobenzoxy-Pro-Ala | − |
| Carbobenzoxy-Phe-Ala | − |
| Gly-Gly | − |
| Leu-Leu | − |
| Phe-Met | − |
| Phe-Val | − |
| Tyr-Gly-Gly | − |
| Leu-Leu-Leu | − |
| Met-Gly-Gly | − |
| Leu-Gly-Gly | − |
| Gly-Val-Phe | − |
| Gly-Pro-Ala-Pro | − |
| Gly-Pro-Gly-Gly | − |
| $N_{succ}$-Ala-Ala-Ala-pNA[c] | − |
| $N_{succ}$-Phe-pNA[c] | − |
| $N_{succ}$-Ala-Ala-Pro-Leu-pNA[c] | − |
| Z-Leu-Gly-Gly-pNA[c] | − |
| Ala-Ala-Ala-Ala-Ala | − |
| Ala-Ala-Ala-Ala-Ala-Ala | − |
| Metenkaphalin | + |
| β-Casomorphin | − |
| Bradykinin | + |
| Substance P | + |
| Glucagon | + |
| Oxidized insulin β-chain | + |
| Neurotensin | + |
| α-Casein | − |
| β-Casein | − |
| κ-Casein | − |
| β-Casein (f33–48)[d] | − |
| β-Casein (f184–202) | + |
| β-Casein (f203–209) | + |

[a] Hydrolysis of the peptides were analyzed by TLC. Hydrolysis of the chromogenic substrates were also measured by $A_{410}$, and hydrolysis of casein was detected by SDS-PAGE. See Materials and Methods for details.
[b] +, hydrolysis; −, no hydrolysis detectable.
[c] Chromogenic substrate; $N_{succ}$, N-succinyl.
[d] Peptide from amino acids 33 to 48 derived from β-casein.

TABLE 3. Effects of potential inhibitors on the endopeptidase activity[a]

| Inhibitor | Inhibition[b] |
|---|---|
| None | − |
| *p*-Chloromercuribenzoate | − |
| 1,10-Phenanthroline | + |
| Mersalyl | − |
| Diethyl-pyrocarbonate | − |
| Ferricyanide | − |
| Phenylarsenoxide | − |
| *N*-Ethylmaleimide | − |
| EDTA | + |
| Cystine | − |
| *p*-Chloromercuribenzenesulfonate | − |
| Thorin | − |
| Plumbagin | − |
| Hydroxylamine | − |
| β-Mercaptoethanol | + |
| Dithiothreitol | − |
| Phenylmethylsulfonyl fluoride | + |

[a] Purified enzyme (4 μg of protein) was preincubated for 10 min at 20°C with 1 mM chemical agent. After the addition of metenkaphalin, the fractions were incubated for 15 min at 30°C. The enzyme activity was tested by TLC.
[b] +, inhibition; −, no inhibition detectable.

TABLE 4. Amino acid composition of endopeptidase[a]

| Amino acid | % of amino acid residues in enzyme | Nearest integer |
|---|---|---|
| Asx | 8.78 | 58 |
| Glx | 7.97 | 52 |
| Ser | 10.25 | 68 |
| His | 2.00 | 13 |
| Gly | 13.46 | 89 |
| Thr | 5.54 | 37 |
| Ala | 9.36 | 62 |
| Arg | ND[b] | ND |
| Tyr | 2.44 | 16 |
| Cys | 2.94 | 19 |
| Val | 4.54 | 30 |
| Met | 1.77 | 12 |
| Phe | 5.54 | 37 |
| Ile | 5.24 | 35 |
| Leu | 9.12 | 60 |
| Lys | 5.98 | 40 |
| Pro | 2.30 | 15 |
| Trp | ND | ND |

[a] Determined as described in Materials and Methods.
[b] ND, not determined.

lysis were analyzed by TLC. The endopeptidase showed a broad substrate specificity and also hydrolyzed two peptides derived from β-casein. Peptides with less than five amino acid residues were not hydrolyzed. The largest peptide hydrolyzed by the endopeptidase was the oxidized β-insulin chain. No hydrolysis of α-, β-, or κ-casein was detected, even after overnight incubation with the purified enzyme (results not shown).

**Effects of various chemical agents.** Table 3 shows that endopeptidase activity was inhibited by EDTA and 1,10-phenanthroline (1.0 mM). The enzyme was also inhibited by phenylmethylsulfonylfluoride, but not by dithiothreitol. β-Mercaptoethanol (1%) treatment of the endopeptidase also inhibited the activity and oxidizing reagents such as ferricyanide, plumbagine, and oxidized glutathione (up to 5 mM) could not restore enzyme activity.

**Effect of metal ions on enzyme activity.** Treatment of the enzyme (4 μg of protein) with 1 mM of $Cu^{2+}$ or $Zn^{2+}$ inhibited the enzyme activity completely. Other cations such as $Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Co^{2+}$, and $Mn^{2+}$ (1 mM) had no effect on enzyme activity. Enzyme activity inhibited by EDTA or 1,10-phenanthroline could be restored by 50 to 300 μM $Co^{2+}$ and not by other divalent cations.

**Amino acid composition.** The amino acid composition of the purified endopeptidase (Table 4) shows a high content of serine and glycine residues. Sulfur-containing amino acids such as cysteine and methionine are present at low concentrations. The molecular mass calculated from the nearest integers of the amino acids was found to be 66 kDa.

## DISCUSSION

A dipeptidase (41), a tripeptidase (3), and an aminopeptidase (38) have already been purified from *L. lactis* subsp. *cremoris* Wg2. During the purification, X-prolyl-dipeptidyl aminopeptidase could also be detected in this strain (results not published). All these enzymes are so-called exopeptidases, and their action will yield amino acids (and dipeptides) as products of hydrolysis. Although the aminopeptidase can hydrolyze larger peptides, its action will be more effective when the large β-casein fragments (25, 26) are first hydrolyzed by an endopeptidase.

In this paper, we described the purification and characterization of an endopeptidase from the crude cell extracts from *L. lactis* subsp. *cremoris* Wg2, which can hydrolyze several large β-casein fragments. The purification of this enzyme involved a five-step procedure. Endopeptidase activity was detected by TLC with the model substrate metenkaphalin. This peptide turned out to be a very good substrate for the screening of peptidase activity. During purification, the activities of other peptidases decreased and fractions eluted from the Mono Q column contained only the endopeptidase activity. An additional purification step was needed to purify the enzyme to homogeneity. The purification, as estimated from the activity, was approximately 600-fold, with a yield of 17%.

The purified endopeptidase differs clearly from the two other endopeptidases, LEP I and LEP II, reported by Yan et al. (48, 49). The enzyme described here is a monomer with a molecular mass of 70,000 Da. The endopeptidase LEP I of *L. lactis* subsp. *cremoris* H61 has a molecular weight of 98,000, and LEP II appears to be a dimer with a molecular weight of 80,000. Differences are also observed with respect to the effects of various metal ions like $Zn^{2+}$ on the peptidase activity. $Zn^{2+}$ inhibits endopeptidase activity completely, while LEP I activity is not affected. The metal-depleted LEP I activity can be restored by $Mn^{2+}$, while $Zn^{2+}$ can even reactivate the EDTA-treated LEP II activity. The pH optima and the pI of the three enzymes also differ: LEP I has its optimum at pH 7 to 7.5 and an isoelectric point of 5.1, whereas our endopeptidase has a pH optimum 6.3 and a pI of 4.1. The enzymes also have different substrate specificities. LEP I cannot hydrolyze glucagon and oxidized insulin β-chain, in contrast to our endopeptidase. Finally, the amino acid compositions (Table 4) of LEP II and our endopeptidase differ significantly. On the other hand, as expected, certain properties of the enzymes such as inactivation by EDTA and the action toward certain peptides are similar.

The specificity of the peptidase was determined by studying the hydrolysis of several peptides of more than four residues. The enzyme cannot hydrolyze dipeptides and

tripeptides and also no activity could be detected toward the specific substrates of aminopeptidase and of X-prolyl-dipeptidyl aminopeptidase (Table 2). The purified protein hydrolyzes Tyr-Gly-Gly-Phe-Met into Tyr-Gly-Gly and Phe-Met (Fig. 3), indicating that it is an endopeptidase.

The enzyme is reversibly inhibited by EDTA, and activity can only be restored by the divalent cation $Co^{2+}$, indicating that this enzyme is a $Co^{2+}$-dependent metallopeptidase. Phenylmethylsulfonyl fluoride also inhibits the peptidase activity in a manner similar to that of a serine proteinase. Sulfhydryl reagents like pCMB(S) and mersalyl have no effect on the activity, while the reducing agent β-mercaptoethanol inhibit the enzyme activity. These observations suggest that the enzyme is active in the disulfide form. However, the disulfide reducing agent dithiothreitol does not inhibit activity. Furthermore, the following observations are also not consistent with an enzyme active in the disulfide form: (i) the enzyme is irreversibly inhibited by β-mercaptoethanol and (ii) oxidizing agents such as ferricyanide and oxidized glutathione are not able to restore the enzyme activity.

Polyclonal antibodies raised against the endopeptidase appear to be very specific. Immunoblotting and CIE indicate that the endopeptidase does not contain immunologically different components as was observed for the proteinase of *L. lactis* subsp. *cremoris* Wg2 (9, 10). Several *Lactococci*, *Lactobacilli*, and *Streptococci* species were found to contain this endopeptidase, indicating that it is of general importance to these organisms.

The purified endopeptidase can hydrolyze small β-casein peptides. It is very likely that this enzyme plays a role in the proteolytic system of *L. lactis* subsp. *cremoris* Wg2. Uptake systems for oligopeptides with more than five amino acids have not been found, and extracellular endopeptidase appears to be essential for casein hydrolysis to transportable amino acids or peptides. The di-, tri-, and aminopeptidase have been purified from the cell extracts. However, solid evidence concerning the exact localization of these enzymes is still lacking. Currently, attempts are made to determine the localization of these enzymes with specific antibodies.

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