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Original article

Hydrolysis of α_{s1}-casein by bovine plasmin

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Summary — *In vitro* hydrolysis of α_{s1}-casein by bovine plasmin was studied by electrophoresis and HPLC. Most of the degradation products were purified by reverse-phase or ion-exchange chromatographic methods and then identified by determination of their N-terminal sequence and amino-acid composition. The cleavage of 7 Lys-X and 4 Arg-X peptide bonds resulted in the formation of 15 peptides, the most hydrophobic of which were insoluble at pH 4.6.

plasmin / milk alkaline proteinase / α_{s1}-casein / HPLC

Résumé — Hydrolyse de la caséine α_{s1} par la plasmine bovine. *L'hydrolyse de la caséine α_{s1} par la plasmine a été étudiée par électrophorèse et par HPLC. La plupart des produits de dégradation ont été purifiés par chromatographie en phase inverse ou par échange d'ions, puis identifiés par détermination de leur séquence N-terminale et de leur composition en acides aminés. La coupure de 7 liaisons peptidiques de type Lys-X et de 4 liaisons Arg-X entraîne la libération de 15 peptides, dont les plus hydrophobes sont insolubles à pH 4,6.*

plasmine / protéase alcaline du lait / caséine α_{s1} / HPLC

INTRODUCTION

The native proteinases of milk can affect the properties of milk and dairy products during storage and also play a significant role during cheese ripening. In bovine milk the main native proteolytic activity is due to the milk alkaline proteinase, plasmin, which originates from blood. This enzyme partly resists UHT treatment and survives to pasteurisation (Driessen and Van der Waals, 1978).

In bovine milk, β and α_{s2}-caseins are rapidly cleaved by plasmin (Snoeren and Van Riel, 1979), α_{s1}-casein is more slowly attacked which κ-casein and whey proteins are very resistant (Eigel, 1977). The 3 main cleavage points in β-casein are well known and lead to the formation of γ-caseins and of some components of the proteose-peptone fraction which are already present in fresh milk. Recently 9 extra plasmin sensitive peptide bonds have been reported by Visser *et al* (1989a) after

digestion of β-casein in a membrane reactor. Under these conditions the plasmin activity was much higher than in milk.

In α_{s2}-casein, 8 peptide bonds are sensitive to plasmin and most released peptides are soluble at pH 4.6 (Le Bars and Gripon, 1989; Visser *et al*, 1989b).

Hydrolysis of α_{s1}-casein was first studied by Eigel (1977) who described the formation of products with a higher electrophoretic mobility than α_{s1}-casein. These products were found (Aimutis and Eigel, 1982) to be analogous to the λ-casein fraction previously described in milk (Long *et al*, 1958; Swaisgood and Brunner, 1961; El Negoumy, 1973).

The aim of the present study was to determine most of the plasmin-sensitive bonds in α_{s1}-casein, and to identify released peptides in order to evaluate plasmin activity in dairy products.

MATERIAL AND METHODS

The bovine plasmin solution (2.5 mg/ml) and carboxypeptidase A (CPA) were obtained from Boehringer GmbH (Mannheim, Germany); trifluoroacetic acid (TFA) came from Sigma (St Louis, USA) and acetonitrile from Baker (Deventer, the Netherlands).

Casein purification

Purified α_{s1}-casein variant B was isolated from homozygous cow's skim milk by isoelectric precipitation and anion exchange chromatography on DEAE–Sepharose according to Mercier *et al* (1968). Residual contaminating α_{s2}-casein was removed by hydrophobic interaction chromato-graphy on Phenyl Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). The column (2.6 x 15 cm) was equilibrated with sodium phosphate buffer (0.8 mol/l, 3.75 mol/l urea, pH 6.0). A total of 300 mg partially purified casein and 2.7 g urea were dissolved in 20 ml initial buffer as described by Chaplin (1986) and injected into the column. α_{s1}- and α_{s2}-caseins were separately eluted within 3 h by a linear gradient of phosphate decreasing from 0.8 mol/l to 0.05 mol/l at a flow rate of 2 ml/min. α_{s1}-casein was then dialyzed against distilled water and freeze-dried.

Hydrolysis of α_{s1}-casein

The substrate solution contained pure α_{s1}-casein (10 mg/ml) in NH_4HCO_3 (0.05 mol/l, pH 8.0), and NaN_3 0.05% (w/v) as microbial inhibitor. Plasmin was added at a final concentration of 12.50 μg/ml and incubation was carried out at 37°C. Aliquots were collected at 1, 2, 3, 4, 5 and 6 h to monitor hydrolysis. Plasmin was inactivated by heating at 100°C for 10 min in a sealed tube and samples were submitted to electrophoresis and RP-HPLC. Peptides were purified from the 6-h hydrolysate. The solution was heated at 100°C for 10 min and the pH was lowered to 4.6 by addition of 1% (v/v) TFA to precipitate largest peptides. After centrifugation the supernatant and the pellet were studied separately.

Analytical procedures

pH 4.6 soluble peptides were separated by RP–HPLC by a gradient system (Waters 600E, Bedford, USA) fitted with a Nucleosil C_{18}, 5 μm, 4.6 x 250-mm column (Shandon, Eragny, France). Two different solvent systems were used :

– a TFA system: solvent A consisted of 0.115% trifluoroacetic acid (TFA) in water: solvent B consisted of 0.10% TFA in 60% acetonitrile;

– a phosphate system: solvent A consisted of 0.05 mol/l potassium phosphate (pH 7.0); solvent B consisted of 60% acetonitrile in solvent A.

Peptides were eluted in a TFA system by a shallow linear gradient of buffer B increasing from 20 to 75% within 110 min. The temperature of the column was 40°C, the flow rate was 1 ml/min and absorbance was read at 214 nm. The main peaks were collected and each of them was then purified with the phosphate solvent system. A last step of purification with the TFA solvent system was then required to remove salts.

pH 4.6 insoluble peptides were separated by ion-exchange chromatography using an FPLC system (Pharmacia, Uppsala, Sweden) fitted with a Mono Q HR 10/10 anion exchange column. Buffer A consisted of 10 mmol/l Tris–HCl, 6 mol/l urea, pH 8.0 and buffer B consisted of buffer A containing molar NaCl. The flow rate was 3 ml/min and elution was obtained by using a triphasic linear gradient; the concentration of buffer B was as follows: 0% at initial time, 8% at 16 min, 18% at 56 min and 40% at 78 min. Absorbance was recorded at 280 nm. Fractions were collected, desalted on C_{18} Sep-Pack (Millipore, Milford, USA), concentrated and chromatographed on RP–HPLC with the TFA system as described above.

Peptides were hydrolyzed by vapor phase hydrolysis (110°C, 24 h) and their composition determined by cation exchange chromatography followed by ninhydrin derivatization on a Biotronik LC5000 amino-acid analyzer (Maintal, Germany).

N-terminal sequence determination was carried out by automatic Edman degradation on an Applied Biosystem, Model 477A protein sequencer (San José, USA).

C-terminal residues were obtained by free amino-acid analysis after hydrolysis by carboxypeptidase A.

Polyacrylamide–gel electrophoresis (PAGE) pH 8.8 was performed with a Mini Protein II apparatus (Bio-Rad, Richmond, USA) on 14% gels containing 4.7 mol/l urea. Proteins were stained with Coomassie blue G250 (Blakesly and Boezy, 1977).

RESULTS AND DISCUSSION

The chromatogram obtained with the 4.6 soluble fraction after 6 h hydrolysis is shown in figure 1b. After elution of a very

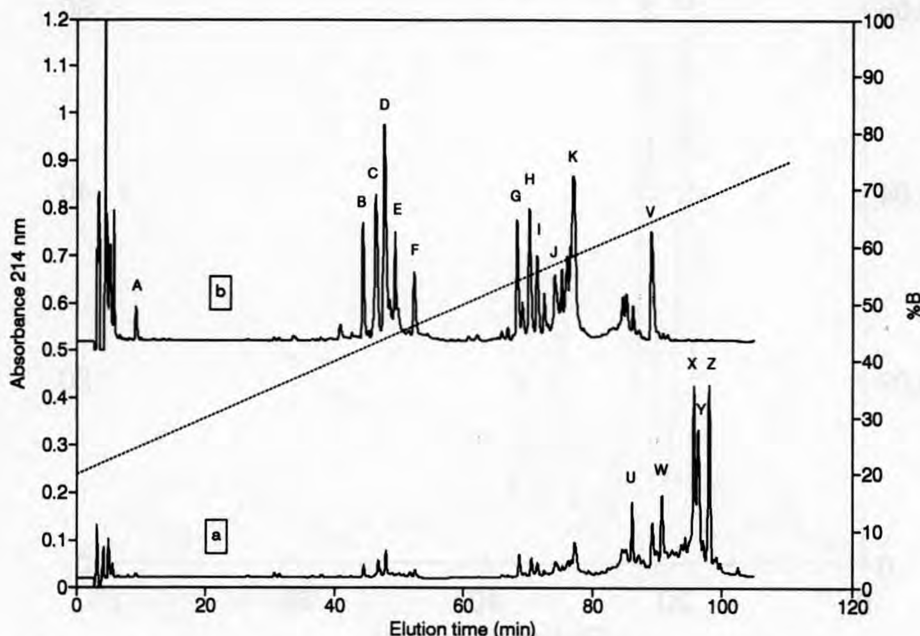

Fig 1. RP–HPLC chromatograms obtained after 6 h incubation at 37°C of α_{s1}-casein with bovine plasmin; (a) whole hydrolysate; (b) pH 4.6 soluble fraction. — A_{214}; - - - solvent B (%).
Séparation par HPLC en phase inverse des produits de l'hydrolyse de la caséine α_{s1} par la plasmine bovine (37°C, 6 h); (a) hydrolysat total, (b) fraction soluble à pH 4,6; — $DO_{214\ nm}$; - - - concentration du solvant B (%).

hydrophilic peptide (peak A), 2 groups of peaks (eluted respectively at 42–46% and 54–59% of solvent B) were observed. When the total hydrolysate was injected, high molecular weight peptides, insoluble at pH 4.6, were eluted by 60–75% of solvent B and only poorly separated (fig 1a). The pH 4.6 insoluble fraction was consequently separately purified by ion exchange chromatography on Mono Q column. Four main peaks were obtained (fig 2).

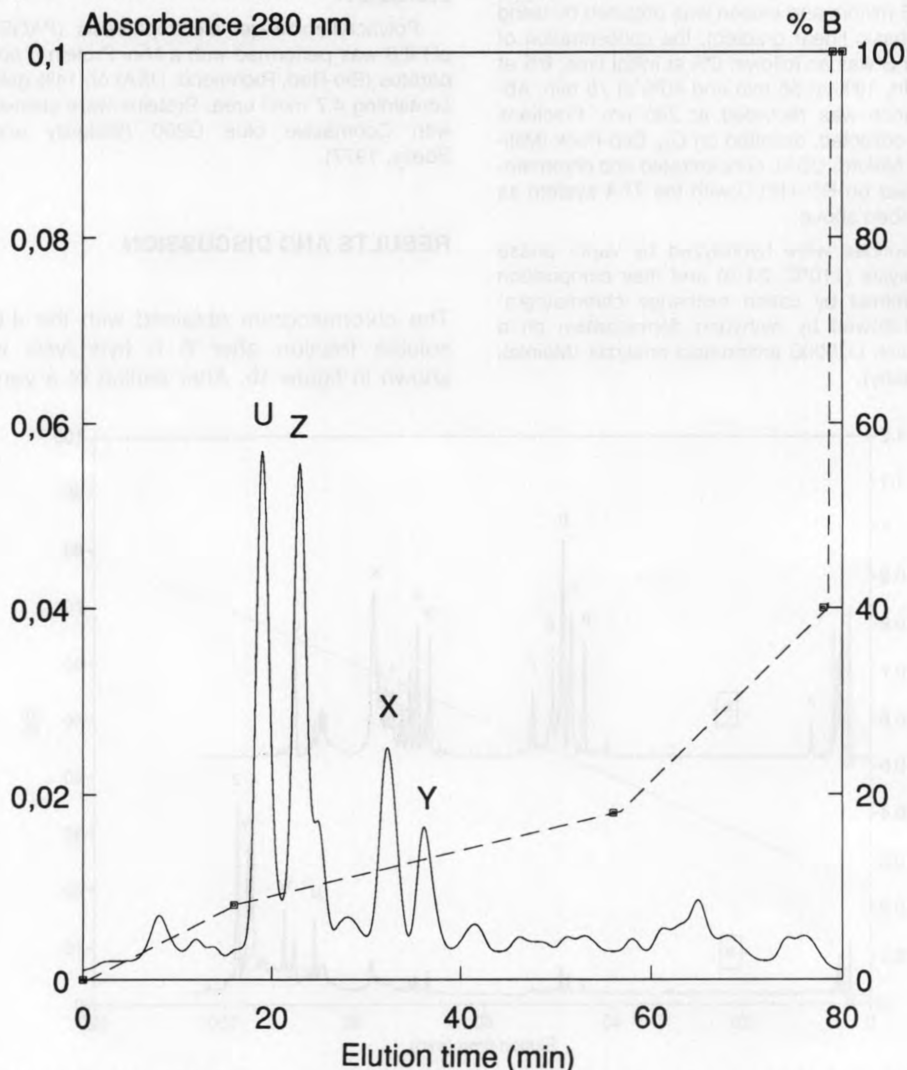

Fig 2. MonoQ chromatogram of insoluble fraction obtained after 6 h incubation at 37°C of α_{s1}-casein with bovine plasmin. — A_{280}; – – – solvent B (%).

Séparation par échange d'ions sur colonne MonoQ de la fraction insoluble obtenue après 6 h d'incubation de la caséine α_{s1} en présence de plasmine bovine. — DO_{280} nm; – – – concentration du tampon B (%).

Eleven peptides from the soluble fraction were identified (table I). They mainly arose from the N-terminal and central part of the α_{s1}-casein; only peptide F was a C-terminal fragment. Their size varied from 6–56 residues. No cleavage occurred in the hydrophilic region 41–80 which contained the phosphoseryl residues. In spite of its size (56 residues), peptide E was eluted by only 46% of B solvent because it contained the most hydrophilic domain of α_{s1}-casein. The 4 peaks (U, X, Y and Z) observed by ion-exchange chromatography corresponded to 4 C-terminal large fragments.

The sequence of the 15 identified degradation products has been presented in figure 3. These products resulted from the cleavage of 11 out of 20 peptide bonds that can be potentially cleaved by plasmin in α_{s1}-casein (fig 3). During digestion of fibrin, plasmin has a clear preference for

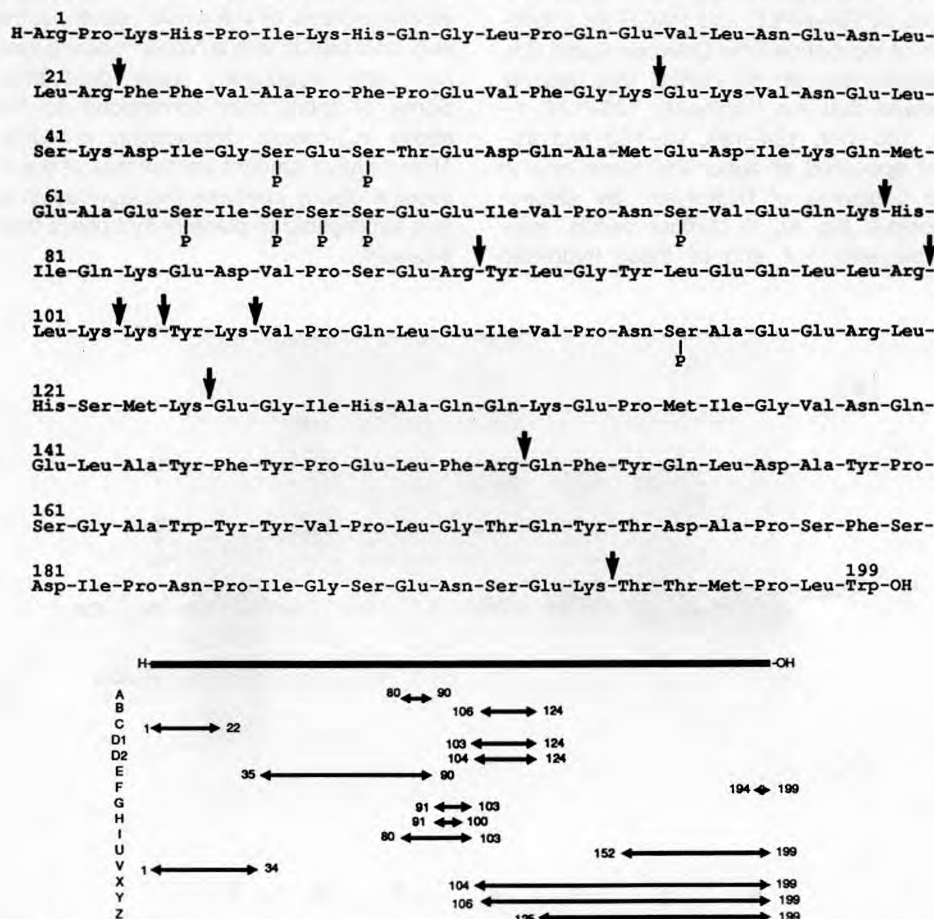

Fig 3. Amino-acid sequence of bovine α_{s1}-casein B (Ribadeau Dumas *et al*, 1973) showing (arrows) peptide bonds hydrolyzed by plasmin and localisation of peptides A to Z.

Séquence de la caséine α_{s1} (Ribadeau Dumas et al, *1973) liaisons peptidiques hydrolysées par la plasmine (flèches) et localisation des peptides A à Z.*

the cleavage of the Lys-X bond over the Arg-X bonds. This preference has also been observed for the hydrolysis of α_{s2}-casein (only one Arg-X labile bond over 7 Lys-X bonds). The difference is not so obvious with α_{s1}-casein (4 Arg-X bonds over 7 Lys-X bonds); this may, however, be due to the higher Arg/Lys ratio in α_{s1}– than in α_{s2}-casein (6/14 and 6/24 respectively).

The cleavage of α_{s1}-casein was monitored by RP–HPLC and PAGE as a function of incubation time between 0 and 6 h. Results from the RP–HPLC (not shown), showed that the fragments 106–124, 1–22, 103–124, 194–199, 91–103 and 91–100 appeared at about the same rate at the beginning of hydrolysis. By electrophoresis (fig 4), 9 distinct bands were visible after 1 h and all these hydrolytic products had a higher mobility than α_{s1}-casein. Between 2 and 6 h, the electrophoretic pattern differed essentially in the intensity of the bands except for the complete disappearance of α_{s1}-casein and a faint band close to α_{s1}-casein. Electrophoresis of the purified fragments from the pH 4.6 insoluble fraction (not shown) indicated that bands X, Y, Z and U corresponded to peptides 104–199, 106–199, 125–199 and 152–199 respectively. After electrophoresis of the whole casein, some very faint bands with a higher mobility than α_{s1}- and α_{s0}-casein were detectable. Some of them may correspond to the above α_{s1}-casein degradation products. Their relative amount *versus* that of the γ-caseins clearly confirms that α_{s1}-casein is less susceptible to plasmin hydrolysis than β-casein.

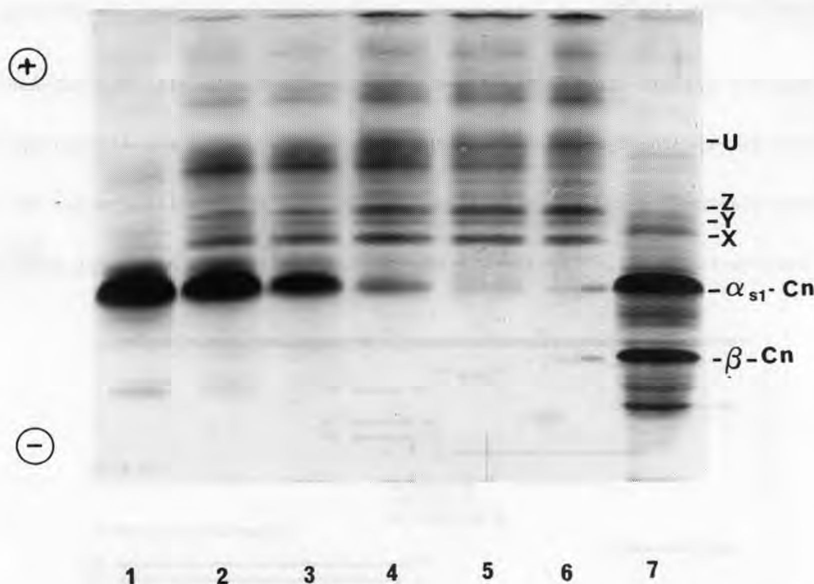

Fig 4. Urea-PAGE of reaction mixture during incubation at 37°C of α_{s1}-casein with bovine plasmin. Lane 1 = intact α_{s1}-casein; lanes 2–6 = 2, 3, 4, 5 and 6 h of incubation respectively; lane 7 = whole casein.

Suivi par électrophorèse en gel de polyacrylamide en présence d'urée, de l'hydrolyse de la caséine α_{s1} par la plasmine bovine à 37°C. Piste 1 = caséine α_{s1} intacte, pistes 2 à 6 = hydrolysat après respectivement 2 h, 3 h, 4 h, 5 h et 6 h, piste 7 : caséine entière.

Table I. Amino acid composition, C- and N-terminal residues and proposed sequences of peptides corresponding to peaks A–Z.
Composition en acides aminés, extrémités C– et N-terminales et séquence proposée pour les peptides correspondant aux pics A et Z.

	A	B	C	D1	D2	E	F	G	H	I	U	V	X	Y	Z
Asp	1.0 (1)	1.0 (1)	2.4 (2)	1.4 (1)	1.4 (1)	6.36 (6)		0.1	0.1	2.0 (1)	4.6 (5)	1.3 (2)	7.1 (7)	6.7 (7)	6.2 (6)
Thr						1.1 (1)	2.4 (2)	0.2	0.4		3.8 (4)		4.0 (4)	3.7 (4)	4.0 (4)
Ser	1.0 (1)	1.5 (2)		2.0 (2)	1.3 (2)	7.2 (9)		0.1	0.2	1.4 (1)	5.3 (5)		6.6 (7)	6.2 (7)	4.7 (5)
Glu	3.1 (3)	3.7 (4)	5.0 (4)	4.5 (4)	4.1 (4)	16.8 (16)		2.0 (2)	2.1 (2)	5.3 (5)	3.9 (5)	4.0 (5)	15.0 (16)	17.7 (18)	12.7 (12)
Gly			1.3 (1)			1.1 (1)		1.4 (1)	1.3 (1)	1.2 (1)	2.9 (3)	2.3 (2)	4.9 (5)	4.5 (5)	4.9 (5)
Ala		1.2 (1)		1.3 (1)	1.0 (1)	2.5 (2)		0.1	0.1		2.8 (3)	1.0 (1)	6.0 (6)	5.8 (6)	5.0 (5)
Cys															
Val	1.0 (1)	1.5 (2)	1.1 (1)	2.3 (2)	1.3 (2)	4.0 (4)		0.3	0.1	0.9 (1)	1.0 (1)	2.7 (3)	3.5 (4)	3.3 (4)	2.3 (2)
Met		1.2 (1)		0.6 (1)	0.7 (1)	1.0 (2)	1.2 (1)				0.3 (1)		3.3 (3)	2.8 (3)	1.8 (2)
Ile	0.6 (1)	0.5 (1)	0.8 (1)	0.4 (1)	0.6 (1)	4.5 (5)				0.3 (1)	2.1 (2)	0.9 (1)	4.0 (5)	3.5 (4)	3.2 (4)
Leu		1.7 (2)	3.6 (4)	2.8 (2)	2.3 (2)	1.3 (1)	1.0 (1)	6.6 (5)	4.3 (4)	3.6 (5)	2.4 (3)	4.0 (4)	6.8 (7)	6.3 (7)	4.9 (5)
Tyr		0.1		2.2 (1)	0.9 (1)			1.9 (2)	1.7 (2)	1.4 (2)	3.8 (5)		7.8 (8)	6.4 (7)	6.6 (7)
Phe								0.1	0.1		1.6 (2)	3.9 (4)	4.1 (4)	3.8 (4)	4.0 (4)
His	0.8 (1)	1.6 (1)	2.6 (2)	1.8 (1)	1.7 (1)	1.0 (1)			0.1	0.5 (1)		1.9 (2)	2.1 (2)	2.0 (2)	1.5 (1)
Lys	1.1 (1)	1.0 (1)	2.0 (2)	3.0 (3)	2.0 (2)	5.0 (5)		1.9 (2)	0.1	3.9 (3)	0.8 (1)	2.4 (3)	4.0 (4)	3.0 (3)	2.6 (2)
Arg	1.1 (1)	0.9 (1)	1.9 (2)	0.9 (1)	1.0 (1)	1.7 (1)	0.9 (1)	1.0 (1)	1.0 (1)	1.9 (2)		1.4 (2)	1.8 (2)	1.7 (2)	1.2 (1)
Pro	0.8 (1)	1.2 (2)	3.3 (3)	2.1 (2)	2.1 (2)	2.6 (2)	1.1 (1)	0.1	0.1	1.3 (1)	5.4 (6)	6.1 (5)	10.6 (10)	10.0 (10)	8.3 (8)
Trp															
N-terminal sequence	His-Ile--Gln-Lys	Val-Pro--Gln-Leu	Arg-Pro--Lys-His	Lys-Tyr--Lys-Val	Tyr-Lys--Val-Pro	Glu-Lys--Val-Asn	Thr-Thr--Met-Pro	Tyr-Leu--Gly-Tyr	Tyr-Leu--Gly-Tyr	X-Ile-Gln--Lys-Glu	Gln-Phe--Tyr-Lys	Arg-Pro--Lys-His	Tyr-Lys--Val-Pro	Val-Pro--Gln-Leu	Glu-Gly--Ile
C-terminal													Trp	Trp	
Proposed sequence	80–90	106–124	1–22	103–124	104–124	35–90	194–199	91–103	91–100	80–103	152–199	1–34	104–199	106–199	125–199

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