



**HAL**  
open science

## Hydrolysis of $\alpha$ s1-casein by bovine plasmin

D Le Bars, Jc Gripon

► **To cite this version:**

D Le Bars, Jc Gripon. Hydrolysis of  $\alpha$ s1-casein by bovine plasmin. *Le Lait*, 1993, 73 (4), pp.337-344.  
hal-00929345

**HAL Id: hal-00929345**

**<https://hal.science/hal-00929345>**

Submitted on 11 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Hydrolysis of $\alpha_{s1}$ -casein by bovine plasmin

D Le Bars, JC Gripon

Station de Recherches Laitières, INRA, 78352 Jouy-en-Josas Cedex, France

(Received 28 January 1993; accepted 31 March 1993)

**Summary** — *In vitro* hydrolysis of  $\alpha_{s1}$ -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 /  $\alpha_{s1}$ -casein / HPLC

**Résumé** — **Hydrolyse de la caséine  $\alpha_{s1}$  par la plasmine bovine.** L'hydrolyse de la caséine  $\alpha_{s1}$  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  $\alpha_{s1}$  / 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,  $\beta$  and  $\alpha_{s2}$ -caseins are rapidly cleaved by plasmin (Snoeren and Van Riel, 1979),  $\alpha_{s1}$ -casein is more slowly attacked which  $\kappa$ -casein and whey proteins are very resistant (Eigel, 1977). The 3 main cleavage points in  $\beta$ -casein are well known and lead to the formation of  $\gamma$ -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  $\beta$ -casein in a membrane reactor. Under these conditions the plasmin activity was much higher than in milk.

In  $\alpha_{s2}$ -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  $\alpha_{s1}$ -casein was first studied by Eigel (1977) who described the formation of products with a higher electrophoretic mobility than  $\alpha_{s1}$ -casein. These products were found (Aimutis and Eigel, 1982) to be analogous to the  $\lambda$ -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  $\alpha_{s1}$ -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  $\alpha_{s1}$ -casein variant B was isolated from homozygous cow's skim milk by isoelectric precipitation and anion exchange chromatography on DEAE-Sephacel according to Mercier *et al* (1968). Residual contaminating  $\alpha_{s2}$ -casein was removed by hydrophobic interaction chromatography 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 in-

jected into the column.  $\alpha_{s1}$ - and  $\alpha_{s2}$ -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.  $\alpha_{s1}$ -casein was then dialyzed against distilled water and freeze-dried.

### Hydrolysis of $\alpha_{s1}$ -casein

The substrate solution contained pure  $\alpha_{s1}$ -casein (10 mg/ml) in  $\text{NH}_4\text{HCO}_3$  (0.05 mol/l, pH 8.0), and  $\text{NaN}_3$  0.05% (w/v) as microbial inhibitor. Plasmin was added at a final concentration of 12.50  $\mu\text{g/ml}$  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<sub>18</sub>, 5  $\mu\text{m}$ , 4.6 x 250-mm column (Shandon, Eragry, 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 (Maital, Germany).

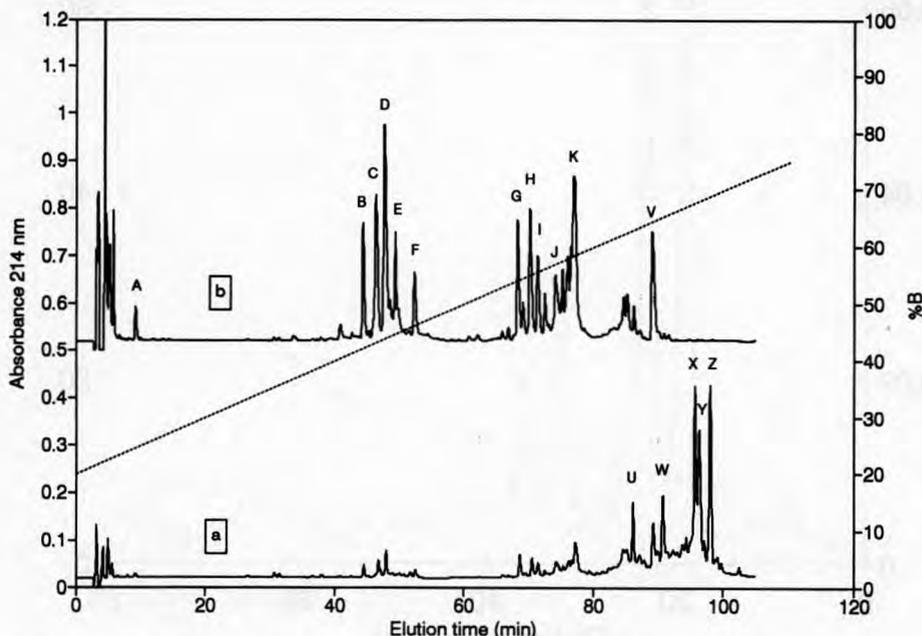
N-terminal sequence determination was carried out by automatic Edman degradation on an Applied Biosystem, Model 477A protein sequencer (San José, USA).

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  $\alpha_{s1}$ -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  $\alpha_{s1}$  par la plasmine bovine (37°C, 6 h); (a) hydrolysate total, (b) fraction soluble à pH 4,6; —  $DO_{214\text{ nm}}$ ; --- 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 sol-

vent 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).

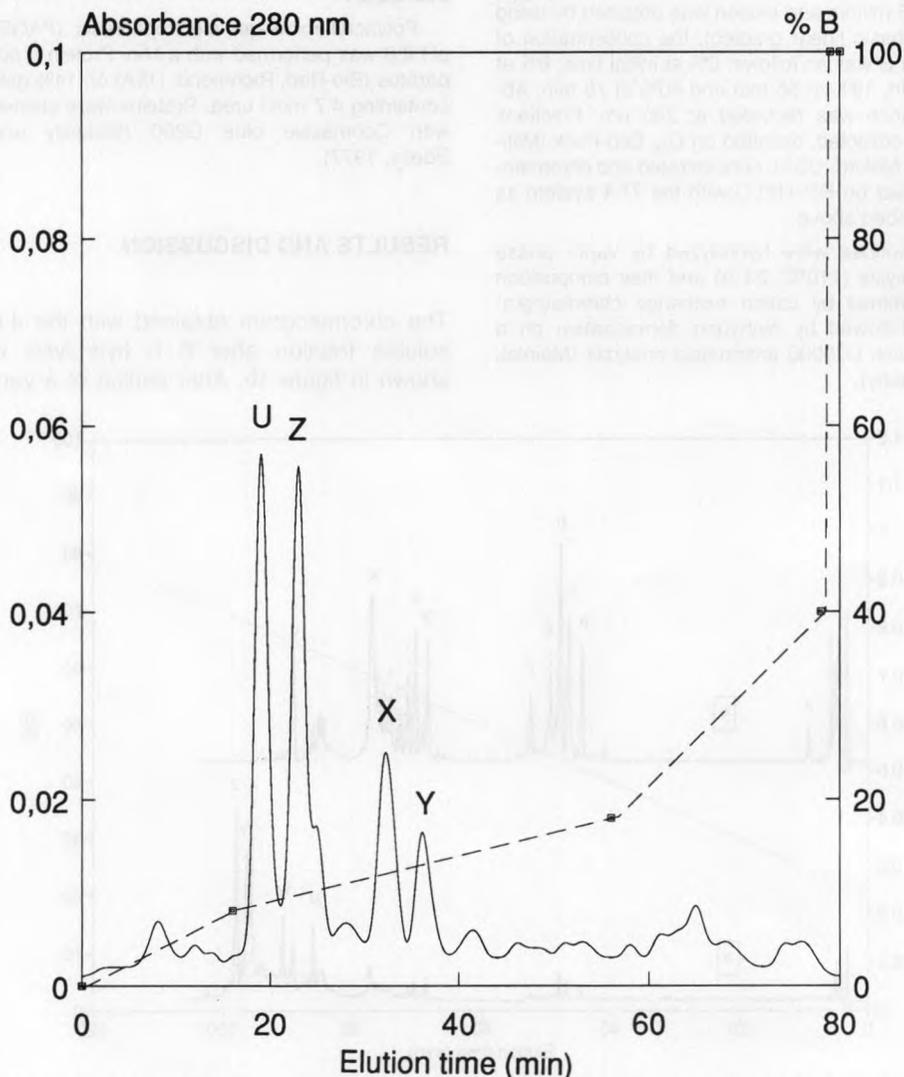


Fig 2. MonoQ chromatogram of insoluble fraction obtained after 6 h incubation at 37°C of  $\alpha_{s1}$ -casein with bovine plasmin. —  $A_{280}$ ; --- solvent B (%).

Séparation par échange d'ions sur colonne MonoQ de la fraction insoluble obtenue après 6 h d'incubation de la caséine  $\alpha_{s1}$  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  $\alpha_{s1}$ -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

$\alpha_{s1}$ -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  $\alpha_{s1}$ -casein (fig 3). During digestion of fibrin, plasmin has a clear preference for

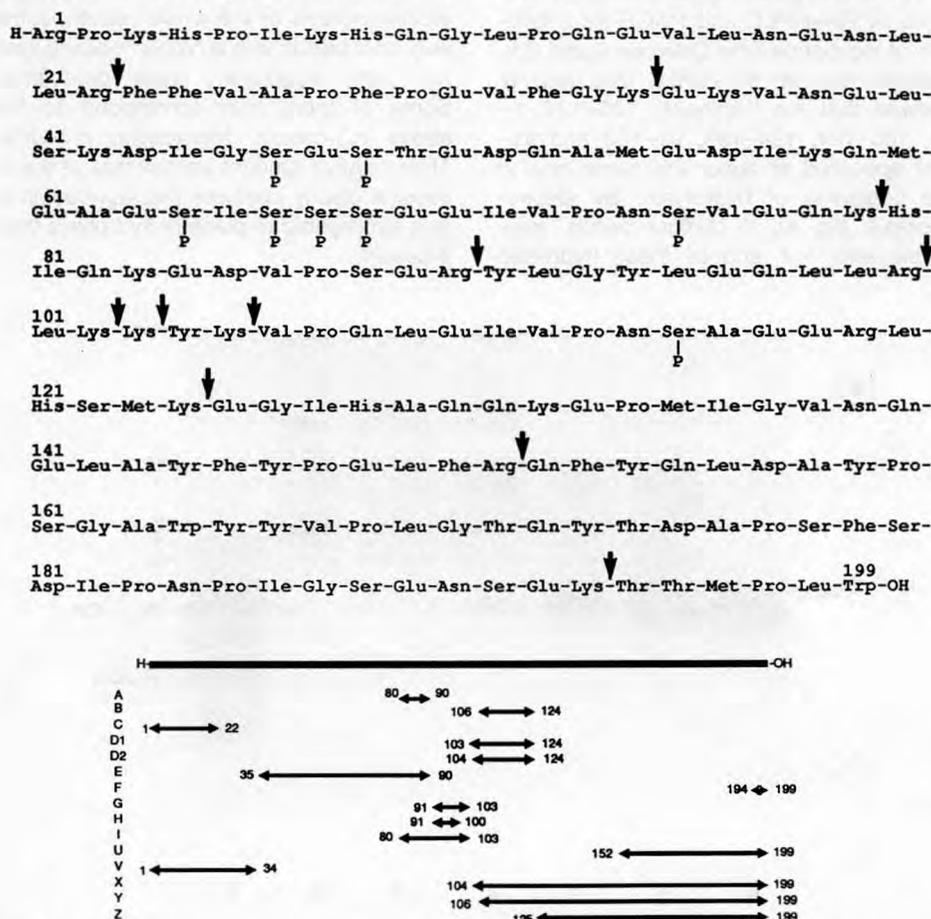


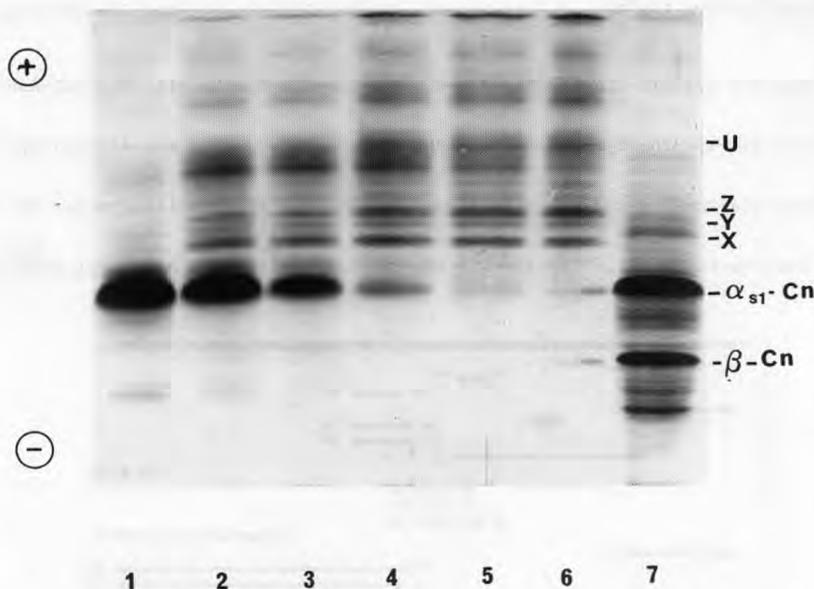
Fig 3. Amino-acid sequence of bovine  $\alpha_{s1}$ -casein B (Ribadeau Dumas *et al*, 1973) showing (arrows) peptide bonds hydrolyzed by plasmin and localisation of peptides A to Z.

Séquence de la caséine  $\alpha_{s1}$  (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  $\alpha_{s2}$ -casein (only one Arg-X labile bond over 7 Lys-X bonds). The difference is not so obvious with  $\alpha_{s1}$ -casein (4 Arg-X bonds over 7 Lys-X bonds); this may, however, be due to the higher Arg/Lys ratio in  $\alpha_{s1}$ - than in  $\alpha_{s2}$ -casein (6/14 and 6/24 respectively).

The cleavage of  $\alpha_{s1}$ -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  $\alpha_{s1}$ -casein. Between 2 and 6 h, the electrophoretic pattern differed essentially in the intensity of the bands except for the complete disappearance of  $\alpha_{s1}$ -casein and a faint band close to  $\alpha_{s1}$ -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  $\alpha_{s1}$ - and  $\alpha_{s0}$ -casein were detectable. Some of them may correspond to the above  $\alpha_{s1}$ -casein degradation products. Their relative amount *versus* that of the  $\gamma$ -caseins clearly confirms that  $\alpha_{s1}$ -casein is less susceptible to plasmin hydrolysis than  $\beta$ -casein.



**Fig 4.** Urea-PAGE of reaction mixture during incubation at 37°C of  $\alpha_{s1}$ -casein with bovine plasmin. Lane 1 = intact  $\alpha_{s1}$ -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  $\alpha_{s1}$  par la plasmine bovine à 37°C. Piste 1 = caséine  $\alpha_{s1}$  intacte, pistes 2 à 6 = hydrolysats 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)	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

**ACKNOWLEDGMENTS**

The authors are grateful to P Anglade for N-terminal sequencing and to A Bourroche for her assistance in the preparation of the manuscript.

**REFERENCES**

- Aimutis WR, Eigel WN (1982) Identification of  $\lambda$ -casein as plasmin-derived fragments of bovine  $\alpha_{s1}$ -casein. *J Dairy Sci* 65, 175-181
- Blakesly RW, Boezy JA (1977) A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G 250. *Anal Biochem* 83, 580-582
- Chaplin LC (1986) Hydrophobic interaction Fast Protein Liquid Chromatography of milk proteins. *J Chromatogr* 363, 329-335
- Driessen FM, van der Waals CB (1978) Inactivation of native milk proteinase by heat treatment. *Neth Milk Dairy J* 32, 245-254
- Eigel WN (1977) Effect of bovine plasmin on  $\alpha_{s1}$ -B and  $\kappa$ -A caseins. *J Dairy Sci* 60, 1399-1403
- El Negoumy AM (1973) Separation of  $\lambda$ -casein and some of its properties. *J Dairy Sci* 56, 1486-1491
- Le Bars D, Gripon JC (1989) Specificity of plasmin towards bovine  $\alpha_{s2}$ -casein. *J Dairy Res* 56, 817-821
- Long J, Van Winkle Q, Gould IA (1958) Isolation and identification of  $\lambda$ -casein. *J Dairy Sci* 41, 317-318
- Mercier JC, Maubois JL, Poznanski S, Ribadeau Dumas B (1968) Fractionnement préparatif des caséines de vache et de brebis par chromatographie sur DEAE cellulose en milieu urée et 2-mercaptoéthanol. *Bull Soc Chim Biol* 50, 521-530
- Ribadeau Dumas B, Mercier JC, Grosclaude F (1973) Amino-acid composition and sequence of bovine  $\alpha_{s1}$ - and  $\beta$ -caseins. *Neth Milk Dairy J* 27, 304-312
- Swaigood HE, Brunner JR (1961) The isolation of a  $\lambda$ -casein from concentrated TCA-urea solutions. *J Dairy Sci* 44, 1163
- Snoeren THM, Van Riel JAM (1979) Milk proteinase, its isolation and action on  $\alpha_{s2}$ - and  $\beta$ -casein. *Milchwissenschaft* 34, 528-531
- Visser S, Noorman HJ, Slangen CJ, Rollema HS (1989a) Action of plasmin on bovine  $\beta$ -casein in a membrane reactor. *J Dairy Res* 56, 323-333
- Visser S, Slangen KJ, Alting AC, Vreeman HJ (1989b) Specificity of bovine plasmin in its action on bovine  $\alpha_{s2}$ -casein. *Milchwissenschaft* 44, 335-339