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Identification of the active site serine of the X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis*

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The active site serine of the X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* (PepX) was identified. The enzyme was labeled by [^{3}H]DFP, treated by CNBr and the resulting peptides were separated by reverse-phase-HPLC. The main radiolabeled peptide was sequenced. Ser-348, in the following sequence, Gly-Lys-Ser-Tyr-Leu-Gly, was identified as the active site serine. A sequence comparison between the active site of PepX and other serine proteases was made, showing only limited sequence homologies in this area. The consensus sequence surrounding the active site serine in the three known X-prolyl dipeptidyl aminopeptidases (mammalian DPPIV, yeast DPAB and PepX) is G-X-S-Y-X-G, where X is a non-conserved amino acid.

X-prolyl dipeptidyl aminopeptidase, Serine protease, Active site, Diisopropylfluorophosphate, Lactococcus lactis

1. INTRODUCTION

Peptide bond involving a proline residue requires specific enzymes to be hydrolyzed. A full spectrum of proline-specific endo- and exopeptidases (including aminopeptidases, dipeptidases or carboxypeptidases) have been described (see [1] for review). X-Prolyl dipeptidyl aminopeptidase which releases X-Pro dipeptides from the amino-terminus of peptide chains has been evidenced in mammals, yeast as well as in bacteria. Recently, an enzyme with such a specificity (named PepX) was purified and characterized from the lactic acid bacteria, *Lactococcus lactis* [2–4]. Lactococci, widely used in the dairy industry, are auxotrophic for several amino acids and their optimal growth on milk requires the hydrolysis of caseins to provide the amino acids necessary for the nutrition of the cell [5]. Since caseins are proline-rich proteins, PepX may be an essential component in their degradation process.

PepX was shown to be inhibited by DFP [2] which indicates that it is a serine protease. However, no homology could be found between the amino acid sequence of PepX deduced from the nucleotide sequence of the cloned gene and protein sequences from the data bases [6,7]. In particular, this enzyme does not appear to belong to one of the two best characterized serine protease families that are represented by trypsin and subtilisin [8].

This paper describes the localization of the active site serine of PepX. The sequence around the active site serine is compared with that found in the other known serine proteases.

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Abbreviations: TFA, trifluoroacetic acid; DFP, diisopropylfluorophosphate; DPAB, yeast dipeptidyl aminopeptidase B; DPPIV; dipeptidyl peptidase IV; PepX, X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis*, PVDF: polyvinylidene difluoride; PTH, phenylthiohydantoin.

2. MATERIALS AND METHODS

2.1. *Purification of PepX*

The enzyme was purified according to [2]. The starting material was a crude powder of *Lactococcus lactis* obtained from Imperial Biotech (UK). After purification, the enzyme was dialysed and concentrated against 20 mM Tris-HCl, pH 7.0, using a Centricon 30 microconcentrator (Amicon, USA).

2.2. *Sequencing of the amino-terminus of PepX*

The purified enzyme (100 pmol) was submitted to SDS-PAGE [9] and was blotted onto PVDF membrane (Applied Biosystems) [10]. After Coomassie blue staining, the band corresponding to PepX was cut out and placed in the cartridge of an Applied Biosystems Model 477 A liquid pulse phase sequencer with on line analysis of PTH-derivatives.

2.3. *Active site labeling*

The enzyme (200 μg, 2.3 nmol) was incubated at room temperature for 1 h with 6.66 MBq of [^{3}H]DFP (370 GBq/mmol; NEN) in 500 μl of 20 mM Tris-HCl, pH 7.0. Unlabeled DFP was then added into the reaction mixture at a final concentration of 1 mM and the incubation was continued at 4°C for 24 h. The unreacted DFP was removed by dialysis and the sample was dried with a Speed-Vac concentrator.

2.4. *Cleavage by CNBr*

The labeled enzyme was cleaved by incubation with CNBr (3 μmol) in 40 μl of 70% TFA [11]. After 24 h at room temperature in the dark, the reaction mixture was dried.

2.5. *Purification of the labeled peptide*

The digest was dissolved in 0.1% TFA, and fractionated on a C_{18}

precolumn Aquapore RP-300, 30x46 mm (Applied Biosystems). The peptides were eluted by a linear gradient from 0.115% TFA (Solvent A) to 60% acetonitrile/0.1% TFA (Solvent B) in 30 min. The flow rate was 1 ml/min and the absorbance of the eluate was monitored at 214 nm. Fractions were collected each minute. The radioactivity distribution was determined by counting 20 μl of each fraction in a liquid scintillation counter Beckman LS1701.

2.6. *Sequencing of the labeled peptide*

Fractions exhibiting the major radioactivity were pooled and dried. They were dissolved in 0.115% TFA and loaded on the sequencer. Only 50 μl out of the total 150 μl conversion product obtained after each step of the Edman degradation were injected for identification of the PTH derivative. The remaining sample (100 μl) was used for the determination of the radioactivity distribution

2.7. *Other methods*

The activity of PepX was assayed with Ala-Pro-paranitroanilide as a substrate [2]. Protein concentration was determined by the BCA method (Pierce) with bovine serum albumine as a standard. Electrophoresis of the DFP-labeled enzyme was performed according to [9] and Coomassie blue R250 was used for the staining. The gel was then soaked in Enhancer (Dupont), dried and fluorographed.

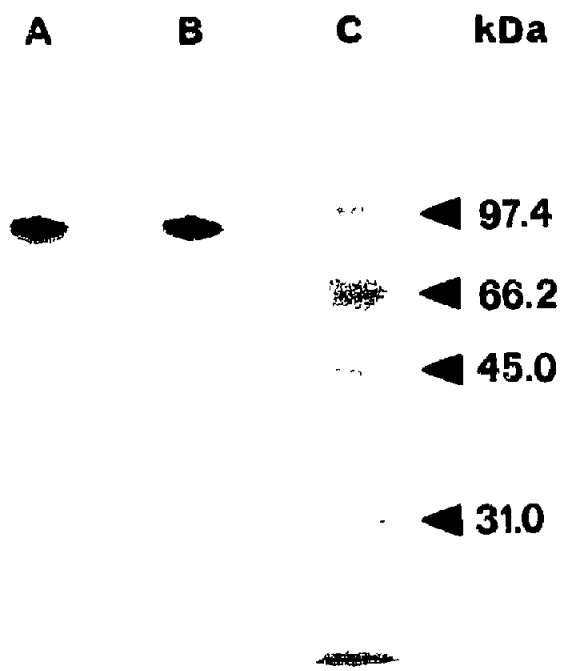

Fig. 1. SDS-polyacrylamide gel electrophoresis of PepX. (Lane A) Purified enzyme (1.3 mg) after Coomassie staining (Lane B) autoradiography of lane A. (Lane C) MW markers.

3. RESULTS AND DISCUSSION

3.1. *Purity and labeling of PepX*

Because the starting material was a commercial lactic acid bacteria extract, it was necessary to check that the X-prolyl dipeptidyl aminopeptidase we purified was PepX. First, we showed that the substrate specificity of our enzyme was similar to that of the previously purified PepX [2,4] (data not shown). Second, our enzyme was found to be made of two subunits with identical apparent MW of 83 kDa by SDS-PAGE (Fig. 1). This value is very close to those previously found by [2] (85 kDa) and [4] (82–83 kDa). Third, we have sequenced the first twelve residues of our enzyme and they were identical to those found for PepX [6]. Therefore we can conclude that the enzyme purified from the commercial powder is PepX.

The purified enzyme was labeled with [^{3}H]DFP and a fraction of the reaction mixture was electrophoresed. After Coomassie blue staining (Fig. 1A), a single band of 83 kDa was visible. After fluorography (Fig. 1B), a radioactive band was perfectly superimposed on the stained band. This shows that the labeled band corresponds to PepX.

3.2. *Sequence determination of the active site peptide*

The [^{3}H]DFP-labeled PepX was cleaved with cyanogen bromide and the resulting peptides were separated by reverse-phase HPLC. (Fig. 2A). Radioactivity was counted in each fraction. One major radioactive peak, well separated from the others, containing 55% of the total recovered radioactivity was found (Fig. 2B). This major product was sequenced. The following sequence was obtained: Thr-Gly-Lys-X-Tyr-Leu-Gly-Thr- (with X representing an unidentified residue). This sequence is identical to the sequence Thr-345 to Thr-352 of PepX, with a Ser in position 348 corresponding to the unidentified residue in fourth position of the sequenced peptide. Moreover, counting the radioactivity released at each step of the Edman degradation showed that the fourth residue is bearing the tritium label. These results indicate that Ser-348 is modified by [^{3}H]DFP and is therefore the active site serine.

The remaining 45% of the recovered radioactivity were present in several minor peaks. They correspond most probably to partial cleavages of the protein by CNBr. Indeed, 6 Met–Thr bonds which are known to be poorly cleaved by CNBr [11], are present in PepX out of 12 Met–X bonds. Two of these bonds preceeds the active site and their incomplete cleavage could lead to radioactive cleavage products of various sizes. Determination of the N-terminal amino acid sequence of the peptide contained in the second radioactive peak (corresponding to 15% of the recovered radioactivity) (Fig. 2A) confirmed this hypothesis. This peptide starts at Thr-311 and its labeling probably results from absence of cleavage at Met-344-Thr-345 bond.

3.3. *Comparison with other serine proteases*

The sequence surrounding the active site serine in PepX, G-K-S-Y-L, was compared with that found in other serine proteases. This sequence differs from the one found in the enzymes of the trypsin family (G-D-S-G-G) or of the subtilisin family (G-T-S-M-A) [12]. It does not fit the general consensus sequence G-X-S-X-G found in the known mammalian serine hydrolases, i.e. serine peptide hydrolases, serine esterases and lipases

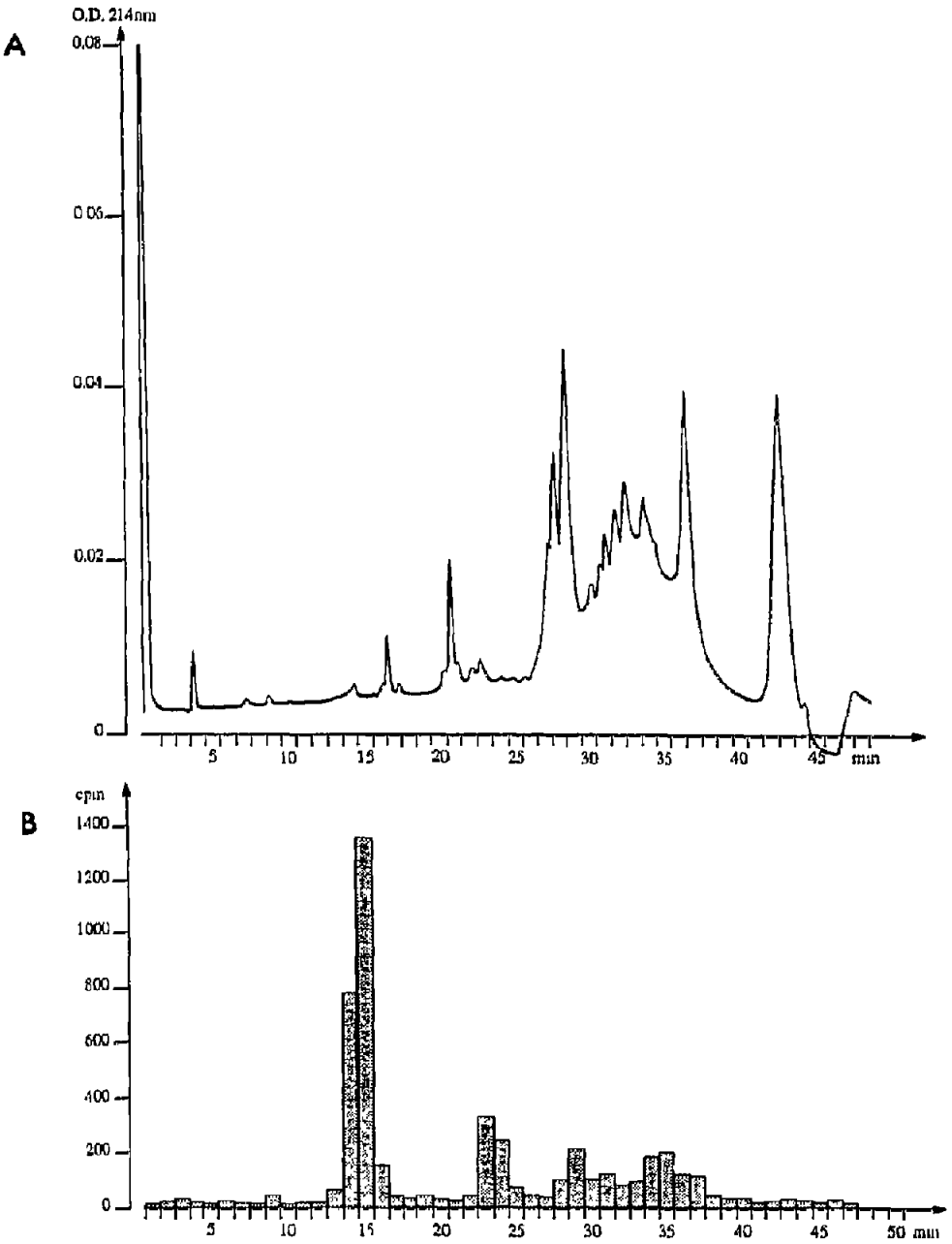

Fig. 2. Separation of the peptides after cleavage by CNBr on a C_{18} precolumn. Fractions of 1 ml were collected and radioactivity distribution was determined by counting 20 ml of each fraction. (A) Elution profile of the peptides monitored at 214 nm. (B) Radioactive profile of the eluted peptides.

[12]. It does not correspond either to the general consensus G-X-S-X-A/S, given for the serine proteases of the subtilisin and carboxypeptidase Y families [13].

The active site sequence of PepX was then compared with that of DPPIV, a mammalian serine protease with X-prolyl-dipeptidyl aminopeptidase activity, which does not present overall sequence homology with any of the three families of serine proteases mentioned above. The active site serine of DPPIV was found in the following sequence: G-W-S-Y-G-G [14]. DPPIV has been proposed to be classified in a new family of serine proteases related to prolyl endopeptidase [15]. This family is defined on the basis of some resemblance in the C-terminal part of the proteins and an active site motif: G-X-S-X-G-G is present in all of them (Table I). However, this motif is not found in PepX. Moreover, the active site of the PepX is not located at the C-terminal part of the protein (Table I). The second Gly of the pattern, absent in PepX and replaced by Leu, has been shown to be necessary for DPPIV activity by site-directed mutagenesis [14]. On the opposite, the third Gly of the motif is retained in PepX, suggest ing a possible role in the active site of all these enzymes. The Tyr residue following the catalytic Ser is conserved in DPPIV and PepX as well as in yeast DPAB [16] which possesses the same enzymatic specificity. Interestingly,

Table I

Sequence comparison of the active site of Pep X and other serine proteases: trypsin from rat [12], subtilisin from *Bacillus licheniformis* [12], yeast carboxypeptidase (CPY) [13], prolyl endopeptidase from *Flavobacterium meningosepticum* (PEP) [17], prolyl endopeptidase from pig brain (POP) [18], yeast dipeptidyl aminopeptidase B (DPAB) [16]

Protease	Size in amino acids	Active site sequence	Position of the serine
		*	
Trypsin	229	GDSGGP	183
Subtilisin	274	GTSMAS	220
CPY	421	GESYAH	146
PEP	705	GRSNGG	536
POP	710	GGSNGG	554
DPAB	841	GWSYGG	678
DPPIV	767	GWSYGG	631
PepX	763	GKSYLG	348

*Active site serine

site directed mutagenesis experiments on DPPIV [14] have shown that this Tyr can be replaced only by a structurally similar residue such as Phe, suggesting that it has an important role in the active site.

In conclusion, PepX appears as an original serine protease. Indeed, the sequence found around the active site serine does not fit any of the active site consensus sequences defined for the different classes of serine proteases. The second residue after the active serine, which is well conserved in the different families of serine proteases, is Leu in PepX, the only bulky amino acid found in this position in the other examined enzyme. The active site sequence is not identical to the one identified in DPPIV although both enzymes have the same specificity. However, the sequence: G-X-S-Y-X-G conserved in lactococcal PepX, mammalian DPPIV and yeast DPAB can be proposed as a consensus for enzymes with X-prolyl dipeptidyl aminopeptidase activity. Further experiments using site-directed mutagenesis would help in determining which residues are important for PepX activity.

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