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Changes in the apparent viscosity profiles of casein suspensions as affected by plant enzymes

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ABSTRACT

The aim of this work was to study the degree of hydrolysis and changes in the apparent viscosity of casein suspensions as a result of various enzymes addition. Suspensions with 3, 12 and 15 g/100 mL of casein, at pH 5.2, 6.0 and 6.5 were prepared in buffer solutions. Previous standardization; plant (papain and bromelain) and animal (chymosin) enzymes were added to hydrolize the casein suspensions. A control with no enzyme addition was used. The rheological behaviour was determined using a rotational rheometer (Haake RV20), with a cone and plate geometry. The Casson and the power law equations were applied to the data. The degree of hydrolysis was a function of the enzyme, pH and casein concentration, presenting chymosin the highest values. All enzymes showed the highest activity at acidic pH. Also, some substrate inhibition was observed. All samples behaved as non-Newtonian, shear-thinning systems with a yield stress value. In all cases, a significant increase in the viscosity was observed when shifting from 3 to 12 g/100 mL. Further increase in concentration caused an opposite effect. Changes in pH of the casein suspensions affected the viscosity, presenting maximum values at pH 6.0. The Casson equation fitted the results better than the power law model.

1. Introduction

Milk coagulation is the basic step in the manufacture of cheese. Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been the most widely used milk-clotting enzyme preparation. Increasing world cheese production has resulted in a bigger demand for rennet, giving as a result, rennet shortage and a rise in the prices. These circumstances has led to the search of substitutes of microbial origin, which has turned out to be a difficult challenge, since the bacterial and fungal proteases have not always been well adapted to the production of cheese (Bruno et al., 2010; Mohamed Ahmed, Babiker, & Mori, 2010). Also, increasing attention has been directed especially in developing countries, toward natural rennet extracted from plants such as *Ananas comosus*, *Carica papaya*, *Sylibum marianum* and *Calotropis porcera* among others (Bruno et al., 2010; Lo Piero, Puglisi, & Petrone, 2002; Yousif, McMahon, & Shammet, 1996). Papain (EC 3.4.22.2) is the term applied both to the raw enzymic preparations obtained from the latex of the in mature fruit of *Carica papaya,* and to different protein fractions. It has good stability at pH 5, but at a pH lower than 3 or higher than 11, the enzyme is very unstable. The papain is a sulfhydryl protease, its optimum pH depends on the substrate, e.g., for egg albumin and casein is about 7 and for jelly is 5. It has a wide specificity, since it can hydrolyse small peptides as well as proteins (Whitaker, 1994, pp. 481–483). The bromelain (E.C. 3.4.22.4) is produced by the plant of the pineapple (*A. comosus*), it is present both in the stem and in the fruit. Since the fruit has a commercial value as a food, only its stem and waste are used for the industrial production of this enzyme. What is known generically as bromelain, has several molecular forms with proteolytic activity, there are about six components obtained from the stem and two derived from the fruit. It is also a sulfhydryl enzyme which usually requires of an activator like cysteine to develop its maximum activity.

The caseins, considered the main target of coagulating enzymes during cheese making, are by definition the phosphoglucoproteins that precipitate from bovine milk at pH 4.6 and 20 °C. When milk is exposed to the action of rennet or chymosin, the casein micelles are destabilized and coagulate. The reaction of hydrolysis of casein by rennet is a complex phenomenon, that has been described in different phases and until today, it has not been totally elucidated (Fox, 1993).

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The concept of rheological behaviour of foods has different applications, foods flow properties determine the processing conditions and the type of equipment most suited to its manufacture. Viscosity measurements give information on both the enzymic reactions and aggregation step of proteins. Rheological models are useful to predict the range of shear, concentration and temperature suitable for food preparation with viscosities that allow industrial handling in processing equipments (Bourne, 2002, pp. 59–63).

The aim of this work, was to evaluate the degree of hydrolysis and the changes in the apparent viscosity of bovine casein suspensions with the plant enzymes papain and bromelain addition, and its comparison with bovine rennet (chymosin), at different substrate concentration and pH values.

2. Materials and methods

2.1. Materials

Acid casein for biochemical use was supplied by Merck. The enzymes papain and bromelain were obtained as purified powders of Sigma Chemical Co., St Louis, MO. Rennet (power 1:10,000) (chymosin) was obtained as a liquid commercial preparation for cheese making from a local company (Cuamex-Mexico).

2.2. Enzymes and samples preparation

Lots of casein suspensions with 3, 12 and 15 g/100 mL, at pH values of 5.2, 6.0 and 6.5 were prepared using buffer phosphates in deionized water. A control without enzyme addition was used. The theoretical effective volume fraction (Ø) of casein suspensions was obtained by multiplying the concentration in g/mL, by a factor **q** of 4.4 that accounts for the voluminosity of casein in the suspending medium (Dahbi, Alexander, Trappe, Dhont, & Schurtenberger, 2010).

2.3. Standardization of the proteolytic enzymes

To adjust the corresponding enzymes, the method of Kunitz (1974) was used. Samples of 1.9 mL of casein suspension in buffer of phosphates 0.05 mol/L, pH 7.6, with 1 g/100 mL, were incubated in a water bath at 36 ± 1 °C, then 100 μL of enzyme dissolved in deionized water were added. The final volume of the reaction was 2 mL, the aliquot of the enzyme had a concentration so that it yielded a final solution concentration of 25 μg/mL. The reaction solutions were incubated (36 ± 1 °C) one at a time by 5, 10, 20, 30, 45, 60, 75 and 90 min respectively, starting from the time when the enzyme was added. To stop the hydrolysis, 1 mL of 5 g/100 mL trichloroacetic acid (TCA) was added. Then, reaction solutions were centrifuged at 1000*g* during 15 min, the optical density of the supernatant was read in a Shimadzu Spectrophotometer model 1211553 at a wavelength of 280 nm. For each test, a control with no casein addition was also run. It was considered that the enzymes were standardized when the enzyme volume used for each case presented the same activity. The proteolytic activity was reported as μg of tyrosine liberated/(g enzyme × h). All tests were run in triplicate.

2.4. Hydrolysis of casein suspensions

To measure the degree of hydrolysis of casein, the above described procedure was followed, 100 μL of previously standardized enzyme solution were added per each 1.9 mL of casein suspension sample with the three different concentrations (3, 12 and 15 g/100 mL), at three pH values (5.2, 6.0 and 6.5) which were incubated in a water bath at 36 ± 1 °C, the reaction was run for 60 min, and then stopped by adding 1 mL of 5 g/100 mL TCA. The degree of hydrolysis was expressed as μg of tyrosine in solution.

2.5. Reological characterization

To obtain samples forrheological tests, the same procedure described above for hydrolysis of casein suspensions was followed, except that the ratio 1 mL of standardized enzyme solution per 19 mL of casein suspension with each different concentration was added. Once the reaction was stopped with TCA 5 g/100 mL, the samples were stored into a fridge at 4 ± 0.5 °C for 15 min, before running the rheological tests.

The rheological behaviour of the casein suspensions was found out using a controlled strain, rotational rheometer (Haake RV20 with accessory M5), with a cone and plate geometry (cone pk 100, needle pk 5, 1.0°, diameter = 40 mm), different values of shear rate (0.3–30 s^{-1}) were run registering the shear stress ($n = 8$), at 25 ± 0.5 °C, which was controlled using a water bath with recirculating water. The samples were left 30 min on the lower (fixed) plate to stabilize the temperature, followed by a conditioning step at 5 s^{-1} for 15 s before running each test that lasted about 5 min. The rheograms (flow and viscosity curves) were obtained from the data displayed by the machine.

2.6. Calculations

The power law equation, $\sigma = \mathbf{K}\gamma'^{\mathbf{n}}$, where **σ** is the shear stress (Pa), **K** is the consistency index (Pa.s^n), γ′ is the shear rate (s^{-1}) and **n** is the flow behaviour index (dimensionless), was applied. The apparent viscosity (**ηap**, Pa s) was obtained from the ratio **σ/γ′**. The Casson equation for non-Newtonian fluids was also applied: $(\sigma)^{0.5} = (\sigma_{0c})^{0.5} + (\eta\alpha\gamma')^{0.5}$; where σ_{0c} (zero-shear rate) and **ηα** (infinite-shear rate viscosity) were calculated from the intercept ($\mathbf{K_{0c}}$, Casson yield stress) and slope ($\mathbf{K_c}$) of a $\sigma^{0.5}$ versus $\gamma'^{0.5}$ plot respectively (Macosko, 1994, pp. 65–98). For the above two models, the determination coefficient ($\mathbf{R^2}$), i.e. the variability explained by the model, was evaluated using Minitab, Ver. 2 (1995).

2.7. Statistical analysis

The effect of the enzyme, casein concentration and pH, on the apparent viscosity, was evaluated by applying one way analysis of variance (ANOVA) (Montgomery, 1984, pp. 43–58), at the significance level of 5% ($^*P < 0.05$) using Minitab, Ver. 2.

3. Results and discussion

3.1. Degree of hydrolysis of casein

In all cases, chymosin hydrolysed casein in a larger extent (more tyrosine produced) than bromelain, which yielded more tyrosine than papain (Fig. 1). Except for papain at pH 6, all plots were similar in shape, the trend showed by chymosin and bromelain was to present maximum activity when the concentration was 3 g/100 mL, irrespective of the pH value, showing no real effect of solids concentration. Then, a high decrease in degree of hydrolysis was observed when shifting to 12 g casein/100 mL; chymosin increased thinly the casein hydrolysis at the last substrate concentration (15 g/100 mL), while in the case of papain this increase was notorious. Bromelain however, presented almost the same level of hydrolysis on the substrate at the highest solids concentration. It can be observed that in all cases, the optimum pH value of the reactions was 6.0, somehow different from the known optimum pH of casein hydrolisis for chymosin, which is between 3.8 and 4.0, but