New Kinetic Approach to the Evolution of Polygalacturonase (EC 3.2.1.15) Activity in a Commercial Enzyme Preparation Under Pulsed Electric Fields

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ABSTRACT: The effect of pulsed electric fields (PEF) on polygalacturonase (PG) activity in an aqueous solution of a commercial enzyme preparation was studied. Monopolar square wave pulses of 4 μs were delivered to the solution, which circulated through tubular chambers with 2 stainless steel electrodes in continuous operation. The electric field intensities (E) and treatment time (t) ranged within 15 to 38 kV cm^{-1} and 300 to 1100 μs, respectively. Although important reduction of the PG activity could be achieved (up to 76.5% at $E = 38$ kV cm^{-1} and $t = 1100$ μs), the experimental data showed certain enhancement of PG activity under soft conditions (up to 110.9% at $E = 15$ kV cm^{-1} and $t = 300$ μs). A kinetic mechanism involving 2 consecutive irreversible first-order steps was developed to describe and explain the experimental data. The tested model exhibited high accuracy in respect of the experimental data and the error of the model was lower than 4.4%. The kinetic rate constant of the first (k_1) and second step (k_2), ratio between the activities of intermediate and native forms of the enzyme (Λ), and other quantities related to the model, were obtained within a Bayesian framework. k_1 resulted independent of the E applied and considerably greater in magnitude order (k_1 mean = 6 μs^{-1}) with respect to k_2, which was dependent on the applied E (mean values ranged from 489E-6 μs^{-1} at $E = 38$ kV cm^{-1} to 1215E-6 μs^{-1} at 38 kV cm^{-1}). The dependency of k_2 toward E was described using an exponential relationship.

Keywords: Bayesian, kinetics, polygalacturonase, pulsed-electric-field, model

Introduction

An important goal of some stages involved in many processes of foods is to reduce the activities of enzymes that could cause negative effects in finished products up to adequate levels. Traditionally, inactivation of enzymes has been carried out by thermal treatment. However, heating brings about unwanted outcomes such as undesirable changes of sensorial properties and losses of nutritional compounds. Pulsed electric fields (PEF) is a nonthermal method that can play an important role among the technologies that have emerged to avoid the drawbacks of the traditional heating methods and, thus, improve quality of finished products.

The effectiveness of PEF to inactivate enzymes has been stated in earlier studies (Bendicho and others 2002; Espachs-Barroso and others 2003; Martín-Belloso and others 2004). However, the amount of data for most enzymes of interest in food processing is still insufficient or even null sometimes. Indeed, to date, and despite its undoubted relevance in fruit and vegetable processing, only one work (Giner and others 2003) focused on the effects of batch PEF treatments on polygalacturonase (PG) enzyme.

On the other side, kinetic models, grasping the mechanism by which PEF inactivation of enzymes occurs, are necessary to analyze and compare available data as well as to provide basis for setting process conditions up. In many cases, the exponential decay model has been found suitable enough to describe the evolution of activity of diverse enzymes as a function of PEF treatment time (t) (Bendicho and others 2003a, 2003b; Giner and others 2003). This kind of model indicates that the process involving the native form (N) and the inactivated form (D) of an enzyme under PEF might be postulated to occur by following a first-order irreversible 1-step mechanism, which can be expressed as

$$N \xrightarrow{k} D \qquad (1)$$

and the rate of change for the concentration of the native enzyme, [N], should be given by

$$\frac{d[N]}{dt} = -k[N] \qquad (2)$$

where k is the inactivation rate constant. Integration of Eq. 2 with the initial condition [N] = [N]$_0$ for $t = 0$ yields

$$[N] = [N]_0 e^{-kt} \qquad (3)$$

Since the amount and concentration of an enzyme is seldom determined, it is rather better to express Eq. 3 in terms related to the activity of the enzyme (A) such as Eq. 4. This expression can be easily obtained considering that A is proportional to the remaining amount of enzyme: [A] = λ N], where λ is the proportionality constant and RA is the residual enzyme activity defined by A/A_0 where

A_0 is the enzyme activity for $t = 0$.

$$RA = e^{-kt} \qquad (4)$$

However, noteworthy deviations from the inactivation pattern given by Eq. 4 are also found in published data that regard inactivation of enzymes by PEF (Yeom and others 2002; Min and others 2003). These deviations usually occur at the beginning of the treatment and mainly consist of occurrences such as unaffected enzyme activity or flat shoulders (Giner and others 2000; Yeom and others 2000; Rodrigo and others 2003), and increase of enzyme activity or activation shoulders (Yang and others 2004; Bendicho and others 2005). After these occurrences, enzyme activity decreases usually according to the pattern described by Eq. 4. Thus, when evolution of enzyme activity under PEF had been modeled, a number of empirical mathematical models such as Hülsheger's (Eq. 5), Fermi's (Eq. 6), or Weibull's equations (Eq. 7) have been also suggested either in addition or alternatively to Eq. 4 and its underlying mechanism (Giner and others 2005). In the case of flat shoulders, these equations improved model accuracy and permitted better description of experimental data by the models than Eq. 4.

Hülsheger's equation is

$$RA = \left(\frac{t}{t_c} \right)^{-(E - E_c)/K} \qquad (5)$$

where t_c is the critical time, E_c is the electric field intensity, and K is an independent constant factor.

Fermi's equation is

$$RA = \frac{1}{1 + e^{-(E - E_h)/a}} \qquad (6)$$

where E_h is the critical level of E where $RA = 50\%$ and a is a parameter which indicates the steepness of the curve around E_h.

Weibull's equation is

$$RA = e^{-\left(\frac{t}{a} \right)^{\beta}} \qquad (7)$$

where α is the form parameter and β is the scale parameter.

Therefore, establishment of PEF technology demands more research in the topic of PG under PEF and, furthermore, suitable kinetic models that grasp inactivation enzyme data in all their complexity.

On the other side, classical frequentist statistical methods have been used to obtain the parameters in the models used to describe the evolution of enzymes under PEF. In these methods, the parameters are considered as fixed quantities to be calculated while the data set are random. In contrast, from the point of view of the Bayesian statistics, the parameters of the models are random variables, with their possible values given as probability distributions conditioned to the fixed data set containing available previous information. The Bayesian approach presents two main advantages: first, generality and coherence, since it only requires the mathematics of probability theory and, second, its ability to incorporate prior information about the parameters considering the data set (Bernardo and Smith 1994).

The objectives of the present work were to study the effect of continuous PEF treatments on PG enzyme and, in addition, present a kinetic mechanism (KM) that, first, was consistent with the experimental data and, second, could explain phenomena of flat and activation shoulders in inactivation curves of PEF-treated enzymes. Computing and estimation of parameters was performed within the Bayesian approach.

Materials and Methods

PG source and medium

Enzyme solution (ES) for PEF treatments was prepared by diluting a commercial enzyme formulation, Pectinex 100 L® (Novo Nordisk Ferment, Neumatt, Switzerland), in distilled water at 2.5% volume fraction. The ES was kept at 4 °C just before PEF treatments. The electrical conductivity of the ES at temperatures within 10 to 40 °C ranged from 0.765 to 0.773 S m^{-1}. The pH of ES at 20 °C was 4.61. The measurements of σ and pH were carried out using a Testo-240 conductivimeter (Testo, Lenzkirch, Germany) and a Crison micropH 2000 pH-meter (Crison, Alella, Barcelona, Spain), respectively.

PG activity measurement

PG activity in PEF-treated ES was determined through the method that was used in a previous work (Giner and others 2003). Briefly, the method was based on performing assays to observe the depletion in viscosity occurring when the enzyme acts on a solution of pectin as substrate. In the present work, the assays for reduction in viscosity were done by mixing 9.5 mL of aqueous solution of pectin (reference P-9561, Sigma Chemical Co., Saint Louis, Mo., U.S.A.) at 2% mass fraction with 0.5 mL of the ES. The PG activity assays were conducted at 50 °C since this temperature was optimal for enzyme activity in previous experiments. The PG assays on treated and untreated samples were done right after the ES had been exposed to PEF.

PEF treatment system

PEF treatments were carried out using a bench-scale continuous system (OSU-4F, Ohio State Univ., Columbus, Ohio, U.S.A.) complemented with a cooling system. The PEF device generated monopolar square wave pulses discharging its 0.5 μF capacitance of capacitor. Pulses of 4 μs were delivered to the 8 co-field flow tubular chambers with a pair of stainless steel electrodes. The inner diameter and the volume of each chamber were 0.115 and 0.012 cm^3, respectively, and the gap of its electrodes was 0.29 cm. Pulses were supplied to 60 mL of ES that recirculated into the PEF system by means of a variable speed pump (model 75210–25, Cole Palmer, Vernon Hills, Ill., U.S.A.) which was set to provide 1 mL s^{-1} flow rate. The repetition rate of pulses was 100 Hz. To avoid possible thermal effects during PEF treatments, stainless steel coils submerged in an ice-water bath cooled the product. The coils were connected just before and after each pair of the treatment chambers. The temperature of the ES was monitored with a thermocouple and never exceeded 25 °C. Six different electric field intensities were explored: 15, 20, 25, 30, 35, and 38 kV cm^{-1}. ES samples were collected for PG activity measurement after exposure to prefixed PEF treatment times (t), which were up to 1100 μs long. Three independent trials, which started with fresh ES, were conducted for each experimental condition on different dates. The mean of 3 PG activity measurements carried out in the untreated fresh ES for each of the assayed electric field intensities was taken as the initial enzyme activity of the ES (A_0).

Kinetic mechanism and its mathematical expression

The inactivation KM for enzymes under PEF is hypothesized to proceed by 2 consecutive irreversible first-order steps (Giner and others 2005) with the presence of intermediate active forms of the enzyme (I) between the native and the completely inactivated enzyme (D). Additionally, the 1st step should be much quicker than the 2nd step. Thus, the scheme for the process will be given by Eq. 8.

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D \qquad (8)$$

The rate of change for N is also given by Eq. 2, while Eq. 9 gives the rate of change for I. The rate constants for the first and second steps of the enzyme inactivation process are k_1 and k_2, respectively.

$$\frac{d[I]}{dt} = k_1[N] - k_2[I] \qquad (9)$$

The solution of the differential equation resulting from substitution of Eq. 2 into 9 with the boundary condition $[I] = [I]_0 = 0$ for $t = 0$ yields Eq. 10.

$$[I] = \frac{[N]_0 \, k_1}{(k_2 - k_1)} (e^{-k_1 \cdot t} - e^{-k_2 \cdot t}) \qquad (10)$$

On the other side, A will be the result of the contributions of the active forms of the enzyme (N and I) for any t, and should be proportional to their concentrations, which is expressed by Eq. 11.

$$A = \lambda_1[N] + \lambda_2[I] \qquad (11)$$

where λ_1 and λ_2 are the proportional constants. Similarly, initial enzyme activity A_0 ($t = 0$) will be expressed by Eq. 12.

$$A_0 = \lambda_1[N]_0 + \lambda_2[I]_0 \qquad (12)$$

The quotient between Eq. 11 and 12 will give RA (Eq. 13),

$$RA = \frac{\lambda_1[N] + \lambda_2[I]}{\lambda_1[N]_0 + \lambda_2[I]_0} \qquad (13)$$

which, regarding that $[I]_0 = 0$ for $t = 0$, yields Eq. 14.

$$RA = \frac{\lambda_1[N] + \lambda_2[I]}{\lambda_1[N]_0} \qquad (14)$$

Defining $\Lambda = \lambda_2/\lambda_1$ and substituting the expressions for $[N]$ and $[I]$ given by Eq. 3 and 10, respectively, into Eq. 14, evolution of RA as a function of t at fixed conditions may be mathematically expressed by Eq. 15.

$$RA = e^{-k_1 \cdot t} - \frac{k_1 \cdot \Lambda}{(k_1 - k_2)}(e^{-k_1 \cdot t} - e^{-k_2 \cdot t}) \qquad (15)$$

The last equation condenses the KM for describing the effect of PEF treatments on PG as well as other enzymes. The meaning of dimensionless parameter Λ is the ratio between the activities of the intermediate and the native forms of the enzyme.

Other parameters of interest involved with the model are the slope of the curve RA against t at $t = 0$ ($RA'|_0$ in Eq. 16) and treatment time for maximum RA ($t_{\max}$ in Eq. 17). Both parameters can be obtained from the first derivative of Eq. 14.

$$RA'|_0 = k_1(\Lambda - 1) \qquad (16)$$

$$t_{\max} = \frac{1}{(k_1 - k_2)} \ln\left[\frac{k_2 + k_1(\Lambda - 1)}{k_2 \cdot \Lambda}\right] \qquad (17)$$

Modeling and calculations

Descriptive statistics (mean, standard deviation, median, 95% credible interval) of the kinetic model parameters (k_1, k_2, and Λ) as conditioned to our experimental data were calculated by non-linear regression within the Bayesian framework of the WinBUGS with DoodleBUGS version 1.4 software (© Imperial College of Science, Technology and Medicine of London and Medical Research Council, Cambridge, U.K.). This software performs Bayesian anal-

ysis of statistical models using Gibbs sampling, Markov chains and Monte Carlo techniques (MCMC; Spiegelhalter and others 2002). Furthermore, the package allows building up models using its special drawing tool for representing models through directed acyclic graphs (the so-named Doodles).

Modeling of data was done assuming that experimentally observed values of RA at fixed parameters followed normal distributions. This is denoted by $RA \sim \text{Normal}\,(mRA, \sigma)$, with mRA and σ being the mean and the standard deviation of the normal distribution, respectively, and, thus, $RA = mRA + \varepsilon_{mRA}$ with ε_{mRA} being a random normal error term of the assayed kinetic model.

A first approach indicated k_1 remained almost unaffected by E whereas increasing of E augmented k_2 values. Therefore, the dependency of k_2 as a function of the electric field intensity was considered throughout an exponential relationship in the model: Eq. 18, in which $k_{2,a}$ and $k_{2,b}$ are terminal parameters to be determined. This kind of relationship has also been used in previous works (Giner and others 2003).

$$k_2 = k_{2,a} e^{k_{2,b} \cdot E} \qquad (18)$$

Figure 1 is the directed acycled graph (Doodle) that, in terms of the WinBUGS package, represents the statistical model used for the estimation of all the quantities of interest concerning the kinetic model. Table 1 shows the descriptions of the links among the variables, the initial distributions for the parameters, and other quantities related
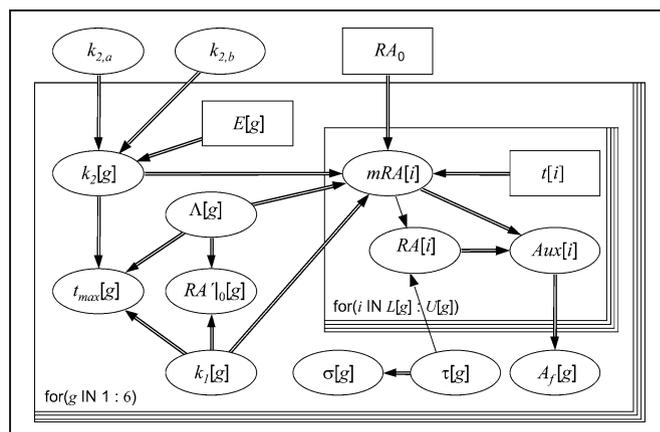
Figure 1 —Directed acyclic graph model (Doodle) for the proposed kinetic mechanism. *Nodes* **represent any of the quantities considered.** *Arrows* **run into nodes from their direct influences (***parents***). The graphical model shows the supposition that each node is independent of all other nodes, given its parents nodes, except its descendants. Rectangles indicate fixed data (for example, treatment time** *t[i]***, electric field intensity** *E[g]***, or constants lying behind the model [initial residual activity,** *RA₀***]). El-lipses depict stochastic nodes, variables that are given as distributions. They may be observed data (for example, residual enzyme activity,** *RA[i]***), unobserved parameters underlying the model (for example, first step rate constant,** *k₁[g]***; ratio between the activities of the intermediate and the native forms of the enzyme** *Λ[g]***; and** *k₂,ₐ* **and** *k₂,ᵦ* **which are parameters in the relationship used for describing the dependence of the second step rate constant,** *k₂[g]***, with the electric field intensity) or nodes resulting from logical functions of other nodes (for example, the mentioned** *k₂[g]***, slope of the curve** *RA* **against** *t* **at** *t = 0***,** *RA′|₀[g]***; and treatment time for maximum activity,** *t₍ₘₐₓ₎[g]***).** *Single arrows* **and** *double arrows* **indicate stochastic and logical link between nodes, respectively.** *Large rectangular boxes* **depict repeated parts of the graph for the range shown at the** *bottom* **(for example,** *g* **IN 1 : 6). Table 1 describes the links among the nodes.**

with the statistical model. Chi-square distributions were chosen to force the distributions for k_1, k_2, $k_{2,a}$, and $k_{2,b}$ to be positive and close to normal. The convergence of the MCMC algorithm was checked by monitoring the modified Gelman and Rubin (Brooks and Gelman 1998) statistics during the adaptation phase for 2 independent MCMC chains initialized with different sets of values. The number of 1000 iterations was enough for this phase. After that, sampling phase for KM parameters and related quantities was performed by running 20000 iterations.

The accuracy factor (A_f) suggested by Ross (1996) was used as a measure of the accuracy of the estimates obtained by the model. A_f was calculated for each assayed electric field intensity (E) and is given by the expression given by Eq. 19.

$$A_f = \exp\left\{ \frac{1}{NO} \sum_1^{NO} \ln\left(\frac{mRA}{RA} \right) \right\} \tag{19}$$

where RA and mRA are the observed and the predicted RA values at the same fixed conditions, respectively, while NO is the number of observations. If there is complete agreement between observed and predicted values, A_f will be equal to 1; otherwise it will yield greater values.

Complementary parameters ($RA|_0$, t_{max}), terminal parameters ($k_{2,a}$ and $k_{2,b}$), and A_f were computed by integrating them within the Bayesian frame of the statistical model built with the mentioned package.

Results and Discussion

Effect of PEF on PG

The effects of PEF treatments using pulses of 4 μs on PG activity in the ES are shown in Table 2. Most of the assayed conditions caused inactivation of the PG activity but certain enhancement of the activity was also detected for a few of them. Maximum inactivation (up to $RA = 23.5\%$) was reached at the highest E (38 kV cm^{-1}) and the longest t (1100 μs). In contrast, augmentation of PG activity (up to 110.9%) was caused by PEF in samples that were exposed to 15 and 20 kV cm^{-1} electric fields for 300 μs. RA in the samples that were treated for t longer than 300 μs decreased generally as E strengthened or t increased. Thus, the experimental data for 15 and 20 kV cm^{-1} electric fields evinced that RA against t curves have a local maximum.

Table 1 – Description of the links and initial distributions for the parameters of the kinetic model shown in Figure 1

Node	Type	Definition
$RA[i]$	Stochastic	$N(mRA[i], \sigma[g]) > 0$
$mRA[i]$	Logical	Eq. 15
$\sigma[g]$	Logical	$1/\sigma^2[g] = \tau[g]$
$\tau[g]$	Stochastic	$N(0, 10^{-6}) > 0$
$k_1[g]$	Stochastic	Dchisqr(3)
$k_2[g]$	Logical	$k_{2,a} \cdot \exp(-k_{2,b} E[g])$
$\Lambda[g]$	Stochastic	Dchisqr(3)
$k_{2,a}$	Stochastic	Dchisqr(3)
$k_{2,b}$	Stochastic	Dchisqr(3)
$RA'|_0[g]$	Logical	Eq. 16
$t_{max}[g]$	Logical	Eq. 17
$A_f[g]$	Logical	Exp(mean($Aux[L[g]:U[g]]$))
$Aux[g]$	Logical	$|ln(\frac{mRA[i]}{RA[i]})|$

RA = residual activity; mRA = mean RA; i = counter denoting observation (i = $L[g]$, ..., $U[g]$); g = counter denoting electric field intensity (g = 1, 2, .., 6); $L[g]$, $U[g]$ = arrays containing the lower and upper values for i depending on the electric field intensity g; $N(m, SD)$ = any normal distribution with initial, or posterior, mean m, and standard deviation SD for the quantity in the node. Dchisqr(c) = any χ-square distribution with freedom degree c for the quantity in node. A_f = accuracy factor.

Table 2 – Residual polygalacturonase activity (RA) in aqueous solution of a commercial enzyme preparation submitted to exponentially decaying electric pulses (4 μs pulse width) at different electric field intensities (E) and treatment times (t)

E (kV cm^{-1})	t (μs)	RA (%)	RA (%)	RA (%)
15	300	108.5	107.8	110.9
	500	95.8	94.4	99.6
	700	91.4	87.9	90.9
	900	81.6	81.5	81.3
	1100	73.1	76.9	71.6
20	300	105.2	100.6	98.9
	500	91.9	96.7	93.5
	700	75.5	70.5	74.6
	900	66.7	63.1	67.8
	1100	56.0	57.9	59.6
25	300	96.9	97.2	91.2
	500	88.3	89.5	86.9
	700	77.4	75.0	76.0
	900	61.3	65.4	63.5
	1100	53.3	52.2	52.9
30	300	85.3	82.4	79.6
	500	74.4	71.7	69.0
	700	62.4	58.7	52.7
	900	55.7	52.1	49.6
	1100	44.3	47.5	45.7
35	300	75.4	84.8	74.0
	500	57.6	59.8	52.5
	700	46.0	49.4	47.9
	900	36.0	40.5	40.4
	1100	30.4	35.5	34.3
38	300	62.8	57.1	62.7
	500	39.5	39.6	41.3
	700	34.7	34.4	34.4
	900	26.4	26.8	25.7
	1100	23.7	23.8	23.5

Notably effectiveness of PEF to reduce PG activity in aqueous solutions from the same commercial enzyme formulation has been also reported previously by Giner and others (2003). These authors, who applied pulses within the range 5.18 to 19.39 kV cm^{-1}, informed inactivation up to 2% RA for $t = 32000$ μs at the maximum E. This range of E values is clearly below the E assayed in the present work. However, PEF treatments in the cited study were considerably longer (up to more than 60000 μs). The higher reduction of PG activity reported in the earlier study compared with the present results should be assigned mainly to the longer PEF exposures, without overlooking different experimental conditions such as pulse width, medium, and pulse system.

KM parameters

Usual kinetic models describing the evolution of RA against t at a fixed E (Eq. 4, 5, and 7) cannot properly contemplate occurrences such as presence of $RA > RA_0$ points as well as existence of local maxima. Since occurrences such as these have been observed in the case of 15 and 20 kV cm^{-1} E, the kinetics of the experimental data required to be analyzed by models, without forcing them, could include the aforementioned facts. The main statistics of the posterior distributions conditioned to the experimental data for the parameters of the KM proposed to describe the effect of PEF treatments (pulse duration time of 4 μs) on PG in solution for PEF treatments, as well as further related quantities of interest, are shown in Table 3. Among other features, the KM considers situations that precedent models were unable to manage and explain properly.

The posterior distributions for k_1 were nearly the same for each of the assayed E. The values of the mean and the median for every value of E were 6 μs^{-1} and 5 μs^{-1}, respectively. Thus, no significant effect

Table 3 — Descriptive statistics (DST) of the posterior distributions for the parameters related to the kinetic model proposed to describe the effect of exponentially decaying electric pulses (4 μs pulse width) at different electric field intensities (*E*) on the residual polygalacturonase activity (*RA*, %) in aqueous solution of a commercial enzyme preparation.

| Parameter | DST | Electric field intensity (kV cm⁻¹) | | | | | |
		15	20	25	30	35	38
k_1 (μs⁻¹)	M	6	6	6	6	6	6
	MD	5	5	5	5	5	5
	SD	3	3	3	3	3	3
	$p_{0.025}$	1	1	1	1	1	1
	$p_{0.975}$	14	14	15	14	14	14
k_2 (μs⁻¹)	M	489E-6	596E-6	726E-6	885E-6	1079E-6	1215E-6
	MD	488E-6	595E-6	726E-6	884E-6	1078E-6	1214E-6
	SD	19E-6	17E-6	18E-6	30E-6	40E-6	60E-6
	$p_{0.025}$	455E-6	564E-6	691E-6	835E-6	995E-6	1104E-6
	$p_{0.975}$	528E-6	630E-6	761E-6	935E-6	1162E-6	1327E-6
Λ	M	1.257	1.184	1.223	1.108	1.04	0.82
	MD	1.257	1.184	1.222	1.108	1.04	0.82
	SD	0.016	0.021	0.017	0.022	0.03	0.03
	$p_{0.025}$	1.228	1.142	1.189	1.066	0.98	0.76
	$p_{0.975}$	1.291	1.225	1.257	1.153	1.10	0.88
$RA'|_0$ (μs⁻¹)	M	1.5	1.1	1.3	0.6	0.22	−1.1
	MD	1.4	1.0	1.2	0.6	0.17	−0.9
	SD	0.9	0.6	0.8	0.4	0.24	0.6
	$p_{0.025}$	0.3	0.2	0.3	0.1	−0.12	−2.7
	$p_{0.975}$	3.7	2.7	3.0	1.6	0.84	−0.2
t_{max} (μs)	M	1.8	1.7	1.7	1.5	1.0	0
	MD	1.4	1.4	1.4	1.2	0.9	−
	SD	1.3	1.2	1.2	1.0	0.8	−
	$p_{0.025}$	0.6	0.6	0.6	0.5	0.0	−
	$p_{0.975}$	5.1	4.7	4.6	4.0	2.9	−
σ	M	1.8	4.4	2.8	3.4	3.5	3.0
	MD	1.8	4.3	2.7	3.3	3.4	2.9
	SD	0.3	0.8	0.5	0.6	0.6	0.5
	$p_{0.025}$	1.3	3.1	2.0	2.4	2.6	2.1
	$p_{0.975}$	2.6	6.2	3.9	4.8	5.0	4.2
A_f	M	1.0160	1.046	1.0290	1.046	1.048	1.059
	MD	1.0150	1.046	1.0290	1.046	1.047	1.058
	SD	0.0013	0.005	0.0011	0.005	0.004	0.004
	$p_{0.025}$	1.0190	1.037	1.0280	1.039	1.044	1.054
	$p_{0.975}$	1.0140	1.057	1.0320	1.057	1.058	1.069

k_1, k_2 are kinetic rate constant of the first and the second stage involved in the model, respectively Λ is ratio between the activities for the intermediate and the native forms of the enzyme $RA'|_0$ is slope at initial time of the residual enzyme activity against treatment time curve σ is error of the model.
A_f = accuracy factor; M = mean; MD = median; SD = standard deviation; $p_{0.025}$, $p_{0.975}$ = 2.5- and 97.5-percentile credible interval, respectively.

of *E* on k_1 was found and the distributions for k_1 exhibited slight skewness to the left. Contrarily, *E* exerted notable effect on k_2. Actually, posterior distributions for k_2 moved toward higher values as *E* increased and thus central measures of tendency such as means and medians increased as well. This fact can be best illustrated regarding that k_2 mean was 489E-6 μs and 1215E-6 μs at 15 kV cm⁻¹ and 38 kV cm⁻¹, respectively. The posterior distribution for k_2 parameter was symmetrical, since the values of the respective mean and median at each fixed *E* were almost the same (Table 3), and the plot of the posterior density function for k_2 obtained running the program exhibited bell-shape. The main statistics of the distributions of the parameters relating the effect of *E* on k_2 (Eq. 18) are shown in Table 4. The values of the pre-exponential factor ($k_{2,a}$) mean and exponential factor ($k_{2,b}$) mean were 271E-6 μs⁻¹ and 0.0396 cm kV⁻¹, respectively. Despite lessening of k_1/k_2 ratio with *E* increasing, the relative magnitude of k_1 means compared with k_2 means always resulted much greater. Thus, the second stage of the KM was the main rate-determining step of the global reaction due to this stage acted as bottleneck or, in other words, since the second step is much slower than the first one, the rate of the overall process for the inactivation of the enzyme under PEF is controlled by the rate of the second stage.

In general, distributions for parameter Λ declined significantly from values most of them greater than 1 toward clearly below this

Table 4 — Descriptive statistics of the posterior distributions for the parameters in the relationship used to describe the effect of electric field intensity on the kinetic rate constant of the second stage (k_2) concerning the proposed kinetic model

Parameter	Mean	Median	Standard deviation	Credible interval [$p_{0.250}$—$p_{0.075}$]
$K_{2,a}$ (μs⁻¹)	271E-6	269E-6	21E-6	[234E-6-317E-6]
$k_{2,b}$ (cm kV⁻¹)	0.0396	0.0396	0.0030	[0.0333-0.0450]

$p_{0.250}$, $p_{0.075}$ = 2.5 and 97.5 percentile, respectively.

value as PEF treatments were carried out at higher *E*. In this way, Λ mean reached 1.257 at the lowest *E* (15 kV cm⁻¹) whereas, at the highest *E* (38 kV cm⁻¹), this parameter was only 0.822. This indicated that PEF treatments during the first step at 15 kV cm⁻¹ yielded intermediate forms up to 25.7% more active in average than the initial native forms of the enzyme. The overall effect of PEF treatments on *RA* (activation, inactivation, or unchanged activity) will depend on the quantity of intermediates that are produced in step 1 by PEF and remain not consumed in the subsequent step 2, which yields final products with null PG activity. However, in case of PEF at *E* = 38 kV cm⁻¹, the reaction intermediates that were generated in the first step already had less activity in average (about 17.8%) than the

initial forms; thus, the overall effect of the PEF treatments always leads to RA lower than RA_0. The means of the distributions for Λ showed a certain decreasing trend with E augmenting within the range of the assayed E in spite of the disagreement at $E = 20$ kV cm^{-1} and $E = 25$ kV cm^{-1}, which should be mainly ascribed to the unavoidable errors of the experimental data.

Intermediate forms with higher activity than the native forms of the enzymes ($\Lambda > 1$) can be explained considering that PEF treatments promote shifts on the molecular structure of the enzyme. In the first stage, the PEF treatments have shifted the structure of the native molecules into a number of intermediate forms with enzyme activity. The activity of these forms can differ from each other. In this way, a fraction of these forms may have structures that may assist progress of the catalytic reaction (enhancement of the enzyme activity). Meanwhile, the rest of the generated intermediate forms have structures that could yield equal and less enzyme activity. As PEF treatments increase in intensity (higher E, longer t), the fraction of intermediates with enzyme activity higher than the native forms of the enzyme decreases. Thus, it is possible that the overall effect of PEF treatments on the measurements of enzyme activity yielded $RA > 100$ if, depending on the conditions (E, t), the remaining quantity of intermediates forms with the enzyme activity higher than the native forms were important enough after occurring the second step, which leads to forms without activity.

KM remarks

Once PEF conditions have been fixed, the evolution of RA against t according to Eq. 15 may take on a variety of shapes depending on the values of KM parameters (k_1, k_2, and Λ). Thus, it is interesting to make some remarks on this equation from a mathematic point of view to know better its properties and implications.

For any set of values for k_1, k_2, and Λ, it results in a curve that starts at $RA_0 = 1$ and, when t tends to infinite, RA decreases asymptotically up to 0 or, in other words, the total inactivation of the enzyme only would be achievable if t become infinite. Regarding Eq. 15 and 16, and considering that $k_1(\Lambda - 1) > 0$, since k_1 and Λ are positive, it is easy to notice that when $\Lambda > 1$ the resulting curve always starts at $RA_0 = 1$ and increases up to a local maximum, which is given by Eq. 17, and then the curve decreases asymptotically up to 0. Contrarily, when $0 < \Lambda \leq 1$ since $RA'|_0 \leq 0$, RA decays continuously from the maximum value ($RA_0 = 1$ at $t = 0$) up to 0 as t increases. Finally, higher values for k_1 and k_2 will always cause decreasing portions in RA against t curves to be sharper.

Most of the last remarks and the effect of E and t on the PG activity in the ES using the obtained KM parameters conditioned to our experimental data are illustrated by Figure 2 and 3. The KM forecasts for PG in ES that treatments at 25, 30, and 35 kV cm^{-1} should have $RA > RA_0$ for $t < 300$ μs since Λ distributed around values greater than 1 and $k_1(\Lambda - 1) > 0$. It is worthy to underline that the experimental data had not evinced RA enhancement by themselves. Similarly, the RA against t curves at 15 and 20 kV cm^{-1} exhibited local maxima for t shorter than 300 μs but, at these E, the experimental data had indicated already the occurrence of the local maxima. The statistics of the distributions for t_{max}, which were obtained according to Eq. 17, are showed in Table 3. Most of the t_{max} values distributed within a 0 to 5.1 ns interval. Although not plainly significant, an increase in E affords likely a drop in t_{max} distributions. In the case of $E = 38$ kV cm^{-1}, the RA against t curve had no real local relative maximum and thus t_{max} should be equal to 0. Finally, the intersection of the curves at $E = 20$ kV cm^{-1} and $E = 25$ kV cm^{-1} according the KM (see Figure 2) is a reflection of what has been already commented on regarding distributions for the Λ parameter in the previous subsec-
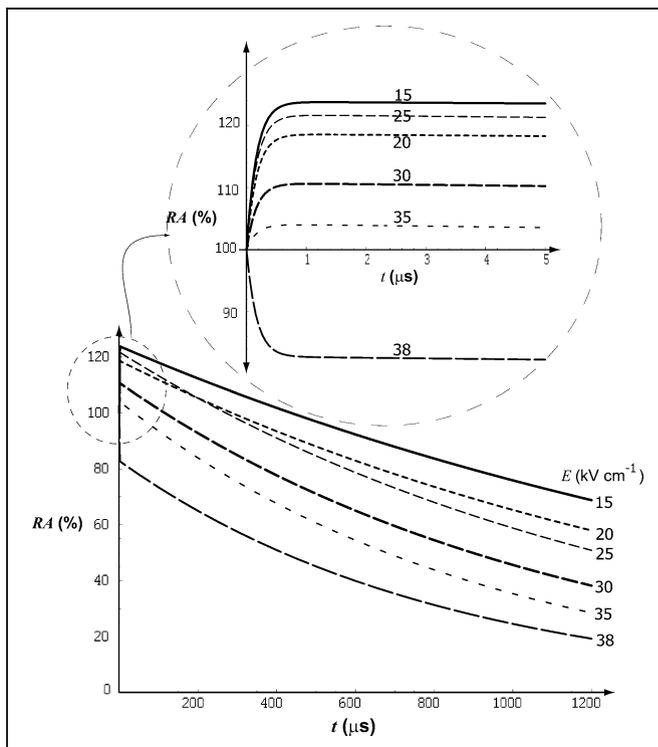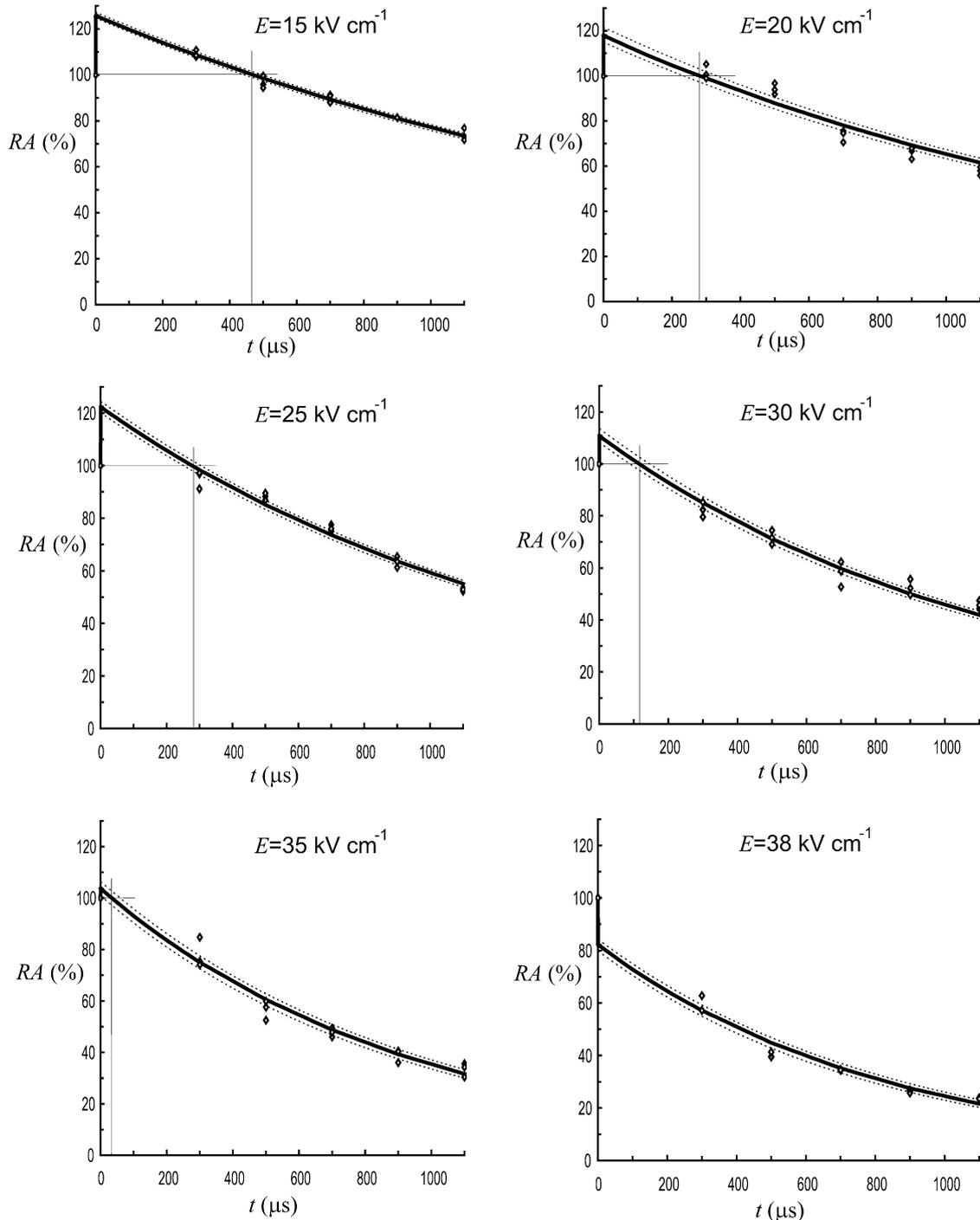

Figure 2 — Profiles according to the kinetic mechanism expressed by Eq. 15 for the treatment time (t) course of the relative polygalacturonase activity (RA) in a commercial enzyme preparation under pulsed electric fields at several electric field intensities (E). The k_1 and Λ mean in Table 3 and the k_2 calculated using Eq. 18, whose parameters are given in Table 4, were used by Eq. 15 to compute the RA values at a given E. RA values were computed using Eq. 15, where k_1 and Λ parameters mean, mean in Table 3 and calculating k_2 with Eq. 18 in which $k_{2,a}$ mean and $k_{2,b}$ mean are given in Table 4.

tion. Additional experiments carried out below 300 μs could help to confirm the predictions at short treatment times by the model from both qualitative and quantitative points of view.

According to the KM scheme suggested for the inactivation of the PG and enzymes under PEF, occurrences of increasing of initial activity at a fixed E suggest that the second stage of the KM scheme had to be slower than the first one. This affirmation should be true because the t intervals showing rising in RA require the disappearance rate of the reaction intermediates showing more activity than the native forms ($\Lambda > 1$) to be happened slower than generation rate. In other words, k_1 must be greater than k_2. The values of the distributions that were obtained for these parameters agreed with these last assertions.

The kinetic models expressed by Eq. 4, 5, 6, and 7 do not contemplate totally the experimental data obtained for the course of RA against t in the ES at a fixed E since their corresponding curve profiles do not explain occurrences such as $RA > RA_0$ points as well as existence of local maxima. Indeed, the initial portions of the RA against t curves having experimental points with $RA > 1$ (cases of 15 and 20 kV cm^{-1} E) cannot be properly matched by these models and even, what happen for Eq. 5, are mathematically undefined for t or E lower than some critical values. After all, in some cases, precedent models may be also useful to give an adequate description of experimental data if RA points are positive values less than 1. In this way, for instance, if treatment time is long enough, the second term in Eq. 15 becomes negligible and thus Eq. 4 might approximate to Eq. 15. Similarly, if $k_1 \cdot \Lambda / (k_1 - k_2)$ became almost null, depending

Figure 3 — Evolution of the relative polygalacturonase activity (*RA*) in an aqueous solution of a commercial enzyme preparation exposed to pulsed electric fields at different electric field intensities (*E*) and treatment times (*t*). Solid lines join the *RA* mean values (◇) in the posterior distributions obtained according Eq. 15. Dashed lines join the $p_{0.025}$ and $p_{0.975}$ percentiles for the *RA* distributions, respectively. Horizontal and vertical straight lines intersect at *t* for *RA* mean equals initial relative enzyme activity (*RA*$_0$ = 100%). According to the kinetic model that have been used, increase (*RA* > 100%) or inactivation (*RA* < 100%) of the activity of the enzyme happens, respectively, when *t* is shorter or higher than the *t* at the crossing point of the lines.

on the relative magnitude of the involved quantities, Eq. 15 could be reduced sometimes into Eq. 4 and, therefore, the course of *RA* at a fixed *E* would follow an exponential decay pattern.

The KM within the Bayesian framework gave information about the variability and uncertainty of the KM parameters as well as the error of the model. The means of the distributions of the error of the model (σ) for *RA* expressed in percentage did not exceed 4.4%.

Furthermore, the KM also showed high accuracy toward experimental data for each of the assayed *E* since its respective posterior A_f means distributed very close to 1. Indeed, A_f means ranged within 1.016 to 1.059. Explorations using the KM (see Figure 3) permitted to draw up the boundaries of *t* that might yield either enhancement (*RA* > 100%) or inactivation (*RA* < 100%) of the PG activity. Plainly, Figure 3 showed that the *t* interval, which can cause enhancement

of PG activity, shortens with the increase in applied E up to 0 in the case of $E = 38\ kV\ cm^{-1}$.

Other biexponential relationships such as Eq. 15, either empirical or phenomenological, might fit the data. The proposed KM harmonizes the prior knowledge on the topic with the set of hypothesis and experimental data. It should be interesting to contrast the proposed model with other different biexponential candidate models that could be mathematically undistinguishable but, certainly, not one was found with the purpose and on the topic in the literature. Comparison with others that could be proposed should be done in terms of reliability as well as ability to conceptualize the knowledge about the process.

Conclusions

Although PEF treatments can reduce efficiently PG activity in the ES, if the combination of applied electric field and treatment time is not severe enough, enhancement of the activity of the enzyme might happen as well. Besides its high accuracy, the proposed KM explains satisfactorily the evolution of the PG activity and permits obtaining valuable information about the conditions for which either inactivation or enhancement of the PG activity may happen. Moreover, the mechanism offers simplicity and flexibility and can explain occurrences such as flat and activation shoulders at the beginning of inactivation curves for enzymes under PEF treatments. Therefore, we believe our kinetic approach might be considered a helpful tool for a better knowledge and understanding of the behavior of other enzymes exposed to electric pulses. Further studies, beyond the present work, are necessary to gain insight into the intermediate states of enzymes, as well as proteins in general, under PEF.

Acknowledgments

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