Debittering and Hydrolysis of a Tryptic Hydrolysate of β-casein with Purified General and Proline Specific Aminopeptidases from *Lactococcus lactis* ssp. *cremoris* AM2

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ABSTRACT: In this study, purified β-casein was hydrolysed with trypsin to produce a bitter substrate. The role of 3 aminopeptidases, a general aminopeptidase lysyl-*para*-nitroanilide hydrolase (KpNA-H), X-prolyl dipeptidyl aminopeptidase (Pep X) and aminopeptidase P (Pep P) each purified from *Lactococcus lactis* ssp. *cremoris* AM2, in the hydrolysis and debittering of the tryptic hydrolysate of β-casein, was then studied. The hydrolysates were analyzed for percentage degree of hydrolysis (DH%) and bitterness score. Results indicate that the hydrolysis and debittering potential of the general aminopeptidase (KpNA-H) is limited in the absence of proline specific aminopeptidases. Statistically significant ($p < 0.001$) reductions in bitterness were obtained following incubation of the tryptic digest of β-casein with specific combinations of the above aminopeptidases.

Key words: debittering, hydrolysates, aminopeptidase

Introduction

HYDROLYSIS OF MILK PROTEINS CAN, UNDER PARTICULAR conditions, lead to improvements in protein solubility, foaming, gelation, and emulsification properties. An undesirable attribute associated with food protein hydrolysis, however, is the development of bitter flavor (Adler-Nissen 1986; Limieux and Simard 1991). Peptides containing hydrophobic amino acid residues are reported to contribute substantially to casein hydrolysate bitterness (Matoba and Hata 1972).

Aminopeptidase activities may be exploited to decrease the bitterness in casein hydrolysates. The utility of general aminopeptidases in hydrolysate debittering was first observed when Clegg (1973), using pig kidney homogenate as a source of exopeptidases, obtained a casein hydrolysate relatively free of bitterness. Umetsu and others (1983) and Umetsu and Ichishima (1988) demonstrated the use of a wheat carboxypeptidase to hydrolyze bitter peptides from casein and soybean hydrolysates. Minagawa and others (1989) showed that aminopeptidase T, a broad specificity aminopeptidase isolated from *Thermus aquaticus* YT-1, could remove the bitterness associated with the peptides present in casein hydrolysates. Tan and others (1993) debittered a tryptic hydrolysate of β-casein using a general aminopeptidase obtained from *Lactococcus lactis* ssp. *cremoris* WG2.

Bouchier and others (1999) reported on the potential hydrolysis routes for casein-derived peptides containing consecutive and nonconsecutive proline residues using combinations of proline-specific and general aminopeptidase from *Lactococcus lactis* ssp. *cremoris* AM2. These synthetic peptide studies clearly demonstrated that extensive hydrolysis of proline-containing peptides required the complementary action of the general and proline specific aminopeptidases. This is due to the fact that the general aminopeptidase from *Lactococcus lactis* ssp. *cremoris* AM2, like other broad specificity aminopeptidases, is unable to cleave the bond which links proline residues to the preceding N-terminal amino acid (McDonnell and others 1999). The mechanism(s) by which proline specific aminopeptidases contribute to hydrolysate debittering has not been extensively researched. The complementary action of Pep N, a broad specificity aminopeptidase from pig kidney cytosol, in conjunction with Pep X from *Lactococcus lactis* ssp. *cremoris* AM2 in the debittering of a β-casein hydrolysate was recently investigated (Barry and others 2000). However, no previous studies appear to have reported on the casein hydrolysate debittering potential of combinations of broad specificity (KpNA-H) and proline specific (Pep X and Pep P) aminopeptidases isolated from Cheddar cheese starter organisms. The present study characterizes the debittering potential of combinations of KpNA-H, Pep X, and Pep P, all isolated from *Lactococcus lactis* ssp. *cremoris* AM2, on a bitter tryptic hydrolysate of bovine β-casein.

Materials and Methods

MILK CONTAINING B-CASEIN VARIANT A^1A^1 WAS OBTAINED from an individual cow in the Teagasc Moorepark herd. N-Tosyl-1-lysine chloromethyl ketone (TPCK)-trypsin (E.C. 3.4.11.1), ortho-phthaldialdehyde (OPA), and N-acetyl-L-cysteine (NAC) were obtained from Sigma Chemical Company (Poole, Dorset, England). Caffeine and urea (Aristar) were obtained from BDH (Poole, Dorset, England). Urea detection kits were from Boehringer Mannheim (East Sussex, England). PD-10 desalting columns were obtained from Pharmacia Biotech, Uppsala, Sweden. All peptides and peptide derivatives were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland).

Purification of β-casein

β-Casein was purified from raw milk using a combination

of urea fractionation (Aschaffenburg 1963) and anion exchange chromatography (Thompson and Pepper 1964) as described by Bouchier (1999).

Purification of aminopeptidases

Purification of KpNA-H, Pep X, and Pep P was performed as described previously (Booth and others 1990; Bouchier and others 1996; McDonnell and others 1997; McDonnell and others 1999).

Determination of aminopeptidase activities

KpNA-H activity was measured according to the method of Exterkate (1975), using Lys-*para*-nitroaniline (pNA) as substrate. Pep X activity was assayed according to the procedure of Kato and others (1978) using Gly-Pro-7-amino 4-methyl courmarin (AMC) as substrate and Pep P activity was measured according to the method of Doi and others (1981) using Arg-Pro-Pro.

Trypsin hydrolysis of β-casein

β-Casein was enzymatically hydrolysed at 37 °C for 6 h with TPCK-treated trypsin. Trypsin was from bovine pancreas and it contained 10,500 N-benzoyl-L-arginine ethyl ester (BAEE) units/mg protein (one unit of activity was defined as that amount of enzyme which gave a change in absorbance at 253 nm of 0.001/min with BAEE as substrate at 25 °C and pH 7.6 (Sigma)). The protein concentration in the hydrolysis reaction was 1% (w/v) and the enzyme to substrate ratio was 1.5% (w/w). The reaction (450 ml) was carried out at pH 7.6. The pH was maintained constant using a pH stat system (Metrohm Ltd., Herisau, Switzerland) loaded with 0.1 M NaOH. The reaction was terminated by heating at 85 °C for 10 min. The hydrolysate was stored at –20 °C prior to subsequent analysis.

Measurement of degree of hydrolysis (DH%)

The degree of hydrolysis for the β-casein hydrolysate was determined using a modification of the OPA method described by Church and others (1983). This involved using NAC as a reducing agent, as described by Garcia Alvarez-Coque and others (1989), and Medina Hernández and others (1991). The equations used to calculate DH % were as follows:

$$DH\ \% = \frac{N}{n} \times 100$$

$$N = \frac{\Delta Abs \times M \times d}{\epsilon \times c}$$

where: n = total number of peptide bonds per molecule of bovine β-casein, that is, 208.

N = average number of peptide bonds hydrolysed

ΔAbs = difference in absorbance of OPA at 340 nm in the presence of β-casein before and after hydrolysis with trypsin and the various aminopeptidases

M = molecular mass of β-casein, that is, 23,980 Da

d = dilution factor

c = protein concentration (g/L)

ϵ = extinction coefficient for OPA at 340 nm, that is, 6,200 $M^{-1}cm$

Triplicate determinations were performed and results expressed as mean DH%. The experimental variation for triplicate determinations using this assay were consistently $< \pm 1\%$.

Incubation of β-casein tryptic hydrolysate with KpNA-H, Pep X and Pep P.

Prior to use, KpNA-H, Pep X and Pep P activities were desalted into 0.02 M sodium phosphate pH 7.5 using PD-10 gel filtration columns following the manufacturers instructions. The enzyme activities were then added to 40-ml aliquots of the diluted β-casein tryptic hydrolysate (0.72% (w/v) protein equivalent) in various combinations and sequences. Generally, 1.2 ml of KpNA-H activity (0.203 μmol/min), 4 ml of Pep P activity (0.044 μmol/min) and 0.8 ml of Pep X activity (273 μmol/min) were added to the diluted β-casein tryptic hydrolysate. The reactions were incubated at 37 °C for 4 h in the presence of 1% (w/v) glycerol. Where aminopeptidase inactivation steps were included during sequential incubations with different aminopeptidases the reaction was heat inactivated at 85 °C for 10 min. At various points throughout the incubations, samples were withdrawn, heat inactivated and stored at –20 °C prior to subsequent analysis. The samples were assayed for DH% as outlined above and evaluated for bitter score using a trained sensory evaluation panel.

Sensory Evaluation

A 4-member taste panel comprising of staff from the Teagasc, Moorepark Research Centre was trained to detect bitterness using caffeine as a bitterness standard (Slattery and FitzGerald 1998). Due to low amounts of purified aminopeptidases and the consequentially small volumes of hydrolysate available for analysis, panelists were evaluated and subsequently trained to detect bitterness in 1 ml volumes (Barry and others 2000). Panelists were presented with 1 ml sample volumes each at a protein concentration equivalent to 0.6% (w/v), which had been withdrawn at various stages during incubation with the various aminopeptidases. An hydrolysate concentration of 0.6% (w/v) was chosen as it had a bitterness score which fell within the panelists maximum and minimum bitterness thresholds. Panelists graded the hydrolysate samples in a range of 0 to 100% bitterness score. With the maximum bitterness of 100% being equal to 0.1% caffeine. All solutions for analysis were presented in a random fashion and in duplicate to the panelists. In between tasting various samples the panelists were instructed to eat a piece of unsalted cracker, to wash their mouths thoroughly with mineral water and to wait 3 min before tasting the next sample. All glassware was acid washed, rinsed twice with distilled water and air dried (Jellinek 1985).

Statistical Methods

Analysis of variance was carried out on the sensory data using GENSTAT version 5 release 3 (Payne 1993).
The model used was:

$$y_{ij} = \mu + ti + pj + eij$$

where y_{ij} = response for the jth panelist (j = 1, 2, 3, 4) to the ith intervention (i = 1, 2)
μ = Constant
t_i = Effect of the ith intervention (i = 1, 2)
p_j = Effect of the jth panelist (j = 1, 2, 3, 4)
e_{ij} = Random error for response y_{ij}

Effects that were statistically significant are stated as such in the results, that is, $p < 0.001$, other increases/decreases that are stated may be indicative of general trends. Standard

errors of differences (SED) are also given.

Protein determination

Protein was determined using the International Dairy Federation (IDF) approved Micro-Kjeldahl Method (IDF 1993).

Results

INCUBATION OF THE TRYPTIC HYDROLYSATE OF B-CASEIN WITH KpNA-H resulted in an increase in DH value, that is, from 9 to 16% and an accompanying decrease in mean bitterness score, that is, from 65 ± 6.3% to 58 ± 6.3% (Figure 1a and 1b). Incubation with either Pep P or Pep X in both cases resulted in further decreases in bitterness score, that is, from 58 ± 6.3% to 46 ± 6.3 and from 58 ± 6.3% to 45 ± 6.3%, respectively. The DH did not change when Pep P was added (Figure 1a), however, it increased from 16 to 18% when Pep X was added to the hydrolysate (Figure 1b). Re-incubation of either hydrolysate with KpNA-H resulted in an increase in DH, that is, from 16 to 20% and a significant ($p < 0.001$) decrease in mean bitterness to 28 ± 6.3% for the incubation sequence KpNA-H, Pep P, KpNA-H (Figure 1 a) and an increase in DH value, that is, 18-24% and a significant ($p < 0.001$) decrease in bitterness to 30 ± 6.3% for the incubation sequence KpNA-H, Pep X, KpNA-H (Figure 1b).

Incubation with buffer instead of Pep P or Pep X, as expected, resulted in no changes in DH or mean bitterness score (Figure 1c). Furthermore, re-incubation with KpNA-H in the sequence KpNA-H, Buffer, KpNA-H resulted in no further changes in DH or mean bitterness score values (Figure 1c).

Incubation of the tryptic hydrolysate of β-casein which had been previously incubated with KpNA-H and subsequently heat inactivated with both Pep P and Pep X gave an increase in DH value from 16 to 18% (Figure 2c) and a decrease in mean bitterness score from 58 ± 6.3% to 48 ± 6.3%. These values were similar to those observed when the KpNA-H incubated tryptic hydrolysate of β-casein was incubated separately with either Pep P (Figure 1a) or Pep X (Figure 1b). Subsequent re-incubation of the above hydrolysate with KpNA-H resulted in an increase in DH value to 24% and a significant ($p < 0.001$) decrease in bitterness to a value of 16 ± 6.3% (Figure 2c). No decrease in bitterness or increase in DH were observed if buffer replaced the second addition of KpNA-H (data not shown). If the initial incubation with KpNA-H was replaced by buffer, subsequent incubation with either Pep P followed by heat treatment and then KpNA-H or Pep X followed by heat treatment and then KpNA-H (Figure 2a and 2b) yielded bitterness values which were comparable to those obtained on incubating the bitter β-casein hydrolysate with KpNA-H alone (Figure 1a and 1b). The DH values obtained following successive incubation with buffer, Pep P, KpNA-H, were also similar to those observed on incubation of the bitter tryptic hydrolysate of β-casein with KpNA-H alone (Figure 2a). Where Pep X replaced Pep P, however, the DH value obtained was higher than the value obtained on incubation of the bitter tryptic hydrolysate of β-casein with KpNA-H (Figure 2b).

Discussion

PREVIOUS STUDIES INDICATED THAT BROAD SPECIFICITY AMINOpeptidases from different sources were able to reduce the bitterness of casein hydrolysates (Clegg 1973; Minagawa and others 1989; Tan and others 1993). In the present study, addition of the broad specificity aminopeptidase, KpNA-H, to a bitter tryptic hydrolysate of β-casein only resulted in

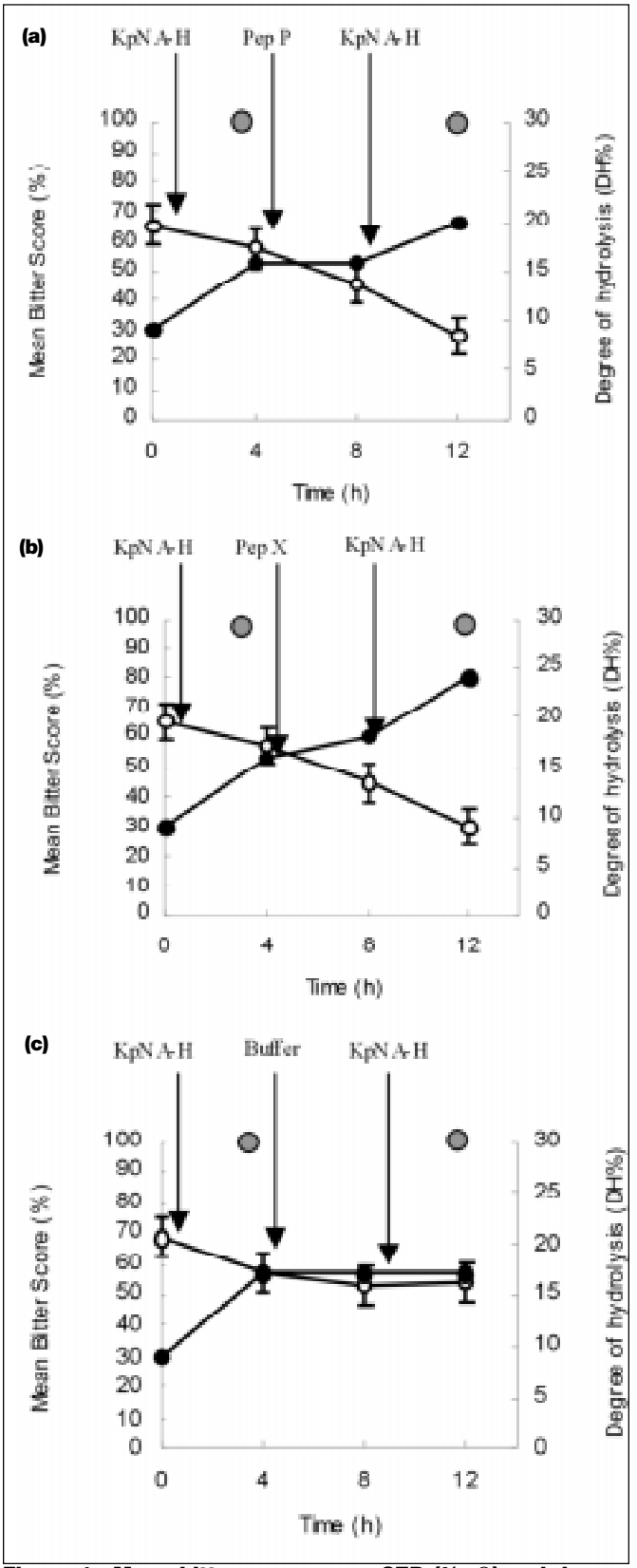

Figure 1—Mean bitterness score ±SED (%, ○) and degree of hydrolysis DH (%, ●) achieved on incubation of a tryptic hydrolysate of β-casein with purified aminopeptidases from *Lactococcus lactis* subsp. *cremoris* AM2. KpNA-H = Lysine-*para*-nitroanilide hydrolase; Pep P = aminopeptidase P; Pep X = X-prolyl dipeptidyl aminopeptidase; Arrows indicate point of addition of aminopeptidase; Heat inactivation (●) at 85 °C for 10 min.

limited hydrolysis and a small reduction in bitterness score (Figure 1a). KpNA-H from *Lactococcus lactis* ssp. *cremoris* AM2, like other broad specificity aminopeptidases, cannot hydrolyse the bond which links proline to the amino acid which precedes it (McDonnell and others 1999). Accordingly, hydrolysis of peptides in the tryptic digest of β-casein by KpNA-H should cease at that residue which precedes a proline residue. Addition of Pep P to the tryptic hydrolysate of β-casein which had previously been incubated with KpNA-H, should remove the N-terminal amino acid preceding the proline (McDonnell and others 1997) leaving a residual peptide commencing with an N-terminal proline. Alternatively, incubation with Pep X of the tryptic hydrolysate of β-casein, which had previously been incubated with KpNA-H removes both the N-terminal amino acid and the following proline residue as a dipeptide. In the present study, the addition of either Pep P (Figure 1a) or Pep X (Figure 1b) to the tryptic hydrolysate of β-casein, which had previously been incubated with KpNA-H resulted in a decrease in bitterness, however, an increase in DH only occurred following Pep X addition. In this context it is interesting to note that while the bitterness score of oligopeptides decreases generally with decreasing molecular mass (Otagiri and others 1985) the amino acid at the C-terminus is reported to exert a greater effect on bitterness than that at the N-terminus (Okai 1976; Ishibashi and others 1988). Studies with purified KpNA-H indicate that it is capable of removing N-terminal proline residues in synthetic peptides following their pre-incubation with Pep P (McDonnell and others 1999). Therefore, incubation of either Pep X or Pep P with the tryptic hydrolysate that had previously been incubated with KpNA-H removes the blockage to KpNA-H hydrolysis presented by the imido bond and allows further hydrolysis by KpNA-H to take place.

This is demonstrated by the observation that a second KpNA-H incubation to either Pep P (Figure 1a) or Pep X (Figure 1b) treated samples brings about a further decrease in bitterness and increase in DH value which are not observed if the second KpNA-H addition is made to the KpNA-H treated tryptic hydrolysate of β-casein which have been incubated with buffer in place of either Pep P or Pep X (Figure 1c). The bitterness score following either of these two incubation sequences, that is, KpNA-H, Pep X, KpNA-H and KpNA-H, Pep P, KpNA-H are not significantly different ($p < 0.001$) from each other, however, the decrease in bitterness following both incubation sequences are significantly different ($p < 0.001$) than when incubated with just KpNA-H alone. This result underlines the importance of either Pep P or Pep X as an adjunct to the broad specificity aminopeptidase in allowing a more complete hydrolysis of proline containing peptides.

Bitterness score and DH values observed after the tryptic hydrolysate of β-casein was incubated with the aminopeptidase sequence buffer, Pep P, KpNA-H (Figure 2a) were the same as had been observed when the same hydrolysate was treated with KpNA-H alone. This result indicates that peptide substrates for Pep P did not exist in the tryptic hydrolysate of β-casein before incubation with KpNA-H. Only one of the peptides expected from the trypsin hydrolysis of β-casein (that is, Gly-Pro-Phe-Pro-Ile-Ile-Val β-CN f(203-209)) contains a proline residue in the second position from the N-terminus. A previous study indicates that while this peptide is not a substrate for Pep P, it is a substrate for Pep X (Bouchier and others 1999). This may therefore explain why the DH value observed after the tryptic hydrolysate of β-casein was incubated with the aminopeptidase sequence shown in Fig-

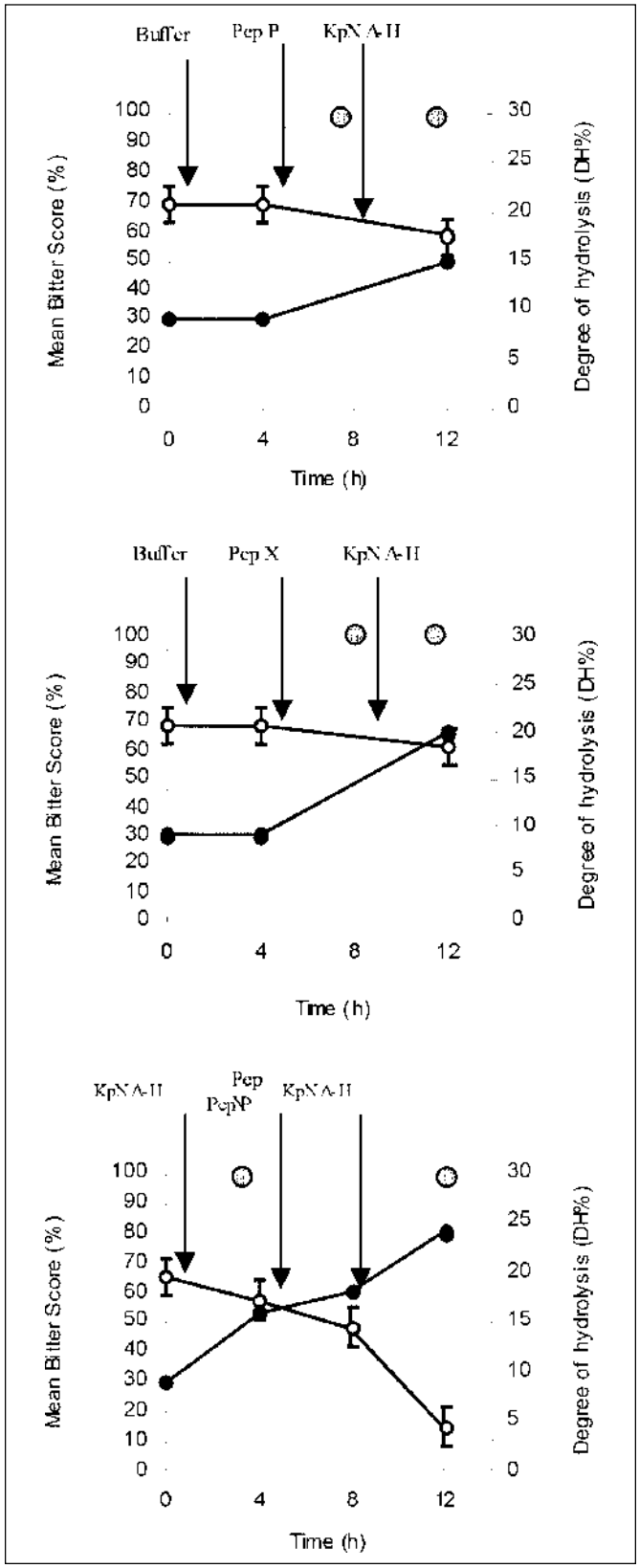

Figure 2—Mean bitterness score ± SED (%, ○) and degree of hydrolysis DH (%, ●) achieved on incubation of a tryptic hydrolysate of β-casein with purified aminopeptidases from *Lactococcus lactis* subsp. *cremoris* AM2. KpNA-H = Lysine-*para*-nitroanilide hydrolase; Pep P = aminopeptidase P; Pep X = X-prolyl dipeptidyl aminopeptidase; Arrows indicate point of addition of aminopeptidase; Heat inactivation (●) at 85 °C for 10 min.

ure 2b, that is, buffer, Pep X, KpNA-H, was higher than that observed after the same hydrolysate was exposed to KpNA-H alone indicating the presence of a substrate for Pep X in the trypsin treated β-casein hydrolysate. β-Casein f(203-209) has been identified in a previous study as bitter tasting (Matoba and others 1970) and it is surprising that the bitterness obtained after the tryptic hydrolysate of β-casein was subjected to the aminopeptidase treatment sequence buffer, Pep X, KpNA-H (Figure 2b) was not greater than the bitterness score observed when the same β-casein hydrolysate was treated with KpNA-H alone. Incubation of the tryptic hydrolysate of β-casein with KpNA-H, Pep P and Pep X, KpNA-H (Figure 2c) resulted in a significantly lower ($p < 0.001$) bitterness score and a higher DH value than those obtained with either KpNA-H, Pep P, KpNA-H (Figure 1a) or KpNA-H, Pep X, KpNA-H incubation sequences (Figure 1b) This may indicate a co-operativity between the hydrolytic capabilities of Pep X and Pep P in removing the blockage to general aminopeptidase action in the hydrolysis of proline containing peptides. Such cooperatively has previously been reported for the hydrolysis of synthetic β-casein sequences which contain sequential proline residues (Bouchier and others 1999).

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