Hydrolysis of α_{s1}- and β-casein-derived peptides with a broad specificity aminopeptidase and proline specific aminopeptidases from *Lactococcus lactis* subsp. *cremoris* AM2

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Abstract Aminopeptidase hydrolysis of α_{s1}- and β-casein-derived synthetic peptides containing non-consecutive and consecutive proline residues was characterised. Aminopeptidase P (Pep P) (EC 3.4.11.9) or post-proline dipeptidyl aminopeptidase (PPDA) (EC 3.4.14.5) along with lysine-paranitroanilide hydrolase (KpNA-H) (EC 3.4.11.1) activities are required in the degradation of peptides containing non-consecutive proline residues. However, both Pep P and PPDA along with KpNA-H are required for hydrolysis of peptides containing consecutive proline residues. The results demonstrate the mechanism by which combinations of purified general and proline specific aminopeptidases from *Lactococcus lactis* subsp. *cremoris* AM2 hydrolyse peptides containing proline residues.

Key words: General aminopeptidase; Post proline dipeptidyl aminopeptidase; Aminopeptidase P; β-Casein derived peptide; Proline enriched peptide

1. Introduction

Casein hydrolysates find application as food ingredients/supplements. Endoproteinase digestion of casein, however, yields hydrolysates having a strong bitter flavour, an undesirable characteristic which militates against their widespread application [1,2]. This bitter flavour has been attributed to the presence of intermediate length peptides many of which contain proline [3,4]. Bovine caseins have high proline contents with β-casein (β-cn), for example, possessing 34 proline residues in a sequence of 209 amino acids [5]. Broad specificity aminopeptidases can release most N-terminal amino acids from peptides, however, these activities are unable to cleave the imido bond and consequently cannot release N-terminal amino acids where proline occurs in the second position [6,7]. Proline-specific aminopeptidases are therefore required to remove the blockage to the further action of broad specificity aminopeptidases as presented by proline residues in the second position from the N-terminus in peptides. Two such activities, i.e. post proline dipeptidyl aminopeptidase (PPDA), which releases amino acyl proline moieties from the N-terminus [8], and aminopeptidase P (Pep P), which removes the N-terminal amino acid where proline is present in the second position [9], have been purified and characterised from *Lactococcus lactis* subsp *cremoris* AM2. While the proteolytic system in Lactococci is well characterised [10], little information exists on the mechanism by which PPDA and Pep P in combination with a general aminopeptidase activity can degrade peptides containing non-consecutive proline residues. Furthermore, no proposed mechanism currently exists for the hydrolysis of peptides containing consecutive proline residues with the above aminopeptidase activities.

This study investigated the hydrolysis of synthetic tryptic β-cn peptides, in addition to other selected synthetic non-tryptic proline-rich peptide sequences arising from both α_{s1}- and β-cn, with various combinations of lysine-paranitroanilide hydrolase (KpNA-H), PPDA and Pep P from *L. lactis* subsp. *cremoris* AM2. The objective therefore was to elucidate the mechanism(s) by which combinations of these aminopeptidases hydrolyse peptides containing consecutive and non-consecutive proline residues.

2. Materials and methods

2.1. Materials

L. lactis subsp. *cremoris* AM2 was obtained from the Teagasc, Moorepark culture collection. Amino methyl coumarin (AMC), Gly-Pro-AMC, Lys-paranitroanilide, Arg-Pro-Pro, Leu-Leu-Leu, Pro-Leu and Leu-Pro were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Val-Pro-Pro-Phe-Ile-Gln, Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Glu-Thr-Pro-Val-Val, Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln and His-Pro-Ile-Lys-His-Gln were obtained from BioResearch Ireland. All other synthetic peptides were obtained from the School of Biomedical Sciences, University of Nottingham, UK.

2.2. Purification of enzymes

Purification of KpNA-H, PPDA and Pep P was performed as described previously [7–9,11].

2.3. Determination of enzyme activities

KpNA-H activity was measured according to the method of Exterkate [12], using Lys-pNA as a substrate. PPDA activity was assayed according to the procedure of Kato et al. [13] using Gly-Pro-AMC as a substrate and Pep P activity was measured according to the method of Doi et al. [14] using Arg-Pro-Pro as a substrate.

2.4. Hydrolysis of synthetic peptides with purified aminopeptidase activities

Synthetic peptides (4 μmol in 2 ml) were incubated with each aminopeptidase i.e. 60 μl KpNA-H (114 nmol/min) or 40 μl PPDA (256 μmol/min) or 200 μl Pep P (27 nmol/min), at 37°C for 4 h. After each aminopeptidase incubation, the reaction was heat inactivated (84°C for 10 min) and an aliquot (500 μl) taken to determine the amino acids released prior to addition of the next aminopeptidase.

2.5. Amino acid analysis

Free amino acids were determined according to the procedure described by Mullally et al. [15].

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Table 1
Amino acids observed following incubation of synthetic peptides (4 μmol in 2 ml) corresponding to tryptic fragments of bovine β-casein with purified Lys-paranitroanilide hydrolase (KpNA-H) from *L. lactis* subsp. *cremoris* AM2

Peptide	Amino acids removed
f(26–28) Ile-Asn-Lys	N.A.A.
f(30–32) Ile-Glu-Lys	N.A.A.
f(33–48) Phe-Gln-Ser-Glu-Glu-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys	N.A.A.
f(98–99) Val-Lys	Val (4.3)
	Lys (3.9)
f(100–105) Glu-Ala-Met-Ala-Pro-Lys	Glu (3.7)
	Ala (4.3)
	Met (3.5)
f(106–107) His-Lys	His (3.1)
	Lys (2.8)
f(108–113) Glu-Met-Pro-Phe-Pro-Lys	Glu (3.9)
f(170–176) Val-Leu-Pro-Val-Pro-Gln-Lys	Val (0.4)
f(177–183) Ala-Val-Pro-Tyr-Pro-Gln-Arg	Ala (2.3)
f(184–202) Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg	N.A.A.
f(203–209) Gly-Pro-Phe-Pro-Ile-Ile-Val	N.A.A.

Values in parentheses represent μmol of amino acid removed. N.A.A. indicates no free amino acids observed.

2.6. Protein determination

Protein was determined according to the procedure of Lowry et al. [16], using bovine serum albumin as a standard.

3. Results

Eleven of the 16 possible peptides/products arising from tryptic hydrolysis of bovine β-cn were tested for their ability to act as substrates for KpNA-H. The results, shown in Table 1, indicate that KpNA-H efficiently hydrolyses β-cn f(98–99) and f(106–107). Hydrolysis of β-cn f(100–105), f(108–113), f(170–176) and f(177–183) by KpNA-H proceeded only as far as the amino acid which preceded the first proline residue in each of these sequences. No hydrolysis was observed with β-cn f(26–28), f(30–32), f(33–48), f(184–202) or f(203–209), (Table 1). Results from the incubation of other β-cn sequences with KpNA-H show that only Leu was removed from β-cn f(140–143) while β-cn f(49–52), f(175–176) and f(182–183) remained unhydrolysed (data not shown).

Table 2 shows that peptides containing non-consecutive proline residues i.e. β-cn f(100–105) and f(108–113) can be hydrolysed on sequential addition of KpNA-H and PPDA or PPDA followed by KpNAH in the case of β-cn f(203–209). KpNA-H removes the N-terminal residue from peptides with proline residues in the third position from the N-terminus, i.e. β-cn f(69–83), f(170–176) and f(177–183).The subsequent addition of Pep P removes the amino acid which precedes the proline residue in the case of β-cn f(170–176) and f(177–183).

Peptides containing proline in the second position from the N-terminus, i.e. α_{s1}-cn f(1–9) and f(4–9) were incubated with Pep P followed by KpNA-H (Table 3). All the peptide bonds in f(4–9) were hydrolysable whereas only Arg was removed from f(1–9) with this combination of exopeptidases. Incubation of α_{s1}-cn f(1–9) with PPDA removes Arg-Pro as evidenced by the removal of Lys on subsequent addition of KpNA-H. Re-addition of PPDA removes His-Pro allowing the appearance of free Ile, Lys, His and Gln on the further addition of KpNA-H (Table 3).

β-Cn f(84–89), which contains consecutive proline residues, was exposed to six different sequential combinations of KpNA-H, PPDA and Pep P. Hydrolysis of this peptide to its constituent amino acids and the Pro-Pro dipeptide was only achievable by sequential incubation with Pep P followed by PPDA followed by KpNA-H (Table 4).

Table 2
Amino acids observed following incubation of a range of β-casein derived synthetic peptides (containing non-sequential proline residues, 4 μmol in 2 ml) with purified Lys-paranitroanilide hydrolase (KpNA-H), post proline dipeptidyl aminopeptidase (PPDA) and aminopeptidase P (Pep P) from *L. lactis* subsp. *cremoris* AM2

Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Glu-Thr-Pro-Val-Val β-casein f(69–83)
(a) +KpNA-H→ Ser (0.04); +PPDA→N.F.A.A.; +KpNA-H→N.F.A.A.;
(b) +KpNA-H→Ser (0.03); +Pep P→N.F.A.A.;
Glu-Ala-Met-Ala-Pro-Lys β-casein f(100–105)
(a) +KpNA-H→Glu (3.9), Ala (4.3), Met (3.5); +PPDA→Lys (2.7);
Glu-Met-Pro-Phe-Pro-Lys β-casein f(108–113)
(a) +KpNA-H→Glu (3.9); +PPDA→Lys (2.9);
Val-Leu-Pro-Val-Pro-Gln-Lys β-casein f(170–176)
(a) +KpNA-H→Val (0.41); +Pep P→Leu (0.11),; +KpNA-H→N.F.A.A.;
Ala-Val-Pro-Tyr-Pro-Gln-Arg β-casein f(177–183)
(a) +KpNA-H→Ala (2.3); +Pep P→Val (0.35); +KpNA-H→N.F.A.A.;
Gly-Pro-Phe-Pro-Ile-Ile-Val β-casein f(203–209)
(a) +PPDA→N.A.A.; +KpNA-H→Ile (1.44), Val (0.032);

Values in parentheses represent μmol of amino acid removed.
+ indicates aminopeptidase addition, 60 μl KpNA-H (114 nmol/min) or 40 μl PPDA (256 μmol/min) or 200 μl Pep P (27 nmol/min). → indicates enzyme incubation at 37°C for 4 h. ; indicates heat inactivation at 84°C for 10 min after which 500 μl was withdrawn for amino acid determination. N.A.A. indicates no free amino acids observed. N.F.A.A. indicates no further free amino acids observed.

Table 3
Amino acids observed following incubation of α_{s1}-casein derived synthetic peptides (containing non-sequential proline residues, 4 μmol in 2 ml) with purified Lys-paranitroanilide hydrolase (KpNA-H), post proline dipeptidyl aminopeptidase (PPDA) and aminopeptidase P (Pep P) from *L. lactis* subsp. *cremoris* AM2

Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln α_{s1}-casein f(1–9)
(a) +Pep P → Arg (0.063); +KpNA-H → N.FA.A.;
(b) +PPDA → N.A.A.; +KpNA-H → Lys (0.32); +PPDA → N.F.A.A.; +KpNA-H → Ile (0.14), Lys (0.15), His (0.09), Gln (0.09);
(c) +KpNA-H → N.A.A.;
His-Pro-Ile-Lys-His-Gln α_{s1}-casein f(4–9)
(a) +Pep P → His (0.44); +KpNA-H → Pro (0.33), Ile (0.35), Lys (0.37), His (0.26), Gln (0.25);

Values in parentheses represent μmol of amino acid removed.
+ indicates aminopeptidase addition, 60 μl KpNA-H (114 nmol/min) or 40 μl PPDA (256 μmols/min) or 200 μl Pep P (27 nmol/min). → indicates enzyme incubation at 37°C for 4 h. ; Indicates heat inactivation at 84°C for 10 min after which 500 μl was withdrawn for amino acid determination. N.A.A. indicates no free amino acids observed. N.F.A.A. indicates no further free amino acids observed.

4. Discussion

The results presented in Tables 1–3 show that KpNA-H from *L. lactis* subsp. *cremoris* AM2 is unable to hydrolyse the imido bond linking proline to the amino acid which proceeds it, as the hydrolysis of proline containing peptides with this aminopeptidase proceeds only as far as the amino acid preceding proline. While KpNA-H appears to be a broad specificity aminopeptidase capable of hydrolysing peptides containing up to 15 amino acids, it does not necessarily hydrolyse all amido bonds in peptides containing less than 15 amino acids (Table 1).

Although KpNA-H cannot hydrolyse imido bonds it can in some instances remove N-terminal proline residues from peptides (Table 3). This is in agreement with previous studies with synthetic peptides which did not correspond to specific casein sequences [17].

The results in Table 3 for α_{s1}-cn f(1–9) and f(4–9) show that hydrolysis of peptides containing non-sequential proline residues can proceed using combinations of KpNA-H and PPDA and/or KpNA-H and Pep P. The removal of Arg-Pro and His-Pro from α_{s1}-cn f(1–9) during sequential incubation with PPDA overcomes the blockage presented by proline residues to extensive hydrolysis of this peptide by KpNA-H. Alternatively, the removal of His from α_{s1}-cn f(4–9) by Pep P also overcomes the blockage presented by imido bonds to extensive hydrolysis of this peptide by KpNA-H. The application of KpNA-H and Pep P in the extensive hydrolysis of peptide sequences containing non-consecutive proline residues is, however, dependent on the ability of KpNA-H to subsequently remove the proline residue exposed at the N-terminus by the action of Pep P. KpNA-H is not capable of removing all N-terminally exposed proline residues as is seen in the case of the N-terminally exposed proline residue following incubation of α_{s1}-cn f(1–9) with Pep P. The hydrolysis route of peptide sequences containing non-consecutive proline residues with combinations of KpNA-H and PPDA and/or KpNA-H and Pep P has been schematically summarised in Fig. 1a,b.

The ability of the sequential combination of Pep P, PPDA and KpNA-H to hydrolyse peptides containing consecutive prolines, as in β-cn f(84–89), is again directly related to the substrate specificity of these aminopeptidases (Table 4). KpNA-H can hydrolyse such peptides up to the amino acid immediately preceding the consecutive proline pair. PPDA cannot remove the N-terminally exposed amino acyl proline as proline is also present in the third position [8]. Pep P, however, can remove the amino acid which precedes the proline pair but cannot then remove proline residues where proline is also in the next position [9]. PPDA can remove the prolyl proline as a dipeptide [8] allowing KpNA-H to continue hydrolysis of the remaining peptide. The hydrolysis route for this peptide is schematically summarised in Fig. 1 c. It is interesting to note that β-cn f(84–89) has previously been identified as a bitter peptide which is present in Gouda cheese [18].

In conclusion, the results indicate that while either PPDA or Pep P are required along with KpNA-H to achieve hydrolysis of peptides containing internally sited, non-consecutive proline residues, both Pep P and PPDA are required along with KpNA-H to achieve hydrolysis of peptides containing a consecutive pair of prolines. Finally, while the results in this study contribute to our understanding of the molecular mechanisms involved in the exploitation of combinations of general and proline specific aminopeptidases in the hydrolysis and potential debittering of casein hydrolysates, the data herein are applicable to our understanding of their general hydrolysis

Table 4
Amino acids observed following incubation of a β-casein-derived synthetic peptide (4 μmol in 2 ml) containing consecutive proline residues β-casein f(84–89), with purified Lys-paranitroanilide hydrolase (KpNA-H), post proline dipeptidyl aminopeptidase (PPDA) and aminopeptidase P (Pep P) from *L. lactis* subsp. *cremoris* AM2

Val-Pro-Pro-Phe-Leu-Gln β-casein f(84–89)
(a) +KpNA-H → N.A.A; +PPDA → N.A.A; +Pep P → Val (0.12);
(b) +KpNA-H → N.A.A; +Pep P → Val (0.16); +PPDA → N.F.A.A.;
(c) +PPDA → N.A.A; +KpNA-H → N.A.A.; +Pep P → Val (0.1);
(d) +PPDA → N.A.A.; +Pep P → Val (0.17); +KpNA-H → N.F.A.A.;
(e) +Pep P → Val (0.22); +KpNA-H → N.F.A.A.; +PPDA → N.F.A.A.;
(f) +Pep P → Val (0.19); +PPDA → N.F.A.A.; +KpNA-H → Phe (0.09), Leu (0.05), Gln (0.05);

Values in parentheses represent μmol of amino acid removed.
+ indicates aminopeptidase addition, 60 μl KpNA-H (114 nmol/min) or 40 μl PPDA (256 μmol/min) or 200 μl Pep P (27 nmol/min). → indicates enzyme incubation at 37°C for 4 h. ; indicates heat inactivation at 84°C for 10 min after which 500 μl was withdrawn for amino acid determination. N.A.A. indicates no free amino acids observed. N.F.A.A. indicates no further free amino acids observed.

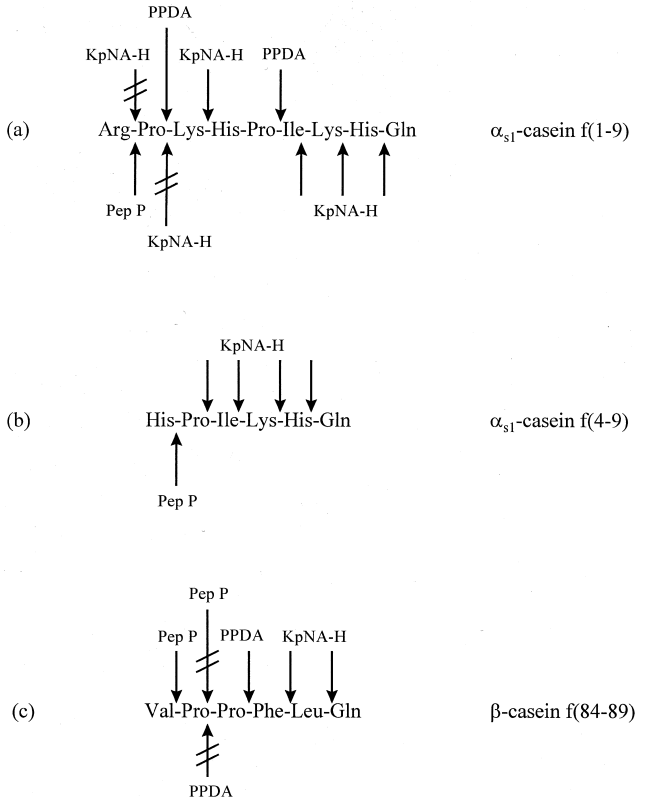

Fig. 1. Schematic representation of the potential routes of hydrolysis for peptides containing non-consecutive proline residues, i.e. (a) α_{s1}-casein f(1–9), (b) α_{s1}-casein f(4–9) and a peptide containing consecutive proline residues, i.e. (c) β-casein f(84–89) using Lys-paranitroanilide hydrolase (KpNA-H), post proline dipeptidyl aminopeptidase (PPDA) and aminopeptidase P (Pep P) from *L. lactis* subsp. *cremoris* AM2.

and potential debittering mechanisms in all food protein hydrolysates.

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