Effect of Peptides from the Sequence 58–72 of β-Casein on the Activity of Endopeptidase, Aminopeptidase, and X-Prolyl-Dipeptidyl Aminopeptidase from *Lactococcus lactis* Ssp. *lactis* MG1363

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The K_i values for the inhibition of the 70 kDa intracellular, *o*-phenanthroline-sensitive endopeptidase from *Lactococcus lactis* ssp. *lactis* MG1363 by bovine β-casein (CN) f58–72, β-CN f58–70, β-CN f60–68, β-CN f60–70, and β-CN f60–66 were 0.018, 0.01, 0.045, 0.150 and 0.28 mM, respectively. The 95 kDa aminopeptidase, which is also sensitive to *o*-phenanthroline, from the cytoplasm of the same microorganism was inhibited by the above peptides but the corresponding K_i values were 3–10 times higher. The X-prolyl-dipeptidyl aminopeptidase from the same source, which is insensitive to *o*-phenanthroline, was not inhibited by any of the above peptides and in fact hydrolyzed β-CN f60–66 and β-CN f60–70. β-Casein f58–72 did not inhibit thermolysin and was degraded by it. The results demonstrate that cheese ripening may be influenced by inhibitory peptides originating from β-casein.

Keywords: *Inhibitory peptides; cheese; lactococcal peptidases*

INTRODUCTION

Proteolysis of bovine or human milk proteins generates a variety of biologically active peptides; e.g. peptides with opiate, angiotensin-converting enzyme (ACE) inhibitory, immunomodulating, or calcium absorption-enhancing activities have been isolated from enzymatic digests of caseins or whey proteins [see Fox and Flynn (1992) and Fiat et al. (1993)]. Biologically active peptides from milk proteins may in the future be used as food additives or in pharmacology (Fiat et al., 1993).

Proteolysis is a major event in cheese ripening. Serine and metallo endo- and exopeptidases from lactic acid bacteria (LAB) are considered as "flavor-generating enzymes", providing peptides and amino acids that directly contribute to flavor or are precursors of complex flavor compounds (Crow et al., 1993). Bacterial metalloendopeptidases, such as thermolysin, intracellular oligopeptidase (PepO) from *Lactococcus* spp. [terminology according to Tan et al. (1993), which replaces the term "LEP" or *Lactococcus* endopeptidase proposed earlier by Yan et al. (1987) for the enzyme which hydrolyzes oligopeptides from caseins but not whole caseins], and mammalian kidney or brain membrane-bound metalloendopeptidases (enkephalinases), preferentially hydrolyze peptide bonds with a hydrophobic amino acid located at the P'_1 position (Almenoff and Orlowski, 1984; Pozsgay et al., 1986; Yan et al., 1987; Tan et al., 1991; Stepaniak and Fox, 1995). The amino acid sequence of PepO isolated from *L. lactis* ssp. *cremoris* by Tan et al. (1991) is similar to a mammalian enkephalinase (Mierau et al., 1993).

Inhibition of mammalian metalloendoproteinases such as enkephalinases or ACE and inhibition of renin or HIV-protease by peptide or peptide analog inhibitors are of medical significance [cf. Pozsgay et al. (1986) and Grant et al. 1993)]. Knowledge of the structure and mechanism of action of natural peptide inhibitors allowed the synthesis of several potent peptide analog inhibitors of ACE or renin (Grant et al., 1993). Despite obvious technological significance, studies on the inhibition of proteolytic enzymes in food systems are limited (Fiat et al., 1993; Haque and Antila, 1993).

We observed that the diluted water-soluble fraction of Cheddar cheese inhibits the hydrolysis of α_{s1}-casein f1–23 and α_{s1}-CN f165–199 (major peptides released from α_{s1}-casein by chymosin) and of methionine enkephalin by a 70 kDa PepO from *L. lactis* ssp. *lactis* MG1363 (Stepaniak et al., 1993; Stepaniak and Fox, 1995). The enzyme is sensitive to *o*-phenanthroline and phosphoramidon and is immunologically identical with the 70 kDa PepO from *L. lactis* ssp. *cremoris* characterized by Tan et al. (1991).

The objective of this study was to isolate from cheese and characterize peptides that are inhibitory to selected intracellular proteolytic enzymes from LAB important for cheese ripening.

MATERIALS AND METHODS

Enzymes and Chemicals. The nomenclature used for proteolytic enzymes from LAB reported or investigated in this study follows that of Tan et al. (1993).

Endopeptidase (PepO) from the cytoplasm of *L. lactis* spp. *lactis* MG1363 was purified by chromatography on DEAE-Sephacel, hydroxyapatite, Mono Q ion exchanger, and Superose 6, as reported by Stepaniak and Fox (1995). General aminopeptidase (PepN) and X-prolyl-dipeptidyl aminopeptidase (PepX) from the same microorganism were purified using the same procedures and conditions as for PepO; chromatography on hydroxyapatite effectively separated the three enzymes.

Acetonitrile (HPLC grade S) was from Rathburn Chemicals Ltd., Walkelburn, Scotland. Ready-cast 12.5% gels for SDS–

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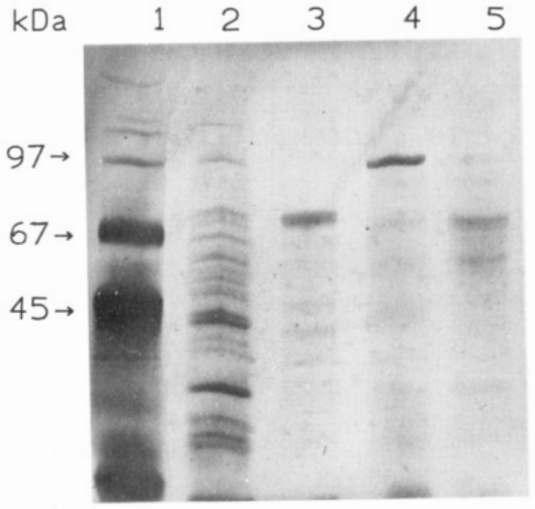

Figure 1. Silver-stained SDS electrophoretogram of enzyme preparations: lane 1, molecular mass marker proteins; lane 2, cytoplasmic fraction before purification; lane 3, purified PepO; lane 4, purified PepN; lane 5, purified PepX.

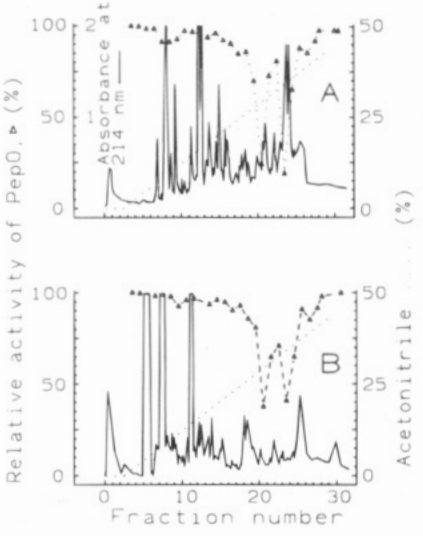

Figure 2. Inhibition of PepO by peptide fractions from Cheddar (A) or Jarlsberg cheese (B).

PAGE, silver staining kit, and the Phast System apparatus were from Pharmacia LKB Biotechnology, Uppsala, Sweden. Thermolysin (type X), methionine enkephalin, aminopeptidase substrates, and all other chemicals were from Sigma Chemical Co., St. Louis, MO.

Determination of Enzyme Activity and Inhibition. Endopeptidase activity was determined on methionine enkephalin (Tyr-Gly-Gly-Phe-Met). Phosphate buffer, pH 6.0, at a final concentration of 50 mM, was used for all analyses except determination of K_i. For inhibition studies, 50 μL of buffer, 25 μL of peptide solution, 100 μL of water, followed by 50 μL of enzyme (10 μg/mL), and 25 μL of 10-fold-concentrated substrate were mixed and incubated for 20 min at 30 °C (total volume, 250 μL). The reaction was stopped by addition of 0.5 mL of 0.4% trifluoroacetic acid. Samples were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a PepRPC HR5/5 column and FPLC equipment (Pharmacia). A Waters Millipore (Milford, MA) integrator, Model 740, was connected to the UV detector, operating at 214 nm. Activity was calculated from the rate of substrate peak reduction (Yan et al., 1987). The separation conditions and the identification of the two peptides released from methionine enkephalin by purified PepO were as described by Stepaniak and Fox (1995). Alternatively, endopeptidase activity was determined on CBZ-Gly-Gly-Phe-pNA (Stepaniak and Fox, 1995).

Table 1. K_i Values for Inhibition of PepO and PepN from *L. lactis* ssp. *lactis* MG1363 by β-Casein Fragments at pH 5.6

peptide fragment	K_i (mM) PepO	K_i (mM) PepN
58–72	0.018	0.44
58–70	0.010	0.35
60–70	0.045	1.80
60–68	0.150	2.10
60–66[a]	0.280	2.50

[a] β-Casomorphin 7.

PepN activity on Leu-pNA, PepX activity on Gly-Pro-pNA, and the effect of *o*-phenathroline and phenylmethanesulfonyl fluoride (PMSF) were determined as described by Stepaniak and Fox (1995). Tri- and dipeptidase activities were determined on 1 mM solutions of Leu-Leu-Leu and Leu-Leu, respectively, using the method of Doi et al. (1981) with cobalt–ninhydrin reagent.

K_i values of inhibitory peptides on PepO and PepN were determined at pH 5.6 (e.g. close to that of cheese) using the universal buffer described by Tan et al. (1991). K_i values were calculated from Dixon plots (Dixon, 1953). Methionine enkephalin was used at final concentrations of 0.25 and 0.5 mM and aminopeptidase substrates at 0.5 and 1 mM. The final concentration of PepN and PepX was 10 μg/mL. K_m values at pH 5.6 for the three enzymes were also determined, using the same substrates as for the inhibitory studies.

Detection and Isolation from Cheese of Peptides Inhibitory for PepO. Water-soluble nitrogen fractions (WSN, 150 μL), prepared according to the method of Kuchroo and Fox (1982) from commercial samples of Cheddar and Jarlsberg cheese, were fractionated by RP-HPLC on the PepRPC column. Fractions (1 mL) were collected and freeze-dried. Part (25%) of each fraction was assessed for inhibition of PepO using methionine enkephalin as substrate.

Inhibitory peptides in the WSN (1 g of freeze-dried preparation) from Cheddar cheese were purified initially by RP-HPLC on a large column (200 × 16 mm) containing C_{18} sorbent (Sigma). The inhibitory fractions were freeze-dried and further purified by gel filtration on Superose 12 (Pharmacia). Finally, inhibitory fractions from Superose 12 were rechromatographed by RP-HPLC on the PepRPC column.

Peptides were sequenced by Edman degradation on an automated liquid-phase protein–peptide sequencer (Model 477A, Applied Biosystems, San Jose, CA).

Peptides homologous to β-casein fragments were synthesized on a peptide synthesizer (Model 431A, Applied Biosystems).

RESULTS AND DISCUSSION

Characterization of Enzymes. The three purified enzymes showed no cross-contamination; aminopeptidases were free of endopeptidase activity determined on CBZ-Gly-Gly-Leu-pNA, while pNA was released from this substrate only in the presence of PepO (which hydrolyzes the Gly–Leu bond) and exogenous aminopeptidase (Stepaniak and Fox 1995). PepN showed activity on Leu-pNA, Leu-Leu-Leu, and Leu-Leu, which is characteristic of this enzyme (Tan and Konings, 1990; Tan et al., 1993), but PepX was active only on Gly-Pro-pNA. However, a silver-stained electrophoretogram showed slight impurities in the PepO and the PepN preparations (Figure 1), and the PepX preparation contained one major and one minor band (Figure 1).

o-Phenanthroline (1 mM) inhibited >90% of PepN activity which was not inhibited by 1 mM PMSF. The opposite response was found for PepX, which is in agreement with all studies on PepN and PepX from LAB reviewed by Tan et al. (1993).

Different molecular masses have been reported for aminopeptidases from *Lactococcus* and *Lactobacillus*.

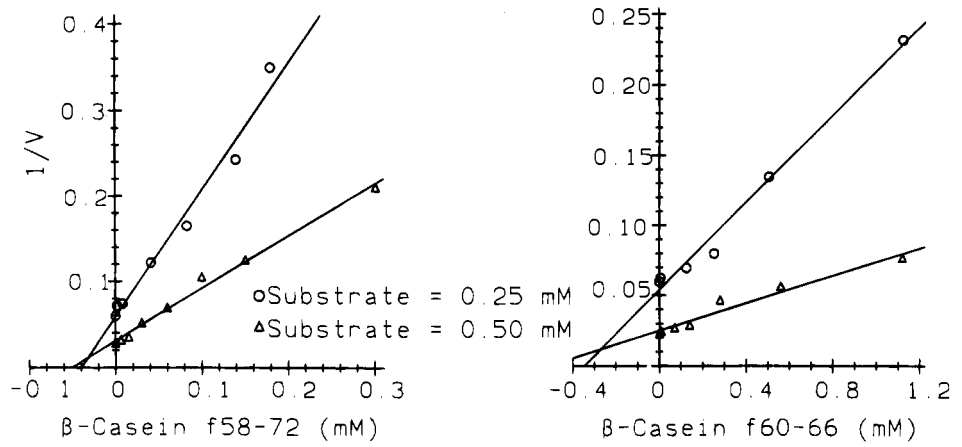

Figure 3. Dixon plot for the inhibition of PepO by β-CN f58–72 and β-casomorphin 7 at pH 5.6

The characterized general aminopeptidases were monomers, while PepXs were dimers (Tan et al., 1993). With one exception, reported values for the M_r of PepN were in the range 78–95 kDa, while subunits of PepX were in the range 72–95 kDa [see Tan et al. (1993)]. In the present study, PepN had an M_r of ca. 95 kDa (Figure 1) and may be identical with the 95 kDa general aminopeptidase isolated by Tan and Konings (1990). The principal band in the PepX preparation had an M_r of ca. 70 kDa (Figure 1).

Isolation of Inhibitory Peptides from Cheese. Figure 2 shows that WSN fractions from Cheddar and Jarlsberg cheeses contained small amounts (compared to other peptides) of inhibitory, partially separated peptides. The inhibition of PepO was less by peptides from Jarlsberg cheese, which contains propionibacteria added to the starter microflora.

RP-HPLC chromatography of the WSN from Cheddar on a large column partially resolved two inhibitory fractions. Further purification on Superose 12 removed a large amount of noninhibitory material from inhibitory fractions which were eluted at a volume corresponding to a molecular mass <3 kDa. Rechromatography of the two inhibitory fractions from Superose 12 by RP-HPLC yielded more highly purified fractions which eluted from the PepRP column at ca. 38 or 41% acetonitrile, but only the fraction eluted at 41% acetonitrile was pure enough to be sequenced.

The fraction contained β-CN f58–72 (Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln) from β-CN genetic variant A^1 and probably peptides truncated at the C or N terminus of the 58–72 fragment. Degradation of β-casein in Cheddar cheese is limited (Fox et al., 1994); thus, the low concentration of peptides originating from β-casein was expected. Although present at low concentrations, the peptides from β-casein strongly inhibited PepO. The weaker PepO-inhibitory effect of the extract of Jarlsberg cheese suggests that propionibacteria may have a different proteolytic system from *Lactococcus*, capable of at least partial degradation of inhibitory peptides.

Characterization of Inhibitory Peptides. Fifty milligram portions of β-CN fragments 58–72, 58–70, 60–70, and 60–68 were synthesized and their effect and that of β-casomorphin 7 (β-CN f60–66) on the three enzymes studied.

PepO and PepN were inhibited by all five peptides, but K_i values found for PepO were 3–10 times lower than those for PepN (Table 1). Dixon plots indicated competitive inhibition of PepO and PepN by all five peptides. Figure 3 shows Dixon plots for two of the peptides on PepO. Fragments beginning with residue 58 were markedly more inhibitory than those beginning with residue 60 (Table 1). Chromatograms in Figure 4 A–C show the inhibitory effect of selected peptides on PepO. The K_i values found for the most inhibitory peptides (β-CN f58–70 and 58–72) were ca. 1000 times higher than that found by Kam et al. (1979) for the inhibition of thermolysin by phosphoramidon and phosphoramidates.

Thermolysin was not inhibited by β-CN f58–72 and in fact degraded this peptide and methionine enkephalin in a mixture at similar rates (Figure 4E). Mammalian enkephalinases and thermolysin are inhibited by phosphoramidon and other peptide analog inhibitors with zinc ligands, but they show different secondary specificities (Almenoff and Orlowski, 1984; Pozsgay et al., 1986). Like thermolysin, PepO is very sensitive to phosphoramidon (Stepaniak and Fox, 1995). The results indicate differences in the structure of the binding sites of the two enzymes.

K_m values found for PepO on methionine enkephalin, for PepN on Leu-pNA, and for PepX on Gly-Pro-pNA were 0.47, 1.63, and 1.12 mM, respectively. PepX was not inhibited by any of the five β-CN fragments at a concentration of 0.25 mM; in fact, it hydrolyzed β-CN f60–70 and β-casomorphin (β-CN f60–66, Figure 4). Degradation of β-casomorphin 7 by PepX from two *Lactococcus* strains was reported by Kiefer-Partsch et al. (1989) and Booth et al. (1990). Interestingly, β-casomorphin was not hydrolyzed by any of the PepO enzymes that have been studied (Yan et al., 1987; Tan et al., 1991; Stepaniak and Fox, 1995).

As reported earlier (Stepaniak et al., 1993), inhibition of the hydrolysis of α_s1-CN f1–23 by PepO by a crude hydrophobic peptide fraction from cheese was pH-dependent. Therefore, the inhibitory fraction was used as an affinity chromatography ligand. However, only part of the enzyme was adsorbed at pH 5.6 and eluted at pH 8.2.

Both the 57–58 and 56–57 bonds of β-CN have been reported to be hydrolyzed by proteinases from lactic acid bacteria [see Visser et al. (1991)]. β-CN f57–70 has also been isolated from yogurt (Brückner and Schieber, 1993) and Cheddar cheese (Fox et al., 1994).

Haque and Antila (1993) found that hydrolysates of sodium caseinate by trypsin, plasmin, and the commercial proteinase preparations Rhozyme and Neutrase depressed the activities of these enzymes by 14–50%; the strongest inhibitory effect was found with the

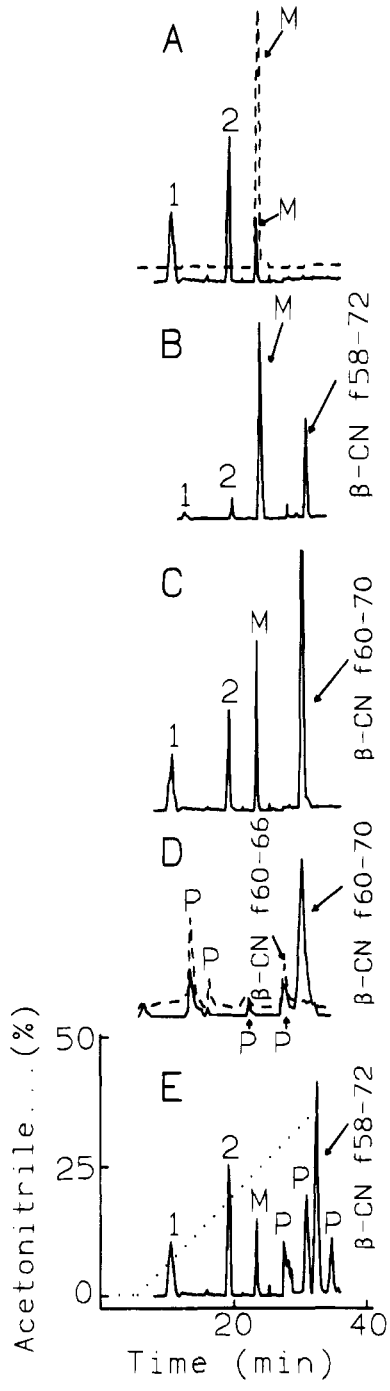

Figure 4. RP-HPLC chromatograms of (A) unincubated control containing 0.2 mg/mL of methionine enkephalin (broken line) or incubated for 20 min with PepO (2 μg/mL) at pH 6.0 (solid line); (B) as "A", incubated but containing also 0.05 mg/mL of β-CN f58–72; (C) as "A", incubated but containing also 0.2 mg/mL of β-CN f60–70; (D) β-CN f60–70, 0.2 mg/mL (solid line) or β-CN f60–66, 0.2 mg/mL (broken line) incubated at pH 6.0 with 10 μg/mL of PepX for 240 min; (E) methionine enkephalin and β-CN f58–72, both at concentrations of 0.2 mg/mL, incubated at pH 6.0 with thermolysin (6 μg/mL) for 20 min. M, methionine enkephalin (Tyr-Gly-Gly-Phe-Met); 1, Tyr-Gly-Gly; 2, Phe-Met; P, peptides released from β-CN f60–70 or β-CN f60–66 by PepX (D) or from β-CN f58–72 by thermolysin (E).

Rhozyme hydrolysate against trypsin. The study indicates that serine proteinases also are inhibited by peptides released from caseins. Booth et al. (1990) identified several competitive (e.g. Arg-Pro-Pro-Gly-Phe, K_i = 1.42 mM) and noncompetitive (e.g. Tyr-Pro-Phe, corresponding to β-CN f60–62, K_i = 0.036 mM) inhibitors of PepX.

Lactic acid bacteria are generally considered as proteolytically weak microorganisms compared with some other species, e.g. *Pseudomonas* spp., which are capable of completely solubilizing casein when grown in milk. The present results indicate that proteolysis in cheese may be limited by inhibitory peptides possibly generated by LAB.

Among natural peptide inhibitors of ACE are β-CN f43–52 and peptides originating from α_{s1}- and κ-casein [see Fox and Flynn (1993) and Fiat et al. (1993)]. Meisel and Schlimme (1994) reported that β-casomorphin, apart from its opiate activity, inhibits ACE, whereas our findings demonstrated that β-casomorphin inhibits the activity of PepO and PepN from LAB.

There is considerable industrial and academic interest in the acceleration of cheese ripening; potential approaches involve the use of exogenous proteinases, including those from LAB (Fox, 1988–89; El Soda and Pandian, 1991). An alternative approach may be engineering of PepO and PepN enzymes by site-directed mutagenesis so as to reduce their affinity for inhibitory peptides. Study of the possible inhibition of chymosin or other aspartate proteinases and cell-bound serine proteinases from LAB by peptides from cheese or milk protein hydrolysates seems to be warranted. Use of specific peptide inhibitors in model studies may be helpful in understanding the role of different proteolytic enzymes in generating the desired flavor, removing the bitterness of cheese, and selecting LAB for starters. Proteolytic enzymes from LAB may have potential in the production of biologically active peptides.

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