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Enzymatic dephosphorylation of caseins and creaming behaviour of o/w emulsions stabilized with dephosphorylated casein fractions

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Summary

α_S- and β-casein were treated with acid or alkaline phosphatase. The dependence of the dephosphorylation on incubation time was investigated by electrophoresis and by determination of the residual phosphate content. The degree of dephosphorylation is correlated with the stability of emulsions prepared with the modified proteins. Incubation of α_S- and β-casein with alkaline phosphatase increased the creaming stability 6- and 10-fold respectively.

Zusammenfassung

Enzymatische Dephosphorylierung von Caseinen und Aufrahmungsverhalten von mit dephosphorylierten Caseinfraktionen stabilisierten O/W-Emulsionen

α_S- und β-Casein wurden mit saurer oder alkalischer Phosphatase behandelt. Die Dephosphorylierung wurde, in Abhängigkeit von der Inkubationszeit, durch elektrophoretische Untersuchungen und Bestimmung des Restphosphatgehaltes charakterisiert. Die Untersuchungen zur Bestimmung des Aufrahmungsverhaltens von mit dephosphorylierten Caseinfraktionen stabilisierten O/W-Emulsionen machten deutlich, daß eine Korrelation zwischen dem Grad der Dephosphorylierung und der Aufrahmungsstabilität besteht. So zeigten Emulsionen mit dephosphorylierten – im Vergleich zu den mit nativen Caseinfraktionen hergestellten Systemen – eine 6-fach (α_S-Casein) bzw. 10fach (β-Casein) verbesserte Stabilität gegen Aufrahmung, wenn die Proteine eingesetzt werden, die nach 42-stündiger Incubation mit alkalischer Phosphatase entstehen.

Introduction

One of the major structural features with respect to the functional properties of milk proteins are the phosphoserines in α_S- and β-casein. These phosphate esters can form calcium phosphate bridges and related non-covalent cross-links resulting in a significant effect on the association behaviour of the caseins. Dephosphorylation of casein causes changes in the functional properties demonstrating the influence of phosphoserines on protein structure.

Enzymatic dephosphorylation leads to a decrease of the net negative charge and the calcium binding ability of the casein fractions, as well as to an increased hydrophobicity of the dephosphorylated proteins [1, 2]. Studies to characterize the lysinoalanine content of acid casein and dephosphorylated acid casein after heat treatment at different pH values demonstrated that under identical experimental conditions the dephosphorylated casein always showed an appreciably lower content of lysinoalanine than the native protein [3].

Investigations on the effect of enzymatic dephosphorylation on parameters of cheese making technology revealed that dephosphorylation of casein increases clotting time and

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reduces the firmness of the curd formed [4, 5]. However, the dephosphorylated casein retained the ability to aggregate, as determined by turbidity measurements during incubation [6].

The present paper describes both the dependence of the dephosphorylation rate of casein on the type of phosphatase used, and the creaming behaviour of o/w emulsions stabilized with these dephosphorylated casein fractions.

Materials and methods

Isolation of α_S- and β-*Casein*

Skim milk at 20 °C was adjusted to pH 4.6 by adding 7 M H_2SO_4 with mechanical stirring. The milk was kept for 15 min. The curd formed was separated from the whey by centrifugation (2000 × g, 20 °C, 30 min). α_S- and β-casein were isolated from whole casein as described by ASCHAFFENBURG [7] and HIPP et al. [8]: the precipitate after isolation of kappa-casein was dissolved in 6.6 M urea and adjusted to pH 7.5 by addition of 1 M NaOH. For precipitation of α_S-caseins the pH of the solution was subsequently lowered to 4.5 by 7 M H_2SO_4. The solution was then diluted with demineralized water to a urea concentration of 3.3 M and allowed to stand for 45 min. The α_S-caseins were removed from the supernatant by centrifugation (14000 × g, 20 °C, 30 min). β-casein was precipitated by adjusting the supernatant to pH 4.9 and diluting with demineralized water to a urea concentration of 1.0 M at 30 °C. β-casein was collected by centrifugation (see above). The precipitates of both casein fractions were dispersed with water, dissolved by adding 1 M NaOH (pH 7.0), dialyzed and lyophilized.

Phosphatases

Alkaline phosphatase (465 U/mg, Art.-No. 108146) and acid phosphatase (60 U/mg, Art.-No. 108197) were purchased from Boehringer (Mannheim, Germany).

Dephosphorylation of α_S- and β-*casein*

Dephosphorylation's were carried out on each substrate with both an acid and an alkaline phosphatase. 1% solutions of the casein fractions were prepared in buffer (acid phosphatase in 0,01 M imidazol, pH 7.0; alkaline phosphatase in 0,01 M Tris-HCl, pH 8.0). The enzymes were added at a level of 0.05 U/mg Protein. The mixtures were incubated at 20 °C either 24 h (acid phosphatase) or 42 h (alkaline phosphatase). Samples were taken from the incubation mixtures after 1, 2, 4, 6, 8 and 24 h (acid phosphatase) or 42 h (alkaline phosphatase), respectively, then analysed by polyacrylamide gel electrophoresis and by phosphorous determination. After incubation the remaining reaction mixtures were heated (80 °C, 5 min), dialyzed (24 h) against demineralized water and lyophilized.

The degree of dephosphorylation was estimated by polyacrylamide gel electrophoresis with urea-containing buffers (REIMERDES et al. [1]) and phosphor determination (IDF-Standard [9]). The phosphor determination involves digesting a weighed quantity of the sample with sulphuric acid in the presence of hydrogen peroxide. The phosphate is treated with sodium molybdate and hydrazine sulphate as a reducing agent. The molybdenum blue so formed is measured photometrically. From this, the phosphorus content is calculated [9].

Emulsifying properties

O/W emulsions with dephosphorylated α_S- and β-caseins were prepared according to REIMERDES et al. [10]. The creaming behaviour was measured by a radioanalytical method described by WIECHEN et al. [11]: the Ra 226 external standard source of a liquid scintillation counter, fixed in the measuring position, was used for the generation of CERENKOV radiation within the emulsions. The amount of

CERENKOV radiation that reaches the photomultiplier of the scintillation counter depends on the turbidity of the emulsion. The course of emulsion turbidity with time is typical and is designated as creaming-up-curve. The time required for 50% creaming up (A/2-value) was used to characterize the creaming behaviour.

Results and discussion

Dephosphorylation with acid phosphatase

The relation between dephosphorylation of casein fractions and incubation time is given in Fig. 1. It can be seen that on incubation the dephosphorylation initially increases rapidly then slows down. After an incubation time of 24 h a dephosphorylation of 94.6% for α_S-casein and 92.8% for β-casein was determined, i.e. 5 – 7% phosphate remained. Compared with the number of phosphoserines (α_S-casein = 8 per molecule, β-casein = 5 per molecule) this corresponds to less than one residue per protein molecule. It seems that none of the phosphate groups is resistant against the enzymatic cleavage.

However in electrophoretic analysis (Fig. 2), even after an incubation time of 24 h, casein bands with a varying number of phosphate groups are visible. The electropherograms also indicate, that the phosphate groups are cleaved rather quickly from the beginning of incubation. The decrease in the dephosphorylation rate with further incubation might be a result of steric hindrance caused by progressive aggregation of the casein fractions [6]. The lanes with a different degree of dephosphorylated β-casein show bands additional to the main casein bands. This may indicate some proteolytic activity of the phosphatase preparation used resulting in the formation of hydrophobic peptides.

Dephosphorylation with alkaline phosphatase

Preceding trials revealed, that an incubation of 24 h with alkaline phosphatase results only in partial dephosphorylation of the casein fractions, especially of β-casein. During the following investigations the incubation time was extended to 42 h. For comparison of the emulsifying properties of casein fractions after a dephosphorylation of 24 h with different phosphatases, samples were also taken after 24 h incubation. Initially the degree of dephosphorylation with alkaline phosphatase is smaller than with acid phosphatase (Fig. 3). After an incubation of 24 h, dephosphorylation was 91.3% for α_S-casein and 84.1% for β-casein. These values increased to 98,1 and 96.6% respectively, after 42 h. Resistence of the phosphate groups to cleavage with alkaline phosphatase was not noticeable.

The electropherograms presented in Fig. 4 also indicate that an incubation of 42 h leads to an almost complete dephosphorylation of α_S- and β-casein because the lanes show mainly one casein band. The α_S-casein incubated for 42 h showed nearly the same electrophoretic mobility as non-dephosphorylated β-casein. The electrophoretic analysis of β-casein showed depending on the incubation time, a greater number of bands than was observed after treatment with acid phosphatase. Proteolysis of β-casein was not detectable.

Creaming behaviour of o/w emulsions stabilized with dephosphorylated casein fractions

In Table 1 the creaming behaviour of o/w emulsions (A/2-values = 50% creaming up) stabilized with native or dephosphorylated casein fractions are summarized. From these

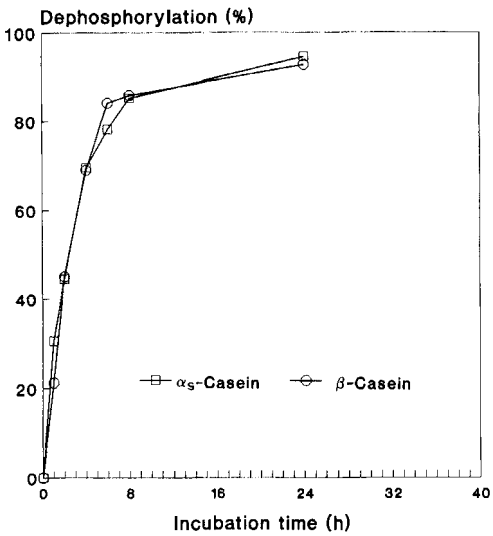

Fig. 1
Relation between dephosphorylation of α_S- and β-casein with acid phosphatase and incubation time

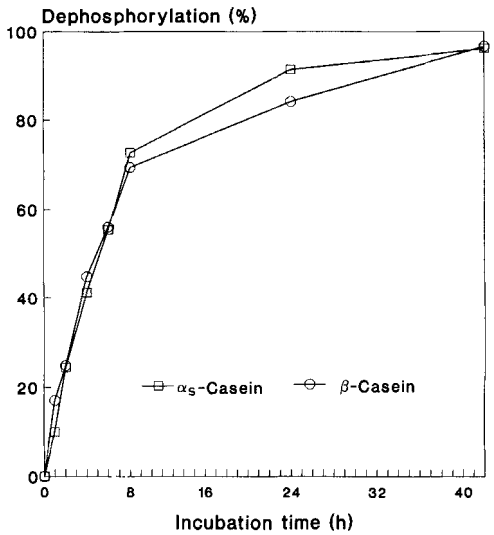

Fig. 3
Relation between dephosphorylation of α_S- and β-casein with alkaline phosphatase and incubation time

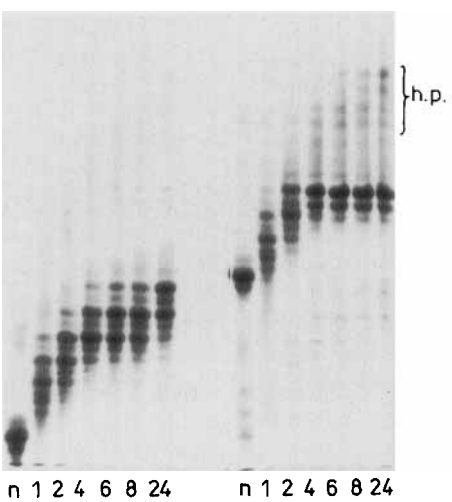

Fig. 2
Urea-polyacrylamide gel electrophoresis of dephosphorylated α_S-(left) and β-caseins (right) in dependence of the incubation time (acid phosphatase). Samples: n = native casein; 1–24 = 1–24 h of incubation; h.p. = hydrophobic peptides

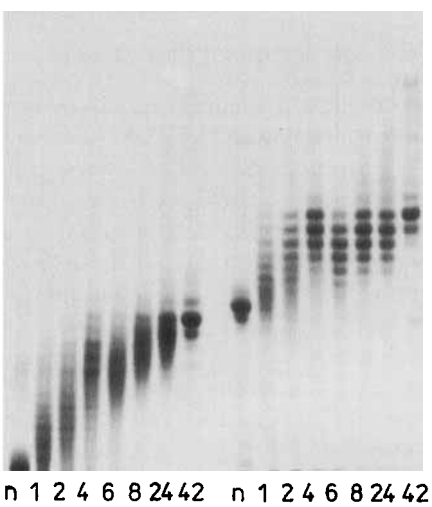

Fig. 4
Urea-polyacrylamide gel electrophoresis of dephosphorylated α_S-(left) and β-caseins (right) in dependence of the incubation time (alkaline phosphatase). Samples: n = native casein; 1–42 = 1–42 h of incubation

Table 1
Creaming behaviour (A/2-values) of o/w-emulsions stabilized with native or dephosphorylated casein fractions

Enzyme	Incubation time [h]	A/2-values [min]	
		α_S-casein	β-casein
None	0	32	16
Acid phosphatase	24	83	84
Alkaline phosphatase	24	75	79
Alkaline phosphatase	42	178	161

results it is obvious that enzymatic modification by phosphatases improves the emulsifying properties of a_S- and β-casein. The degree of dephosphorylation is correlated with the stability of emulsions prepared with the modified proteins. After an incubation of 42 h with alkaline phosphatase as a biocatalyst a 6-fold increase for dephosphorylated α_S- and a 10-fold increase in the A/2-values for dephosphorylated β-casein in relation to systems stabilized with the native proteins were found. With nearly the same degree of dephosphorylation, differences in the emulsifying properties of casein fractions found with the type of phosphatase used for incubation might be related to the specificity of these enzymes.

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