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# Influences on the film thickness in the enzymatic autodeposition process of casein

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ABSTRACT

A novel technique for the formation of biological coatings is presented. An enzymatic reaction is used to trigger the coagulation and specific deposition of protein particles on a support surface. As a model system, the major milk protein casein and the protease chymosin are used for a controlled deposition of casein biocoatings. The presented system is based on the natural enzymatic reaction used in cheesemaking and allows for a high control over the deposition process. For this purpose, chymosin is physically immobilized onto the support material and cleaves casein micelles in close proximity to the surface, initializing deposition of casein. This method shows characteristics of the autodeposition process of latex particles. Casein deposition takes place until the enzyme molecules are covered by cleaved casein micelles and are no longer able to cleave additional casein micelles. This paper reports the influence of reaction parameters such as reaction time, pH value, and casein concentration on the film thickness in the enzymatic autodeposition process. Film thickness of the deposited casein films increases up to 90 min of deposition time. After that, no significant increase of film thickness is observed. Higher casein concentrations and deposition in the acidic range of pH also result in higher film thickness.



## 1. Introduction

### 1.1. General remarks

Coatings based on biopolymers attained more and more attraction in the last years, since lots of efforts have been put forth into replacing fossil fuel-based polymers with polymers which are derived from natural and renewable resources [1–3]. This is evident from the fact that governmental recommendations regarding evaluation of environmentally friendly resources as well as a growing general awareness of sustainability are present [4]. Furthermore, biopolymers exhibit superior properties compared to synthetic polymers in many applications, such as biocompatibility, multifunctional surfaces, and biodegradability [5–7].

In this study, a new approach for the formation of protein films, by means of an enzymatic reaction, is presented. It uses the change in the solubility of certain biopolymers by an enzymatic reaction. This enzymatically controlled deposition of biopolymer films exhibits a great potential as a new kind of material design [8–10].

The herein described system is based on the milk protein casein and the aspartic protease chymosin. Casein is the predominant protein in milk of mammals and has a very long tradition for coating applications which go back as far as the time of ancient Egypt [11]. It consists of four main proteins, namely $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein [12]. In aqueous environments, caseins form complex micelles. The size distribution of casein micelles is naturally highly polydisperse and at the pH value of milk the diameter of casein micelles varies between 50 nm and 500 nm [13]. The inner core of the casein micelle consists of the predominantly hydrophobic $\alpha_{s1}$-, $\alpha_{s2}$-, and β-casein molecules. κ-casein which has a very amphiphilic structure is located on the surface of the micelle and provides the stability of the entire micelle [14]. The protruding hydrophilic part of κ-casein which is called caseinomacropeptide can be cleaved off by chymosin. The remaining hydrophobic para-κ-casein stays within the micelle [15]. Chymosin is known to be able to specifically cut the $Phe^{105}$-$Met^{106}$ peptide bond in κ-casein and drastically change the solubility of casein micelles [16,17]. By this cleavage reaction, casein micelles become hydrophobic and aggregation and precipitation of casein micelles result. This enzymatic process is used in industry for cheesemaking and occurs also as a natural process in the stomach of young mammals.

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Casein films for coating applications are basically obtained by conventional methods such as solution casting and spin coating applications [18–21]. Products made of casein provide good mechanical and gas barrier properties which are comparable to those of other used protein films, e.g. soy protein isolate and wheat gluten films [22]. Compared to other proteins, caseins have very specific physicochemical properties that are based on the very unique distribution of hydrophilic and hydrophobic regions in their protein chains. In combination with their flexible and mobile structure, caseins are very well suited for coating applications [23]. Caseins find application as biocompatible and protective layers in packaging, as adhesive layers in gluing applications, as binder for coatings, and exhibit a promising potential as biomaterial for medical applications, such as drug delivery systems [11,24,25].

### 1.2. Concept

In comparison to the aforementioned techniques, an autodeposition process for the formation of casein coatings is presented. A controlled deposition of casein and a subsequent film formation require an immobilization of chymosin onto the support surface. Thus, cleavage reactions of casein micelles occur only in close proximity to the support surface. By this, uncontrolled and unintended aggregation and precipitation of casein micelles are restricted. Due to hydrophobic interactions cleaved casein micelles deposit on the support, instead of precipitating in the bulk phase. Since chymosin is reversibly immobilized onto the support, diffusion of enzyme molecules into the solution takes place. As an advantage of this, chymosin is able to cleave casein micelles in a greater distance to the support surface. The amount of deposited casein increases with the ability of chymosin molecules to diffuse. Therefore, the film thickness of the casein coating is controllable by time of reaction, because with increasing reaction time a higher degree of diffusion of enzyme molecules occurs. Consequently, the amount of deposited casein micelles increases. Due to the characteristics of this process and with respect to the conventional autodeposition process of latex particles by dissolving metal ions of the support, we called this biobased process for the formation of biocompatible films *enzymatic autodeposition process*. The general concept is illustrated in Fig. 1.

Using the enzymatic autodeposition process, continuous casein films can be obtained on glass supports. Since the deposition of the casein micelles is enzymatically controlled, cleavage of the casein micelle's stabilizing κ-casein layer has taken place. The cleaved casein micelles show an increased hydrophobicity and are stable towards washing with deionized water, as previously shown in our publication [8]. In this complementary study, the dependence of the film growth from characteristic reaction parameters, which are time of reaction, pH value, and casein concentration, is investigated. The presented approach brings new aspects to the formation of casein coatings, because it utilizes the natural cleavage reaction of casein micelles by chymosin, offering a high degree of control over the deposition process.

## 2. Materials and methods

### 2.1. Materials

Casein from bovine milk and hemoglobin were purchased from Sigma-Aldrich and used without further purification. Calf rennet powder from Renco New Zealand was provided by the Swiss supplier Bichsel AG. The enzyme content of the provided calf rennet powder was 1.6 wt%, as detected by thermogravimetric analysis. In order to enhance the efficiency of the conducted deposition experiments, the chymosin content of the rennet powder was drastically

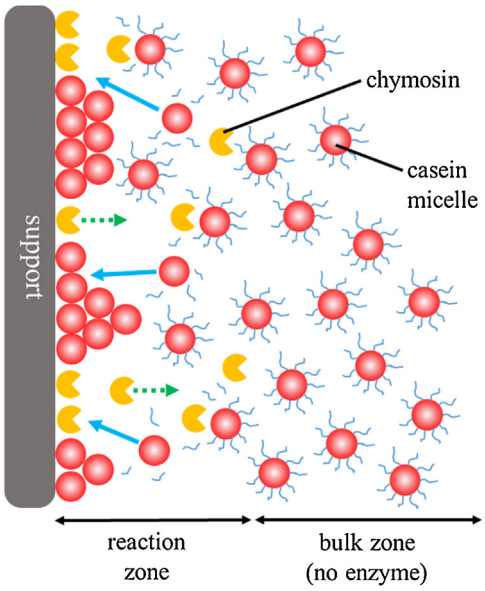


**Fig. 1.** General illustration of the enzymatic autodeposition process. Reversibly immobilized chymosin molecules have the ability to diffuse. The area where chymosin is present defines the reaction zone. Casein micelles which enter this zone can be cleaved by chymosin, become hydrophobic, and eventually deposit on the support surface. Dashed lines represent diffusion of chymosin molecules and solid lines depositing casein micelles, respectively.

increased by reduction of the salt content via ultrafiltration. The rennet powder was dissolved in deionized water at a concentration of 100 g/L and centrifuged three times at 5000 × *g* and 25 °C for 2 h, using Amicon® Ultra-15 Centrifugal Filter Devices with a cutoff of 3000 g/mol. By this, the enzyme content was increased up to 67 wt%. Microscope cover glasses were obtained from VWR and used as support material for casein deposition. All other chemicals were obtained from local suppliers and used without further purification.

### 2.2. Determination of enzymatic activity

The pH dependence of enzymatic activity of native chymosin was investigated in a range of pH 2–8 with hemoglobin as substrate in sodium hydrogen phosphate/citric acid buffers. Hemoglobin solutions with a concentration of 1% (w/v) were prepared by dissolving hemoglobin at the particular pH value at 25 °C in an ultrasonic bath for 15 min. The enzymatic reactions were started by the addition of 250 μL enzyme solution (0.1% (w/v)) to 500 μL hemoglobin solution. Reactions were carried out at 40 °C for 10 min and stopped by the addition of 500 μL trichloroacetic acid (10% (w/v)). After centrifugation of the reaction mixtures at 13,000 rpm for 15 min, 1 mL of the colorless supernatant was removed for absorbance measurements at 325 nm. Relative enzymatic activity was calculated by comparison with control samples which were prepared in the same manner as the test samples, except for that addition of enzyme solution to the hemoglobin solution was done after the reaction time and after the addition of trichloroacetic solution to the glass vessel.

### 2.3. Autodeposition of casein films with adsorbed chymosin

Microscope slides were cut into smaller pieces (15 mm × 75 mm) and cleaned with ethyl acetate and deionized water. Adsorption of chymosin was achieved by drying 600 μL of a solution of chymosin in deionized water (3 g/L) on a particular area (15 mm × 25 mm) of the glass support. After that, the glass slide with adsorbed chymosin was placed in a holder