Lait (1989) 69, 451-459

Original article

Hydrolysis of α-lactalbumin by chymosin and pepsin. Effect of conformation and pH

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(received 21 March 1989, accepted 26 June 1989)

Summary — The correlation between change of conformation of α-lactalbumin and its degradation by gastric enzymes was verified. With citrate buffer (0.1M), the modification of α-lactalbumin conformation occurred when the pH value was below pH 4.0. This conformational change was influenced by buffer composition and ionic strength. However, the presence of EDTA in the buffer did not modify the pH value at which the change of conformation of the protein occurred. Concomitantly, hydrolysis of α-lactalbumin by bovine and porcine pepsins A and rennet was observed at pH values corresponding to the change in conformation of the protein. However, with chymosin, under the same pH and ionic strength conditions there was no significant hydrolysis of α-lactalbumin.

α-lactalbumin (bovine) — conformation — hydrolysis — pepsin — chymosin — rennet

Résumé — Hydrolyse de l'α-lactalbumine par la chymosine et la pepsine : effet de la conformation et du pH. *Nous avons vérifié la corrélation entre le changement de conformation de l'α-lactalbumine et sa dégradation par les protéases gastriques. En présence de tampon citrate (0,01 M) le changement de conformation de l'α-lactalbumine se produit à des valeurs de pH inférieures à 4,0. Le changement de conformation est influencé par la nature du tampon et par la force ionique de ce dernier. Toutefois la présence d'EDTA dans le tampon ne modifie pas la valeur du pH à laquelle se produit le changement de conformation de la protéine. Parallèlement, nous avons observé une hydrolyse de l'α-lactalbumine par les pepsines A bovine et porcine et par la présure aux valeurs du pH auxquelles le changement de conformation de l'α-lactalbumine intervient. Cependant avec la chymosine bovine, aux mêmes conditions de pH et de force ionique, nous n'avons pas observé d'hydrolyse significative de l'α-lactalbumine.*

α-lactalbumine (bovine) — conformation — hydrolyse — pepsine — chymosine — présure

INTRODUCTION

α-lactalbumin is a calcium binding protein. This protein, in conjunction with a galactosyl transferase, forms the lactose synthetase (E.C. 2.4.1.22) which is involved in the biosynthesis of lactose. The primary structure of bovine α-lactalbumin has been established (Brew *et al.*, 1970). The conformation of this protein is pH-dependent (Kronman *et al.*, 1965; Kuwajima, 1977; Contaxis & Bigelow, 1981). Bovine α-lactalbumin is resistant to *in vitro* proteolysis by pepsin at pH 6.0, which is the pH of calf abomasum contents immediately after feeding milk, but is degraded at pH 2.0 (Jenkins *et al.*, 1980). *In vivo* experiments on pre-ruminant calves (Yvon *et al.*, 1984) and on rats (Fushiki *et al.*, 1986) have also shown that α-lactalbumin is hydrolyzed by gastric proteinases concomitantly with the acidification of the stomach's content. However, ≈ 50% of the ingested α-lactalbumin leaves the stomach before being proteolyzed by gastric enzymes (Yvon *et al.*, 1984).

The aim of our study was to ascertain that hydrolysis of α-lactalbumin was correlated to the modification of its conformation.

MATERIAL AND METHODS

α-lactalbumin preparation

Bovine α-lactalbumin was prepared by the method of Aschaffenburg & Drewry (1957) and then purified by chromatography on an Ultrogel AcA 54 column (Gaye *et al.*, 1982). To eliminate effects of possible association, α-lactalbumin was dissolved at a concentration of 0.08 g/100 ml (Kronman & Andreotti, 1964). Citrate or citrate-phosphate buffers (0.01 M) were used at different pH values : 2.50—6.50 at different ionic strengths (0, 0.51 and 0.86 M NaCl).

Gastric proteinases

Bovine pepsin A was purified from commercial powder (Chr. Hansen's Laboratory Ltd.) according to Martin *et al.* (1980). The proteolytic activity of the preparation was measured on a synthetic hexapeptide : Leu-Ser-Phe(NO_2)-Nle-Ala-LeuOMe (Bachem, CA, USA) at 30 °C, in a sodium acetate buffer 0.1 M, pH 4.7 (Martin *et al.*, 1980). The result, expressed as hydrolyzed peptide (μmol mg^{-1} s^{-1}), was 2 540 ± 40. Porcine pepsin A (ref : P6887) and bovine chymosin (rennin) (ref : P7751) were obtained from the Sigma Chemical Co. (USA). Liquid rennet (520 mg chymosin, 290 mg pepsin/l) was obtained from Boll-Hansen (France).

Change of conformation

Modification of the conformation of α-lactalbumin was observed by measurement at 291 nm of the difference absorption of tryptophan residues at the different pH values and referred to the absorbance of the reference solution at pH 6.50 (Kuwajima, 1977). The absorbance was measured in citrate or citrate-phosphate buffers (0.01 M) with or without EDTA (0.001 and 0.01 M).

Hydrolysis of α-lactalbumin

Hydrolysis of α-lactalbumin by gastric proteinases was performed at 37 °C with an E/S ratio of 1/2 500 (w/w) and stopped by adjusting the pH to 9.0 with NH_4OH. Hydrolysis of the protein was verified by SDS—gradient polyacrylamide gel electrophoresis (SDS—GPGE) (Trieu-Cuot and Gripon, 1981). The kinetics of disappearance of the protein were checked on a FPLC system (Pharmacia, Uppsala, Sweden) by anion exchange chromatography on a Mono Q column (Pharmacia, Uppsala, Sweden). The column was equilibrated with Tris—HCl buffer, 10^{-2} M,

pH 8.0. The peptides were eluted in a 20 min linear gradient of NaCl from 0.05 to 0.22 M. Elution rate was 1 ml/min.

Some particular peptides, soluble in 2% trichloroacetic acid (TCA), liberated during hydrolysis were purified on a Spectra Physics LC system. The column (μ-Bondapack C18 column, Waters) was equilibrated in solvent A (0.15% TFA), and the peptides were eluted by a linear gradient of 0—100% solvent B (0.10% TFA, 60% CH_3CN). Peptides were detected at 220 nm. The column was kept at 40 °C in a water bath. The elution rate was 2 ml/min. These peptides were identified by their amino acid composition, after acid hydrolysis (110 °C, 24 h, 5.7 N HCl, under vacuum) according to Spackman *et al.* (1958). The N-terminal amino acids were identified by recurring Edman degradation using the technique of Tarr (1982).

RESULTS AND DISCUSSION

Modification of the conformation of α-lactalbumin

In citrate buffer (Fig. 1a) the absorbance of α-lactalbumin solution decreased at pH values below pH 4.0, which indicated a change of conformation of the protein below this pH value. With citrate-phosphate buffer (Fig. 1a) the decrease of the absorbance occurred below pH 3.5. With citrate buffer, in presence of EDTA (Fig. 1b) there was no modification of the pH value at which change of conformation occurred. When the ionic strength of the citrate buffer was increased by adding NaCl (0 to 0.86 M) the change of conformation occurred at a higher pH value (Fig. 1c) (pH 4.50 with 0.17 M of NaCl). With 0.51 and 0.86 M of NaCl at the pH value near the isoelectric pH of α-lactalbumin (Kronman *et al.*, 1965), partial precipitation of the protein occurred. These results showed that the pH at which the protein changed con-

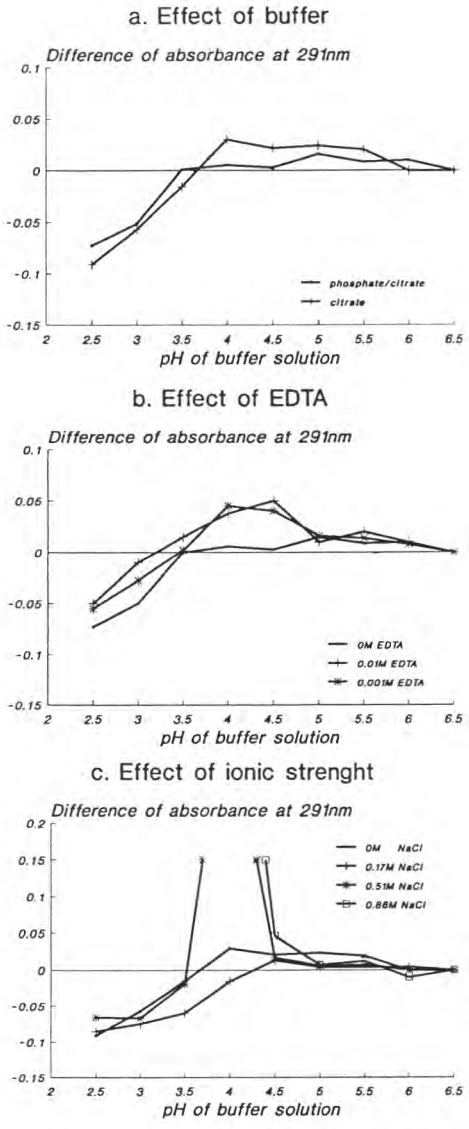


Fig. 1. Effect of buffer (a), EDTA (b) and ionic strength (c) on the change of conformation of α-lactalbumin (0.08 mg/100 ml) at different pH values.
Effet de la nature du tampon (a), de la présence d'EDTA (b) et de la force ionique (c) sur le changement de conformation de l'α-lactalbumine (0,08 mg/100 ml) en fonction de la valeur du pH.

formation depended on the nature and on the concentration of the salts in the buffer and that the presence or the absence of Ca^{2+} did not influence the pH value at which change of conformation occurred.

Electrophoretic study of α-lactalbumin hydrolysis

Using SDS—GPGE it was observed that α-lactalbumin was proteolyzed *in vitro* by bovine pepsin A when the pH of the solution was below pH 4.0 (Fig. 2a). However, in the presence of 0.51 M NaCl, α-lactalbumin was hydrolyzed by pepsin between pH 2.5 and 4.5 (Fig. 2b). These results show that there is a correlation between the change of conformation of the protein and its ability to be proteolyzed by bovine pepsin A.

Kinetics of hydrolysis of α-lactalbumin

The hydrolytic action of bovine and porcine pepsin A, bovine chymosin and rennet were tested on α-lactalbumin solution in citrate buffers (pH 2.5—6.5). With bovine and porcine pepsin A (Fig. 3a, b), the kinetic curves obtained at different pH values confirmed that pepsin A completely hydrolyzed α-lactalbumin at pH values < pH 4.0. At these pH values, both enzymes are near their optimum for activity. The disappearance of α-lactalbumin was faster at pH 3.0 than at pH 2.5 or pH 3.5. pH 3.0 corresponds to the activity maxima of both pepsins A on this protein. However, under the same conditions, much faster hydrolysis by porcine pepsin A was observed. With chymosin (Fig. 3c), only a small degree of proteolysis (≈ 10%) occurred when the pH was < 4.0. Although chymosin is a less active proteinase compared to pepsins, in the pH range between 3.0—4.0, chymosin shows maximum proteolytic activity, but minimum stability which may presumably be accounted for by autolysis of the enzyme (Foltmann, 1966). Similar autolytic decomposition of proteinase is known in the case of pepsin ≈ pH 2.0 (Foltman, 1966). These properties could explain why at pH values < 4.0 only a little α-lactalbumin could be proteolyzed by chymosin in spite of its conformational change. With rennet (Fig. 3d) the observed kinetics of hydrolysis were similar to those for bovine pepsin A.

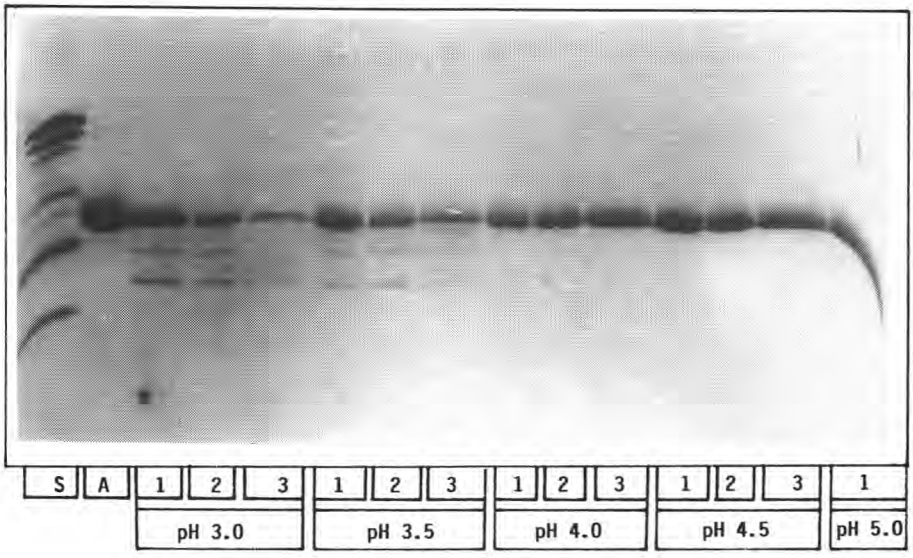
Characterization of some hydrolysis products

During hydrolysis of α-lactalbumin by bovine pepsin A, 3 peptides, soluble in 2% TCA, appeared rapidly (Fig. 4a) and remained as major products until the total disappearance of the protein (Fig. 4b). These peptides were analyzed to identify their sequence (Table I). They corresponded to the sequences 41-52, 40-52 and 41-53 of the protein.

CONCLUSION

Our results show that the modification of α-lactalbumin conformation is pH-dependent and occurs at pH values < 4.0. However, this conformational change is influenced by buffer composition and ionic strength but is not influenced by the presence or the absence of Ca^{2+}. α-lactalbumin hydrolysis by porcine and bovine pepsins A and rennet also occurred only at pH values < 4.0. At higher pH, al-

a. Citrate buffer



b. Citrate buffer in presence of 0.51M NaCl

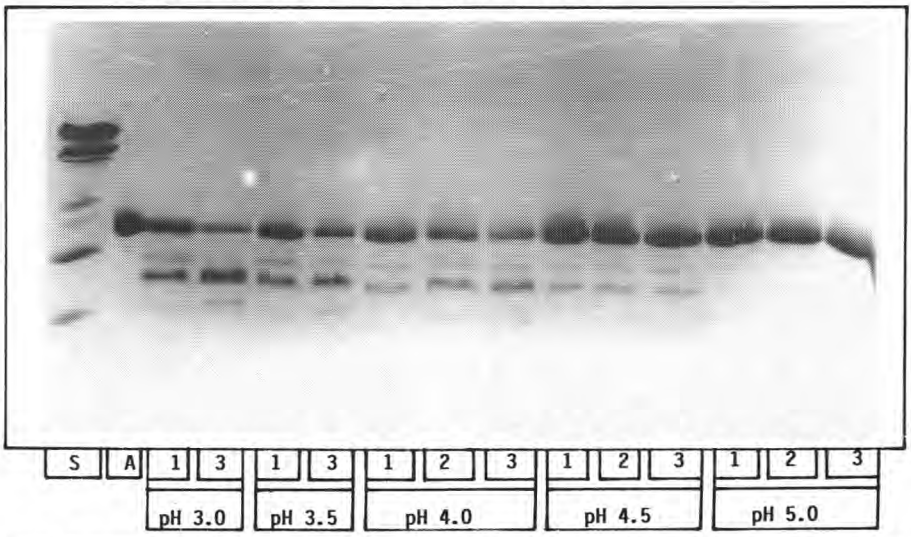


Fig. 2. SDS-GPGE of α-lactalbumin hydrolysed by bovine pepsin A at different pH values with and without NaCl (0.51 M). S = molecular weight standard solution; A = α-lactalbumin; 1, 2, 3 = *In vitro* hydrolysate of α-lactalbumin by bovine pepsin A (citrate buffer 0.01 M, 37 °C, E/S = 1/2 500) at different pH values for 40, 80 and 120 min hydrolysis, respectively.

Electrophorèse SDS d'hydrolysats d'α-lactalbumine par la pepsine bovine A, à différents pH, en présence et en absence de NaCl (0,51 M). S = standard de poids moléculaires. A = α-lactalbumine. 1, 2, 3 = hydrolysats in vitro d'α-lactalbumine par la pepsine bovine A (tampon citrate 0,01 M, 37 °C, E/S = 1/2 500) à différents pH, après respectivement 40, 80 et 120 min d'hydrolyse.

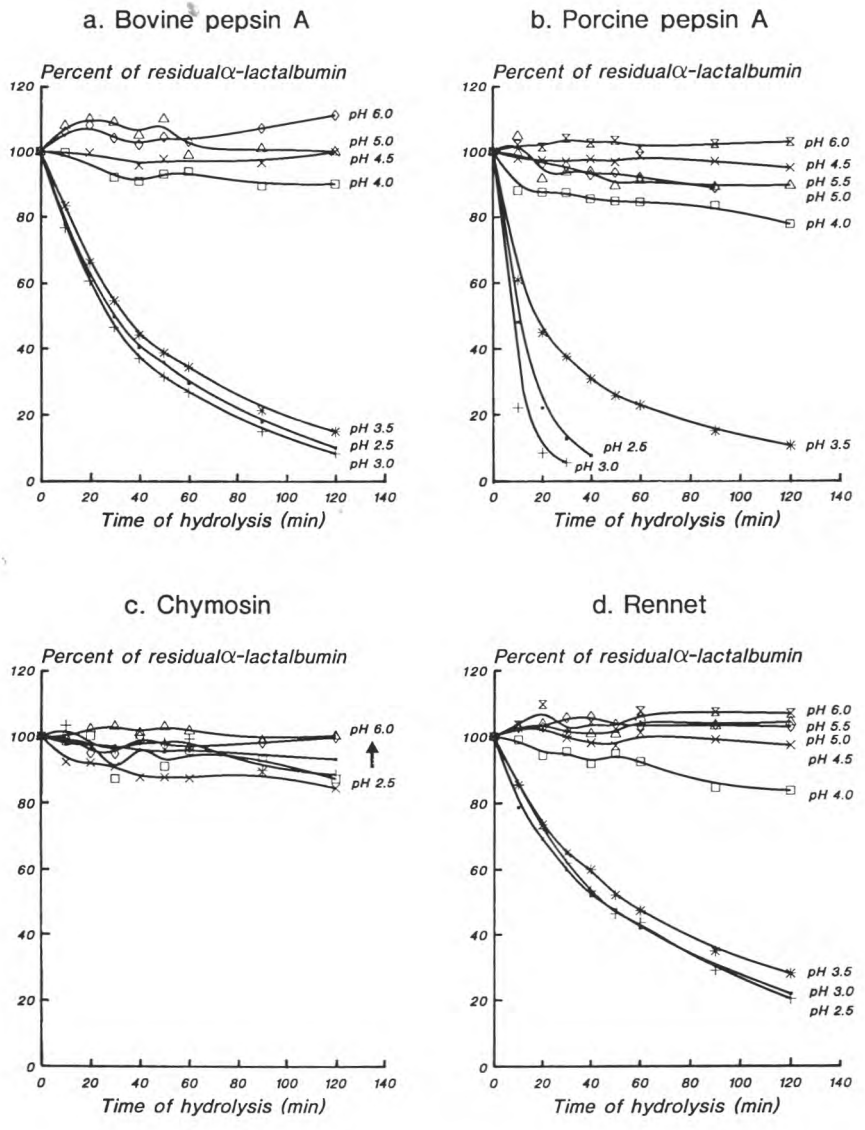


Fig. 3. *In vitro* hydrolysis (citrate buffer 0.01 M, 37 °C, E/S = 1/2 500) of α-lactalbumin solution (0.08 mg/100 ml) at different pH values by bovine (a) and porcine (b) pepsins A, chymosin (c) and rennet (d). Results were expressed as percent of residual α-lactalbumin during hydrolysis.
Hydrolyse in vitro (tampon citrate 0,01 M, 37 °C, E/S = 1/2 500) de l'α-lactalbumine à différents pH, par les pepsines A bovine (a) et porcine (b), la chymosine (c) et la présure (d). Les résultats sont exprimés en pourcentage d'α-lactalbumine résiduelle au cours de l'hydrolyse.

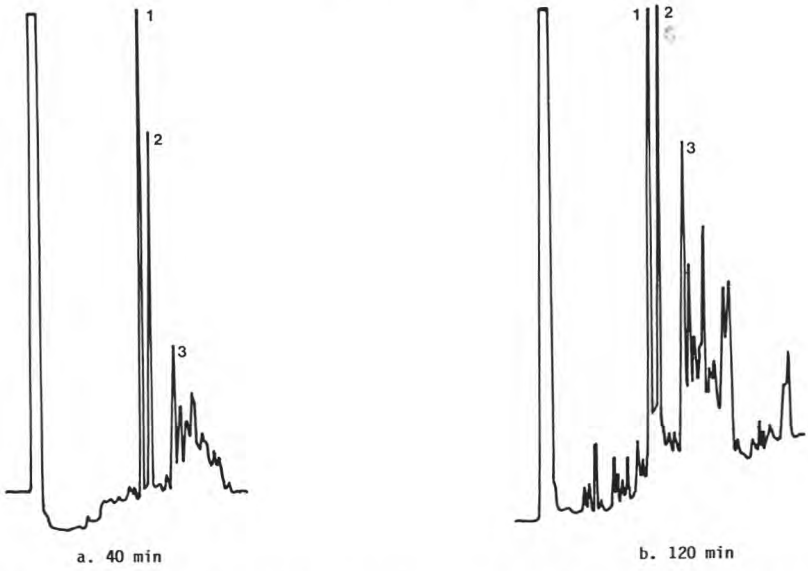


Fig. 4. RP—HPLC of the 2% TCA soluble fraction of α-lactalbumin hydrolysed by bovine pepsin A (citrate buffer 0.01 M, pH 3.2, 37 °C, E/S = 1/2 500) for 40 (a) and 120 (b) min of hydrolysis. RP—HPLC conditions : solvent A : TFA/H_2O (1.15/1000); solvent B : TFA/H_2O/CH_3CN (1/400/600); gradient : 0—100% B in 20 min; temp; 40 °C; λ = 220 nm. 1 = peptide 41—52; 2 = peptide 40—52; 3 = peptide 41—53.

RP-HPLC de la fraction soluble en TCA 2% final d'un hydrolysat d'α-lactalbumine par la pepsine bovine A (tampon citrate 0,01 M, pH 3,2, 37 °C, E/S = 1/2 500) après 40 (a) et 120 (b) min d'hydrolyse. Conditions en RP-HPLC : solvant A : TFA/H_2O (1,15/1000); solvant B : TFA/H_2O/CH_3CN (1/400/600); gradient : 0 à 100% B en 20 min; temp : 40 °C; λ = 220 nm. 1 = peptide 41-52; 2 = peptide 40-52; 3 = peptide 41-53.

though they are not at their optimal of activity, these proteinases are always active and hydrolyse proteins such as caseins (Pélissier, 1984). This is also the case for chymosin. These results suggest that the change of conformation of α-lactalbumin is responsible for the susceptibility of α-lactalbumin at these pH values and that the native protein is resistant to proteolysis. In contrast with chymosin, there was no significant hydrolysis of α-lactalbumin regardless of the protein conformation. These results can be explained by a lower activity of chymosin compared to pepsins, and by a loss of activity at a pH at which chymosin has a minimum stability. All these results suggest that the *in vivo* hydrolysis of α-lactalbumin observed in the calf only when the pH value of the stomach's content is < 4 (Yvon *et al.*, 1984), occurs specifically by the action of pepsins. In other animal species, α-lactalbumin could also be degraded by pepsins. But, as these enzymes may only act when the stomach pH is low, a large part of the α-lactalbumin could arrive intact in the gut.

Table I. Amino acid compositions and N-terminal amino acid sequences of peptides 1, 2 and 3 (see Fig. 4). Results (mol/mol) were obtained after 24 h acid hydrolysis. Figures in parentheses are theoretical values.
Compositions en acides aminés et séquences N-terminales des peptides 1, 2 et 3 (voir Fig. 4). Les résultats (mole/mole) ont été obtenus après hydrolyse acide de 24 h. Les chiffres entre parenthèses correspondent aux valeurs théoriques.

Peptides	*1*	*2*	*3*
Asx	2.3 (3)	2.2 (2)	2.3 (3)
Thr	0.8 (1)	0.7 (1)	0.7 (1)
Ser	0.9 (1)	1.0 (1)	0.9 (1)
Glx	2.0 (2)	2.0 (2)	2.0 (2)
Pro	—	—	—
Gly	0.9 (1)	0.9 (1)	1.3 (1)
Ala	—	0.6 (1)	—
Val	0.6 (1)	0.6 (1)	0.6 (1)
Met	—	—	—
Ile	0.6 (1)	0.6 (1)	0.9 (1)
Leu	1.0 (1)	0.9 (1)	1.1 (1)
Tyr	0.6 (1)	0.7 (1)	0.6 (1)
Phe	—	—	1.2 (1)
His	—	—	—
Lys	—	—	—
Arg	—	—	—
N-ter	—	Ala-Ile	—
Origin	41–52	40–52	41–53

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