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EFFECT OF HIGH VOLTAGE ELECTRIC FIELD ON FOOD GRADE PAPAIN

EFECTO DEL CAMPO ELÉCTRICO DE ALTO VOLTAJE EN PAPAÍNA GRADO ALIMENTARIO

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Abstract

In this study, the enzyme papain was treated under different conditions of High Voltage Electric Field (HVEF), where three main factors were varied: frequency (0, 180 and 360 Hz), electric field strength (1, 5, and 9 kV / cm), and processing time (10, 20 and 30 minutes). The objective was to determine the changes in the activity of the enzyme and in its structure. The results mainly showed an effect of reduction on the residual activity (RA) of the enzyme papain. The best effect on the RA (47.9%) was achieved under the most intense treatment conditions of EF (9 kV/cm) and time (30 min). The results in the structure analysis of papain by fluorescence spectroscopy showed that HVEF led to denaturation and possibly aggregation of the enzyme. Therefore, HVEF can be considered as an efficient alternative nonthermal technology that can be used for partial enzyme inactivation.

Keywords: papain, residual activity, high voltage electric field, frequency, time.

Resumen

En el presente trabajo la enzima papaína fue tratada bajo diferentes condiciones de Campo Eléctrico de Alto Voltaje (CEAV), donde se aplicaron tres factores principales: frecuencia (0, 180 y 360 Hz), intensidad de campo eléctrico (1, 5, y 9 kV/cm) y tiempo de proceso (10, 20 y 30 minutos). El objetivo fue determinar los cambios en la actividad de la enzima, así como en su estructura. Los resultados principalmente mostraron un efecto de reducción en la actividad residual (AR) de la papaína. El mejor efecto en la AR (47%) se logró bajo las condiciones de tratamiento más intensas de CE (9kV/cm) y tiempo (30 min). Los resultados del análisis en la estructura de la papaína por espectroscopia de fluorescencia demostraron que el CEAV propició desnaturalización y posiblemente agregación de la enzima. Por lo tanto, se puede concluir que el CEAV es una tecnología no térmica alternativa eficaz para una inactivación enzimática parcial.

Palabras clave: papaína, actividad residual, campo eléctrico de alto voltaje, frecuencia, tiempo.

1 Introduction

Papain (EC 3.4.22.2) is a proteolytic enzyme found in the latex of the tropical fruit papaya (Carica papaya), which is grown intensively in Mexico and other tropical countries (Carballo-Sanchez *et al*., 2016). The enzyme papain is probably the most studied member of the family of cysteine proteases and was the first enzyme of its kind whose tridimensional structure was determined (Rawlings & Salvesen, 2013). It was shown that the polypeptide chain is constituted by two domains of approximately equal size but different conformation. The L domain consists of residues 10-111 and 208-212, and is mainly composed of alpha helix, while the domain R consists of residues R 1-9 and 112-207 (Kumar *et al*.2009),

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and it is mainly made of antiparallel β sheets in its structure. The active site is formed by the catalytic triad Cys-25, His-158 and Asn-175 (Kumar, Sathish, & Prakash, 2009).

In regards to the applications of the enzyme, papain has had an impact on textile, pharmaceutical, detergent and food industry (Llerena-Suster *et al*., 2012). In the food industry, papain has been applied as a meat tenderizer, as a clarifying agent in beer, in the digestion of proteins, in pharmaceutical preparations, and in confectionery for the production of sweets and desserts (Amri & Mamboya, 2012). Due to its multiple applications and the emergence of new processing technologies, the enzyme has been studied in its pure form under different conditions by non-thermal technologies such as Pulsed Electric Fields (PEF), High Hydrostatic Pressure and Ultrasound in order to determine changes in activity and structure. (Yeom *et al*., 1999, Katsaros *et al*., 2009 and Zhi-Long *et al*., 2014). High Voltage Electric Field (HVEF) is a relatively new non-thermal technology that has the following general characteristics: a) treatment time measured in minutes, hours or days, b) use of an electrostatic field generated by corona discharge, electrical or electro hydrodynamic field, and c) it does not increase the temperature in the treated sample (Palanimuthu *et al*., 2009, Zaho *et al*., 2011 and Hsieh *et al*., 2008). This technology has been applied in apples resulting in a delay in CO2 production, a suppression of the climacteric peaks and changes in the respiration rates (Electric Field Amplitude 600 kV/m; 7 days of treatment; 0, 10 and 23 °C and relative humidity of 75%). In the emblica fruit or Indian gooseberry, benefits were also observed, such as a decreased weight loss and increased shelf life after treating the fruit with HVEF (EF 430 kV/m, 2 hours, relative humidity=65% and treatment temperature of 25 °C). In carrot juice treated with HVEF, promising results were also obtained in the content of phenolic compounds, and in the case of pears, plums and bananas, this treatment slowed the respiration rate during the climacteric periods (Atungulu *et al*., 2004; Bajgai *et al*., 2006; Hsieh & Ko, 2008; Esehaghbeygi & Basiry, 2011). Other examples of the application of HVEF are on rice, wheat, wine, cranberry, avocado and food enzymes (Cao *et al*., 2004, Zeng *et al*., 2008, Palanimuthu *et al*., 2009, Ariza-Ortega *et al*., 2013). In the latter, the effect of the electric field on polyphenol oxidase of fungal origin (10 kV/cm, 0-6 minutes, 0-950 Hz) and polyphenol oxidase from avocado (9 kV/cm, 3 min, 0-760 Hz) were analyzed. The RA values of 12% and 15% were respectively obtained after treatment (Castorena-García *et al*., 2013a). For the enzyme polygalacturonase, the effect of the electric field (25, 45, 550 Hz; 5, 10 and 15 minutes; 15 kV/cm) was also determined, finding the best effect (39% of RA) at 45 Hz, 15 kV/cm and 5 minutes (Castorena-García *et al*., 2013b).

In recent years, the use of HVEF at a pilot scale in various food products has increased because of its effectiveness. However, despite advances in research, there is currently little information about the application of this technology in proteolytic enzymes, and in particular in papain. Of the latter enzyme, there is only information about partial inactivation by PEF (Yeom *et al*., 1999). Therefore, in this work, the effect of HVEF on the residual activity (RA) and the possible structural changes in the papain were assessed.

2 Materials and methods

2.1 Materials

Maltodextrin-standardized food grade papain with an activity between 90-110 UTyr (Enmex, S.A. de C.V., Tlalnepantla, Mexico) was used for the study. All reagents used in this investigation were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2 Methods

2.2.1 Sample preparation

Food grade papain was used at a concentration of 25 mg/mL, and deionized water were used to prepare the working solution. The protein concentration (6.8%) in the commercial enzyme was determined by the Kjeldahl method.

2.2.2 High Voltage Electric Field equipment

The equipment used in this research was designed and built by the staff at the Center for Research in Applied Biotechnology (CIBA-IPN) as previously described (Castorena-García *et al*., 2013a). Experiments were performed without allowing electric arcing. The distance between electrodes was constant and it was determined according to the height of the sample holder box. It is important to emphasize that in this equipment, the sample to be treated does not touch the electrodes and, consequently, no increase

in temperature is generated (Castorena-García *et al*., 2013a).

2.2.3 Determination of enzyme activity

The enzyme activity was determined by the method of Kunitz (Kunitz, 1947) using casein as substrate and following the release of aromatic amino acids by UV spectrophotometry (UV-VIS spectrophotometer, Genesys 10S, Thermo Scientific) at 280 nm in the 5% trichloroacetic acid supernatants after centrifugation of the incubation mixtures. Results were expressed as a percentage of the original activity (residual activity or RA).

2.2.4 Fluorescence spectroscopy

2.2.4.1 Surface hydrophobicity

The determination of surface hydrophobicity (H0) was performed on the treated and untreated samples. For the treated samples, only those which were observed to have the lowest, intermediate and highest effect on the RA of papain were analyzed. To determine H0, the 1-anilinonaphthalene-8-sulfonate (ANS) hydrophobic probe was used. For the fluorescence determinations, an ISS K2 Spectrofluorometer (Champaign, IL USA) equipped with a Peltier type temperature controller was used, which was also coupled to a Fisher Scientific Recirculating Water Bath (Model 90). The protein was used at a 1 μM concentration in deionized water. Emission excitation was at 380 nm and emission spectra were recorded between 400 and 600 nm with a slit width of 16 and 8 nm for excitation and emission respectively (Edwin & Jagannadham, 1998).

2.2.4.2 Intrinsic fluorescence

For the intrinsic fluorescence (IF) determinations, the ISS K2 Spectrofluorometer equipped with a Peltier type temperature controller was used, which was also coupled to a recirculating water bath from Fisher Scientific (Model 90). In this case, the excitation was performed at 282 nm, the emission was recorded in the 285-500 nm region with a slit width of 16 nm for excitation and 8 nm for emission (Edwin & Jagannadham, 1998).

2.2.5 Statistical analysis

A completely randomized 3^3 factorial experimental design was used with the following factors: electric Field (1, 5 and 9 kV/cm), frequency (0, 180 and 360 Hz) and time (10, 20 and 30 minutes). For the regression analysis, Minitab® v17.3.1 software was used to determine the effect of HVEF process variables on the RA of papain. All the tests were performed using a significance level $\alpha = 0.05$.

3 Results and discussion

3.1 *Effect of HVEF on the RA of papain*

Non-thermal processes have gained importance in recent years as potential technologies to replace or complement the traditional thermal processes. Compared to thermal processes, non-thermal processes offer the advantage of handling relatively low processing temperatures, lower energy usage, retention of flavors, nutrients and freshness in addition to being able to inactivate microorganisms and enzymes, which is critical in the processing and preservation of foods. There are non-thermal technologies, such as PEF, capable of decreasing the enzymatic activity. Thus, there are reports on PEF-susceptible enzymes such as peroxidase, polyphenoloxidase and vegetable-origin pectin methylesterase, where RA percentages of only 0-15% have been reached. On the other hand, there are more resistant enzymes such as ascorbic acid oxidase and carrot peroxidase in which case, the observed RA was of 50-80% (Zhao *et al*., 2012; Leong *et al*., 2014). The results obtained in this study with HVEF treatment can be seen in Table 1 and Fig. 1, where a reduction in RA is observed in most cases. Only two cases where there was an increase in RA were observed: the case of 104.1% obtained at 1 kV/cm, 360 Hz and 10 min of treatment, and the case of 103.4% at 1 kV/cm, 0 Hz and 10 min. In yeast enolase, an increase in RA was also observed (approximately 107%) by applying 13 kV/cm and 150 seconds of PEF treatment (Ohsima *et al*., 2007). However, although the interaction between treatment and enzymes generate an interesting increase in RA, the activation mechanism is still not clear in the literature (Ohsima *et al*., 2007). In regards to the variation of papain RA in this study, a progressive decrease was observed when EF and time increased. Shorter times and lower EFs resulted in a minor effect on the RA. For example, in the case where 5 kV, 180 Hz and 20 minutes were applied, an 86% of RA was obtained; and as the EF and time increased, the effect on RA was higher (9 kV, 180 Hz and 20 minutes, 80.8% of RA). The most important result obtained in

this research was a 47.9% of the RA (9 kV/cm, 180 Hz, 30 minutes). Comparing these results to those obtained by Yeom *et al*. (1999) where the following measures were used: a field of 10-50 kV/cm, pulse duration of 4 μs, 1500 pulses, flow rate of 0.77 mL/s stored for 0, 24 and 48 hours, obtaining a 10% of RA at the time of 48 hours; the importance of using common factors, such as the electric field and the time to achieve effectiveness in inactivation, is denoted. Data were adjusted to a 3FI quadratic model, which was highly significant ($p \leq 0.01$) with an adjusted coefficient of determination R^2=0.7. See Eq. (1).

$$RA = 101.076 - 0.357EF - 0.00051F + 0.061t - 0.129EFt \quad (1)$$

In this model, the interaction between EF and time (t) was highly significant ($p \leq 0.01$) and had a 97% contribution to the variability of the response (RA). In the literature, it has been stated that the enzymatic activity of the pepsin begins to decline when the treatment time with high density pulsed electric field is increased, which is considered as the critical treatment time. This behavior is consistent with the information obtained in this work (Zhao & Yang, 2009). As shown in Table 1 and Fig.1, the effect in the RA of papain was higher during the most prolonged treatment time and at the highest field intensity, indicating that the HVEF technology could be also an important alternative for enzyme inactivation.

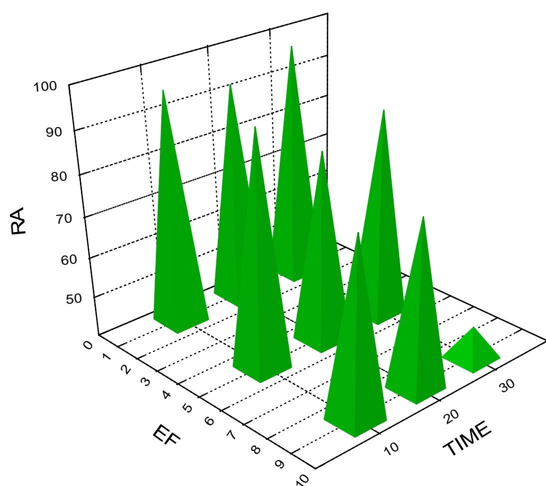

Fig. 1. Changes in the residual activity (RA) (%) of papain with time(min) and electric field intensity (kV/cm) at a frequency of 180 Hz.

Table 1. Residual activity (RA) of HVEF treated papain.

EF (kV/cm)	Frequency (Hz)	time (min)	RA (%)
0	0	0	100.0
1	0	20	98.0
1	0	30	99.0
1	0	10	103.4
1	180	20	92.9
1	180	10	96.1
1	180	30	97.5
1	360	20	97.3
1	360	30	98.3
1	360	10	104.1
5	0	30	79.5
5	0	20	87.2
5	0	10	94.7
5	180	20	86.0
5	180	30	90.3
5	180	10	96.5
5	360	20	78.2
5	360	30	91.8
5	360	10	96.2
9	0	20	71.3
9	0	30	79.8
9	0	10	83.0
9	180	30	47.9
9	180	20	80.8
9	180	10	83.4
9	360	30	58.9
9	360	20	78.2
9	360	10	91.2

3.2 Fluorescence spectroscopy

3.2.1 Surface hydrophobicity

The surface hydrophobicity of proteins (H0) is a function-structure relation dependent on the size and shape of the protein molecule, the sequence and composition of amino acids, and the presence of intramolecular or intermolecular cross-linking. H0 is one of the most important properties of proteins, for it allows the detection of changes in the distribution of hydrophobic groups on the surface of such proteins. These changes are caused by alterations in the structure of these molecules with different degrees of denaturation.

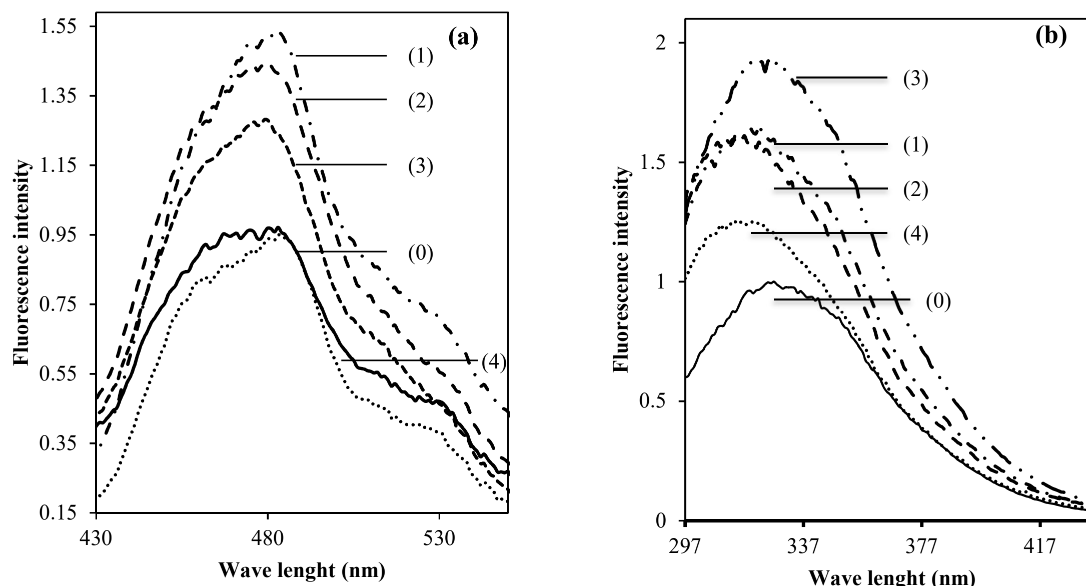

Fig. 2. Effect of the HVEF treatment on the (a) surface hydrophobicity and (b) intrinsic fluorescence of food-grade commercial papain in deionized water. 0. (—) Blank 1. (— ·) 9kV/cm, 0 Hz 20 min, RA= 70.7%, 2. (- - -) 9 kV/cm, 180 Hz, 30 min, RA=47%, 3. (– ··) 1.0 kV/cm, 360 Hz, 10 min, RA= 104.2 %, 4. (· · ·) 9kV/cm, 360 Hz, 30min, RA= 58.2%. In all cases protein concentration was 1 μM and the spectrum areas were normalized to 1.

H0 is an index of the number of hydrophobic groups present on the surface of a molecule; therefore, hydrophobic interactions are critical for stability, conformation and function of proteins (Chandrapala *et al*., 2011; Wang *et al*., 2014).

Fig. 2a shows the binding of ANS to food grade papain as measured by fluorescence spectroscopy, before and after treatment with HVEF. Fluorescence intensity decreased in the 9 kV/cm, 360 Hz, 30 min treatment with respect to the control (untreated samples). In the literature, the decrease in fluorescence intensity in hydrophobicity is interpreted as a low frequency or presence of hydrophobic groups. The sudden decrease in H0 is called hydrophobic collapse and it is an indication that the protein has an aggregation by self-association, as a result of an increase in protein-protein physical contact via hydrophobic interaction (Zhao & Yang, 2009; Wang *et al*., 2014). Significantly, the sample in which fluorescence intensity decreased the most was the one in which the frequency and time were applied at the highest levels, which could be interpreted as factors influencing the hydrophobic aggregation of papain. In the other treatments, fluorescence intensity increased to a peak of intensity at 9 kV/cm, 0 Hz and 20 minutes of treatment. When the treated samples were compared with the control, an increase in fluorescence intensity was observed in the samples exposed to HVEF, with the exception of the sample mentioned above, which may indicate that the papain hydrophobic groups moved to the outside of the molecule. This might be because the HVEF broke some internal hydrophobic interactions, resulting in increased exposure of these areas at the surface of the molecule. Therefore, HVEF treatment is capable of generating some degree of unfolding in the structure of papain (Jiang *et al*., 2014). The results of residual activity showed that frequency is not a crucial factor for the loss of activity; and in the case of denaturation, the sample that reached the greatest fluorescence intensity was the one that had a frequency of zero, thus corroborating the importance of the electric field and the time in RA. Results of Ho showed changes in denaturation and formation of aggregates in the papain treated with HVEF.

3.2.2 Intrinsic fluorescence

Currently, the determination of intrinsic fluorescence (IF) is an effective indicator of changes in the tertiary structure of proteins. It is known that the fluorescence emission of a protein is primarily due to its aromatic amino acid residues (Zhi *et al*., 2014). The IF of proteins originates from the presence of aromatic amino acids such as tryptophan (Trp),

tyrosine (Tyr) and phenylalanine (Phe). Tryptophan residues are the most important due to its absorbance values and its emission in the far UV spectrum of proteins. Tyrosine has a quantum yield similar to tryptophan, but its emission spectrum is closely distributed in the wavelength scale at 303 nm and is relatively insensitive to polar solvents. The use of fluorescence techniques in the far UV spectrum is often applied to proteins, because within biopolymers, they are the only ones that have this feature. Among the characteristics of this technique, there is its high sensitivity, low sample concentration, and the presence of only one or a few residues of tryptophan facilitates the interpretation of its absorption spectrum (Lakowicz, 2006). Given the above, intrinsic fluorescence is an important tool for determining some structural changes in proteins, since a conformational change in proteins results in a change in intrinsic fluorescence (Tetsuro, 1993). In the results of this study, an additional exposure of tyrosine in the samples treated with HVEF was detected (Fig. 2b). In the native folded state of the protein, Trp and Tyr are generally buried within the molecule, while in the state of partial folding or denaturation, the residues are exposed to the solvent. The maximum fluorescence intensity was obtained after a 1.0 kV/cm, 360 Hz and 10 min of treatment. The increase in fluorescence intensity may be due to ionization of some of the chains leading to the exposure of the hydrophobic core to the aqueous solvent (Edwin *et al*., 1998). The sample treated with 9 kV/cm, 360 Hz and 30 minutes had the lowest fluorescence intensity, which is consistent with the results of surface hydrophobicity, where it also showed the lowest fluorescence intensity. The above shows that the treatment with HVEF generated aggregation and a certain degree of denaturation in papain. The changes in the tertiary structure were determined by fluorescence fluctuations. Probably, in the case of papain, a disruption in secondary structure might occur because this enzyme possesses a strong dipole moment due to the presence of the α-helix, which has the potential to be affected by external electric fields. These changes in structure can profoundly affect the physical properties and reactivity of the dissolved molecules. Local electrostatic fields are of fundamental importance in proteins, including folding, molecular recognition and catalytic functions. The high intensity external electric field affected the local electrostatic fields in papain and then interrupted electrical interactions of peptide chains, which can be one of the reasons why a conformational change of partial unfolding or denaturation is generated under treatments with HVEF (Zhao *et al*., 2012).

Conclusions

From the results obtained in this study, it is possible to conclude that the HVEF generated a significant reduction in the RA of papain through structural changes. The main variables identified as responsible for the HVEF effect on the enzyme were the electric field intensity and processing time. Among the main structural changes, alterations in surface hydrophobicity and in intrinsic fluorescence were observed, which resulted from structural changes indicating a partial denaturation and a possible aggregation in papain. Therefore, the HVEF is a good alternative to achieve a partial inactivation through structural changes of enzymes such as papain. Finally, this method does not consume large amounts of energy and temperature, and during the treatment, it does not increase by joule effect. Therefore, HVEF can be used for application in different foods or food products without appreciable changes.

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Notation

3FI	three factors with interactions
EF	electric field (kV/cm)
H0	surface hydrophobicity
HVEF	high voltage electric field (kV/cm)
IF	intrinsic fluorescence
PEF	pulsed electric field (kV/cm)
RA	residual activity (%)
t	processing time (min)

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