ABSTRACT

High-intensity pulsed electric field (HIPEF) is a non-thermal food processing technology that is currently being investigated to inactivate microorganisms and certain enzymes, involving a limited increase of food temperature. Promising results have been obtained on the inactivation of microbial enzymes in milk when suspended in simulated milk ultrafiltrate.

The aim of this study was to evaluate the effectiveness of continuous HIPEF equipment on inactivating a protease from *Bacillus subtilis* inoculated in milk. Samples were subjected to HIPEF treatments of up to 866 μs of squared wave pulses at field strengths from 19.7 to 35.5 kV/cm, using a treatment chamber that consisted of eight colinear chambers connected in series. Moreover, the effects of different parameters such as pulse width (4 and 7 μs), pulse repetition rates (67, 89, and 111 Hz), and milk composition (skim and whole milk) were tested.

Protease activity decreased with increased treatment time or field strength and pulse repetition rate. Regarding pulse width, no differences were observed between 4 and 7 μs pulses when total treatment time was considered. On the other hand, it was observed that milk composition affected the results since higher inactivation levels were reached in skim than in whole milk. The maximum inactivation (81%) was attained in skim milk after an 866-μs treatment at 35.5 kV/cm and 111 Hz.

(Key words: high-intensity pulsed electric field, protease, enzyme, milk)

Abbreviation key: HIPEF = high intensity pulsed electric field, SM = skim milk, SMUF = simulated milk ultrafiltrate, WM = whole milk.

INTRODUCTION

The high-intensity pulsed electric field (HIPEF) process can achieve high levels of microorganism destruction in milk and dairy products (Dunn et al., 1987; Zhang et al., 1995; Pothakamury et al., 1995ab; Qin et al., 1998), maintaining the original flavor and nutrient content (Qin et al., 1995; Grahl and Märkl, 1996; Bendicho et al., 2002a). So, a great part of HIPEF research is focused on studying the feasibility of using this technology to pasteurize milk and dairy products.

Most of the studies carried out on dairy products have been performed to evaluate HIPEF effect on microorganisms. The level of microbial inactivation achieved with HIPEF treatment mainly depends on field strength and treatment time (Qin et al., 1995; Martín et al., 1997; Pothakamury et al., 1997; Reina et al., 1998). In relation to its effect on enzymes, some controversial results have been obtained. In several cases, large inactivation has been achieved, whereas in other cases no effect or even enhancement of initial activity has been detected (Vega-Mercado et al., 1995; Bendicho et al., 2001; Castro et al., 2001; Vega-Mercado et al., 2001; Bendicho et al., 2002b,c). However, various process factors have been reported as influencing enzyme activity. The variation in enzyme activity following HIPEF treatment depends on the field strength, treatment length, treatment temperature, equipment characteristics, type of enzyme, and the media containing the enzyme (Vega-Mercado et al., 1995; Castro et al., 2001; Vega-Mercado et al., 2001; Bendicho et al., 2002b,c). Some dairy enzymes whose behavior under HIPEF treatment has been evaluated suspended in simulated milk ultrafiltrate (SMUF) or in milk are alkaline phosphatase, plasmin, peroxidase, and microbial enzymes, such as proteases and lipases from *Pseudomonas fluorescens* or *Bacillus subtilis* (Vega-Mercado et al., 1995; Grahl and Märkl, 1996; Bendicho et al., 2001; Castro et al., 2001; Vega-Mercado et al., 2001; Bendicho et al., 2002b,c; Van Loey et al., 2002).
Castro et al. (2001) achieved up to 65% inactivation of alkaline phosphatase in SMUF or skim milk (SM) while just up to 59% in 2% fat milk or whole milk (WM), although Grahl and Märkl (1996) and Van Loey et al. (2002) did not observe any significant activity depletion in this enzyme after a HIPEF process. Grahl and Märkl (2002) did not observe any significant activity depletion of HIPEF on peroxidase, another milk enzyme, and observed no significant effects on its activity after subjecting the raw milk to different HIPEF treatments. On the other hand, the activity of a dairy protease, such as plasmin could be reduced up to 90% in SMUF by applying continuous flow HIPEF treatments (Vega-Mercado et al., 1995). These studies demonstrated that depending on the enzyme itself and the configuration of the HIPEF equipment used to treat the samples, enzyme activity can be highly modified or not.

Milk and dairy products may contain psychrotrophic microorganisms, which can cause important problems in the dairy industry since they can grow and maintain activity even at refrigeration temperatures. Among gram-negative bacteria, the genera Pseudomonas, especially strains of Pseudomonas fluorescens, are the most commonly encountered psychrotrophs in dairy products; Bacillus is representative of gram-positive psychrotrophic bacteria (Muir et al., 1979; Richard, 1981; Cousin, 1982; Driessen, 1983; Deeth and Fitz-Gerald, 1983). These species may secrete enzymes, such as lipases or proteases, that remain active even after the pasteurization process. The presence of lipases in milk causes a highly unpleasant rancid flavor, mainly due to the liberation of butyric acid after the hydrolysis of triglycerides (Andersson et al., 1981). On the other hand, proteases degrade caseins, implying losses in the yield of cheese, an increase of nitrogen content in the whey, and technologically, reduction of milk’s thermal stability (Gebre-Egziabher et al., 1980; Veisseyre, 1988). Bendicho et al. (2002b) studied the effect of HIPEF processing on a lipase from Pseudomonas fluorescens suspended in SMUF. A maximum of 62.1 and 13% inactivation was reported for a batch and continuous process, respectively, applying similar levels of energy densities. On the other hand, a protease also from Pseudomonas fluorescens was studied by Vega-Mercado et al. (2001), who achieved up to 60% inactivation in SM, but no significant effects were observed when the enzyme was suspended in a casein-Tris buffer.

The behavior of a protease from Bacillus subtilis after exposure to HIPEF also was evaluated (Bendicho et al., 2001; Bendicho et al., 2002c). It is known that this enzyme can be near completely inactivated (96%) after a heat pasteurization treatment (75°C, 15 s; Bendicho, 2002). However, inactivation levels achieved by HIPEF were not very high for this enzyme in preliminary studies made in SMUF and milk, applying HIPEF treatments in batch or continuous mode at energy densities of up to 500 kJ/L (Bendicho et al., 2001). Further investigations made with continuous flow equipment applying much higher input energy densities achieved very promising results on the inactivation of this protease in SMUF (Bendicho et al., 2002c). The aim of this paper is to elucidate the effectiveness of high-energy density continuous flow HIPEF treatments on inactivation of this protease inoculated in SM or WM. The effects of media composition and HIPEF parameters (treatment time, field strength, pulse width, and frequency) were also evaluated.

**MATERIALS AND METHODS**

**Sample Preparation**

This study was performed in SM and WM provided by Granja Castelló S. A. (Mollerussa, Spain). Protease from Bacillus subtilis [EC 3.4.24.28] was purchased in liquid commercial form (Aldrich, Steinheim, Germany). According to the supplier information, it contained 728 U/ml protease activity. Enzyme activity was expressed in milliunits per milliliter after verifying a linear correlation between enzyme activity in milliunits per milliliter (from commercial information) and enzyme concentration in milligrams per milliliter.

Before the application of treatments, milk was added to 50 mU/ml of protease.

**Enzyme Determination**

**Reagents.** Azocasein was obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dihydrogenphosphate, disodium hydrogenphosphate, and trichloracetic acid were from Prolabo (Fontenay S/Bois, France).

**Solutions.** Before running the analysis to determine protease activity, a pH 7.2 buffer solution was prepared mixing 36 ml of sodium hydrogenphosphate 5 mM and 14 ml of sodium dihydrogenphosphate 5 mM and diluting with distilled water until 1000 ml. This buffer was used to prepare a 1% azocasein solution. A 5% TCA aqueous solution was also required.

**Protease activity determination.** The assay for proteolytic activity, using azocasein as substrate, was described by Christen and Marshall (1984) and has been validated for use in SMUF, SM, and WM by Bendicho et al. (2002d). It consisted of combining 1 ml of azocasein solution with 100 μl of the sample containing the enzyme. The contents of the tubes were mixed and incubated at 35.5°C for 15 min. The reaction was stopped by adding 2 ml of 5% TCA. The absorbance of the supernatant fraction was read at 345 nm in a...
spectrophotometer UV/Visible (CECIL, CE 1021, England).

**Pulsed electric field treatments.** HIPEF treatments were carried out using a continuous bench scale system (OSU-4F, Ohio State University) using square wave pulses. The treatment chamber consisted of eight colinear chambers in series, and each contained two stainless steel electrodes separated by a gap of 0.29 cm whose treatment volume was 0.012 cm³. The flow rate of the process was adjusted to 60 ml/min and was controlled by a variable speed pump (model 75210-25, Cole Palmer, Vernon Hills, IL). The product was refrigerated in the space provided between the chambers by means of ice water. The temperature of the entrance and exit of the treatment chamber was recorded by the equipment and never exceeded 46°C.

Experiments were first carried out in SM to discover the conditions that reached the maximum levels of enzyme inactivation. Samples were subjected to monopolar HIPEF treatments of up to 866 μs at field strengths from 19.7 to 35.5 kV/cm. The effectiveness of two different pulse widths (4 or 7 μs) and three pulse repetition rates (67, 89, and 111 Hz) were also tested. The effect of milk composition was evaluated by comparing the results obtained with SM to results obtained exposing the WM samples up to 866 μs at maximum field strength (35.5 kV/cm) using 7-μs pulses at the three pulse repetition rates evaluated (67, 89, and 111 Hz).

The input energy density (Q, J/L) supplied to the samples can be computed by [1] (Martín et al., 1994):

\[
Q = \frac{V_0 \cdot I \cdot t}{v}
\]

where \(V_0\) is the peak voltage (V), \(I\) the intensity of the current (A), \(t\) the treatment time, and \(v\) the combined volume of all treatment chambers (l).

**Statistics.** Each processing condition was assayed in duplicate and enzyme determination was also performed in duplicate. Therefore, the results were the averages of four measurements. Enzyme activity was expressed as relative activity [RA(%)] computed using [2]. The percentage of enzyme inactivation I(%) can be calculated by [3]:

\[
RA = 100 \cdot \frac{A_t}{A_o}
\]

\[
I(\%) = 100 - RA
\]

where \(A_t\) is the enzyme activity in the sample after treatment, and \(A_o\) is the enzyme activity of the untreated sample, which was determined immediately after processing to avoid the effects of storage.

Analysis of variance was used to determine significant differences among the treatments (\(P < 0.05\)) and was performed by Statgraphics plus version 3 for Windows package (Statistical Graphics Co., Rockville, MD).

Experimental values were adjusted to a first order equation related to energy density [4]:

\[
RA = RA_0 \cdot e^{-k_1 \cdot Q}
\]

where \(RA_0\) is the intercept of the curve (100%), \(k_1\) (l/ kJ) a first-order constant, and \(Q\) the input energy density (kJ/l).

**RESULTS AND DISCUSSION**

Studies to evaluate the influence of several HIPEF parameters (field strength, treatment time, treatment energy, pulse duration, and pulse frequency) on the effectiveness of HIPEF were initially performed in SM to avoid possible problems due to the fat presence. After establishing the suitable conditions for inactivating the enzyme in SM, HIPEF treatments were applied to WM.

The activity of the enzyme in skim milk decreased up to 81.1% after applying the most severe evaluated treatment, 866 μs at 35.5 kV/cm and 111 Hz. So, the evaluated HIPEF process conditions led to considerable levels of enzyme inactivation. Thus, HIPEF caused enough changes in the enzyme configuration to reach denaturation, and due to alteration in the enzyme shape, the substrate could not fit the active site, preventing conversion of the substrates into products. Changes in enzyme structure might be caused by changes in the conformational state of proteins due to the application of electric fields, since charged groups and structures are highly susceptible to various types of electric field perturbations, and these changes cause a loss of its structure and consequently the loss of activity due to difficulty assembling the substrate with the active site (Tsong, 1990). The resistance of the enzyme may depend on the number of hydrogen bridge bonds, the amino acids that contains the structure that can vary its hydrophobicity and the volume of several parts of the molecule, and on the perturbations around the site of the metal (Ca²⁺) that needs the enzyme to be active.

**Effects of Field Strength, Treatment Time, and Input Energy Density**

Protease activity varied with change in treatment time and electric field intensity (\(P < 0.05\)) when SM samples were exposed to HIPEF treatments at 67 Hz.
Inactivation obtained at 19.7, 23.7, and 27.6 kV/cm showed analogous behavior patterns ($P < 0.05$), reaching a maximum of 14.8% inactivation after the 866-μs treatment. The maximum level of protease inactivation at 67 Hz was 37.9%, achieved after the 866-μs treatment at 35.5 kV/cm (Figure 1).

Bendicho et al. (2002c) studied the effects of similar HIPEF treatment on this protease suspended in SMUF, also observing that, in general, enzyme activity decreased with field strength and treatment time, although in that case the maximum inactivation achieved at a pulse repetition rate of 67 Hz was 48% after treatment of 895.8 μs at 35.5 kV/cm. Other enzymes studied in milk or SMUF, such as a lipase from *Pseudomonas fluorescens*, plasmin, or phosphatase alkaline showed analogous patterns, since when subjected to HIPEF-processing activity decreased with increase of field strength and treatment time (Castro et al., 1994; Vega-Mercado et al., 1995; Bendicho et al., 2002b). However, the effect of HIPEF on other enzymes, such as proteases from *Pseudomonas fluorescens* or *Bacillus subtilis* did not show the same patterns, since depending on the HIPEF conditions, the effect varied from some decrease to no significant variation or even enhancement of the enzyme activity (Bendicho et al., 2001; Vega-Mercado et al., 2001).

The most severe evaluated treatment at 67 Hz (35.5 kV/cm for 866 μs) delivered 6560 kJ/L to the samples, and led to maximum levels of enzyme inactivation (37.9%) (Figure 2), whereas in SMUF a similar treatment of 6787 kJ/L achieved up to 48% inactivation (Bendicho et al., 2002c). However, treatments of lower energy (<500 kJ/L) did not achieve large levels of enzyme inactivation (0 to 13%) in milk or in SMUF (Bendicho et al., 2001). The activity of a lipase from *Pseudomonas fluorescens* also decreased with increase of input energy density. After a batch or a continuous flow mode treatment of 504.97 kJ/L and 424.36 kJ/L, respectively, up to 62.1 and 13% inactivation was achieved, in which case the treatment temperature never exceeded 35°C (Bendicho et al., 2002b).

The decrease of relative protease activity in SM could be successfully related to the supplied energy densities ($Q$) using a first-order kinetic model [4], leading to a k-value of $6 \times 10^{-5}$ L/kJ (Figure 2):

$$RA = 100 \cdot e^{-6 \times 10^{-5} Q} \quad (R^2 = 0.846). \quad [5]$$

The decrease of protease activity with increase of input energy when the enzyme was suspended in SMUF was also studied by Bendicho et al. (2002c). In that case, the activity also lowered exponentially with the applied energy density leading to a k-value of $7 \times 10^{-5}$ L/kJ, a value similar to that obtained when protease was suspended in SM. Decreased enzyme activity with input energy density after HIPEF treatments of another microbial enzyme, lipase from *Pseudomonas*, also fitted a first-order model (Bendicho et al., 2002b). In that case, the k-values obtained were $1.9 \times 10^{-3}$ L/kJ for a batch-mode treatment and $2 \times 10^{-4}$ L/kJ for the continuous one, indicating that protease is more resistant to HIPEF treatment since it requires higher energy density to achieve the same levels of inactivation than lipase.

**Effects of Pulse Duration**

To evaluate the influence of pulse duration on HIPEF effectiveness, treatments at 35.5 kV/cm up to 866 μs and 67 Hz using pulses of 4 or 7 μs were conducted. No significant change in enzyme inactivation was observed when total treatment time was considered, whereas
when enzyme activity was related to the number of pulses, there was a significant difference between the inactivation achieved using 4- or 7-μs pulses. It should be noted that the 4-μs-pulse-width process requires a lot more pulses than the 7-μs pulse width one to achieve similar cumulated treatment time. Thus, longer pulses might be considered more effective because similar inactivation can be reached with the application of fewer pulses (Figure 3). Bendicho et al. (2002c) did not observe any significant difference between protease inactivation achieved subjecting SMUF samples to processes of 4- or 7-μs pulses.

Effects of Pulse Frequency

The effectiveness of HIPEF at different frequencies was studied in SM and WM. While maintaining a constant electric field strength (35.5 kV/cm) and applying the same treatment times, different pulse repetition rates (67, 89, and 111) were tested. For both SM and WM, when similar treatments in cumulated time and field strength were applied, it was observed that the higher the frequency pulse rate the higher the inactivation achieved.

In SM, inactivation levels varied from 37.9 to 81.1%, depending on whether treatments of 35.5 kV/cm for 866 μs were performed at 67 or 111 Hz. In WM, both mentioned treatments led to 37.9 and 57.1% inactivation, respectively.

Temperature might not be the cause of increased inactivation as other authors suggested (Van Loey et al., 2002), since maximum treatment temperature achieved at 67-Hz treatments was 34 to 37°C and for 89 and 111 Hz was 39 to 44°C and 41 to 46°C, respectively. These increases are not enough to justify the rise in enzyme inactivation, since previous studies on thermal inactivation of protease in SM showed that treatments of up to 30 min at 50°C only reached inactivation levels of about 12 to 13% (Bendicho et al., 2001).

A similar study performed in SMUF held a similar trend, since the higher the frequency rate of pulse application the higher the inactivation obtained for treatments of equal field strength and treatment time. In that case, the maximum inactivation was reached after a 111-Hz treatment at 35.5 kV/cm and 866 μs, which was 62.7% (Bendicho et al., 2002c).

Bendicho et al. (2001) also reported a similar pattern of inactivation of the protease suspended in SMUF applying treatments of much lower input energy densities using continuous flow equipment provided for a coaxial treatment chamber. In that case, treatments up to 500 kJ/L and pulse repetition rates from 2 to 4 Hz were applied. When subjecting the samples to treatments of similar energy, it was observed that the higher the frequency, the higher the inactivation levels achieved. Other authors also reported differences in enzyme inactivation between treatments applied at different frequencies. Vega-Mercado et al. (2001) reported a reduction of up to 60% for a protease from *Pseudomonas fluorescens* suspended in milk using low field strengths (14 to 15 kV/cm) and frequencies of 1 and 2 Hz, whereas at lower frequencies (0.6 Hz) and higher field strengths (25 kV/cm), an enhancement of proteolytic activity was observed. For a lipase from *P. fluorescens* suspended in SMUF, when applying processes of similar energy, Bendicho et al. (2002b) observed that the higher the pulse repetition rate of treatment the higher the level of enzyme inactivation. An 80-pulse treatment at 37.3 kV/cm and 2 Hz led to nearly 7% inactivation, whereas applying the same electrical conditions at 3.5 Hz resulted in decreased activity up to 13%.

Effects of Milk Composition

The effectiveness of HIPEF on inactivating this protease was evaluated in SM and WM by applying treatments of up to 866 μs at 35.5 kV/cm at three different frequencies (67, 89, and 111 Hz).

At the lower frequency (67 Hz), no significant differences were observed among the results of inactivation achieved with SM or WM ($P < 0.05$). This similarity among inactivation results for the two different media might be due to the low levels of inactivation achieved under the HIPEF treatment conditions (35 to 38%). In previous studies performed on this enzyme suspended in SMUF, Bendicho et al. (2002c) reported a similar inactivation pattern of inactivation after treatments under similar conditions of treatment time and field strength, although the maximum inactivation achieved
Figure 4. Inhibition of relative protease activity (RA) in skim milk (black) and whole milk (white) exposed to 67, 89, and 111-Hz high-intensity-pulsed-electric field treatments. Treatments were performed at 35.5 kV/cm using pulses of 7 μs.

in SMUF was slightly higher (48%) than that obtained in milk (P < 0.05).

When HIPEF was applied at intermediate frequencies (89 Hz) and at the highest frequencies (111 Hz), more differences in the effectiveness of treatment were observed, in which the inactivation obtained with WM was lower than that with SM. It should be the fat content that interferes with the process, protecting the enzyme and making its structure more stable, whereas in WM up to a 38.9% inactivation was attained after an 866-μs treatment at 89 Hz, with SM up to 64.4% inactivation was reached. The highest levels of enzyme inactivation for both media were achieved when samples were subjected to 111-Hz treatments. In that case, samples of WM led to inactivation levels of about 57.1%, whereas in SM up to 81.1% inactivation was observed (Figure 4).

Other authors also reported differences among enzyme inactivation in different media. Castro et al. (1994) studied the HIPEF inactivation of alkaline phosphatase in SMUF, nonfat milk, 2% fat milk, and WM. HIPEF treatment was able to reduce up to 65% of alkaline phosphatase activity after 70 pulses at 22 kV/cm in SMUF and at 18.8 kV/cm in SM. When 2% milk and WM were treated, alkaline phosphatase activity was reduced up to 59% after a 70-pulse treatment at 18.8 kV/cm. For microbial proteases, Vega-Mercado et al. (2001) achieved up to 80% inactivation of an extracellular protease from Pseudomonas fluorescens when the enzyme was suspended in tryptic soy broth enriched with a yeast extract after a HIPEF treatment of 20 pulses at 18 kV/cm at 0.25 Hz. Inactivation levels changed significantly when the media was SM, in which low field strengths (14 and 15 kV/cm) and frequencies of 1 and 2 Hz reached up to 40 and 60% reductions after 32 and 98 pulses, respectively. No significant inactivation of protease or significant change in susceptibility of the casein to proteolysis was observed. This indicates that casein has a protective effect against HIPEF treatment.

For microorganism destruction, proteins have been reported to have a protective effect (Martín et al., 1997). However, protease inactivation was more effective in a medium with proteins, since with SMUF (fat- and protein-free solution) and SM (fat-free emulsion) the highest levels of enzyme inactivation after similar treatments were achieved in SM. Therefore, the presence of proteins may enhance the effect of HIPEF treatments (Bendicho et al., 2002c). However, the fat content has been demonstrated to diminish the effect of HIPEF, since in WM less reduction of enzyme activity was observed than in SM after the same HIPEF treatments.

This work has proven that the activity of this bacterial enzyme in milk can be reduced by HIPEF treatment. Among the conditions evaluated, maximum inactivation (81.1%) was reached when applying up to 6560 kJ/L (866 μs at 35.5 kV/cm) at 111.11 Hz. However, further investigation is required to achieve more effective treatments and to evaluate how HIPEF affects the internal configuration of the enzyme to decrease its enzymatic activity.

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