Inhibition of Proteolytic Enzymes from Pseudomonas fluorescens ATCC 948 and Angiotensin I-Converting Enzyme by Peptides from Zein, Hordein, and Gluten Hydrolysates

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ABSTRACT

Peptides inhibitory to partially purified endopeptidase and crude proteinase from Pseudomonas fluorescens ATCC 948 were isolated from tryptic hydrolysates of zein and hordein by reversed-phase fast protein liquid chromatography and identified by sequencing. The sequences are Ser-Ala-Tyr-Pro-Gly-Gln-Ile-Thr-Ser-Asn and Gln-Val-Ser-Leu-Asn-Ser-Gly-Tyr-Tyr for peptides from zein and hordein, respectively. Inhibitions of >85% and from >50 to >85% were determined on endopeptidase and proteinase by peptides from zein and hordein. Kᵦ values ranged from 4 to 32 μM. The same peptides also showed inhibition of the angiotensin I-converting enzyme. The concentrations of peptides providing 50% inhibition of angiotensin I-converting enzyme were 7 and 23 μM for the decapptide and nonapeptide, respectively. Other fractions containing peptides with less inhibitory activity were detected in the zein as well as in the gluten tryptic digests.

Key words: Proteinase, Pseudomonas fluorescens, angiotensin I-converting enzyme, inhibitory peptides

Endogenous opioid peptides know as enkephalins were first isolated in 1975 and shortly afterwards, in 1979, exogenous peptides derived from the partial enzymatic digestion of bovine casein were shown to have opioid activity (30). Peptides produced from milk proteins, and especially from β-casein (β-casomorphins), have been shown to possess opiate, antithrombotic, antihypertensive, immunomodulating and enhancing mineral utilization properties (26). Fragments of human β-casein also have opioid activity (4); synthetic peptides of human β-casein inhibited angiotensin I-converting enzyme (19); and a group of opioid peptides (cytochrophins and hemorphins) have been produced from enzymatically treated bovine blood (5, 6). The in vivo formation of opioid peptides and caseinophosphopeptides have been found in the duodenal chyme of young pigs after ingestion of a diet containing casein (24, 25). Opioid peptides with unknown primary structures have also been derived from vegetable proteins such as gluten, zein, hordein, and soy α-protein (36). Schlimme and Meisel (30) recently reviewed the structural, physiological, and analytical aspects of bioactive peptides.

The identification of several bioactive sequences in food proteins introduced a new criterion in evaluating the nutritional value of a protein: peptides which are hidden in an inactive state within the protein sequence may be released by proteolytic processes during in vivo digestion and also during food processing. Bioactive peptides are generated in fermented milk (29) and ripened cheese (33) and from casein hydrolysis by caseolytic microorganisms (17) or by purified proteinase from lactic acid bacteria (35). Bioactive peptides from tryptic digests of caseinomacropetide (3) and from β-casein-chymosin hydrolysate are produced in continuous-stirred-tank membrane reactors. The functionalities and uses of protein hydrolysates in nutritional products have been considered with particular reference to the synthesis of bioactive peptides (2).

Since the greater part of the bioactive effects are mainly based on the mechanism of peptide analog inhibitors of proteolytic processes and since the multifunctional role of some bioactive peptides has been demonstrated (β-casomorphins and casokinins in concomitance inhibited mammalian metalloendopeptidases such as enkephalinase and angiotensin I-converting enzyme) (16, 28), the bioactive peptides produced during food processing could also play a significant role in the enzymatic processes involved in food preparations. Recent studies (14, 18, 31) have shown that peptides produced by in vitro proteolysis of bovine caseins with chymosin, trypsin, chymotrypsin and pepsin or peptides produced during cheese ripening (15) had inhibitory activities towards animal and microbial proteolytic enzymes. These peptides contained part of the sequences of bioactive peptides. Highlighting these properties, bioactive peptides could be used for inhibiting spoilage microbial enzymes such as proteinases from Pseudomonas fluorescens.

In this study peptides inhibitory to proteinase and endopeptidase from P. fluorescens ATCC 948 and to angio-
tensin I-converting enzyme have been isolated from tryptic hydrolysates of zein, hordein, and gluten.

**MATERIALS AND METHODS**

**Enzymes**

*Pseudomonas fluorescens* ATCC 948 was maintained and propagated in nutrient broth. Five liters culture were shaken at 160 rpm at 28°C until an absorbance (620 nm) of 1.1 to 1.4 (stationary phase) was reached. Cells were harvested by centrifugation at 6,000 × g for 15 min and crude proteinase was solubilized by repeated washing in a calcium-free buffer (0.05 M Tris-HCl, pH 7.5). The supernatant containing proteinase activity was concentrated 10-fold by freeze drying (Edwards MOD EIPTB; Edwards, Milan, Italy). Proteinase activity was optimal at pH 7.5 and 37°C and was markedly inhibited by EDTA.

Cells were then resuspended (10^11 CFU/ml) in 0.05 M phosphate buffer, pH 7.0, and the suspension was treated by an Ultrasonic A 180 G Instrument (PBI International, Milan, Italy) at 20 kc/s for 30 min at 5-min intervals. The cell homogenate was centrifuged (30 min at 15,000 × g), and the supernatant filtered (0.22-μm-pore-size filter; Syrfil Filter, Nucleopore, Costar Corporation, Cambridge, MA) and applied to a Q-Sepharose HR 16/50 column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. Proteins were eluted at a flow rate of 12 ml/h with a linear NaCl gradient from 0.1 to 0.5 M. Fractions (9 ml) with endopeptidase activity were pooled, dialyzed for 24 h at 4°C against 0.05 M phosphate buffer, pH 7.0, concentrated 10-fold by freeze-drying, and resuspended in the same buffer. The pooled fractions were loaded onto a Fast Protein Liquid Chromatography (FPLC) Superose 12 HR 10/30 column (Pharmacia). Elution was made with 50 ml of 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.0, at a flow rate of 0.4 ml/min and fractions (1 ml) with endopeptidase activity were pooled and concentrated 10-fold by freeze-drying. The partially purified endopeptidase had optimal activity at pH 7.0 and 30°C, was EDTA-sensitive and free from aminopeptidase and di- or tri-peptidase activities.

Trypsin (salt-free lyophilisate preparation from bovine pancreas, ca. 110 U/mg) and angiotensin I-converting enzyme (ACE; porcine lyophilized preparation, ca. 42 U/liter) were from Boehringer Mannheim, Milan, Italy and Sigma Chemical Company (St. Louis, MO, USA), respectively.

**Preparation of zein, hordein, and gluten hydrolysates**

Trypsin was chosen to produce the hydrolysates since it is considered safe in food applications and has been extensively studied. Trypsin was added at a concentration of 4 mg/ml to a 1% (wt/vol) suspension of commercial gluten, zein (Sigma), or hordein, extracted from barley (Trebbia variety) by the method of Crosatti et al. (7), in 0.05 M phosphate buffer pH 8.0. Preliminary assays were conducted to have a concentration of trypsin which caused a moderate hydrolysis of proteins. After incubation with shaking (180 rpm) at 37°C for 90 min, the enzyme was inactivated by heating at 80°C for 10 min. Unhydrolysed vegetable proteins were removed by centrifugation at 12,000 × g for 10 min and trifluoroacetic acid (TFA) was added to the supernatant to a final concentration of 0.2% (vol/vol). After another centrifugation (7,000 × g for 10 min) the supernatant was the source of peptides released by trypsin.

The degree of proteolysis of the vegetable hydrolysates was determined by the method of McKellar (23) using 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma).

**Isolation of inhibitory peptides**

Peptides from vegetable hydrolysates were separated by reversed-phase FPLC using a PepRPC HR 5/5 column and FPLC equipment (Pharmacia) with a UV detector operating at 214 nm. A 0.5-ml sample was loaded onto the column and eluted as reported by Stepaniak and Fox (32) using an acetonitrile gradient from 0 to 45% between 5 to 60 min, from 45 to 60% between 60 to 73 min and from 60 to 100% between 73 to 78 min. For preliminary screening, thirty 2-ml fractions were collected and TFA, acetonitrile, and water were removed by freeze drying. Fractions containing peptides which inhibited both proteinase and endopeptidase from *P. fluorescens* ATCC 948 were twice rechromatographed and then the centers of inhibitory peaks were collected, freeze dried and, re-injected onto the PepRPC HR 5/5 column to check the chromatographic purity. The separation of the most inhibitory peptide from zein required additional purification by gel filtration on Superose 12 HR 10/30 using the conditions previously described. Enough material for characterization of peptides was accumulated by multiple separations of 0.25-ml samples.

Peptides were sequenced at the Biotechnology Centre, University of Oslo, Norway, by Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (Applied Biosystems Inc., Foster City, CA, USA; model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives using a model 120 A analyzer (Applied Biosystems).

**Alignment of the peptides with zein and hordein sequences**

The amino acid sequences of the two isolated peptides were aligned with the sequences of zeins and hordeins obtained from the translation of the nucleic acid sequences contained in the EMBL data bank by using the analysis program “aautransl” which is part of the pc/gene program (IntelliGenetics, Inc., Mountain View, CA). To align the protein sequences the analysis program “palign” was utilized with the following parameters: structure-genetic matrix, open gap cost seven and unit gap cost one.

**Determination of proteinase and endopeptidase activities and inhibition**

Proteinase and endopeptidase activities were determined by RP-FPLC (27) using casein and methionine enkephalin (Sigma), respectively. Reaction mixture for the determination of the proteinase activity consisted of 45 μl of concentrated enzyme solution, 30 μl of 6.7% (wt/vol) buffered (10 mM phosphate buffer, pH 7.5) and dialyzed casein containing 0.07% (wt/vol) sodium azide and 25 μl of peptide fraction or water. After incubation for 15 h at 37°C, the mixture was diluted 1:1 with 24% TCA, centrifuged at 12,000 × g for 10 min and the supernatant was diluted with 300 μl of 0.26% TFA. The pH was then adjusted to 2.0 to 3.0 by 5 M NaOH. Inhibition was calculated by the reduction of the major peptide peak areas which appeared as a result of casein hydrolysis.

The incubation mixture for endopeptidase assays contained 15 μl of 1.25 mM methionine enkephalin, 35 μl of 10 mM phosphate buffer, pH 7.0, 30 μl of peptide fraction or water and 30 μl of partially purified endopeptidase. After incubation at 30°C for 45 min, the reaction was stopped by adding 500 μl of 0.26% TFA. The samples were centrifuged at 13,000 × g for 10 min and used for RP-FPLC analysis. Activity was calculated from the rate of reduction of the substrate peak area (32) and the partially purified endopeptidase was standardized by dilution so as to reduce the area of methionine enkephalin peak by ca. 80%.

To ascertain heat stability, portions (200 μl) of the partially purified endopeptidase in 0.05 M phosphate buffer, pH 7.0, were heated at different temperatures (70 to 90°C) for 1 to 5 min in 0.5-ml glass tubes. At intervals, 25-μl samples were cooled, and the
remaining activity was measured using the procedure described previously.

**Determination of ACE activity and inhibition**

The ACE activity and inhibition were determined by the method of Nakamura et al. (29) with few modifications. Hip-His-Leu was dissolved (0.05 M) in 0.1 M Na-borate buffer, pH 8.3, containing 0.3 M NaCl. Two-hundred microliters of Hip-His-Leu solution were mixed with 60 μl of peptide fraction or water and 40 μl of ACE (0.1 U/ml of water) and the mixture was incubated for 45 min at 37°C. The reaction was stopped with 250 μl of 1 N HCl, hippuric acid liberated by ACE was extracted with 1.7 ml of ethyl acetate and, after removal of ethyl acetate by vacuum evaporation, diluted in 1 ml of distilled water and determined spectrophotometrically at 228 nm. The percent of inhibition was calculated as follows: (B - A)/[(B - C) X 100, where A = optical density in the presence of both ACE and peptide fraction, B = optical density without peptide fraction, and C = optical density without ACE.

The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC<sub>50</sub> value.

**RESULTS**

**Isolation of inhibitory peptides**

Chromatograms of peptides produced from zein, hordein, and gluten by trypsin are shown in Figure 1. Thirty fractions were collected from each hydrolysate. On the basis of the amino acid analysis, the amount of liberated and isolated fragments was calculated to be in the range of 0.026 to 0.042 mg/g of proteolyzed protein. Despite the differences in the peptide profiles, for zein and hordein the peptides inhibitory either to endopeptidase and proteinase from *P. fluorescens* ATCC 948 or to ACE were in fraction 15, indicating a certain degree of hydrophobicity. Fraction 21 from gluten hydrolysate showed the highest inhibition on endopeptidase and ACE.

Two inhibitory peptides were then isolated and purified to homogeneity from fraction 15 of zein and hordein hydrolysates. The peptide purified from the zein digest inhibited >85% of endopeptidase and proteinase activity in methionine enkephalin and casein, respectively. Figure 2 shows the degradation pattern of methionine enkephalin by the endopeptidase of *P. fluorescens* ATCC 948 and the relative inhibition by the purified peptide from zein. The enzyme showed identical peptide cleavage (Gly-Phe bond) of the purified PepO from *Lactococcus lactis* subsp. *lactis* (32). The inhibition of the extracellular proteinase activity was shown by the marked reduction of the area of peptides produced by the casein hydrolysis in the absence of the inhibitory peptide (Figure 3). While the purified peptide from zein also inhibited >78% of the ACE activity (data not shown), the purified peptide from hordein showed about the same inhibition on endopeptidase (Figure 2), but had a reduced effect on proteinase (Figure 3) and ACE activities (>50% and >62% of inhibition, respectively). No hydrolysis of the inhibitory peptides from zein and hordein was observed after incubation at 30 and 37°C for 2 and 24 h with the endopeptidase and proteinase from *P. fluorescens* ATCC 948, respectively (data not shown). Other peptide fractions, 24 and 21, from zein and hordein, showed inhibition to the endopeptidase activity (>60%) but had low effect on proteinase and ACE (data not shown).

The peptide isolated and purified from fraction 21 of the gluten digest inhibited about 50% of endopeptidase and
FIGURE 3. RP-FPLC chromatograms showing inhibition of proteinase from Pseudomonas fluorescens ATCC 948 on whole casein. A, unincubated control; B, incubated control; C, incubated with the peptide isolated from zein; and D, incubated with the peptide isolated from hordein.

FIGURE 2. RP-FPLC chromatograms showing inhibition of endopeptidase from Pseudomonas fluorescens ATCC 948 on methionine enkephalin. A, unincubated control; B, incubated control; C, incubated with the peptide isolated from zein; and D, incubated with the peptide isolated from hordein. 1, Phe-Met; 2, Tyr-Gly-Gly; and S, residual unhydrolyzed methionine enkephalin (Phe-Met-Tyr-Gly-Gly).
ACE activities but did not affect the hydrolysis of casein by proteinase from *P. fluorescens* ATCC 948 (data not shown). Due to its low inhibitory activity this peptide was not characterized further.

**Identification and characterization of the inhibitory peptides**

The characteristics of the two isolated inhibitory peptides from zein and hordein are summarized in Table 1. The sequences are Ser-Ala-Tyr-Pro-Gly-Gln-Ile-Thr-Ser-Ser-Asn and Gln-Val-Ser-Leu-Asn-Ser-Gly-Tyr-Tyr for peptides from zein and hordein, respectively. The purity of the peptides was confirmed by there being no significant background interference during amino acid sequencing. The *K*ₘ calculated from Dixon plots (9) (data not shown) indicated competitive inhibition of *P. fluorescens* endopeptidase and proteinase by both peptides; values ranged from 4 to 32 µM. The IC₅₀ values of the decapptide from zein and the nonapeptide from hordein were 7 and 23 µM, respectively.

The best alignment for the peptide from zein was achieved with the 16 kDa zein C1 (accession number of the nucleotide sequence NZMZE16) showing an identity of 30% with the protein plus a similarity of 10% (similar amino acids are those that can interchange with each other without modifying the biological activity of the protein). The peptide from hordein had an identity of 33.3% and a similarity of 10% when compared with the δ-hordein (accession number of the nucleotide sequence HVDNAHOR3). These percentages are not surprising due to the complex protein matrix used and due to the high number of different zeins and hordeins occurring in maize and barley, respectively. α-Zeins as well as hordeins are coded by multigene families (10, 34) and only some protein sequences are reported in the data banks.

**DISCUSSION**

Milk proteins are currently the main sources of various biologically active peptides (opioids, immunostimulants, antithrombotics, antihypertensives and mineral carriers) (30). To our knowledge, only a few studies have been done on the synthesis of bioactive peptides from vegetable proteins (1, 21, 36). Recently, some studies have shown that peptides containing a bioactive sequence are inhibitory to enzymes of technological significance. The β-casein fragment 58 to 72 which contains the sequence of β-casomorphin-7 is produced during Cheddar ripening and has been shown to selectively inhibit PepO, PepN, and PepX from lactic acid bacteria involved in cheese making (33). β-Casein fragments 193 to 209, 69 to 97, 141 to 163, and 69 to 84 were produced by chymosin, trypsin, and chymotrypsin and have shown inhibition to PepO and PepN of *L. lactis* subsp. *lactis* MG1363 (31). Amphipathic peptides produced from enzymatic hydrolysis of sodium caseinate were shown to be inhibitory to several proteinases used in cheese making (18).

Unknown hydrophobic peptides produced during ripening of several Italian cheeses inhibited the intracellular endopeptidase activity of dairy starters (15). High specificity of the peptide analog inhibitors is responsible for the variations in sensitivity of enzymes belonging to the same biochemical class (14, 16). From vegetable protein digests we isolated two peptides inhibitory to proteolytic enzymes involved in dairy product spoilage. Endopeptidases, but especially thermostable proteinases from psychrotrophic *P. fluorescens*, cause bitterness in UHT milk (12), contribute, along with plasmin, to age gelation of unconcentrated UHT (ultra-high temperature treated) milk (8) and drastically reduce the shelf life. Modified UHT treatments or monitoring of the cold storage of raw milk are not completely satisfactory for controlling the production and the activity of these enzymes (12). The proteinase used in this study is thermostable (13) and also the partially purified endopeptidase has shown a Δ*GBC* of 2 min. The decapptide from zein and the nonapeptide from hordein inhibited >85% and 54 to >85% of the endopeptidase and proteinase activity of *P. fluorescens* ATCC 948. The inhibitory peptides show the same N-terminal residue (Gln and Ser) of those produced by trypsin on β-casein which inhibited the PepO and PepN of *L. lactis* subsp. *lactis* MG1363 (31); they have a certain degree of hydrophobicity as shown for inhibitory peptides produced during ripening of several cheeses or by in vitro degradation of sodium caseinate (15, 18); and the peptide from zein contains six of the amino acid residues present in the sequence of β-casomorphin-11 from which the main bioactive peptides originated (27).

Like the proteinase from *P. fluorescens* ATCC 948, ACE is a metalloproteinase which uses a general acid-base mechanism to catalyze the hydrolysis of the inactive angiotensin I in the hypertensive peptide hormone angiotensin II (11). The peptides from zein and hordein isolated in this study were both inhibitory to the proteolytic enzymes of *P. fluorescens* ATCC 948 and to ACE activity. While a broad substrate specificity characterizes the bacterial proteinase, a narrowly defined function is attributed to ACE. However, a multifunctional role of some bioactive peptides or part of them has been demonstrated: β-casomorphins and casoxins have been shown to have opiate properties and to specifically inhibit mammalian metalloendopeptidases such as enkephalinase or ACE (16, 28). Inhibitors of ACE were first obtained from the venom of snakes such as *Bothrops jararaca* and *Agristodon halys blomhoffii* (21) and afterwards Maruyama et al. (22) isolated angiotensin I-converting enzyme inhibitors (CEI) from an enzymatic hydrolysate of bovine casein. Even though a considerable number of peptide ACE inhibitors have shown a proline reach sequence in their C-terminal part, several authors (11, 20, 22) have reported that other consensus sequences may inhibit the potency of ACE. The decapptide and nonapeptide...
tide from zein and hordein do not have Pro at their C-terminus and possess a very different sequence. Similarly, heterogeneous peptides inhibitory to ACE were produced from α-zein digests (1) and from a thermolysin hydrolysate of γ-zein (21). Recent studies, conducted by Nakamura et al. (29) and Yamamoto et al. (35), have shown the presence of tripeptides inhibitory to ACE in a Japanese sour milk and the production of several inhibitor oligopeptides, not containing Pro at the C-terminus, from the casein hydrolysate by an extracellular proteinase of Lactobacillus helveticus CP790. The IC₅₀ values determined for zein and hordein peptides (7 and 23 µM, respectively) compared well with the range (4 to 28 µM) reported for the most active inhibitors of these studies (29, 35).

This report has shown the inhibition of proteolytic spoilage enzymes from P. fluorescens by vegetable peptides which also possess antihypertensive activity. The use of these peptides as food additives could be warranted as well as further studies on the production of bioactive peptides from vegetable proteins.

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REFERENCES