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FACULTY OF CHEMISTRY

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DOCTORAL THESIS SUMMARY

KINETICS OF ENZYME INACTIVATION MODULATED BY ADDITIVES

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(Pages, chapters, figures, tables, schemes, equations and references numbering corresponds to the thesis)

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INTRODUCTION

The enzymes are widely used in many fields including medical and pharmaceutical research, clinical diagnosis, industry (pharmaceutical, chemical, food and textile), and wastewater treatment. Advantages such as: high specificity, high activity under mild environmental conditions, catalytic efficiency, and biodegradable nature has made it possible to use enzymes as modern and efficient alternative for the conventional catalysts.

Various factors such as temperature and chemical additives lead usually to inactivation of enzymes. The thermo stability increase and operational stability of enzymes can be achieved with low cost by adding in the reaction mixture of various chemicals: salts, polyols, dextran, bovine serum albumin, polyethylene amine, polyelectrolytes, organic osmolytes, organic solvents, carbohydrates and other additives [5]. A preliminary investigation of enzyme inactivation kinetics is first of all necessary in order to understand the stabilization mechanism of different additives. There are few studies regarding this topic [11], and enzyme inactivation phenomenon is usually treated more or less empirically. Detection and measurement of enzyme inactivation under normal reaction conditions may be an important tool for describing the stabilization of enzymes in the presence of various additives.

Although the potential use of oxidoreductases is very high, their applications in industry are limited due to inactivation by substrates and/or by reaction products and their low stability in aqueous solutions. Therefore, improving the stability of oxidoreductases is a major objective for biotechnology. Because of their ability to remove the free radicals from the reactive oxygen species and of the reactive nitrogen species, catalase and peroxidase were included in the class of enzymes that protect the cell against the oxidative stress. The oxidative stress is also compensated in vivo by nonenzymatic systems including some natural flavonoids. *In vitro* studies indicate that flavonoids have antioxidant capacity [34-42]. Quercetin and catechin possesses structural elements and the ability to destroy *in vitro* the reactive oxygen species and singlet oxygen radicals besides the radicals of other origins. Flavonoids such as capsaicin found in peppers of the genus Capsicum have been studied due to their antioxidant properties. Another flavonoid, with simple structure, common in edible plants is the guaiacol, which has been assigned a cellular antioxidant activity and ability to counteract the effects of pathogens. [45].

The main objective of this thesis is to study the kinetics of reactions catalyzed by some oxidoreductases in order to obtain simple and plausible kinetic models and find the best modulators able to increase their operational stability and thermo stability. Among the multitude of enzymes with potential for use in industry, two were chosen: catalase (enzyme with high substrate specificity) and peroxidase (enzyme with low specificity, able to oxidize a wide range of phenolic derivatives). These enzymes have Fe in their active site structure. By analyzing the interactions between modulators and active site of the enzyme is expected to develop a strategy for selecting an optimal stabilization for each enzyme.

The main issues pursued in this work were:

- To study the effect of solvent, ionic strength and pH on the stability and inactivation of bovine liver catalase in the decomposition reaction of hydrogen peroxide.
- To study the effect of additives on thermo stability of catalase in the absence and in the presence of substrate.

- To study the influence of substrate nature on the kinetics of their oxidation with hydrogen peroxide in the presence of horseradish peroxidase and its inactivation using the isoconversional method.
- To study the kinetics of quercetin oxidation with various oxidizing agents: oxygen (auto-oxidation) in different environments, hydrogen peroxide (chemical oxidation), hydrogen peroxide and horseradish peroxidase (enzymatic oxidation) and to identify the resulting oxidation products.
- To study the oxidation kinetics of catechin with various oxidizing agents: oxygen (auto-oxidation), hydrogen peroxide (chemical oxidation), hydrogen peroxide and horseradish peroxidase (enzymatic oxidation) and to identify the resulting oxidation products.
- To identify the oxidation products of catechin and quercetin and to establish plausible kinetic models.

The present PhD thesis includes a literature part that describes the current state of knowledge in the field of study and a second part, the original contributions, based on the experimental results obtained during the course of research. Several relevant aspects connected with the thesis are summarized in the literature part: kinetics of enzyme catalyzed reactions (chap. I.1), enzyme inactivation (chap. I.2), ways to reduce the oxidative stress using enzymatic and non-enzymatic systems (chap. I.3), catalytic activity of beef liver catalase and of horseradish peroxidase (HRP) (chap. I.4). The original part of the thesis contains kinetic studies regarding both the stability and inactivation of catalase (chap. II.1) and the inactivation of peroxidase accompanying the oxidation of substrates like catechin, quercetin, guaiacol and capsaicin (chap. II.2).

II. ORIGINAL CONTRIBUTIONS

II.1.a. Solvent effect on the kinetics of H_2O_2 decomposition in the presence of catalase

This study aimed to investigate the kinetics of hydrogen peroxide decomposition in the presence of catalase in partially aqueous solutions. The non-aqueous components were chosen as mono and polyhydroxylic alcohols, in the attempt to change the hydroxyl group concentration but keeping unchanged the chemical nature of the medium. A series of correlations were found between the reaction rates or the rate constants and some relevant solvent parameters like the dielectric constant (D), hydrophobicity (log P), polarizability (α), Kamlet-Taft parameter (β), Kosover parameter (Z), hydroxyl group concentration (OH). The hydrogen peroxide decomposition in the presence of catalase was monitored using a spectrophotometric method in different binary water - alcohol media (methanol, ethanol, 1-propanol, 1-butanol, 2-propanol, 1,2-ethanediol, 1,2,3-propanetriol). The extended kinetic curves - unreacted hydrogen peroxide concentration [S] versus time - were calculated from the measured absorbance in time according to Lambert-Beer law, after subtracting the enzyme and solvent absorbance.

The illustrative integral kinetic curves for the hydrogen peroxide decomposition in the presence of catalase in water-ethanol mixtures with different compositions are given in Figure (II.1.2.). Similar results were also obtained for all other water-alcohol mixtures.

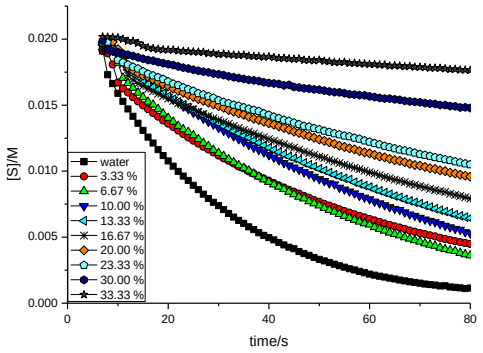


Figure II.1.2. Integral kinetic curves in ethanol-water mixtures (%vol alcohol)

The instant (v_R) and initial (v_R^0) reaction rates were estimated from such integral kinetic curves [S] = f(time) by fitting four parameter (a,b,c,d) functions (II.1.1) and calculating numerically their derivatives:

$$y = ae^{-bx} + ce^{-dx} \quad \text{(II.1.1)}$$

One obtains:

$$v_R = \frac{1}{v_S}\frac{d[S]}{dt} = abe^{-bt} + cde^{-dt} \quad \text{(II.1.2)}$$

$$v_R^0 = \left(\frac{1}{v_S}\frac{d[S]}{dt}\right)_{t=0} = ab + cd \quad \text{(II.1.3)}$$

The calculated initial reaction rates are given in Figure (II.1.8).

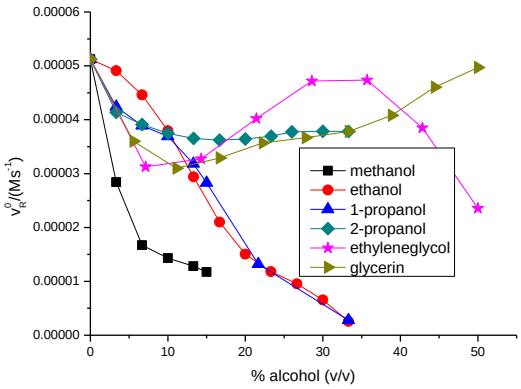


Figure II.1.8. Initial reaction rates variation with the alcohol content (%vol)

It was found that the initial reaction rates decrease in water-alcohol mixtures as compared to water. To explain the enzyme inactivation during the course of the reaction, a kinetic model (II.1.6) implying a slow enzyme inactivation step was used [125].

$$\begin{cases} E + S \xrightarrow{1} X_1 \\ X_1 + S \xrightarrow{2} E + P \\ E \xrightarrow{3} E_{in} \end{cases} \quad \text{(II.1.6)}$$

where E_{in} represents an inactive form of catalase.

The literature data showed that the first step of this kinetic model is diffusion controlled and consequently k_1 is practically constant [68]. In order to estimate the other rate constants, k_2 and k_3, the system of differential equations describing the time evolution of the system composition [114] was solved numerically. The system of 5 differential equations corresponding to the kinetic model (II.1.6) gives the rates of transformation for all 5 components:

$$\begin{cases} \frac{d[E]}{dt} = -k_1[E][S] + k_2[X_1][S] - k_3[E] \\ \frac{d[S]}{dt} = -k_1[E][S] - k_2[X_1][S] \\ \frac{d[X_1]}{dt} = k_1[E][S] - k_2[X_1][S] \\ \frac{d[P]}{dt} = k_2[X_1][S] \\ \frac{d[E_{in}]}{dt} = k_3[E] \end{cases} \qquad \text{(II.1.8)}$$

Using the initial conditions ($k_1=1.4\cdot10^7$ $M^{-1}s^{-1}$ and $[E]_0=3\cdot10^{-8}$ M, $[S]_0=0.021$M), the estimates of the rate constants k_2 and k_3, leading to the best superposition of experimental and calculated data, were obtained. The inactivation rate constant k_3 is situated within the range 10^{-2}-$10^0$$s^{-1}$, proving the existence of a slow enzyme inactivation; k_3 increases with the nonaqueous component concentration for all binary mixtures; for those mixtures containing 2-propanol or 1,2,3-propanetriol, the change is smaller.

The effect of several solvent properties on the kinetic parameters v_R^0, k_2 şi k_3 was subsequently analyzed and discussed. One of the most widely used solvent properties, in terms of the influence on the kinetics of chemical reactions, is the dielectric constant (D). In order to understand the effect of solvent composition on the kinetics of hydrogen peroxide decomposition in the presence of catalase, a dielectric solvation model advanced by Hiromi [128,129] was used. This model assumes that the solvent is a continuous and isotropic medium with the dielectric constant D, and the charged component (reactant or transition state) is embedded in a spherical cavity of radius b and internal dielectric constant D_i, where M discrete, point charges e_1, e_2…e_M are specifically distributed. The reference medium is a hypothetical medium having an infinite dielectric constant. The previously proposed kinetic model (II.1.6) can be detailed taking also into account the formation of the corresponding activated complexes:

$$\begin{aligned} &E + S \overset{1}{\longleftrightarrow} X_1 \\ &X_1 + S \overset{2'}{\longleftrightarrow} ES^{\neq} \xrightarrow{2} P + E \\ &E \overset{3'}{\longleftrightarrow} E^{\neq} \xrightarrow{3} E_{in} \end{aligned} \qquad \text{(II.1.9)}$$

where $ES^{\neq}$ and $E^{\neq}$ are the activated complexes (transition states) implied in the elementary steps 2 and 3.

Using the Hiromi model and assuming that the molecular radii of X_1 and $ES^{\neq}$ equal the corresponding radius of the free enzyme (since the hydrogen peroxide molecule has a much smaller radius as compared to enzyme) one can approximate $b_{X1} = b_{ES^{\neq}} = 4.2\ 10^{-10}$ m [129]; with these approximations the sought equations correlating the variation of the rate constants k_2 and k_3 with the dielectric constant and charge distribution are obtained.

According to these equations, when the solvation is preponderantly dielectric, a linear correlation between log k and 1/D holds. From the plots ln k_2 = f(1/D) and ln k_3 = f(1/D), one can conclude on the charge configuration in the complex X_1 and transition state $ES^{\neq}$ and in

enzyme E and transition state $E^{\neq}$, respectively. The variations of the rate constants k_2 and k_3 with 1/D are apparently quasi-linear or exhibit quasi-linear domains are illustrated in Figures (II.1.12.) and (II.1.13. These results were used to evaluate the charge dispersion in the implied transition states as compared to the corresponding initial states.

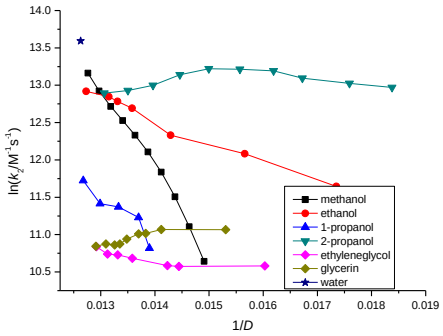


Figure II.1.12. The variation of ln k_2 against 1/D

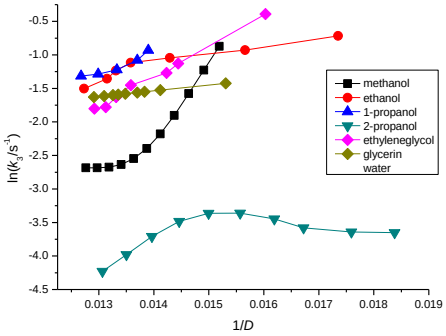


Figure II.1.13. The variation of ln k_2 against 1/D

To find out if the solvent effect can be explained only on the basis of dielectric solvation, the kinetic parameters v_R^0 , k_2 şi k_3 were interpolated for an isodielectric medium with D=76. The results are given in Figure (II.1.14.). The same trend was also found for other isodielectric media. Since the obtained values are not constant, one can conclude that the kinetics of this reaction is significantly influenced by some other specific interactions (hydrophobic, acid-base, etc.).

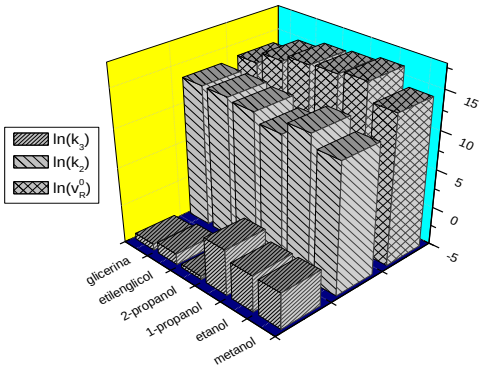


Figure II.1.14. Variation of the kinetic parameters in an isodielectric medium (D=76)

Consequently, the influence of other solvent properties was considered. One of the most widely used solvent properties necessary to take into account the hydrophobicity/lipophilicity of a solvent is log P, representing the partition coefficient of a compound between two immiscible phases [132]. For binary mixtures log $P_{mixture}$ was calculated as [133]:

$$\log P_{mixture} = x_w \log P_w + x_s \log P_s \qquad \text{(II.1.14)}$$

where x_w, x_s, P_w and P_s are the molar fractions and partition coefficients of water and added solvent. For all binary mixtures, except 2-propanol, the variation of the kinetic parameters is monotonous. When mixtures containing the same molar fraction of the nonaqueous component (e.g. x_S = 0.06) are considered, the dependence is not longer monotonous, as seen in Figure (II.1.18.).

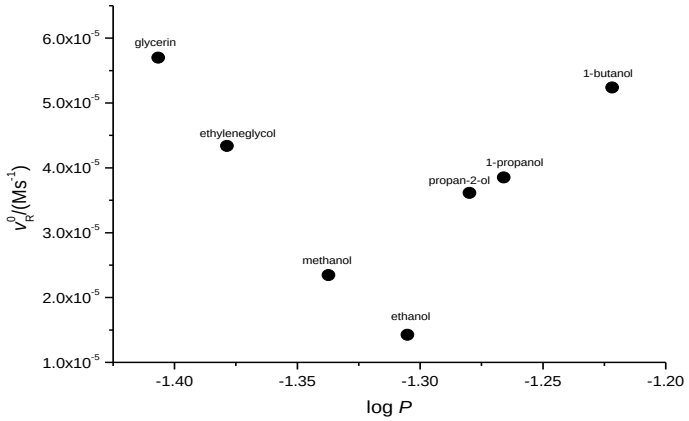


Figure II.1.18. Variation of the initial rate with hydrophobicity constant composition (x_S = 0.06)

Since the initial rates for 1-propanol and 2-propanol were higher, it was considered interesting to find the trend for higher normal alcohols; consequently the experiments were extended to water/1-butanol mixtures (up to the solubility limit). The results indicate that the variation of the initial rate with respect to solvent hydrophobicity, expressed as log P, can be divided in two distinct zones: from lower values up to log P = -1.30 the catalase activity decreases linearly, while for higher values the activity increases linearly. For polyols (ethylene glycol, glycerin) and short chain monohydroxy alcohols (methanol, ethanol), the catalase activity decreases with the solvent hydrophobicity, while for longer chain monohydroxy alcohols the variation is in opposite direction. When these results are compared with those referring to the dielectric constant effect, one can conclude that for those mixtures containing alcohols exhibiting preponderantly dielectric salvation, the catalase activity decreases with the increase of log P, while for those mixtures containing alcohols exhibiting also significant specific solvation effects, the trend is in the opposite direction.

Data from the literature show that the number of hydroxyl groups, especially for polyols, have a significant effect on the stability and activity of some enzymes [134]. This is why it was considered useful to calculate the hydroxyl group concentration [OH] from the available density data [135] and the corresponding molar fractions, for all studied water-alcohol mixtures. It was found that the initial reaction rate increases with [OH], for all systems under investigation. For the primary lower alcohols (methanol, ethanol, 1-propanol) the variation is approximately linear, with slopes of roughly 10^{-7} s^{-1}. For polyols, the variation is still linear but with slopes 10 times smaller than for monohydroxy alcohols, while for 2-propanol the variation is no longer linear. It seems that the primary hydroxyl groups have the most important effect on the catalase activity.

Since the analysis of the solvent effect using only one solvent parameter was not sufficient to establish the weight of each solvent property on the kinetic parameters of the hydrogen peroxide decomposition in the presence of catalase, a multilinear correlation, taking into account the cumulative activity of several properties, was tried. Data of the literature show that numerous physical and empirical properties of solvent can be used for a multivariate analysis of a chemical reaction in different organic solvents [136]. For a rational and reliable approach, it is necessary to choose a reduced set of representative parameters, independent of each other. There are many parameter sets used in literature. Among these, a frequent choice in

chemical kinetics [137, 138] is a set of four characteristic parameters of which two characterize the nonspecific interactions and the other two the specific solute-solvent interactions. The characteristic multilinear equation, which can be applied to reaction rate or to kinetic parameters variation using four linearly independent parameters, is of the form:

$$A = A_0 + yY + pP + eE + bB \qquad \text{(II.1.15)}$$

where A is the dependent (or response) variable (the initial rate or a rate constant), A_0 is the dependent variable value in the reference medium, Y and P are nonspecific solvent properties describing the electrostatic solvation, E and B are specific solvent properties describing the Lewis acidity and basicity (electrophilic and nucleophilic solvation), while y, p, e and b are the regression coefficients measuring the sensitivity of the response variable with respect to the four independent variables [136].

For the reaction under investigation the following solvent descriptors were chosen: the Kirkwood function $(D-1)/(2D+1)$ for Y, the polarizability α for P, the Kamlet – Taft parameter β, for E, and the Kosower number Z for B; with these descriptors the equation (II.1.14) takes the form:

$$A = A_0 + y \cdot \frac{D-1}{2D+1} + p \cdot \alpha + e \cdot \beta + b \cdot Z \qquad \text{(II.1.16)}$$

This equation was used for each water-alcohol mixture, of variable composition. Very good correlations were obtained for mixtures containing methanol, ethanol, 1-propanol. The determination coefficient R^2 decreases slightly for mixtures with 2-propanol and ethylene glycol, while for glycerin the determination coefficients are very small. In order to check the effect of all these overall descriptors on all mixtures, the initial rate and rate constants were estimated at a same solvent molar fraction ($x_S = 0.06$).

It can be seen that the sensitivity of the initial reaction rate is of the same order of magnitude for Kirkwood parameter, α, and β, but smaller for Z. Except for (D-1)/(2D+1), the initial reaction rate increases with the increase of all descriptors. Taking into account that the initial reaction rate is independent of the enzyme inactivation, the same correlation is also expected for k_2. The results given in Table (II.1.5) show that k_2 increases with all descriptors and is preponderantly influenced by (D-1)/(2D+1) and α . On the other hand the correlation involving the inactivation constant k_3 is poor.

II.1.b. pH and ionic strength effects on the kinetics of H_2O_2 decomposition in the presence of catalase

This chapter presents the kinetic study on the decomposition of hydrogen peroxide in the presence of catalase for ionic strength between 0.002M and 0.1M and for pH between 4 and 11. The ionic strength was modified by using different concentrations of citrate, phosphate and carbonate buffers. It was observed that the catalase activity did not vary significantly with pH, except for very acid pH values. The initial reaction rates calculated using the equation (II.1.3) are given in Figure (II.1.25.).

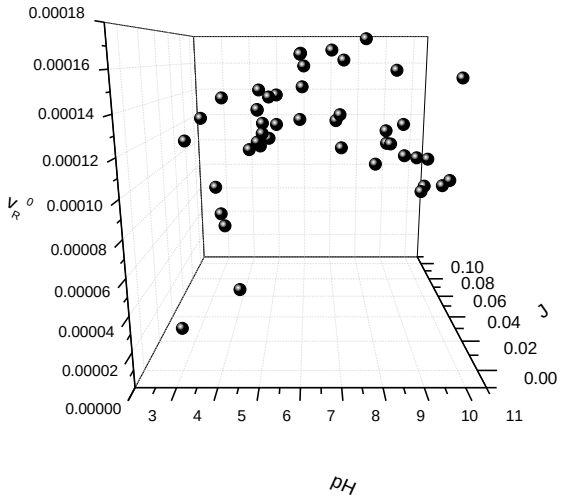


Figure II.1.25. Variation of the initial reaction rate with pH and ionc strength

It can be seen that there is a bell-shaped variation in the initial rate of reaction with respect to pH, while there is not a definite dependence with respect to ionic strength. For pH = 6 two different buffers were used (citric acid/sodium phosphate and monosodium phosphate/disodium phosphate) to check if the buffer components can modify significantly the catalase activity. For lower ionic strengths it was shown that the reaction rate is independent of the buffer presence and nature. The rate constants of the component steps were estimated on the basis of the kinetic model (II.1.6) solving numerically the corresponding system of differential equations which describes the time evolution of system (II.1.8). For pH centered on the neutral values, there are no significant changes of the rate constants with pH. For pH = 4, at extreme ionic strengths lower values of k_2 are found. For k_3 the lowest values were found for the 6 – 8 pH range. For pH = 4 and large ionic strength a strong catalase inactivation occurs due to its denaturation [139].

II.1.c. The effect of some additives on the thermal stability of catalase

A simple way to stabilize the enzymes against their thermal inactivation is the addition in the reaction medium of some simple compounds: salts, polyols, carbohydrates, other organic compounds [111,126,144]. To this end, the thermal inactivation rate constant of catalase during the hydrogen peroxide decomposition was evaluated in the presence of different additives such as ethanol, ethylene glycol, glycerin and sucrose [134,146 -148]. The catalase thermal inactivation was investigated in two distinct conditions: i) in the presence of substrate measuring the hydrogen peroxide consumption in time at different temperatures between 30 and 60^{o}C; ii) incubating the enzyme in the absence of substrate at different temperatures and different times followed by kinetic runs at room temperature, after different incubation times [154]. For aqueous solutions without additives supplementary kinetic runs were made at constant substrate concentration and variable enzyme concentrations between 10^{-7} and 10^{-6} M (in protein) at several temperatures between 30 and 55^{o}C. These measurements were necessary for the estimation of the overal inactivation constant using the isoconversional method [148]. The initial reaction rates calculated using the equation (II.1.3) and given in Figure (II.1.42.) for the catalase inactivation in the presence of substrate exhibit a maximum around 40^{o}C for water and water-ethylene glycol and water-glycerin mixtures, 43^{o}C for water NaCl and 45^{o}C for water-sucrose mixture.

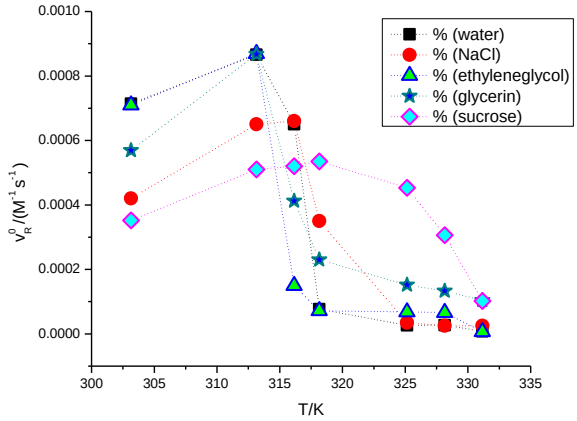


Figure II.1.42. Variation of the initial reaction rate with system temperature

In the presence of NaCl or sucrose, the initial reaction rates are smaller than in other media for temperatures lower than 45^{o}C. At higher temperatures in the presence of sucrose or of glycerin the thermal inactivation is less apparent. Taking into account that the measurements were obtained at different temperatures, all the rate constants from the model (II.1.6) are also temperature dependent. All the rate constants were estimated using the numerical solutions of the system of differential equations (II.1.8). The results for aqueous medium are given in Figure (II.1.43).

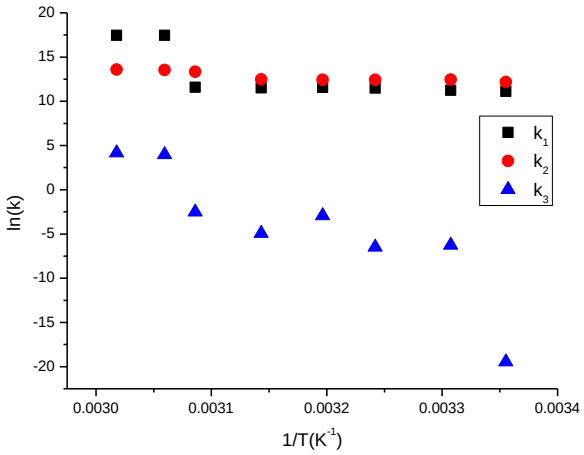


Figure II.1.43. Temperature variation of the rate constants in aqueous medium

It can be seen that the largest variation is exhibited by the inactivation constant k_3.
For each step the corresponding activation energy was estimated from the linear regression ln k_i = f(1/*T*). The results indicate that the thermal inactivation is a slow process of reduced probability with activation energy 50 times greater than the activation energies of the other two steps.

A useful but qualitative method to detect if an enzyme suffers inactivation was proposed by Selwyn [149]; it requires making several kinetic runs for the same substrate concentration and different enzyme concentrations. On the basis of this procedure, an isoconversional method has

been proposed [148], allowing the estimation of the inactivation constant. A series of kinetic measurements in aqueous solutions were performed at different temperatures within the range 35 to 55^{0}C using enzyme concentrations within the range 1 10^{-7}–1 10^{-6} M. For each temperature the inactivation occurrence was ascertained using the plot [S] =f ($[E]_0$·t). It was noticed that the inactivation becomes more important when the temperature increase, as seen in Figures (II.1.47.) and (II.1.48.).

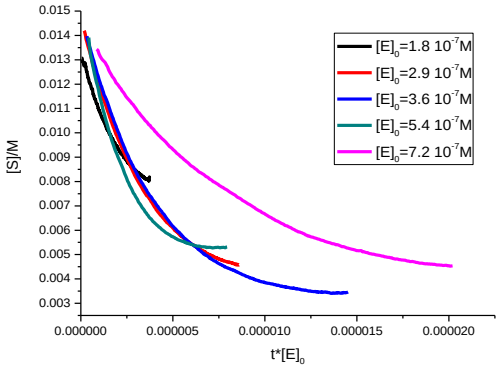


Figure II.1.47. Selwyn curve for water at 55^{o}C

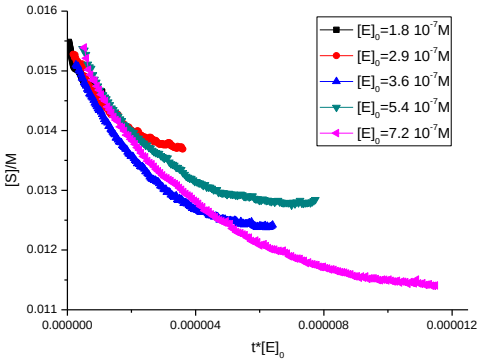


Figure II.1.48. Selwyn curve for water at 35^{o}C

Once the inactivation process was detected, the kinetic curves [S] = f(time) for different $[E]_0$, were used to determine the reaction times corresponding to a selected substrate concentration as well as the derivatives d[S]/dt at these times. The procedure consists in fitting a function of the type (II.1.1) on the experimental data, followed by an interpolation and a numerical derivation at the corresponding times. For a first order inactivation, the derivatives $(d[S]/dt)_i$ for a selected substrate concentration (isoconversional condition) depend on the isoconversional times t_i according to equation (II.1.16):

$$\frac{d[S]}{dt} = f([S])[E]_{0,i}e^{-k_{in}t_i} \qquad \text{(II.1.16)}$$

From this equation, the overall inactivation constant k_{in} can be estimated through linear regression on equation (II.1.17):

$$\ln\left[\left(\frac{d[S]}{dt}\right)_i \frac{1}{[E]_{o,i}}\right] = \ln f([S]) - k_{in}t_i \qquad \text{(II.1.17)}$$

when the slope equals the inactivation constant. The results are given in Figure (II.1.49). It is observed that the obtained values are of the same order of magnitude for temperatures up to 45^{o}C, while at 55^{o}C significant deviations exist; these deviations can be attributed to the errors accompanying the estimation procedure based on the kinetic model which contains many parameters.

The activation energy for enzyme inactivation was estimated from the linear regression ln k_{in} = f(1/T). The obtained value Ea = 136 kJ/mol is similar to the estimates determined using the kinetic model (II.1.6).

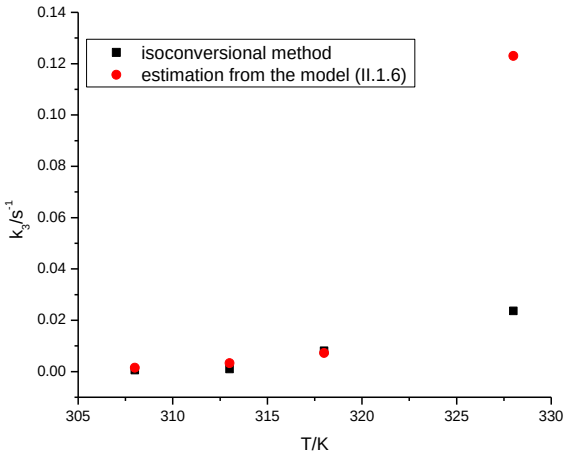


Figure II.1.49. Comparative values of the inactivation constant in aqueous medium

Thermal inactivation in the presence of substrate

The initial reaction rates were calculated according to equation (II.1.3), taking into account their dependence on the additive nature, incubation time and temperature. The relative reaction rates were calculated according to equation:

$$v_R^{rel} = \frac{(v_R^0)t_{in}}{(v_R^0)t_0} \qquad \text{(II.1.18)}$$

where $(v_R^0)t_{in}$ is the initial reaction rate after the incubation time t_{in} and $(v_R^0)t_0$ is the initial reaction rate before incubation. The variation of the relative reaction rates show that the inactivation degree increases with the temperature increase for all investigated additives. If these figures for systems with different additives at each temperature are compared, one can observe that the addition of NaCl or ethylene glycol has no significant effect on the enzyme thermal stability while the glycerin and sucrose protect the enzyme against thermal inactivation. When the temperature increases over the optimum activity temperature of catalase, an inactivation effect of ethylene glycol occurs, while the glycerin and sucrose are still good stabilizers.

To find out the best model for catalase inactivation, the available kinetic equations frequently reported in literature [142,150], were fitted on the experimental data giving the relative initial reaction rate as a function of the incubation time. These models were chosen in accord with the observation that the experimental data show an exponential decay, characteristic for first order processes and are given in Table (II.1. 9.). Three distinct models were selected: a first order model with the formation of a totally inactive enzyme (D), a first order model with the formation of a partially active enzyme (E^*), and a model where the formation of the inactive enzyme occurs in two successive first order steps. (k_1 is the rate constant of the first inactivation step, k_2 is the rate constant of the second inactivation step, and β^* is the initial partial enzyme activity). The estimation of the kinetic parameters of the inactivation process was based on the nonlinear regression analysis and for the model discrimination both statistical criteria and some physical restrictions were used. The confidence interval of the estimated parameters, the quality of fit expressed as the adjusted coefficient of determination (R^2_{adj}), the sum of the squared residuals (SS) and their distribution were included among the statistical criteria. The Fischer test F was also given, considering the highest values exceeding the critical threshold for the number of experimental data and the number of estimated parameters.

Table II.1.9. Thermal inactivation models and their characteristic kinetic equations

Kinetic model	Kinetic equation
E→D (total inactivation)	$v_R^{rel} = \exp(-k_1 \cdot t)$ (II.1.19)
E→E* (partial inactivation)	$v_R^{rel} = (1-\beta^*)\exp(-k_1 \cdot t) + \beta^*$ (II.1.20)
E→E*→D (successive inactivation)	$v_R^{rel} = \exp(-k_1 \cdot t) + \beta^* \frac{k_1}{k_2 - k_1}(\exp(-k_2 t) - \exp(-k_1 \cdot t))$ (II.1.21)

The physical restrictions imposed positive values for the evaluated kinetic parameters, without exceeding the maximum (limiting) values predicted by the transition state theory.

Depending on the additive nature, the best fitted model proved to be given by partial enzyme inactivation in pure water and in the presence of NaCl or of sucrose, by total enzyme inactivation in the presence of ethylene glycol and by successive enzyme inactivation in the presence of glycerin.

The relative half times, calculated as the ratio of half time in the presence of additive and in pure water, are illustrated in Figure (II.1.55). At 30°C each additive has a stabilizing effect, at 45°C only the ethylene glycol leads to catalase inactivation and at 60°C the best thermal stabilizer is sucrose.

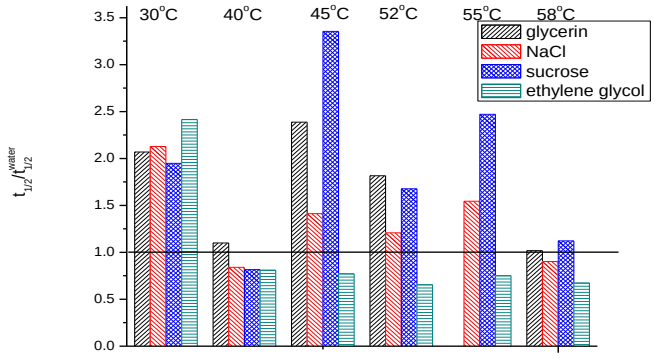


Figure II.1.55. The relative half times in the presence of additives

For a more comprehensive characterization of the overall thermal stabilization ability of the investigated additives, the corresponding activation energies of inactivation were also estimated using the linear regression on the equation:

$$\ln(k_1) = \ln(A) - \frac{E_a}{R}\frac{1}{T} \quad \text{(II.1.23)}$$

The results, given in Table (II.1.11) indicate that the best thermal stabilizers are glycerin and sucrose.

Table II.1.11. Activation energies in the presence of additives

Aditive	E_a(kJ/mol)
-	106.2±15.1
NaCl	115.4±18.1
ethane-1,2-diol	100.7±15.5
propane-1,2,3-triol	125.9±14.7
sucrose	127.6±12.2

II.2.a. The use of isoconversional method to detect the peroxidase inactivation

II.2.a1. Oxidation of capsaicin

In order to investigate the kinetics of capsaicin oxidation a spectrophotometric method was also used; capsaicin was provided as an alcoholic extract from hot pepper (*Capsicum*). The UV-VIS spectra were recorded and the wavelength λ = 262 nm, where the reaction products absorb, was chosen for quantitative measurements. To verify if the enzyme is inactivated during the catalytic process, a Selwyn test was performed which confirmed the existence of the inactivation. The isoconversional method was subsequently applied and the isoconversional times for the product absorbance A = 1.8 and the corresponding derivatives dA/dt were evaluated. From the linear regression ln $[(dA/dt)_i \cdot 1/[E]_{0,i}] = f(t_i)$ illustrated in Figure (II.2.3) the inactivation constant was evaluated to be (2.87±0.16) 10^{-3} s^{-1}.

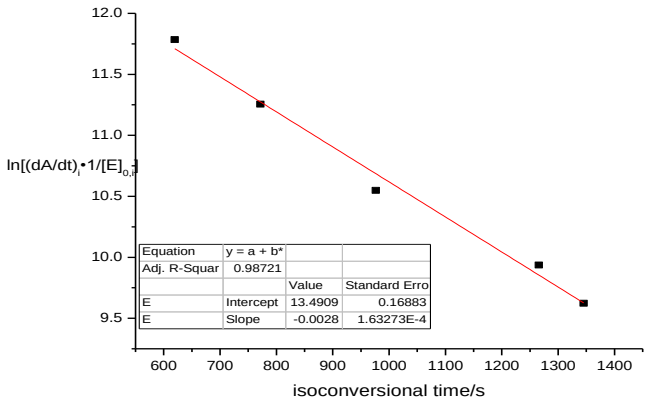


Figure II.2.3. Estimation of the inactivation rate constant for oxidation of capsaicin

II.2.a2. Oxidation of guaiacol

For the oxidation of guaiacol a similar spectrophotometric method was also used. The inactivation of peroxidase was confirmed using the Selwyn test and the isoconversional method was used subsequently. The plot ln $[(dA/dt)_i \; 1/[E]_{0,i}] = f(t_i)$ was linear and the inactivation rate constant was (9.22±0.28) 10^{-4} s^{-1}.

II.2.a3. Oxidation of catechin

Using a similar procedure the average inactivation rate constant was (2.32±1.28) 10^{-5} s^{-1}.

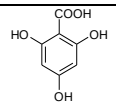
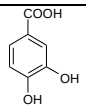
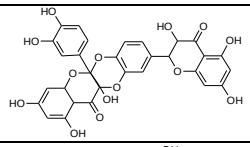
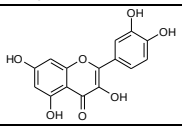
II.a4. Oxidation of quercetin

The oxidation reaction of quercetin was investigated both in ethanol and methanol to find out if there is a significant solvent effect on the peroxidase inactivation. The values of the inactivation constant obtained in methanol are larger than in ethanol.

II.2.b. Stability and inactivation of horseradish peroxidase in the presence of quercetin and hydrogen peroxide

The identification of the reaction products resulted from the auto-oxidation and enzymatic oxidation of quercetin was realized during a training period at *Mediteranean Agronomic Institute of Chania(M.A.I.C.H),* in the department of *" Food Quality and Chemistry of Natural Products*" under the guidance of *Dr. Panagiotis Kefalas.* Using the chromatographic techniques LC-MS and HPLC, the auto-oxidation and enzymatic oxidation of quercetin was investigated in the presence and in the absence of hydrogen peroxide in ethanolic solution of quercetin with addition of phosphate buffer. The identified compounds are presented in Table (II.2.3). The results indicate that the products from auto-oxidation and enzymatic oxidation of quercetin are similar, but the reaction mechanism is dependent on the oxidizing agent [164, 168].

Table II.2.3. The identified compounds using the LC-MS analysis for enzymatic oxidation of quercetin in the presence and in the absence of hydrogen peroxide

Structure	Name	$[M+H]^+$
	2,4,6-trihidroxibenzoic acid	170
	3,4-dihidorxibenzoic acid	154
	Dimers of quercetin	602.8
	Quercetin	303.5

The enzymatic oxidation results in breaking of flavonoid skeleton with the formation of cathechuic and gallic acids, accompanied by dimers and trimers formation.

Kinetics of quercetin oxidation

The oxidation reaction of quercetin with different oxidants (oxygen, hydrogen peroxide) in the presence and in the absence of HRP was investigated using a spectrophotometric method. In the absence of the enzyme, both the auto-oxidation (oxidation with dissolved oxygen) and oxidation with hydrogen peroxide were investigated. The enzymatic oxidation was investigated in the presence of hydrogen peroxide. To obtain some new information regarding the effect of the reaction medium on these reactions, the measurements were done in several different media:

water, buffer solutions, presence of surfactants (non-ionic Brij, cationic hexadecylpyridinium chloride (HDPC), alkyl polyglucoside (APG)), alcohols (methanol, ethanol). In solutions containing Brij or HDPC, two absorption peaks belonging to quercetin, centered at 260 nm and 375 nm, were detected. The experimental data indicated that the auto-oxidation reaction can be neglected in comparison with oxidation in the presence of other oxidizing agents and surfactants. The decrease of the absorption bands at 260 nm and 375 nm confirmed the oxidation of quercetin in all the investigated media. Excepting for ethanol, in all other media an increase of the absorbance at 320 nm was observed; it was attributed to the products formation with heterodimer as the preponderant species. Since the measured absorbance resulted through the superposition of at least two components, the kinetic analysis was performed for the initial reaction rates at λ= 370 nm for a preponderant absorption of quercetin and at λ= 330 nm where the dimers absorb [167]. For the wavelength corresponding to the absorption maximum of band III (λ= 370 nm), the necessary calibration curves were plotted through successive quercetin dilution. For the calculation of the initial reaction rates, only the absorbance at 370 nm and at 320 nm were used; the equation (II.2.1) followed by the calculation of the first order derivatives at t = 0 [equation II.2.2] was used for a qualitative and comparative analysis:

$$[Qu] = ae^{-bt} + ce^{-dt} \qquad (II.2.1)$$

$$v_R^0 = \left(\frac{1}{v_S}\frac{d[Qu]}{dt}\right)_{t=0} = ab + cd \qquad (II.2.2)$$

The initial reaction rates are presented in Table II.2.5. It can be seen that that the rate of auto-oxidation is higher in alcoholic media and increases with the pH of the added buffer. The same order was observed for the dimers formation.

Table II.2.5. Initial reaction rates for quercetin auto-oxidation in different media

Reaction medium	$v_R^0\ 10^8$/M s^{-1}
Water without disolved O_2 (Ar purge)	0.232
buffer pH=5.7	0.967
APG	0.658
ethanol - water	0.869
ethanol – pH 8	1.730
methanol-pH 8	2.110
methanol-pH 10	9.720

For the investigation of the chemical and enzymatic oxidation of quercetin, only alcoholic solutions were used due to its higher solubility, subsequently mixed with aqueous buffers.

Quercetin oxidation with hydrogen peroxide

The ethanolic and methanolic solutions of quercetin were oxidized with hydrogen peroxide in the presence of phosphate buffer with pH 8. The time evolution of the system was monitored spectrophotometrically within the range 250-450 nm. The results are given in Figure (II.2.40).

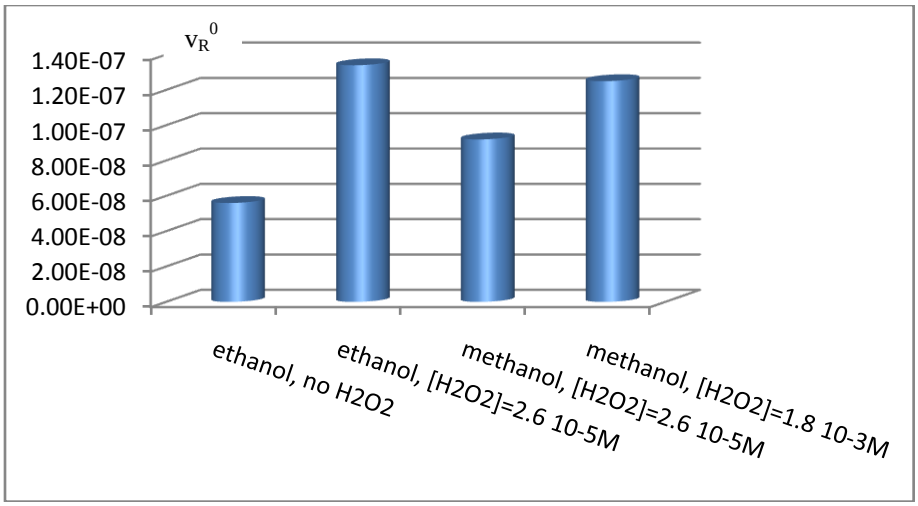


Figure II.2.40. Initial reaction rates for quercetin oxidation with H_2O_2 in alcoholic media

Influence of the reactant concentration on quercetin oxidation with hydrogen peroxide

To see the influence of the concentration of reactants and to determine the reaction partial orders, determinations were made in methanol using the isolation method. The concentrations of the unreacted quercetin were calculated from the consecutive recorded spectra. The following partial reaction orders were determined: I for quercetin and 0 for hydrogen peroxide. The shape of the dimers kinetic curves suggest that for higher reactant concentrations the dimers are accumulated to a certain concentration followed by a subsequent transformation in benzoic acids proved by gallic and protocatechuic acids identification.

Quercetin oxidation with hydrogen peroxide in the presence of peroxidase

The kinetics of quercetin oxidation using hydrogen peroxide in the presence of peroxidase was monitored spectrophotometrically. Different kinetic runs were performed successively varying the concentration of one component while the concentrations of the other components were kept constant; for quercetin solubilization, the reaction medium was changed by adding methanol, ethanol, buffer solutions having different pH values or surfactants. The following results for the partial reaction orders were found: $\alpha_{Qu}=1$, $\alpha_{H2O2}=0.35$, $\alpha_{HRP}=0.11$. For H_2O_2 and peroxidase the coefficients of determination were very small indicating that the reaction is not a simple one, a conclusion confirmed also by other kinetic data and by the chromatographic analysis of the reaction products.

II.2.c. Stability and inactivation of peroxidase in the presence of catechin and hydrogen peroxide

The products identification for the auto-oxidation and enzymatic oxidation of catechin was performed under conditions similar to those discussed for quercetin oxidation. The auto-oxidation and enzymatic oxidation (in the presence or in the absence of hydrogen peroxide) was investigated using ethanolic solutions of catechin with addition of phosphate buffer. The analysis of the reaction mixture indicated the presence of catechin dimers (dehydrocatechin A and B) having similar structures and properties.

Kinetics of catechin oxidation

The catechin oxidation was investigated spectrophotometrically in various conditions (aqueous or alcoholic media, in the presence or absence of buffer). Both the auto-oxidation (with the dissolved atmospheric oxygen) and enzymatic oxidation using hydrogen peroxide were investigated. Auto-oxidation was studied in aqueous solution, in acid and basic media. The characteristic absorbance of catechin and reaction products indicated a bathochromic shift with the pH increase. The dimers concentrations were calculated from their absorbance. They suffer other reactions leading to a decrease of the measured absorbance. Using the initial linear dependence of the kinetic curves [Dimers] = f(t) it was possible to estimate the initial reaction rates for the auto-oxidation of catechin in ethanolic solutions with buffers addition having various pH values. The variation of the initial reaction rate with the pH of the buffer is given in Figure (II.2.94).

Enzymatic oxidation of catechin

The reaction was investigated spectrophotometrically. Different kinetic runs were performed successively varying the concentration of one component while the concentrations of the other components were kept constant. The spectra of the reaction mixture were recorded within the interval 200-800 nm, for 800 – 2,000 seconds.

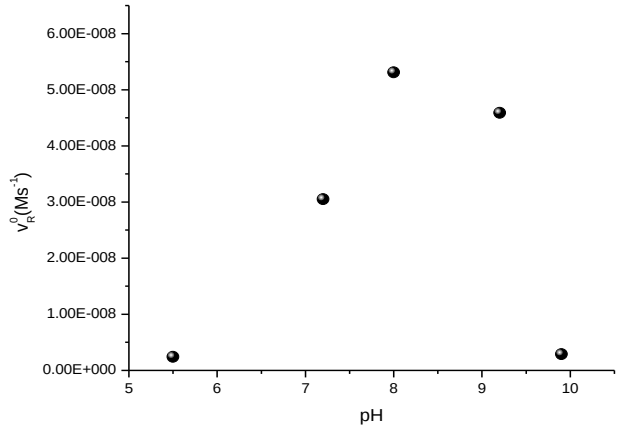


Figure II.2.94. Variation of the initial reaction rate with the pH of the added buffer for the auto-oxidation of ethanolic solutions of catechin

Influence of catechin concentration

Using the initial linear dependence of the kinetic curves [Dimers] = f(t), the initial reaction rates were estimated for different catechin concentrations and given in Table (II.2.7).

Table II.2.7. Variation of the initial reaction rates with the initial catechin concentration

[Catechin]/M	v_R^0 10^8(Ms^{-1})
5.00 10^{-5}	(2.68±0.15)
7.50 10^{-5}	(4.40±0.39)
1.25 10^{-4}	(9.25±0.78)

The results are greater than those for the auto-oxidation reaction. The partial reaction order with respect to catechin was determined from the regression ln v_R^0 = f(ln [Dimers]) and is 1.35±0.06, with a coefficient of determination R^2 = 0.9978.

Influence of hydrogen peroxide concentration

The obtained results are given in Table (II.2.8).

Tabel II.2.8 Variation of the initial reaction rates with hydrogen peroxide concentration

$[H_2O_2]10^5$/M	$v_R^0\ 10^8$(Ms^{-1})
5.5	(6.11±0.33)
6.0	(7.69±0.32)
7.3	(7.85±0.45)
8.0	(5.44±0.23)

The results are also greater than those for the auto-oxidation reaction; when the hydrogen peroxide concentration increases over $7 \cdot 10^{-5}$ M a decrease of the initial reaction rate occurs, which can be attributed to inactivation of peroxidase. From the plot ln v_R^0 = f (ln [Dimers]) the partial reaction order with respect to hydrogen peroxide was evaluated as 1.01±0.25 with a coefficient of determination R^2 = 0.9678.

Influence of enzyme concentration

The results are given in Table (II.2.9).

Table II.2.9. Variation of the initial reaction rate with enzyme concentration

[HRP]10^9/M	$v_R^0\ 10^8$(Ms^{-1})
6.75	(8.41±0.75)
7.89	(9.63±0.12)
11.5	(13.8±1.20)
14.1	(9.97±0.96)

The results are also greater than those for the auto-oxidation reaction; when the hydrogen peroxide concentration increases over 10^{-8}M, a slight decrease of the initial reaction rate occurs, which can be attributed to enzyme concentration. From the plot ln v_R^0 = f (ln [Dimers]) the partial reaction order with respect to peroxidase was 0.98±0.12 with R^2 = 0.9997.

Influence of pH

The ethanolic solution of catechin was oxidized enzymatically with hydrogen peroxide with addition of pH buffers. The buffers were monosodium phosphate/disodium phosphate for pH 7.2 and 8.0, or sodium carbonate/sodium bicarbonate for pH 9.9. From the spectra obtained for different pH values, the dimers concentrations were calculated using the measured absorbance at λ = 440 nm. The initial reaction rates were estimated using the initial linear dependence of the kinetic curves [Dimers] = f(t). The results are given in Table (II.2.10).

Table II.2.10. Variation of the initial reaction rate with pH

pH	$v_R^0\ 10^8(Ms^{-1})$
5.5	(1.22±0.05)
7.2	(5.48±0.46)
8.0	(7.53±0.55)
9.9	(1.37±0.10)

The comparative values of the initial rates of dimers formation are illustrated in Figure (II.2.103)

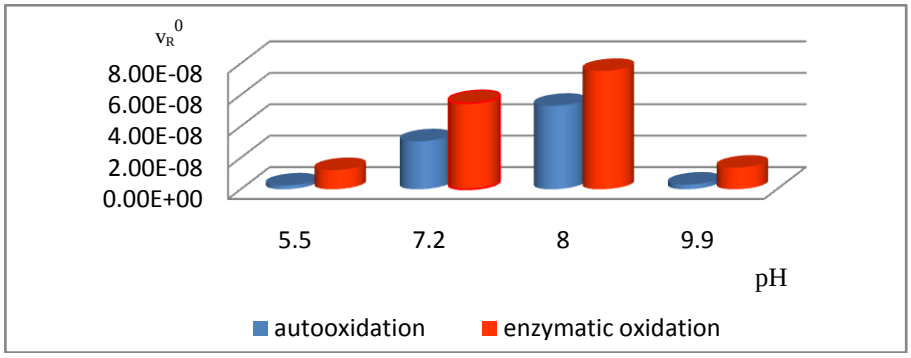


Figure II.2.103. Initial reaction rates for catechin oxidation in different conditions

At pH values close to the optimum peroxidase pH, the dimers formation rate is higher, but the enzyme is still a good catalyst for catechin oxidation even at extreme pH values.

General conclusions

In order to study the inactivation of enzymes in the presence of some additives, two oxidoreductases were chosen: catalase, an enzyme with high substrate specificity, which catalyzes the decomposition of a simple substrate – the hydrogen peroxide – and peroxidase, an enzyme with low specificity, able to oxidize a wide range of phenolic derivatives.

For the model system catalase-hydrogen peroxide, the effects of several factors on the enzyme inactivation and thermo inactivation were investigated:

- ✓ It was studied the effect of pH and ionic strength on the inactivation of catalase. A simple kinetic model was proposed, implying a first order inactivation step; the model allows a better estimation of the involved parameters and suggests that the pH influence is the result of the enzyme inactivation through its transformation into an inert compound, a fact confirmed also by the variation of the inactivation constant as a function of pH. The ionic strength resulted from the buffer components has a significant effect on the catalase activity especially at extreme pH values. The analysis of the data obtained in buffers with different components and in non buffered aqueous solutions showed that in water the inactivation is lower.
- ✓ The solvent effect (for binary water-alcohol mixtures) on the initial reaction rate and on the rate constants characteristic for a simple three step kinetic model was analyzed and discussed on the basis of several characteristic solvent properties: dielectric constant, polarizability, hydrophobicity, hydroxyl group concentration [OH], and some empirical parameters (Kamlet-Taft parameters and Kosower number). For the mixtures containing

methanol, ethanol, 1-propanol, and 1,2-ethanediol good correlations between ln v_R^0, ln k_2 and ln k_3 and the Kirkwood function (D-1)/(2D+1) were obtained, suggesting that the solvent effect has an important dielectric component. When mixtures containing the same molar fraction of the nonaqueous component (e.g. $x_S = 0.06$) are considered, for the multi-hydroxilic alcohols (1,2-ethanediol, 1,2,3-propanetriol) and short chain lower alcohols (methanol and ethanol) the catalase activity decreases with the solvent hydrophobicity while for longer chain monohydroxilic alcohols (1-propanol, 2-propanol, 1-butanol) the catalase activity increases with the solvent hydrophobicity. On the other hand, the greater the hydroxyl group concentration, the greater the initial reaction. By using the multilinear regression with four solvent descriptors – the Kirkwood function (D-1)/(2D+1), polarizability, Kamlet-Taft parameter and Kosower number – for constant molar fractions of alcohols, it was found that the kinetic parameters v_R^0, k_2, and k_3 depend significantly on the chosen descriptors.

- ✓ The kinetics of catalase inactivation in the presence and absence of hydrogen peroxide was investigated at temperatures between 30°C and 60°C. The reaction was investigated in aqueous solution and in the presence of some stabilizers proved to be efficient for many enzymes: NaCl, 1,2- etanediol, 1,2,3- propanetriol and sucrose. Using the extended kinetic curves (substrate concentration against time) and applying kinetic models from literature for the enzyme incubation in the absence of substrate, followed by measuring the hydrogen peroxide decomposition after different incubation time, the inactivation parameters (rate constants, half times and activation energies) were estimated. The stabilizing effect is temperature dependent, sucrose being the best stabilizer for catalase. Due to occurrence of both thermal and substrate inactivation, the first order rate constant for inactivation is larger in the presence of substrate than in its absence.

For the model system peroxidase - phenolic derivatives with anti-oxidant properties, the effects of several factors on the enzyme inactivation and stability were investigated:

- ✓ For the investigation of peroxidase inactivation by hydrogen peroxide, the overall enzyme inactivation constants during the oxidation of several antioxidant phenolic substrates (capsaicin, guaiacol, catechin, and quercetin) were evaluated using an isoconversional method developed in the laboratory of chemical kinetics.
- ✓ The inactivation is of the first order for all the investigated substrates. For capsaicin, guaiacol, and quercetin the overall inactivation constants have the same order of magnitude (10^{-3} s^{-1}) while in the case of catechin oxidation the inactivation is much slower (10^{-5} s^{-1}) showing that this substrate has a protecting effect for peroxidase inactivation by hydrogen peroxide.
- ✓ The oxidation of quercetin was investigated in different experimental conditions: auto-oxidation, oxidation with hydrogen peroxide in the absence and presence of peroxidase. The reaction was studied in aqueous solutions, in buffered aqueous solutions, in partially alcoholic solutions and in micellar solutions (using either anionic or non-ionic surfactants). The identification of the reaction products resulted from the auto-oxidation and enzymatic oxidation of quercetin was performed using LC-MS and HPLC techniques during a training period at "Mediteranean Agronomic Institute of Chania" (M.A.I.C.H), in the department of "Food Quality and Chemistry of Natural Products" under the guidance of Dr. Panagiotis Kefalas. With these techniques it was possible to identify as reaction products benzoic acids (catechuic and gallic acids) as well as quercetin dimers

with similar structures and properties. The products of auto-oxidation and oxidation were practically the same, but their quantities are dependent on the oxidizing agent. For the auto-oxidation in the presence of methanol and phosphate buffer the formation of benzoic acids is favored, while for enzymatic oxidation the dimers formation is favored. Peroxidase is inactivated in the presence of high concentrations of hydrogen peroxide, a behavior also encountered for other substrates.

- ✓ The oxidation of catechin was investigated in different experimental conditions: auto-oxidation, oxidation with hydrogen peroxide in the presence of peroxidase. The reaction was investigated in aqueous solutions, in buffered aqueous solutions, in partially alcoholic solutions (methanol or ethanol). The identification of the reaction products resulted from the auto-oxidation and enzymatic oxidation of catechin was achieved as with quercetin. The analysis of the reaction mixture indicates the presence of catechin dimers (dehydrocatechin A and B) with similar structures and properties. The mixture of the two dimers was obtained but their separation was not possible. From the analysis of the UV-VIS absorption spectra, the dimers characteristic bands were identified and used for the kinetic analysis. It was found that for the enzymatic oxidation the dimer formation is favored even at extreme pH values. The results are in agreement with other literature data confirming an oxidation mechanism implying dimers as products, with intermediate formation of semiquinons.

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