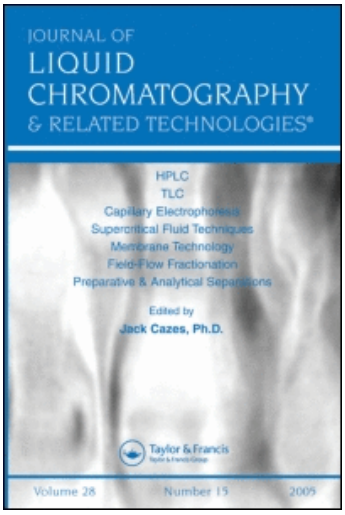

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INFLUENCE OF PHOSPHORYLATION ON PROTEOLYTIC CLEAVAGE

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INFLUENCE OF PHOSPHORYLATION ON PROTEOLYTIC CLEAVAGE

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ABSTRACT

Phosphorylation of an amino acid residue close to the peptide bond, which is a target of a specific proteolytic enzyme, seems to cause inhibition of such a cleavage. This finding may have a number of important consequences.

The effect of phosphorylation of a model peptide on its proteolytic cleavage by trypsin was studied. The velocity of peptide bond cleavage of nonphosphorylated synthetic peptide was compared with cleavage velocity of the same bond of synthetic peptide phosphorylated on serine residue located in closed proximity of the specifically cleaved peptide bond. It was shown that the enzymatic cleavage was inhibited by phosphorylation of mentioned amino acid residue. Reversed-phase high-performance chromatography (RP-HPLC) was used for the fast and reliable analysis of reaction mixtures.

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Influence of the addition of alkaline phosphatase on proteolytical cleavage of casein was also investigated. RP-HPLC peptide maps of β-casein cleaved by trypsin in the presence and absence of alkaline phosphatase were compared. It was proven that the action of acid phosphatase affects the composition of the resulting proteolytic digest.

Key Words: Proteolytic cleavage; Trypsin; Phosphoproteins; Casein; Cheese ripening

INTRODUCTION

Phosphorylation of proteins and peptides is very common and significant posttranslational modification dramatically changes the properties of modified molecules.

One, from a huge number of examples, is the role of the degree of β-casein phosphorylation during cheese ripening. The degree of phosphorylation of β-casein and peptides, as products of its enzymatic hydrolysis,[1] depends mainly on the activity level of acid phosphatase during cheese processing. Low activity of acid phosphatase is related to a higher degree of casein phosphorylation and, thus, could cause inhibition of proteolytical cleavage of this protein.[2] It means that lower activity of acid phosphatase maintains a higher degree of casein phosphorylation, and the phosphorylation of peptides formed by proteolytical cleavage of casein during cheese maturation as well. On the other hand, a higher activity of acid phosphatase causes a decrease (decline, drop) in phosphorylation of the mentioned peptides. Probably, the higher degree of casein phosphorylation (when activity of phosphatase is low) also causes inhibition of enzymatic cleavage of some (phosphorylated) peptides. Under these conditions the accumulation of longer phosphopeptides may occur and negatively affect the final organoleptic properties of cheese. It seems that the activity of acid phosphatase during cheese processing may significantly influence proteolysis of β-casein and, in this way, it plays an important role in the creation of the final cheese quality. Influence of protein phosphorylation on proteolytical cleavage may have various consequences. It was shown, for instance, that changes in phosphorylation degree of pepsinogens may be linked to carcinogenesis.[3–5]

The first goal of the experiments presented in this paper was to investigate the influence of phosphorylation of an amino acid residue close to the peptide bond, split specifically by selected proteolytic enzyme. The second goal was modelling of cheese ripening.

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EXPERIMENTAL

Chemicals

Model peptides Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu and Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu (phosphorylated form) were synthesized in the Polypeptide Laboratories (Prague, Czech Republic). The purity of both peptides was higher than 95%. Alkaline phosphatase (EC 3.1.3.1) (activity 23 units/mg solid), trypsin (EC 3.4.21.4) TPCK treated from bovine pancreas (activity 10,000 units/mg solid), and β-casein from bovine milk were obtained from Sigma (Prague, Czech Republic). Trifluoroacetic acid (HPLC grade) was obtained from Fluka (Buchs, Switzerland) and acetonitrile (HPLC grade) from Merck (Darmstadt, Germany).

Proteolytic Digestion of Model Peptides

An amount of 2 mg of model peptide with sequence Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu was diluted in 30 mL of 0.1 M ammonium hydrogen carbonate (pH 8.0). Then 280 μL of trypsin solution (0.2 mg/mL solution in the same buffer; activity 10,000 units/mg solid) was added. Afterwards, the reaction mixture was incubated at 37°C for different time intervals since addition of trypsin. The reactions were stopped by acidification of reaction mixtures to pH 4.5 using 50% acetic acid. Phosphorylated peptide Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu was digested under the same conditions.

Digestion of β-Casein in the Presence of Alkaline Phosphatase

Digestion of β-casein with TPCK treated trypsin in the presence of alkaline phosphatase was performed in 0.01 M *tris*-HCl buffer (pH 8.0) with 0.02 M magnesium chloride concentration of β-casein was 1 mg/mL. Stock solutions of alkaline phosphatase in concentration of 1 mg/mL and trypsin in concentration 1 mg/mL were prepared in the same buffer. Stock solutions of trypsin (4 μL) and alkaline phosphatase (2 μL) were added to 1 mL of β-casein solution. Only stock solution of trypsin (4 μL) was added to another 1 mL of β-casein solution. The mixture of 1 mL of the *tris*-HCl buffer without β-casein and with 4 μL of stock solution of trypsin and 2 μL of stock solution of alkaline phosphatase was also prepared as a blank. All three reaction mixtures were then incubated at 22°C. Samples of all reaction mixtures were analyzed after 12 and 48 hours by RP-HPLC.

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Reversed-Phase HPLC Separation

RP-HPLC was performed on Hewlett-Packard 1090 Series II Liquid Chromatograph, on reversed-phase column (250 × 4 mm I.D.) (OD-584, HP) LiChrospher 100 RP-18 (5 μm). Injected volume was 200 μL, flow-rate 1.0 mL/min.

Solvent A = trifluoroacetic acid–water (0.1 : 99.9 v/v), solvent B = solvent A–acetonitrile (20 : 80 v/v). Linear gradient: 100% A for 7 min, 0–70% B generated over 30 min for experiments with synthetic model peptides and 0–75% B generated over 60 min for β-casein digests. Peptides were detected at 220 nm.

Separation of digests: solvent A = trifluoroacetic acid–water (0.1 : 99.9 v/v), solvent B = solvent A–acetonitrile (20 : 80 v/v). Linear gradient: 100% A for 7 min, 0–80% B generated over 60 min for digests of β-casein. Detection was performed at 220 nm.

RESULTS AND DISCUSSION

Influence of Phosphorylation on Model Peptide Cleavage

It was supposed, that phosphorylation of an amino acid residue located near the peptide bond, which is normally specifically enzymaticaly cleaved, can cause inhibition of such cleavage. For this experiment, to prove this assumption, peptides Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu and Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu (phosphorylated form) were synthesized. The TPCK treated trypsin, which cleaves very specifically the peptide bond between lysine and serine, was used for proteolytical cleavage of these artificially prepared peptides.

Figure 1 shows the peptide digest of Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu after 1 min incubation with trypsin. The highest peak, with retention time 26.636 min, corresponds to the original peptide (Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu), which is prevailing in the mixture. Trypsin cleaves the bond Lys-Ser and, thus, two peaks with retention times 19.847 and 23.603 corresponding to two peptides Phe-Ile-Val-Lys and Ser-Ala-Phe-Glu, were found in the digest. Figure 2 shows the composition of the reaction mixture after 15 min of incubation with trypsin. After 15 min of enzymatic cleavage, no original peptide was present in the mixture, while areas of two peaks with retention times 19.948 and 23.471 corresponding to two above mentioned peptides are much larger in comparison with Fig. 1, and demonstrates total proteolytic cleavage.

The chromatogram given in Fig. 3 shows peptide Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu after 8 hours of incubation with trypsin. Only one significant peak, with retention time 25.440 related to the original peptide (Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu), was found in the chromatogram. A slightly lower retention time of the phosphorylated form of this peptide on reversed-phase

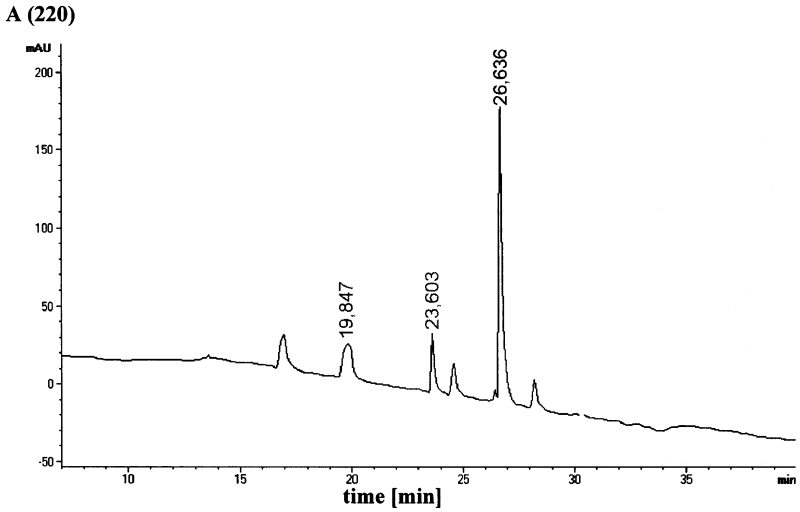

Figure 1. RP-HPLC evaluation of model peptide Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu cleavage by trypsin for 1 min. A_{220} = absorbance at 220 nm. For separation conditions see the text.

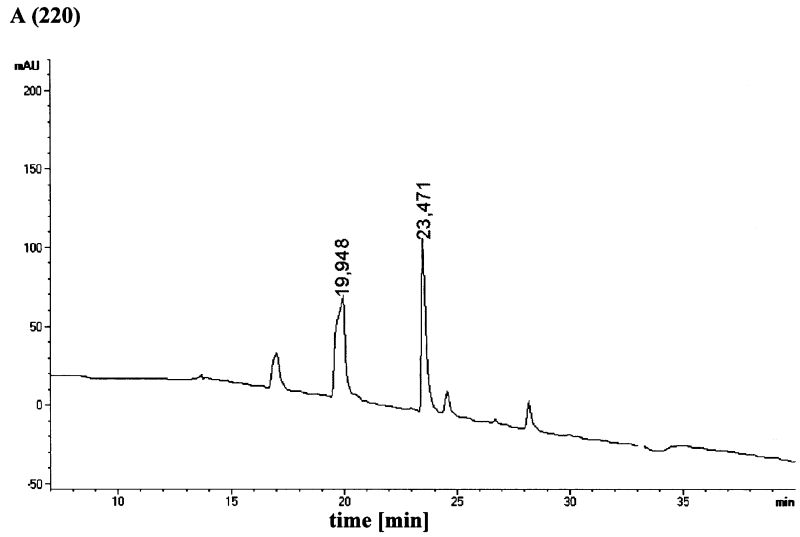

Figure 2. RP-HPLC evaluation of model peptide Phe-Ile-Val-Lys-Ser-Ala-Phe-Glu cleavage by trypsin for 15 min. A_{220} = absorbance at 220 nm. For separation conditions see the text.

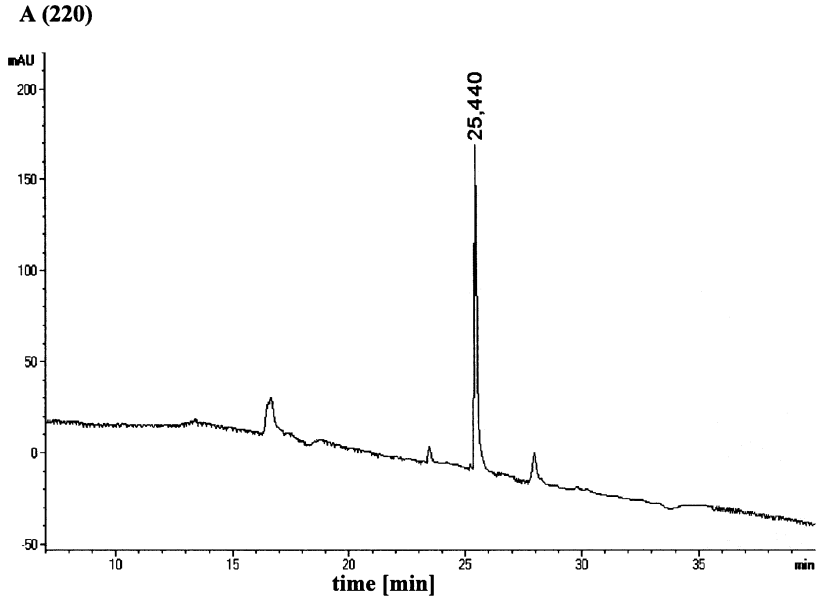

Figure 3. RP-HPLC evaluation of model peptide Phe-Ile-Val-Lys-Ser(PO_3H_2)-Ala-Phe-Glu cleavage by trypsin for 8 hours. A_{220} = absorbance at 220 nm. For separation conditions see the text.

(Fig. 3) in comparison with nonphosphorylated form (Fig. 1), is due to its lower hydrophobicity caused by the presence of phosphate group. It is evident, that even after 8 hours of incubation of phosphorylated peptide with trypsin no cleavage products were identified (see Fig. 3). This is in strong contrast with easy cleavage of nonphosphorylated peptide, which was completely hydrolyzed within 15 min of incubation with trypsin (Fig. 1). While peptide bond Lys-Ser is easily cleaved by trypsin, enzymatic cleavage of the peptide bond Lys-Ser(PO_3H_2) is almost impossible. This experiment provides clear evidence that the phosphorylation of certain amino acids may inhibit proteolytical breakdown.

Cleavage of β-Casein with Trypsin in the Presence of Alkaline Phosphatase

The phosphorylation degree of β-casein is considered to be an important factor affecting the degradation rate of β-casein during cheese ripening. To verify this assumption, β-casein proteolysis by trypsin was performed both in the presence

and absence of an alkaline phosphatase. Trypsin and alkaline phosphatase were chosen for the following reasons: they have similar pH optima and their high activities allow use of their very low concentrations, which do not affect the subsequent analysis. In addition, trypsin has the same hydrolytic selectivity as plasmin, which is a major proteinase in milk (plasmin splits the peptide bond from the carboxylic side of arginine or lysine if proline is not the next amino acid).

Chromatograms on Fig. 4 compare RP-HPLC profiles of peptide mixtures, which were formed by enzymatic cleavage of β-casein. A blank experiment proved (is not shown in figure) that autoproteolytic products of trypsin, as well as proteolytic products of alkaline phosphatase, did not cause interfering contamination. Figure 4 compares composition of the reaction mixtures after 12 hours of incubation (proteolytic hydrolysis) in the absence (a) and in the presence (b) of alkaline phosphatase. Figure 4(c, d) show the same comparison after 48 hours of hydrolysis. Activity of alkaline phosphatase was probably reduced during this long incubation period due to its proteolytic cleavage by trypsin. Despite it, we can observe certain differences in composition of reaction mixtures. The faster degradation of some peptide fragments, eluted between 50–60 min, can be observed in the presence of alkaline phosphatase. Fragments eluted at such higher

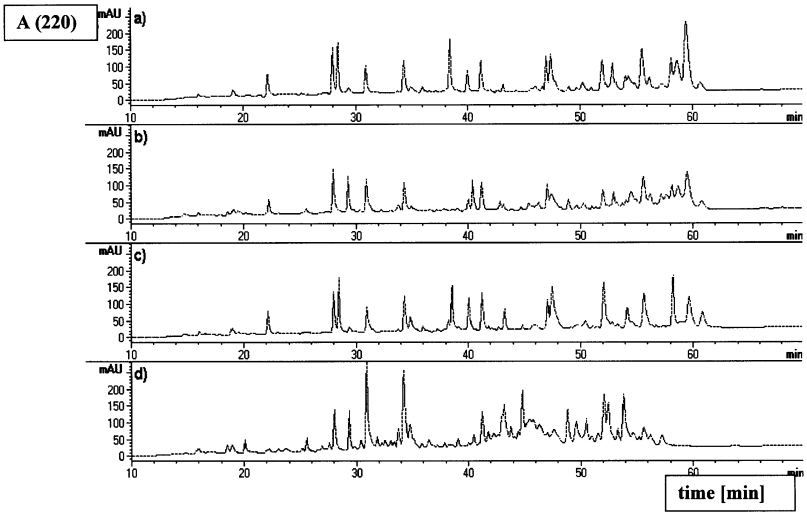

Figure 4. Comparison of RP-HPLC profiles of peptide mixtures formed from β-casein by proteolytical cleavage with trypsin. (a) Incubation for 12 hours without alkaline phosphatase, (b) Incubation for 12 hours with alkaline phosphatase, (c) Incubation for 48 hours without alkaline phosphatase, (d) Incubation for 48 hours with alkaline phosphatase. A_{220} = absorbance at 220 nm. For separation conditions see the text.

retention times are probably longer and hydrophobic peptides. The presence of alkaline phosphatase caused the dephosphorylation of casein and its fragments, which enables further proteolytical cleavage of the longer hydrophobic peptides. These results seem to verify that the higher phosphorylation of β-casein (caused by the low activities of phosphatases during cheese ripening) affects peptide mixture composition. However, it should be mentioned that the conditions during cheese ripening are different from those of described experiments. During cheese ripening, the pH is lower and, thus, acid phosphatase plays a more important role in casein dephosphorylation. Cheese ripening is a very complex process where a number of proteinases are involved in protein degradation.

CONCLUSIONS

The results obtained by the experiments with the model peptides demonstrate that phosphorylation of certain amino acids may play a pivotal role in the inhibition of peptide bond cleavage. The comparison of β-casein cleavage by trypsin in the presence and absence of alkaline phosphatase shows the influence of phosphatase on peptide mixture composition, which is formed by proteolytical cleavage. Owing to the importance of phosphorylations in the number of biological processes, the obtained results may have a wide range of applications.

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REFERENCES

1. Addeo, F.L.; Chianese, L.; Salzano, A. J. of Dairy Res. **1992**, *59*, 401.
2. Dulley, J.R.; Kitchen, B.J. Austr. J. of Dairy Tech. **1973**, *28*, 114.
3. Akimuru, K.; Utsumi, B.; Sato E.F.; Klostergaard, J.; Inoue, M.; Utsumi, M. Arch. Biochem. Biophys. **1992**, *298*, 703.
4. Huang, X.P.; Fan, X.T.; Desjeux, J.F.; Castagna, M. Int. J. Cancer **1992**, *52*, 444.
5. Boulikas, T. Int. J. Oncology **1995**, *6*, 271.

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