Université d'Ottawa • University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

MARTINEZ BELTRAN, Emerson Rafael

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M.A.Sc. (Chemical Engineering)

GRADE - DEGREE

Department of Chemical Engineering)

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Decrease of the Chlorogenic Acid Content in Sunflower Meal Using and Enzyme Preparation Secreted by the White-Rot Fungus *Trametes versicolor*

Zdravko Duunjak

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

D. McLean .....

J. Thibault .....

.....

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES — SIGNATURE — DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

DECREASE OF HT CHILOROGENIC ACID CONTENT IN SUNFLOWER MEAL USING AN ENZYME PREPARATION SECRETED BY THE WHITE-ROT FUNGUS *TRAMETES VERSICOLOR*

by

Emerson R. Martinez Beltran

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of

MASTER OF APPLIED SCIENCE

in the Department of Chemical Engineering

University of Ottawa

April 2003

Your file Votre référence
ISBN: 0-494-05675-4
Our file Notre référence
ISBN: 0-494-05675-4

ABSTRACT

This project describes the enzymatic degradation of chlorogenic acid (CGA) in a model system with pure CGA and in a sunflower meal (SFM) system. The effects of several parameters, such as pH, temperature, and substrate, enzyme and sunflower meal concentrations, on the decrease of the CGA concentration were studied. The optimum pH was found to be 4.3 for the model system and 3.4 for the SFM system; the difference in pH was due to the buffering capacity of the meal in the SFM system. The optimum temperature was 45°C for both systems. There is a linear relationship between the enzyme concentration and the initial reaction rate for low enzyme concentrations. That linearity disappears when the enzyme concentration was higher than 1 nkat/mL and 2.2 nkat/mL for the model and SFM systems, respectively. Regarding the effect of the substrate concentration, a saturation with substrate was noticed when its concentration was 0.13 mM. Further concentrations of substrate gave unreliable results in the spectrophotometer. The effect of mass transfer was also investigated. By changing the agitation speed of the system, the rate of the CGA decrease changed. However, the results of the effect of particle size of SFM on the rate of the CGA decrease did not show any pattern. In this project, it was also shown that the thermal stability of the enzyme increased in the presence of both sunflower meal and bovine-albumin.

Kinetic studies were also carried out by following the CGA decrease with time at different pH values, temperatures and enzyme concentrations. From these studies, it can be concluded that a 98% conversion of CGA can be achieved in three hours for the model system and in four hours for the SFM system. When the kinetics of the CGA decrease in the SFM system was investigated at different ratios of continuous phase to meal, it was

found that the rate of the CGA decrease was greater in the presence of a large amount of liquid phase in the system. This indicates that under these conditions, a faster extraction of CGA occurs and therefore its availability to the enzyme increases. A spectrophotometric and an HPLC technique were used to measure the CGA content in both systems. According to the obtained results, the spectrophotometric technique gave reliable results only when the initial reaction rates were measured.

A mechanistic model was proposed to model both the simultaneous effect of pH, temperature, substrate and enzyme concentrations, and the dynamic of the CGA transformation in the model system. The results showed that the mechanistic model describes the experimental results quite well.

RÉSUMÉ

Ce projet décrit la diminution par enzyme de l'acide chlorogénique (ACG) dans un système modèle d'ACG pur ainsi que dans un système de sémoule de tournesol (ST). Les effets de plusieurs paramètres sur l`abaissement de l' ACG ont été étudiés tel que l'effet du pH, de la température, du substrat, d'enzymes et de différentes concentrations de sémoule de tournesol. Le niveau du pH optimal était de 4.3 pour le système modèle et de 3.4 pour le système ST; la différence de pH s'explique par la capacité tampon du sémoule dans le système ST. La température optimale dans les deux systèmes étaient de 45°C. À basse concentration d'enzymes, il existe une relation linéaire entre la concentration d'enzymes et la vitesse initialle de la réaction. Par contre, cette relation n'est plus linéaire lorsque la concentration d'enzyme est plus grande que 1 nkat/mL et de 2.2 nkat/mL pour les systèmes modèle et ST respectivement. Pour ce qui est de l'effet sur la concentration du substrat, une saturation avec substrat a été observée lorsque la concentration de celle-ci atteint 0.13 mM. L'effet de transfert de masse a aussi été étudié. Le changement de la vitesse d'agitation du système a eu pour effet de changer la vitesse de la réduction de l' ACG . Cependant, les résultats sur l`effet de grandeur des particules ST sur le taux de diminution de l' ACG n`a pu démontrer une tendance. La stabilité thermique des enzymes a augmenté en présence de ST et de l`albumine bovin.

Des études cinétiques ont aussi été faites en observant les dynamiques de la décroissance de l' ACG avec le temps pour différents niveaux de pH, de températures et de concentrations d'enzyme. D'après ces études à des conditions précises, une conversion de 98% de l' ACG a été obtenue après trois heures pour le système modèle et de quatres heures pour le système ST. Les cinétiques de la décroissance de l`ACG dans

le système ST ont aussi été étudies à différentes proportions de la phase continue (liquide) par rapport à la sémoule. D'après ces tests, la vitesse de décroissance de l'ACG était plus grande lorsqùil y avait une grande quantité de liquide dans le système. Ceci indique que sous ces conditions, une extraction plus rapide de l' ACG se produit par l'augmentation de sa disponibilité aux enzymes. Deux techniques ont été utilisées pour mesurer le contenu de l'ACG dans les deux systèmes. D'après les résultats obtenus, la technique spectrophotométrique a donné des bons résultats seulement lorsque les taux de réaction initiaux furent mesurés.

Un mécanisme a été proposé pour la modélisation de l'effet simultané du pH, de la température, de la concentration de substrat et d'enzyme ainsi que de la dynamique de la transformation de l'ACG dans le système modèle. Les résultats ont démontré que la modélisation mécanistique décrit plutôt bien les résultats expérimentaux.

Acknowledgments

I would like to give my gratitude to Dr. Z. Duvnjak for his guidance and financial support during the course of this project and for helping me to overcome many of the problems encountered during this project. I also would like to thank Archer Daniels Midland (ADM), Decatur, IL, for donating the sunflower meal. I am very grateful to Dr. Hasan Atiyeh for his help and insights into many of the initial problems found in this project as well as for his companionship. I also would like to thank my lab colleagues, Nilesh Patel, Sriram Kumarappan, and J. Shen for their advice, help and companionship in the last three years. The technical assistance given by Mr. L. Tremblay, Mr. F. Ziroldo, and Mr. G. Nina during the last three years is also appreciated.

I would like to thank God for giving me health and strength to complete this goal in my life. It is also a joy to express my gratitude to my parents and family, especially my grandma, for their support and encouragement, without which this work would not have been possible. And last, but not least, I would like to thank the Bannister's and the Santana's families for their support, love, and understanding.

NOMENCLATURE

ABBREVIATIONS

GABA	- Acetylcholine and Gamma-Aminobutyric Acid
ATCC	- American Type Culture Collection
CA	- Caffeic Acid
CM	- Canola Meal
CGA	- Chlorogenic Acid
FA	- Ferulic Acid
GLC	- Gas Liquid Chromatography
HPLC	- High Performance Liquid Chromatography
I.D.	- Internal Diameter
LP	- Lignin Peroxidases
LC-MS	- Liquid Chromatography-Mass Spectroscopy
MnP	- Manganese-Dependent Peroxidases
MtOH	- Methanol
MW	- Molecular Weight
PPO	- Polyphenol Oxidase
PPO-L	- Polyphenol Oxidase from leaves
PPO-E	- Polyphenol Oxidase from fruit endosperm
QA	- Quinic Acid
SA	- Sinapic Acid
SAE	- Sinapic Acid Esters
SALD	- Sinapaldehyde

SIN	- Sinapine	
SFM	- Sunflower Meal	
TCA	- Trichlroacetic Acid	
UV-VIS	- Ultra-Violet Visible	

SYMBOLS

A_{ini}	- initial absorbance	
A_t	- final absorbance	
A(w)	- absorbance at wavelength w	
Δt	- enzymatic assay time	[s]
ΔS^*	- entropy change of a reaction	[J mol^{-1} K^{-1}]
ΔG_i^0	- standard Gibbs energy of reaction i	[J mol^{-1}]
ΔA_i	- constant specific for i^{th} reaction	[-]
ΔB_i	- constant specific for i^{th} reaction	[K^{-1}]
ΔC_i	- constant specific for i^{th} reaction	[K^{-2}]
ΔD_i	- constant specific for i^{th} reaction	[K^2]
[CGA]	- chlorogenic acid concentration	[mM]
[S]	- substrate concentration	[mM]
[ES]	- concentration of the enzyme substrate complex	[mM]
[E]	- concentration of enzyme	[mM]
[EP]	- concentration of the enzyme product complex	[mM]
[P]	- product concentration	[mM]
c	- concentration of absorbing species	[mM]
Dil	- dilution factor	[-]

E^0	- protonated form of the enzyme	
E^{-2}	- deprotonated form of the enzyme	
E_a^-	- active form of enzyme	
E_d^-	- thermally deactivated form of enzyme	
ES	- enzyme substrate complex	
EP	- enzyme product complex	
$E_{a,i}$	- activation energy for reaction i	[kJ mol^{-1}]
E_d	- energy of enzyme deactivation	[kJ mol^{-1}]
e_0^-	- initial concentration of the active protonated form of the enzyme	[mM]
$e_a^-(t)$	- concentration of active form of the protonated enzyme after time t of incubation	[mM]
$\langle e_a^- \rangle$	- average concentration of the active form of enzyme during the reaction	[mM]
$[e_0]$	- total initial concentration of the enzyme	[mM]
e(w)	- proportionality constant (molar absorptivity)	[moles/liter]
h	- Planck constant	[J s]
H^+	- concentration of hydrogen ions	[M]
I_i	- constant specific for i^{th} reaction	
J_i	- constant specific for i^{th} reaction	[J mol^{-1}]
Km	- Michaelis-Menten constant	[mM]
K_{ms}	- Michaelis-Menten constant for substrate S	[mM]
K_{mp}	- Michaelis-Menten constant for product P	[mM]

K_i	- protonation or deprotonation dissociation constant	[M]
K_1	- protonation dissociation constant	[M]
K_2	- deprotonation dissociation constant	[M]
k_d	- enzyme decay/deactivation constant	[s^{-1}]
k_{cat}	- turnover number	[s^{-1}]
k_1	- second order rate constant	[mM^{-1} min^{-1}]
k_2	- first order rate constant	[min^{-1}]
k_3	- second order rate constant	[mM^{-1} min^{-1}]
k_{-1}	- first order rate constant	[min^{-1}]
k_{-2}	- second order rate constant	[mM^{-1} min^{-1}]
k_{-3}	- first order rate constant	[min^{-1}]
k_B	- Boltzman constant	[JK^{-1}]
k_i	- rate constant for reaction i	[min^{-1}] or [mM^{-1} min^{-1}]
$k_{0,i}$	- frequency factor for i^{th} rate constant	[mM^{1-no} s^{-1}]
k_i^*	- arbitrary rate constant at central or average temperature T*	[$mM^{1-no}s^{-1}$]
l	- length of the path	[mm]
R	- universal gas constant	[J mol^{-1} K^{-1}]
r_A	- reaction rate	[mM/min]
t	- time	[s]
t_r	- reaction time	[s]
T	- temperature	[K]
T^*	- central or average temperature	[K]
V_{max}	- maximum velocity (activity)	[nkat]

V_{max}^{opt}	- maximum activity at optimum pH	[nkat]
V_{max_f}	- maximum forward velocity (activity)	[nkat]
V_{max_r}	- maximum backward velocity (activity)	[nkat]
V_{sys}	- volume of the system	[ml]
V_e	- volume of the enzyme added	[ml]
ν	- enzyme activity	[nkat/ml]
υ_f	- forward initial rate (activity)	[nkat]
υ_r	- backward initial rate (activity)	[nkat]
υ	- initial reaction rate/activity/velocity	[nkat]
υ_{pH}^{opt}	- initial reaction rate at optimum pH	[nkat]
Y_c	- corrected initial reaction rate	[nkat]

GREEK SYMBOLS

α	- ratio of the volume of the bulk liquid over the volume of SFM	
β	- proportionality constant	$[mmol_{enz}mL_{enz}^{-1}]$
ε	- molar absortivity	$[M^{-1}\ cm^{-1}]$

Table of Contents

List of Figures

List of Tables

CHAPTER 1. INTRODUCTION

Sunflower seeds are an important source of vegetable oil. The oil is considered a premium oil because of its light colour, high level of unsaturated fatty acids and lack of linolenic acid, insipid flavour, and high burn points. Sunflower accounts from about 14% of the world production of seed oil and about 7% of the oilseed cake and meal produced from oilseeds (Putnam et al., 1990). Sunflower meal, which is a by-product from the oil extraction, can be used as a protein source for human and animal consumption. The potential uses of the meal have, however, been limited by the presence of some polyphenolic substances, particularly chlorogenic and caffeic acids, and by the presence of reducing sugars (Pomenta and Burns, 1971; Sabir et al., 1974; Milic et al., 1968). Depending on the extraction conditions (pH, oxidation and heat), these polyphenolic substances can cause significant darkening of the products and reduce its acceptability. A decrease in the nutritional value (Sripad et al., 1982; Jung and Fahey, 1981) of the proteins may also result due to the interaction of polyphenols with residues such as lysine, cysteine and methionine (Pierpoint, 1970) as well as alanine, phenylalanine and glutamic acid (Sripad et al., 1982). Therefore, the degradation of these phenolic compounds is desirable in order to use the meal in a greater extent as well as replacing some other more expensive protein sources, such as soybean and canola meal, in livestock diets.

Removal of these polyphenolic substances from sunflower meal has been attempted using aqueous, organic and mixtures of aqueous and organic solvents (Smith and Johnsen, 1948; Joubert, 1955; Mikolajczak et al., 1970; Pomenta and Burns, 1971; Sosulski et al., 1972, 1973; Fan et al., 1976; Sodini and Canella, 1977; Dominguez et al.,

1996). A complete removal of the polyphenolic substances has not been achieved by using these methods. This is because the hydrogen bond between hydroxyl groups of phenolic compounds and peptide bonds in proteins is strong and the equilibrium in aqueous solutions favours the formation of complexes between phenols and proteins (Loomis and Battaile, 1966). The most effective method to date to remove polyphenolic substances from canola meal is an enzymatic process, in which a polyphenoloxidase from the white-rot fungus *Trametes versicolor* was used to decrease the phenolic content by over 98% (Lacki, 1997). Due to the fact that canola meal and sunflower meal have similar properties, and that phenolic acids and their esters are common microconstituents in most oilseed, legume and cereal seeds, the enzymatic process proposed by Lacki and Duvnjak (1996a,b,c) for canola meal was proposed as a possible solution to remove the polyphenolic substances present in sunflower meal.

The main goal of this project was to study the enzymatic degradation of chlorogenic acid (CGA) in both a model system with pure CGA and a sunflower meal (SFM) system using an enzyme preparation secreted by the white-rot fungus *Trametes versicolor* ATCC 42530 strain. In addition, this project was also aimed to the development of a mechanistic mathematical model, which could describe both the dynamics of the enzymatic decrease of chlorogenic acid and the simultaneous effects of pH, temperature, substrate and enzyme concentrations when pure CGA is used (model system). From these objectives, it was expected to have a better understanding of the main factors that affect the enzymatic transformation of CGA in both systems and to predict the effect that any change in pH, temperature, substrate and enzyme concentration will have on the enzyme activity.

The thesis is organized in seven chapters. The first and second chapters describe the objectives of the present study and a literature review. The third chapter explains the methodology used to carry out this project and a description of the analytical techniques used for the analysis of samples. In the fourth chapter, the results obtained during the execution of the project are explained in detail and a discussion of each result is made. The fifth chapter contains the mechanistic mathematical model proposed to describe the enzymatic transformation of CGA and the simultaneous effect of pH, temperature, substrate and enzyme concentrations in a model system. In the sixth and seventh chapters, some conclusions are drawn and recommendations are given. The thesis finishes with a series of appendices, which contain additional results as well as some theoretical aspects of the work.

CHAPTER 2. LITERATURE REVIEW

2.1 Sunflower seed

The sunflower (*Helianthus annuus*) is believed to have originated in the Mexico area. It was introduced in Europe in the 16th century and it became very popular as an ornamental flower. In Eastern Europe, it was established as an oilseed crop. Two centuries later, it was imported into Russia from Holland and over the period 1830-40, it began to be used in the production of commercial oil. Since then, the crop steadily grew in importance and soon became one of the world's most important oilseeds. The first commercial use of the sunflower crop in the US was as a feed source for poultry. Canada started the first official government sunflower breeding program in 1930. The basic plant breeding material utilized came from Mennonite (immigrants from Russia) gardens. Because of the oil demand, the cultivation of sunflower seeds was spread and by 1946, a small crushing plant was built by Canadian farmers. Acreage spread into Minnesota and North Dakota and in 1964 a Russian cultivar called Peredovik was licensed by the Government of Canada. This seed produced high yields and high oil content. The cultivation of sunflower increased in the US with commercial interests in the production of sunflower oil. In the middle of the seventies, sunflower was hybridized providing additional yield and oil enhancement as well as disease resistance.

Sunflower plants can grow up to 10 feet tall. The flowers have a giant size, and a yellow colour and they always keep their face towards sun. Most sunflower seeds are commonly crushed for their oil. The rest, less than 25% of the total, are for animal consumption. Oil seeds are generally small and black and very rich in oil. The

confectionary (or food) seeds, which are the ones that are edible, are typically larger than the oilseed variety and they are usually stripped.

2.1.1 Sunflower seed processing

The sunflower seed is more or less four-sided and flattened. The seed has a fragile pericarp or hull, which encloses a kernel. Oil and proteins in sunflower are located in the kernel while the pericarp consists of fibers. Hulls in sunflower seeds constitute from 35 to 45% of the weight of the seeds; therefore they must be removed before processing if it is desirable to obtain a maximum yield of oil and a meal with high protein content. The process description for the oil extraction from the seed is shown in Figure 2-1. This

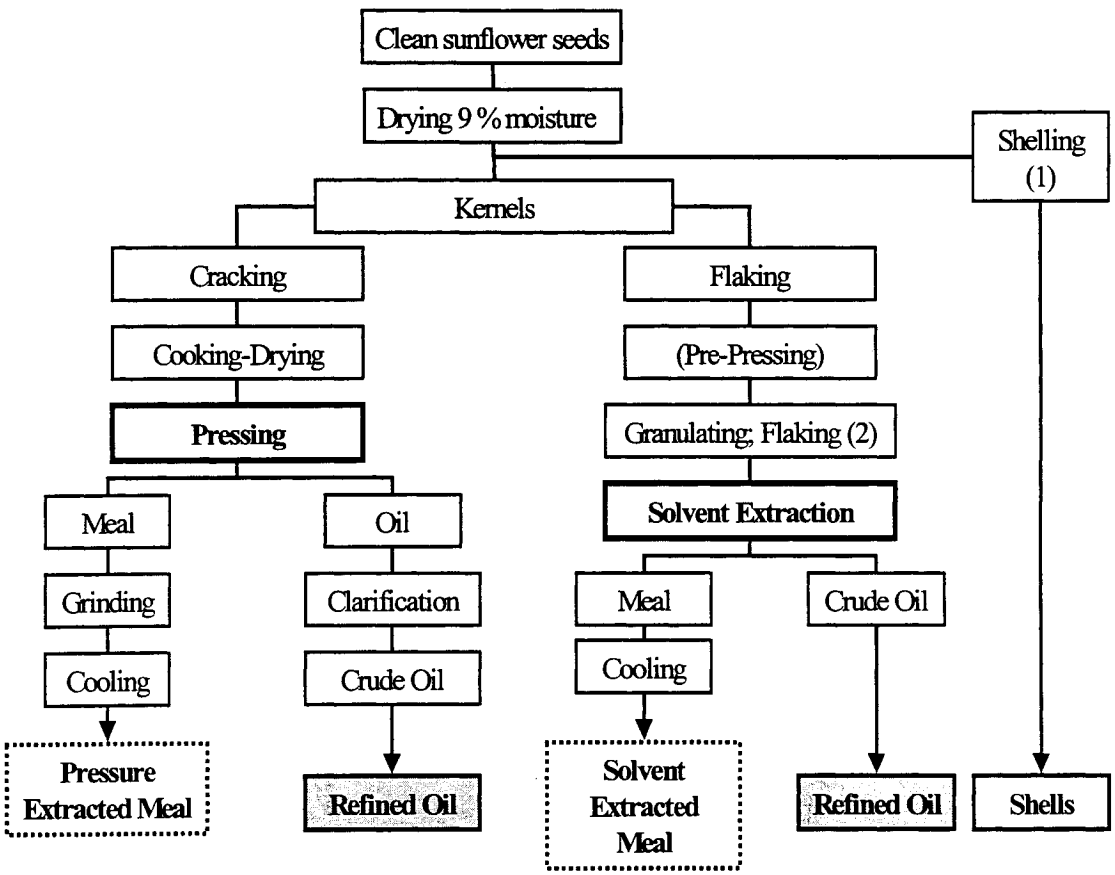


Figure 2-1. Oil extraction process[FAO/EBRD, 1999]

process consisted of:

1) Drying and storage
    - Seeds are dried (with less than 9% moisture content) and stored.
    - Drying temperatures of 50 – 70°C are suitable and do not affect the oil content.
2) Crude oil extraction
    - Separation of shells can be done in crackers and bed screens. About 8 to 12% of shells are in the kernels while 1.5% of kernels are in the shells. Shells are compacted and used as a fuel for boilers.
    - After a secondary drying, the kernels are flaked to break down the seed cells and to increase the oil extraction yield.
    - Crude oil can be obtained either through pressing or solvent extraction using hexane as a solvent. About 80% and 99% of the oil is extracted from the kernels by pressing and solvent extraction, respectively.
    - The meal obtained by pressing contains more residual oil than the one obtained by solvent extraction (1% or less residual oil for the last one).
3) Refining
    - Oil obtained by solvent extraction can be refined using the following steps: degumming, neutralizing, drying, bleaching, deodorizing, and dewaxing. When the oil is obtained by pressing, only dewaxing is necessary.
    - Degumming can be affected by adding hot water and by centrifuging.
    - The oil is neutralized by adding caustic soda and mixing under controlled conditions, which neutralizes the free fatty acids.

- Bleaching (colour removal and purification of oil) is achieved with neutral clay or bentonite (acid-activated earth). Deodorization consists of a steam-stripping of the oil at high temperature and under high vacuum.
- Dewaxing is done by cooling the oil to crystallize the wax and to remove the wax crystal by filtration.

2.1.2 Sunflower meal

Sunflower meal is obtained as a by-product of the oil extraction as shown in Figure 2-1. The total world production of sunflower meal was 11 million metric tonnes in 2000. The major producers of sunflower meal are the former Soviet Union, Argentina, Eastern Europe, USA, China, France and Spain producing 84% of the world's production. Sunflower meal, non-dehulled or partially dehulled, has been substituted for soybean meal in isonitrogenous (equal protein) diets for ruminant animals, as well as for swine and poultry feeding. The meal is higher in fiber and in methionine, but it is lower in energy value and in lysine than soybean meal. The protein percentage of sunflower meal ranges from 28% for non-dehulled seeds to 42% for completely dehulled seeds.

2.1.2.1 Composition: main components in the meal

a) General

The composition of the sunflower meal depends on several factors such as the quality of the original seed, the method of processing and whether or not the seeds are dehulled prior to oil extraction. The main compounds in the meal are proteins. Sunflower kernels are well above average in protein, phosphorus and iron concentration. They are very rich sources of B vitamins. Table 2-1 shows the composition of sunflower meal, and of the corresponding protein concentrate and isolate. The determination of moisture,

proteins, lipid, crude fiber, and ash contents of sunflower meal and of protein concentrate and isolate was done as mentioned somewhere else (Sodini and Canella, 1977).

Table 2-1. Composition of a specific sunflower meal and of the corresponding protein concentrate and isolate obtained after acidic 1-butanol treatment (Percent moisture-free basis)[a]

Description	Meal	Protein concentrate	Isolate
Moisture	10.00	10.40	2.90
Protein (N x 6.25)	60.75	67.62	93.52
Lipid	1.47	0.92	0.18
Chlorogenic acid	2.59	≤ 0.05	≤ 0.05
Total sugar	9.12	4.28	0.71
Crude fiber	4.33	4.87	0.19
Ash	7.25	8.07	1.36

[a] Sodini and Canella (1977)

b) Carbohydrates

There are no complex carbohydrates such as starch, dextrin, or inulin in sunflower meal (Altschul, 1958). The only carbohydrates present are mono-, di-, and trisaccharides, some of which are reducing or which yield reducing sugars in hydrolysis. The carbohydrate content in two sunflower meals is shown in Table 2-2.

Table 2-2. Carbohydrate content in sunflower meal[a]

	Flour (%)	Hulled oil meal (%)
Crude fiber	4.1	7.8
Nitrogen-free extract	16.8	19
Absorbable polysaccharides	14.6	13.2

[a] Altschul (1958)

c) Minerals

The macro and trace mineral content of sunflower meal is fairly high and should be accounted for when determining mineral requirements. On a dry matter basis, sunflower meal contains approximately 1.10, 1.95 and 0.65% of phosphorus, potassium, and magnesium, respectively; and 33, 43, 1.00 and 123 ppm of copper, manganese, molybdenum and zinc, respectively. Sunflower meal has a relatively high phosphorus content compared to the calcium content; therefore, the meal needs to be mixed with feedstuffs lower in phosphorous, but higher in calcium when used as a protein source. The calcium content in the flour meal is 0.36% while the phosphorus is 1.05% (Altschul, 1958).

Calcium and phosphorus are required in the greatest amount in the diets of swine. The dietary requirements for Ca are between 0.50 and 0.90% and for P are between 0.40 and 0.70% depending upon age, weight and purpose (growth, gestation, or lactation). The requirement for Ca is about 1.2 times the requirement for P.

d) Amino acids

The amino acids composition for an unextracted meal and a meal extracted with different solvents is showed in Table 2-3. This composition was reported by Sripad and Narasinga (1987) and it is in agreement with the compositions reported by different authors such as Sabir et al. (1973, 1974); Sosulski and Fleming (1977); Sodini and Canella (1977); Bau et al. (1983). These authors also found that there was no significant difference in the amino acid compositions of several extracted meal proteins compared to that of unextracted meal proteins.

Table 2-3. Amino acid composition of sunflower meals[a] (g AA/16 g N)

Amino acid	Unextracted meal[b]	Extracted with			
		acetone	ethanol	water	methanol
Aspartic acid	9.03 ± 0.4	8.95	8.89	9.13	8.88
Threonine	3.42 ± 0.08	3.39	3.42	3.63	3.55
Serine	4.64 ± 0.13	4.6	4.56	4.8	4.55
Glutamic acid	20.62 ± 0.68	21.05	21.89	19.77	19.76
Proline	3.78 ± 0.37	4.07	4.29	4.29	4.26
Glycine	5.46 ± 0.13	6.05	5.81	5.5	5.22
Alanine	4.15 ± 0.24	3.99	4.22	4.55	4.33
Cystine	1.10 ± 0.10	0.97	1.09	0.94	1.00
Valine	3.54 ± 0.41	3.51	3.53	3.63	3.77
Methionine	1.71 ± 0.20	1.57	1.6	1.76	1.55
Isoleucine	2.89 ± 0.36	2.78	2.96	2.93	3.00
Leucine	5.73 ± 0.25	5.69	5.81	5.97	5.99
Tyrosine	1.83 ± 0.20	2.3	2.51	2.46	2.44
Phenylalanine	4.03 ± 0.35	4.24	4.33	4.45	4.32
Histidine	2.20 ± 0.18	2.42	2.17	2.22	3.11
Lysine	3.42 ± 0.15	3.39	3.19	3.51	3.55
Arginine	9.00 ± 0.38	8.95	8.66	8.66	8.44

[a] Sripad and Narasinga (1987); [b] each value represents the mean ± standard deviation of three experiments

e) Vitamins

Solvent-extracted sunflower meal has been reported to be richer in B vitamins than defatted wheat germ, corn germ, or defatted soybean meal. Sunflower meal contains approximately 150% more preformed nicotinic acid than did the best grade of wheat

germ analyzed and was approximately 500% richer in this vitamin than corn germ or solvent-extracted soybean meal (Altschul, 1958). Altschul (1958) also reported that the pantothenic acid content of sunflower meal was similar to that of solvent-extracted soybean meal and 60% greater than that of soybean meal produced by the screw-press process. Corn germ and wheat germ were found to have less pantothenic acid than sunflower meal.

Sunflower seeds are a rich source of thiamin and niacine. Thiamin (Vitamin B1) may enhance circulation, helps with blood formation and the metabolism of carbohydrates. It is also required for the health of the nervous system and is used in the biosynthesis of a number of cell constituents, including the neurotransmitter acetylcholine and gamma-aminobutyric acid (GABA). It is also excellent for the brain and may help with depression and assist with memory and learning. Niacin, or Vitamin B-3, is necessary for normal breakdown of fats and fatty acids and the release of energy from carbohydrates. Lack of these vitamins will produce deficiency like beriberi and pellagra.

2.1.2.2 Others compounds present in the meal: Phenolic compounds

Sunflower proteins have exceptional organoleptic and functional characteristics that would make them useful in processed foods (Sosulski, 1979). Because of its high protein content, which have a good nutritional value (Clandinin, 1958), sunflower meal can be used as a protein source for human and animal consumption. The main problem in using the meal in human diets is the presence of hulls and polyphenols in the seed. The hulls contribute to high crude fiber content of the meal while the polyphenols interact with proteins via oxidation and the products formed, which are responsible for the dark

colour, decrease the available lysine content (Loomis and Battaile, 1966; Sodini and Canella, 1977) and therefore the nutritive quality of the meal. This decrease is due to the binding of these products to the amino, thiol and methylene groups of the proteins (Sabir et al., 1974; Hurrell and Finot, 1985; Shamanthaka and Sastry, 1990). The presence of polyphenols in the meal also inhibits proteases (Sastry, 1984) so they must be removed before their use as a substrate for production of enzymatic protein hydrolysates. In addition, because of the high polyphenol content, inhibition in the growth of some microorganisms (Parrado et al., 1991) can occur, including genetically engineered microorganisms, which limit its use as a nitrogen source for formulation of fermentation media. These problems could be overcome by different pretreatments. According to Bau et al. (1983), 79% of the polyphenols found in sunflower meal were soluble while 21% were protein-bound phenolics.

The major polyphenols found in sunflower meal are chlorogenic acid (CGA), caffeic acid (CA), and quinic acid (QA). The content of CGA, which forms nearly 70% of the total polyphenols, ranges from 1.42 to 4% (Dorrel, 1976). CGA (3-{(3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl)oxy}-1,4,5 trihydroxycyclohexane-carboxylic acid) is mainly located in the aleurone or protoplasts, and is linked to the 11S sunflower storage protein by hydrogen bonding (Shamanthaka and Sastry, 1990). CGA inhibits activity of digestive enzymes including trypsin, chymotrypsin, amylase and lipase (Cheeke and Shull, 1985). Because CGA is uncondensed and non-hydrolyzable, its content of 1% or more of a total of 3-3.5% phenolic compounds in sunflower meal is not reported in tannin assays. Additions of methionine and choline are required to counteract

the effect of chlorogenic acid. The basic phenolic compounds found in oilseed products are showed in Figure 2-2.

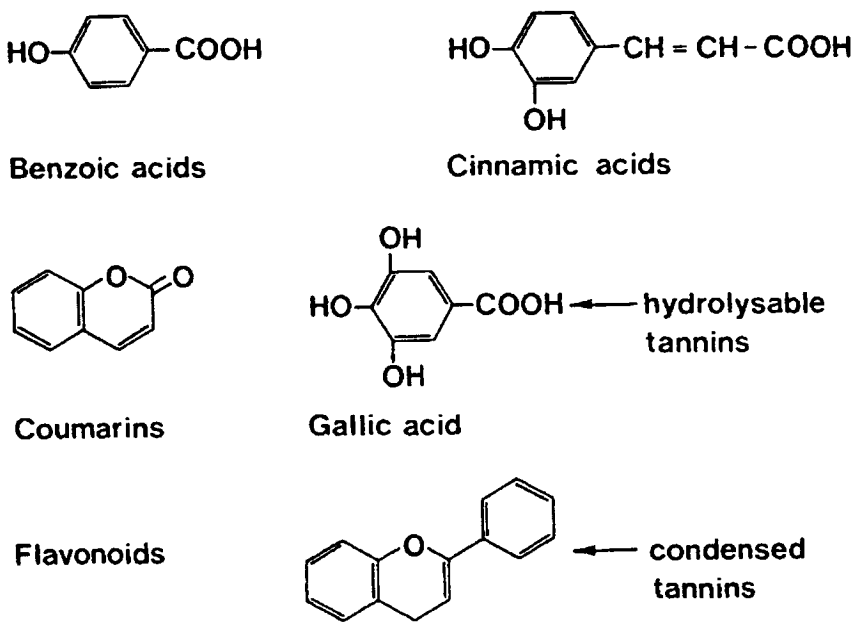


Figure 2-2. Structures of the basic phenolic compounds in oilseed protein products.

Phenolic acids include the benzoic acid ($C_6 + C_1$) and cinnamic acid ($C_6 + C_3$) based phenolics (Figure 2-3). The simpler types of benzoic acids include p-hydroxybenzoic, protocatechuic, vanillic, gallic and syringic acids plus the o-hydroxy salicylic and gentisic acids. The cinnamic acids p-coumaric, caffeic, ferulic, and sinapic are found in most oilseeds and occur frequently in the form of esters with quinic acid or sugars (Figure 2-3). Chlorogenic acid is an ester of caffeic and quinic acids and it is found in several isomeric and derivatized forms. Figure 2-4 shows the structures of chlorogenic and quinic acids. The coumarins occur as glucosides and they are less

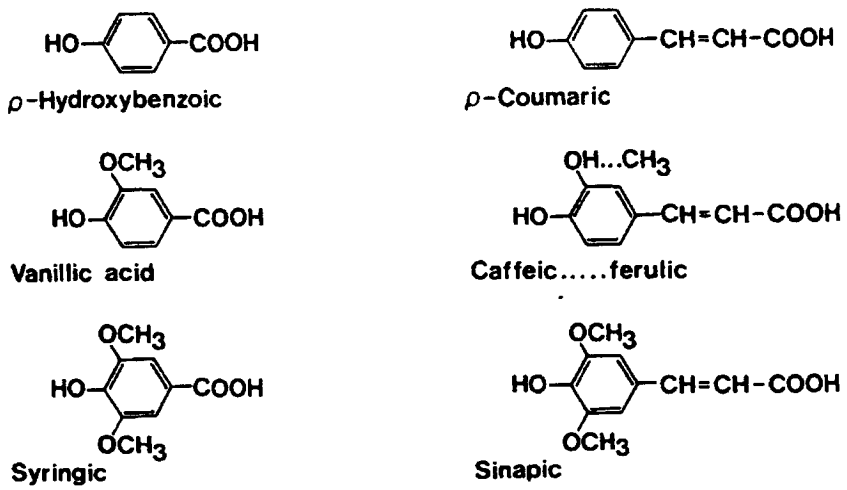
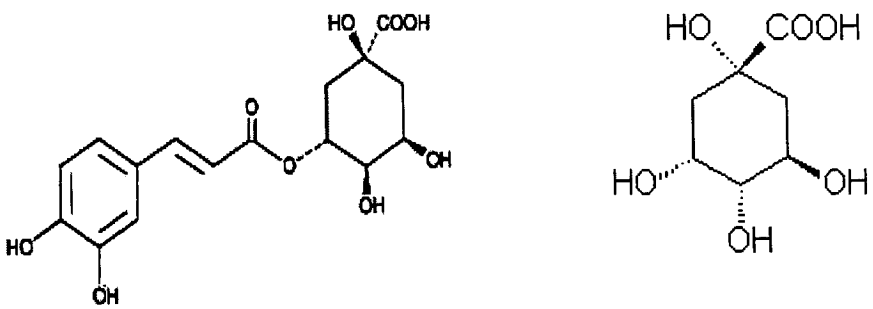


Figure 2-3. Structures of common phenolic acids in oilseed protein products.



Chlorogenic acid

Quinic acid

Figure 2-4. Structures of chlorogenic and quinic acids.

distributed in seeds. They also show limited chemical reactivity and have a very small effect on organoleptic or nutritive value of seed proteins products. Regarding the flavonoids, they are by far the most common. They do not occur as glycosides but they

show greater reactivity especially through polymerization into "condense tannis" as shown in Figure 2-2.

2.1.2.3 Interaction of phenolic compounds with proteins and amino acids

Enzyme-catalysed oxidation of phenols in seeds results in the formation of quinones and hydrogen peroxides. Both products can damage labile amino acids, denature proteins and inhibit enzymes such as indole acetic acid oxidase (Sosulski, 1979), trypsin and lipase (Milic et al., 1968), and arginase (Sosulski, 1979). Cinnamic acids and their esters are of great significance in oilseeds because they are the favour substrates for phenol oxidase (phenolase, polyphenoloxidase) (Sripad and Narasinga, 1987). The oxidation of *o*-dihydroxyphenols, especially caffeic and chlorogenic acids, to *o*-quinones is carried out by copper-containing enzymes as showed for the caffeic acid case in Figure 2-5.

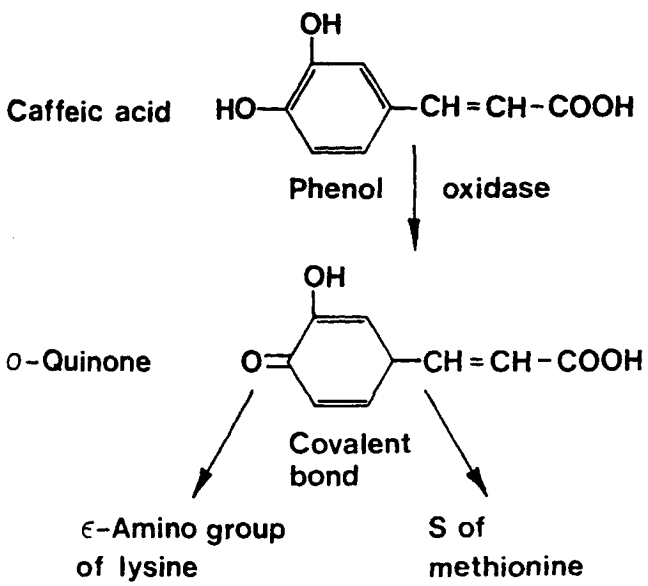


Figure 2-5. Enzyme-catalysed oxidation of caffeic acid to caffeo-quinone followed by autolytic bonding to amino and thiol groups in proteins.

Once the *o*-quinones (e.g., chlorogenoquinone) are formed, they are reduced or bond covalently to amino, thiol and methylene groups. The ∈-amino group of lysine and the thioether group of methionine are commonly attacked to make them nutritionally unavailable to the monograstric system. The quinoidal formation in sunflower meal is a serious problem due to the high content of chlorogenic and caffeic acids present in the meal (3 to 3.5 g / 100 g of flour).

Sunflower flours have much blander flavours and odours than soybean, cottonseed, or peanut flours despite its high concentration in chlorogenic acid. Due to the oxidation of polyphenolic compounds, sunflower protein solutions develop dark green and brown colours at alkaline conditions and when the meal proteins are precipitated at pH 4.7, it is impossible to wash out the green colour from the protein isolate (Sosulski, 1979). However, the nutritive values of sunflower flour and concentrate are proportional to their lysine contents (Sosulski and Fleming, 1977). When sunflower proteins are extracted with neutral salt solutions, all the major protein fractions are free of phenolic associations (Sosulski, 1979) and about one-third of the chlorogenic acid is bound to low molecular weight polypeptides and oligonucleotides (MW ≤ 5000). The colour problem with chlorogenic acid under alkaline conditions represents an impediment in using the protein products in the food industry. Cater et al. (1972) reported that chlorogenic acid in sunflower seed was responsible for the green to brown colour of alkaline extracts while caffeic produced only a slight pink. Quinic acid did not have any effect on colour. Besides chlorogenic and caffeic acids, Sabir et al. (1974a) also identified several other phenolic compounds, such as *trans*-cinnamic, *p*-coumaric, isoferulic, and sinapic acids, in the meal using GLC methods.

2.1.2.4 Uses of sunflower meal

The nutritive value of sunflower meal as a feedstuff for poultry and other livestock, as previously mentioned, depends of the quality of the seed, the completeness of the hulling operation, and the conditions to which the oil is extracted from the seeds. Different scientists have investigated the use of sunflower meal in human, poultry, swine, and cattle nutrition.

a) Human nutrition

The high protein content present in sunflower meal and its digestibility make the meal a suitable food for using in infant nutrition. It is not, however, recommended as a milk substitute (Altschul, 1958). The addition of sunflower seed flour to chocolate cakes, muffins, and yeast rolls was tested by the Home Economics Department of the University of Illinois (Altschul, 1958). Many of the products containing 10% of the sunflower seed flour were reported as being delicious.

b) Poultry nutrition

When the meal was included in a semi-synthetic chick starter ration to supply 20% protein, it was found that the meal was a complete required source of amino acids for growth (Grau and Almquist, 1945). Several researchers reported experiments dealing with the use of commercial sunflower meal in poultry rations to replace other protein supplements. Sunflower meal was a satisfactory substitute in chick starter rations when replacing not more than one-third of the total animal protein mixture (Altschul, 1958). A normal growth in chicks up to 7 weeks of age was reported when using a 2% sunflower meal in a chick starter ration containing 7.5% meat meal, 2% fish meal, and 2% skim milk powder, whereas 10% of meal combined with 3.75% meat meal in absence of fish

meal and skim milk powder resulted in slower-than-normal growth (Altschul, 1958). Using results obtained in feeding trials, Nikolaiczuk et al. (1948) concluded that sunflower meal provides a nutritionally incomplete protein for chick growth but that it is superior to soybean meal as a source of proteins for the chick.

On the other hand, sunflower meal was a satisfactory protein supplement for the growth of turkeys when used at the level of 21% or less in a 28% protein ration (Altschul, 1958).

c) Swine nutrition

Sunflower meal can be used as a part of a protein-vitamin supplement for dry-lot hog feeding to replace a part of the soybean meal and/or meat meal (Altschul, 1958). Gains were unaffected, when 10% meat meal was replaced. When both meat meal and soybean meal were replaced, the rate of gain was significantly decreased. Pigs digest very well the protein, fat, and nitrogen-free extract of sunflower meal (Altschul, 1958). Sunflower meals that varied in protein content from 31.4 to 49.1% and in fat from 1.5 to 15.6% were fed to pigs weighing about 26 pounds. Digestion values for protein, fat, and nitrogen-free extract ranged from 77 to 81%, 28 to 63%, and 87 to 92%, respectively.

d) Cattle nutrition

In Europe, sunflower meal is said to be a popular feed for all classes of livestock, especially for dairy cows. The uses of several oilseed cakes as feed for cattle have been reported (Altschul, 1958). He stressed the desirable characteristic (high protein content and ease feeding) of linseed oil cake and sunflower meals. Sunflower meal was compared with cotton meal as a source of supplementary protein by doing two experiments involving a total of 22 steers (Altschul, 1958). The results indicated that the two protein

supplements were about equal for fattening steers from the standpoint of average daily gain, feed requirement per unit of gain, and dressing percentage.

2.2 Enzymatic processes

The use of enzymes in the agro-industrial processing operations has the potential to increase productivity, efficiency and quality output in these operations. Enzyme-catalyzed processes generally require low capital investment and low energy consumption when compared to other methods of food processing.

Enzymes vary in quality from crude preparations in the form of leaves, plant exudates, chopped fruits and pounded grains to highly purified commercial preparations. Enzymes can be used in several applications such as the enhancing of extraction and separation processes, elimination of toxic and anti-nutritional factors, catalyzation of carbohydrate, protein and lipid conversions, and utilization as non-thermal food preservatives through their antioxidant and biocatalytic activities. Enzymatic reactions in foods can be controlled by manipulating temperature, pH, water activity and oxygen levels, or by using chemical inhibitors or inactivators. Novel technologies such as the use of ionizing radiation, protein inhibitors and "killer enzymes" which are enzymes capable of inactivating other enzymes, are all mechanisms which are being increasingly used for controlling enzymatic reactions in food systems.

Enzymes produced commercially can originate from different natural resources such as plant, animal and microbial (Crueger and Crueger, 1984). Microorganisms are by far the primary source of industrial enzymes, but several animal-derived enzymes and a smaller group of enzymes derived from plants are still being applied (Whitehurst and

Law, 2002). Microbial enzymes are commercially produced either through submerged fermentation (SMF) or solid substrate fermentation (SSF) techniques.

2.2.1 Enzymatic treatment of oilseed meals

Few studies related to the enzymatic treatment of oilseed meals have been done. Among these studies, the use of an enzymatic treatment for processing rapeseed/canola seeds and meal has been studied by several researchers (Sosulski and Sosulski, 1990; McCurdy and March, 1992; Jensen et al., 1990). These studies were done with the objective of altering the structural properties of the meals or seeds, by using enzymatic treatments, rather than decreasing antinutritional compounds present in the meals. The results of these treatments were meals with a higher accessibility in terms of further processing, such as oil and protein extraction. Sunflower and palm kernel meals were also treated by applying methods based on these principles (Dusterhoft et al., 1993; Dominguez et al., 1995).

2.2.2 Enzymatic treatment of phenolic compounds

The term "phenolic compound" includes a wide range of plant substances, which possess an aromatic ring bearing one or more hydroxyl substituents. The flavonoids are the largest single group of phenolic compounds. They are C15 compounds composed of two phenolic rings connected by a three carbon units. Cinnamic acid and its derivatives are simple phenolic acid. They are derived primary from the shikimate pathway via phenylalanine or tyrosine (Mann, 1978) and major examples are coumaric and caffeic acids.

Phenolics are common constituents of many oilseed meals. It was reported that they have a negative effect on the nutritional value of meal products used as animal feed.

Phenolic compounds can be attacked by different enzymes, which, in most cases, utilize molecular oxygen to break the aromatic nucleus (Sariaslani, 1989). To have these reactions, the aromatic structure has to be first modified by the action of oxidizing enzymes such as laccases and polyphenol oxidases (PPO) (Higuchi, 1982; Butt, 1980). These enzymes can simultaneously be found in preparations obtained from white rot fungi cultures. According to Morohoshi et al. (1987), these enzymes are secreted by the white rot fungi and they can be used to degrade lignin.

The enzymatic treatment of polyhenols by enzymes secreted by the white rot fungus *T. versicolor* has been studied previously by Lacki and Duvnjak (1996a,c, 1997). In these studies, it was reported that PPO obtained from *T. versicolor* could transform sinapic acid and its derivatives in a model enzymatic system. It was also shown that the enzyme preparation could decrease the concentrations of phenolic compounds in canola meal (Lacki and Duvnjak, 1996a,b) including tannins (Lorusso et al., 1996). Therefore, by using the same enzyme preparation and the proper conditions, it could be expected to have a decrease in the content of the phenolic compounds present in sunflower meal.

2.2.3 Polyphenol oxidase

Polyphenol oxidases (PPO) or tyrosinases are a group of copper proteins that are widely distributed from bacteria to mammals (Robb, 1984). These proteins catalyze the oxidation of hydroxyphenols to their quinone derivatives, which subsequently polymerize, at expenses of oxygen (O_2). Three types of proteins are related to PPOs, those are catechol oxidase, laccase, and cresolase (Yelena and Brudvig, 1996). They catalyze two reactions: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase

activity). PPOs produce enzymatic browning in fruits and vegetables; and it is very often unwanted and responsible for disagreeable sensory qualities and losses in nutrient quality (Sanchez-Amat and Solano, 1997). The enzyme has been obtained in purified form from mushrooms (Mason, 1956; Kertesz and Zito, 1965; Ponting, 1960) and it can be inhibited by substances which form stable complexes with copper, such as KCN, sodium azide, hydrogen sulfide, and diethyldithiocarbamate (Reed, 1966).

An enzymatic mechanism of PPOs found in plants using potatoes as a source of the enzyme and pyrocatechol (catechol) as the substrate is shown in Figure 2-6

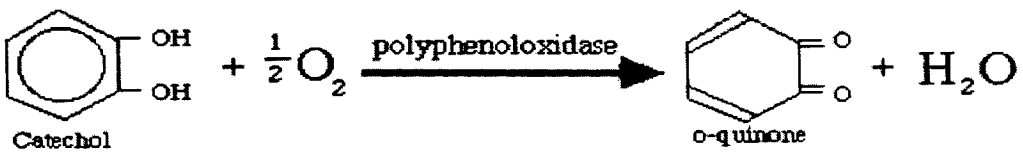


Figure 2-6. Enzyme-catalysed oxidation of catechol to o-quinone.

The reaction converts catechol (a POLYPHENOL) to an oxidized form with the aid of the enzyme. The ortho-quinone is rapidly converted to red products that can be measured in a spectrophotometer at 540 nm. Since these subsequent reactions are relatively very rapid, the measurement of the red products at time intervals gives an immediate indication of the rate of the enzyme-catalyzed reaction.

Polyphenol oxidase activity is important in some fermentation processes, for instance, in tea fermentation, and the enzyme can be used in different applications of the food industry such as the production of coloured end products and of the particular flavours associated with the browning reaction.

2.2.4 *Trametes versicolor* and its enzymes

The majority of white rot fungi produce laccase, frequently as the dominant extracellular enzyme in laboratory liquid cultures (e.g for *T. versicolor* and *P. ostreatus*). Besides laccase, *Trametes versicolor* produces the oxidative enzymes manganese peroxidase and lignin peroxidase. All these microbial extracellular oxidases (laccases, peroxidases) have been identified from wood-degrading basidiomycetes; terricolous basidiomycetes; ectomycorrhizal fungi and soil-borne microfungi and actinomycetes. According to Dehorter and Blondeau (1992), they are induced by humic acids and normally serve either in a protective (Mn-peroxidase, laccase) or in a degradative (lignin peroxidase) capacity. In the case of Mn-peroxidase and laccase, they normally oxidize phenols and anilines with a standard redox potential of up to 1.1 V to non-toxic polymerised products, while lignin peroxidase oxidizes non-phenolic compounds with redox potentials in excess of 1.4 V; this latter characteristic grants the white-rot fungi the ability to depolymerise lignin in lignocellulosic materials.

Khan and Overend (1990) reported that the white rot fungi *T. versicolor* ATCC 42530 strain, possesses an enzyme capable of transforming cathecol into muconic acid semialhehyde. This enzyme had two important characteristics: one, it was produced late in the growth cycle and two, it did not require addition of an inducer, which is important for the production of a peroxidase and laccases as shown by Paszczynski and Lobarzewski (1984) and Leonowicz et al. (1978) when using ferulic acid as an inducer. The enzyme obtained by Khan and Overend (1990) reached its maximum activity, 50 nkat/mL, after 18-23 days of incubation. When the growth time was longer, the enzyme activity decreases; however, in other studies with the white-rot fungi, this condition

facilitated the obtaining of peroxidases with a maximum activity (Szklarz et al., 1989; Leonowicz and Trojanowski, 1975). Similar results were reported for laccases (Rogalski et al., 1990; Fahraeus, 1952; Fahraeus and Reinhammar, 1967) and for Mn-dependent peroxidases (Szklarz et al., 1989; Archibald and Roy, 1992; Collins and Dobson, 1995).

2.2.4.1 Laccase from *T. versicolor*

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to a family of blue multicopper enzymes (oxidases), which are widely distributed in many plants and fungi. Laccases catalyze the oxidation of a range of inorganic and aromatic substances by the removal of electrons with the simultaneous reduction of O_2 to water (Youn et al., 1995; Xu, 1996). Laccases are regulated during fungal development (Ross, 1982; Choi, 1987), and show diverse functions in different fungi such as controlling pathogenicity in *Cryptococcus neoformans* (Salas et al., 1996), and conidial pigment synthesis in *Aspergillus nidulans* (Clutterbuck, 1972). Laccases from white-rot fungi are intimately involved in lignin- and recalcitrant-degradation (Roy-Arcand and Archibald, 1991; Rodriguez et al., 1999). Several reports have been published on the purification and biochemical characterization of fungal laccases from several basidiomycetes: *Agaricus bisporus* (Perry et al., 1993), *P. ostreatus* (Garzillo et al., 1992; Palmieri et al., 1997), *Trametes versicolor* (Bourbonnais et al., 1995) and *T. villosa* (Yaver et al., 1996). *Trametes versicolor* laccase is a globular protein of about 500 amino acids; its active center contains only four copper atoms.

D'Souza et al. (1999) has reported the induction of the lignin-degrading enzymes, lignin peroxidase, manganese peroxidase and laccase, by the addition of lignin components to *G. lucidum*. Lundquist and Kristerrson (1985) and Ishihara and Ishihara

(1976) reported the formation of quinones from benzoic and cinnamic acid derivatives, the main constituents of canola meal (CM) phenolics, when using laccase of white-rot fungus. Laccases from *T. versicolor* can catalyze the transformation of benzoic and cinnamic acids with the formation of quinones (Bollag et al., 1982; Leonowicz et al., 1984; Katase and Bollag, 1991).

At present, there is no study related to the use of *T. versicolor* laccase in the enzymatic decrease of chlorogenic acid. However, some authors have reported enzymatic treatments of chlorogenic acid using laccase obtained from other sources. For instance, Piacquadio et al. (2002) reported that the concentration of chlorogenic acid was decreased when apple juice was treated with laccase immobilized on copper-chelate carrier. The enzymatic browning in apricots (Prunus armeniaca L) was found to be catalysed by a catechol oxidase (o-diphenol oxidiase) and a laccase (p-diphenol oxidase), both acting on catechin and chlorogenic acid as the natural substrates and the levels of catechin and chlorogenic acid were found to be reduced in the rotted tissue (Dijkstra and Walker, 1991)

2.2.4.2 Peroxidases from *T. versicolor*

Lignin is an aromatic polymer occurring in the woody tissue of higher plants. This compound is poorly biodegraded by most microorganisms because of its hydrophobicity and complex random structure lacking of hydrolysable bonds. White rot fungi, that produce extracellular peroxidases, are the best degraders of lignin (Kirk and Farrell, 1987). These enzymes are involved in the initial attack of the lignin polymer (Hammel et al., 1993). The most ubiquitous ligninolytic enzymes produced by these fungi are lignin peroxidases (LP), manganese-dependent peroxidases (MnP), and laccases (phenol

oxidases). According to Mester and Tien (2000), the peroxidases are heme-containing enzymes having typical catalytic cycles, which are characteristic of other peroxidases as well. One molecule of hydrogen peroxide oxidizes the resting (ferric) enzyme withdrawing two electrons. Then, the peroxidase is reduced back in two steps of one electron oxidation in the presence of appropriate reducing substrate. The range of the reducing substrates of the two peroxidases is very different due to their altered substrate binding sites. LP is able to oxidize various aromatic compounds, while MnP oxidizes almost exclusively Mn (II) to Mn (III), which then degrades phenolic compounds. One of the most often encountered peroxidases among white rot fungi is (MnP) (Mester and Field, 1998). This peroxidase, MnP, is a glycosylated hemeprotein occurring mostly as a number of isozymes (Kirk and Farrell, 1987). Studies in crystallographic and site-directed mutant confirmed the presence of a unique manganese-binding site in MnP from the best-studied white rot fungus, *Phanerochaete chrysosporium* (Sundaramoorthy et al., 1994, 1997). Some organic acids have shown to have an important role in the mechanism of MnP and lignin degradation (Wariishi et al., 1992; Mester and Field, 1998). Organic acids strip Mn (III) from the enzyme, forming Mn (III)-organic acid complex, which acts as a diffusible mediator in the oxidation of lignin by MnP (Wariishi et al., 1992; Mester and Field, 1998). Mn (III) can also oxidize organic acids, yielding radicals (Khindaria et al., 1994).

Some researchers have published about the oxidation of chlorogenic acid using peroxidases, which were obtained from several sources. Felton and Duffey (1991) reported that tomato foliar peroxidases oxidize an array of endogenous compounds including caffeic acid, chlorogenic acid, rutin, coumaric acid, cinnamic acid, and

guaiacol. Joo et al. (1991) found that the peroxidase activity of rabbit hemoglobin catalyzes the oxidation of caffeic acid, chlorogenic acid and indole-3-acetic acid quite rapidly compared to other naturally occurring phenolic compounds such as scopoletin, esculetin, ferulic acid and p-coumaric acid.

In summary, it is said that sunflower have been originated in the Mexico area. The crop is an important source of cooking oil. Sunflower oil can be obtained either by solvent extraction or by pressing. After the oil is extracted from the seeds, the residue is called sunflower meal. This meal is rich in proteins and other components such as carbohydrates, minerals, and vitamins. Unfortunately, the meal also contains antinutritional compounds such as polyphenols, which after interacting with proteins, produce products that decrease the nutritional quality of the meal. The main polyphenols present in the meal are chlorogenic acid (CGA), caffeic acid (CA), and quinic acid (QA). CGA represents nearly 70% of the total polyphenols. Polyphenols can be attacked by different enzymes such as laccases and polyphenol oxidases (PPO). These enzymes are secreted by the white rot fungi. Nowadays, the more efficient method to remove polyphenols from oilseed meals is the enzymatic method. This method consists in using enzymes secreted by fungi such as the white rot fungus *Trametes versicolor*.

CHAPTER 3. MATERIALS AND METHODS

This chapter contains the experimental procedures that were used for the completion of this project.

3.1 Growth Conditions and Enzyme Preparation

3.1.1 Growth conditions

The white-rot fungus *Trametes versicolor* ATCC 42530 was kept on agar slants consisting of (% w/v): malt agar (4.5), glucose (0.5), and yeast extract (0.5). The medium was boiled, to dissolve the agar, and volumes of 5 mL of that medium were transferred to 23 mL tubes and covered. The tubes were autoclaved at 121°C for 20 minutes. After autoclaving, they were placed with a slight inclination angle and allowed to cool overnight prior to inoculating with a piece of culture from a previously-prepared slant. After inoculation, the tubes were placed in an incubator at 28°C until the surface of the slant was covered with mycelium and afterwards they were stored in a refrigerator at 4°C before transferring a piece of mycelium to new prepared agar slants.

Once grown on agar slants, the fungus was used to inoculate a partially defined medium having the following composition (% w/v): glucose (2.0), yeast extract (0.5), nutrient broth (0.8) and Vogel's trace minerals (0.5). The pH of the medium was adjusted to 5.5 by using hydrochloric acid and 4N sodium hydroxide. One hundred mL of this medium was prepared in a five hundred mL flask and it was autoclaved at 121°C for 20 minutes. Once the medium was cooled, it was inoculated with a piece of mycelium and the culture was incubated in a rotary shaker at 160 rpm and 28°C for up to 8 days. After 7-8 days, the contents of several flasks were combined and homogenized for 30 seconds using a Sunbeam Osterizer 8 blender set on "liquefy". The blended culture was

used as an inoculum for the enzyme preparation. Vogel's mineral solution consisted of (% w/v): citric acid monohydrate (5), zinc sulphate heptahydrate (5), ferrous ammonium sulphate hexahydrate (1), cupric sulphate pentahydrate (0.25), magnesium sulphate monohydrate (0.05), boric acid (0.05) and sodium molybdate (0.05) (Vogel, 1959).

3.1.2 Enzyme preparation

The liquid medium for the enzyme preparation consisted of the same composition mentioned in the section "growth conditions" (glucose, yeast extract, nutrient broth, and Vogel's minerals). Seventy-five mL of that medium was placed in a 250 mL flask. Several flasks were prepared in order to collect enough enzyme for several days. The flasks were autoclaved at 121°C for 20 minutes and then they were cooled. Once cooled, 5 mL of the previously blended culture was used to inoculate each 75 mL of autoclaved fresh liquid medium. When all the flasks were inoculated, they were incubated in a rotary shaker at 28°C and the enzyme activity was monitored in each flask every day using ferulic acid (FA) as substrate (Khan and Overend, 1990). Once the enzyme activity reached its maximum point, the media were filtered and the supernatants were collected in different flasks and frozen. They were used to study the enzymatic decrease of the chlorogenic acid content in both the model and the sunflower meal systems.

3.2 Model enzymatic system

3.2.1 Characterization of the enzyme: a steady-state approach

The activity of the enzyme was measured spectrophotometrically by following the loss in absorbance of the chlorogenic acid at 324 nm using a Beckman DU 640 spectrophotometer equipped with a temperature controller. The enzyme activities were expressed in terms of nanokatals (nmol of substrate converted/s). The reaction system

consisted of 2.72 mL citrate phosphate buffer solution of a chosen pH, 0.1 mL of an enzyme preparation solution and 0.18 mL of substrate solution (usually 2 mM). This system was called the "model" system. All reaction conditions were specified in each figure and table. Results of the enzymatic reactions were obtained by following the decrease of the concentration of chlorogenic acid with time. Enzymatic reactions were stopped using trichloro acetic acid solution (TCA) with a concentration of 66% (w/v).

The enzyme activities with each substrate were evaluated from the linear part of the progress curves by using Eq. (3.1)

$$v = 10^6\left(\frac{A_{ini} - A_t}{\Delta t}\right)\frac{1}{\varepsilon}\frac{V_{sys}}{V_{enz}}\frac{1}{60}DIL \qquad 3.1$$

where v is the enzyme activity (nkat/mL), A_{ini} and A_t are the initial and final absorbencies, respectively, Δt is the reaction time (s), V_{sys} is the volume of the system (mL), V_{enz} is the volume of the enzyme added (mL), DIL is the enzyme dilution factor, ε is the molar absortivity for CGA (1.854 x 10^5 M^{-1} cm^{-1}). The results of the effect of each variable on the enzyme activity are shown as the mean of two measurements and their standard deviations from the mean.

3.2.1.1 Effect of pH and temperature

To study the effect of pH and temperature on the enzyme activity, a system similar to the previously mentioned "model system" was used. Buffer solution was used as a blank and the enzyme activities were measured by changing either the pH from 2.6 to 6.0 or the temperature from 25 to 75°C depending on the variable that was studied. The pH values were measured using a digital Fisher accumet pH meter, AR 50. On the other

hand, the stock enzyme concentration was determined using ferulic acid as a substrate in a system consisted of 2.7 mL citrate phosphate buffer (pH 3.5), 0.2 mL ferulic acid (2 mM), and 0.1 mL of an enzyme preparation.

3.2.1.2 Effect of the substrate concentration

The effect of the substrate concentration on the enzyme activity was studied in a system which contained 2.72 mL citrate phosphate buffer solution of a chosen pH, 0.1 mL of an enzyme preparation solution and 0.18 mL of a substrate solution of a specific concentration. The pH and temperature used were 4.3 and 25°C, respectively. The enzyme activities were measured for substrate concentrations in the system between 0 and 0.14 mM. With higher substrate concentrations, the spectrophotometer readings were unreliable and data were scattered. In the study of the substrate concentration, different concentrations of substrate were prepared in order to get the CGA concentration wanted in the system.

3.2.1.3 Effect of the enzyme concentration

The effect of the enzyme concentration on the initial reaction rate of the CGA decrease was carried out at two different temperatures, 35 and 55°C, with an enzyme concentration that changed from 0 to 3 nkat/mL. The substrate concentration in the system was kept constant (0.12 mM) at the zero-order kinetics. The pH of the system was 4.3.

3.2.2 Enzyme thermal stability

To study the enzyme thermal stability, tests were done at 55°C in a custom made temperature controlled air chamber, which was equipped with a rotary tube rack mixer, Fisher Roto-Rack model 440. The stock enzyme preparation was first diluted, in 15 mL

tubes with screw caps, using citrate phosphate buffer solution of a chosen pH. Later on, the tubes were placed in the temperature-controlled air chamber and, after a predetermined time, samples were withdrawn and their activities were measured using chlorogenic acid as a substrate. The results are expressed as a percentage of the initial enzyme activity. In certain cases, the thermal stability of the enzyme was determined when an overheated enzyme preparation was added to a system containing buffer solution and substrate. In these cases, the loss in absorbance of the chlorogenic acid was found by doing wavelength scans with time, from 250 nm to 800 nm. The overheated enzyme preparation was obtained by exposing a stock enzyme solution to a certain temperature in a hot water bath.

3.2.3 Comparison of techniques in the determination of the CGA

The CGA content in the model system was determined using either the spectrophotometric or the HPLC technique and in some cases both techniques. These techniques are explained in more detail in section 3.4.2. To compare the results obtained by the spectrophotometric and the HPLC techniques, the CGA concentration decrease was followed with time in a system containing an initial CGA concentration of either 0.13 or 0.07 mM. The comparison was done at two enzyme concentrations, 0.06 and 0.12 nkat/mL, for each initial substrate concentration used. These experiments were carried out at pH 4.3 and 35°C.

3.2.4 Dynamics of the CGA decrease in the model system

The dynamics of the CGA transformation was studied by following the changes in its concentration with time. This enzymatic transformation was carried out at 35°C in 125 mL flasks using a temperature controlled water bath, Fisher A22. The system

consisted of 37.7 mL of citrate phosphate buffer solution (pH 4.3), 12 mL of CGA (0.5 mM), and 0.3 mL of an enzyme preparation. Each flask contained this system. The flasks were incubated in the water bath and after a period of time, 5 mL of sample was taken from each flask and 0.3 mL of TCA (66% w/v) was added to each sample to stop the enzymatic reaction. The CGA present in each sample was determined using the HPLC technique explained in section 3.4.2.3.

3.3 Sunflower meal system (SFM)

3.3.1 The decrease of chlorogenic acid content in sunflower meal

The decrease of the chlorogenic acid content in sunflower meal was done in two different systems:

System A consisted of 2.5 g sunflower meal, 22.5 mL of citrate phosphate buffer solution, of a chosen pH, and 2.5 mL of an enzyme preparation (a desired enzyme concentration was obtained by diluting the stock enzyme with de-ionized water). The enzyme was first mixed with the buffer solution in a 125 mL flask, using a magnetic stirrer and then a weighted amount of sunflower meal was added into the system and the pH was checked. In most cases a 10% (w/v) sunflower meal concentration was used. Several flasks containing system A were prepared and placed in a temperature controlled water bath shaker, Fisher A22. After a predetermined period of time, 2.5 mL of TCA (66% w/v) was added to stop the enzymatic reaction. The slurry was then centrifuged for 20 minutes at 5000g and 5°C. The supernatant (number one) was separated from the residue by decantation. The residue was oven-dried overnight at 100°C. About 0.1 g of the dried residue (number one) was extracted in 20 mL of aqueous methanol (MtOH-Water: 20-80) at room temperature for half an hour in the temperature controlled water

bath shaker mentioned previously. The extract was centrifuged for 10 minutes at 2000g and 5°C. The CGA left in the supernatant (number two) was measured using HPLC while the residue (number two) from this centrifugation was extracted again in aqueous methanol until all the CGA was removed. Two to three extractions, 20 mL aqueous methanol each extraction, were enough to extract the chlorogenic acid left in the initial residue (number one). On the other hand, the amount of chlorogenic acid present in the supernatant (number one) was measured after the decantation process using the HPLC technique explained in section 3.4.2.3. Controls, which contained water instead of enzyme preparation, were used to calculate the percentage of CGA converted after the enzymatic treatment.

The second system (system B) consisted of 1 g sunflower meal, 9.25 mL of buffer solution of a chosen pH, and 0.75 mL of an enzyme solution of desired activity. The enzymatic process was carried out in 15 mL screw caps tubes. The tubes were placed in the custom-made temperature controlled air chamber. The reaction was stopped by adding 0.75 mL of TCA. The suspension was centrifuged for 20 minutes at 5000g and 5°C and the CGA present in the supernatant was determined, as in system A, using HPLC while the residue was oven-dried (100°C) and the next day the CGA content in the residue was determined in the same way as in system A.

3.3.1.1 Dynamics of the CGA concentration decrease in the SFM system

The decrease of the CGA concentration in the SFM system was studied using two methods. The first method consisted of using system A with meal and enzyme concentrations of 10% (w/v) and 3 nkat/mL, respectively. In this case, the decrease of the chlorogenic acid concentration was followed with time and samples were withdrawn in

duplicates from the system. Control samples, which did not contain enzyme, were also taken. The second method consisted of keeping the ratio of continuous phase to meal constant and following the decrease of the chlorogenic acid concentration with time. In this method, three different ratios and several enzyme concentrations were used. The procedure to quantify the CGA content in both methods was the same as in system A. In the tests of the second method, three series of experiments at different meal and enzyme concentrations were considered and in each series the meal concentration varied from 6.67 to 20% (w/v). The first series corresponded to an enzyme concentration in the continuous phase of 4 nkat/mL; the second series corresponded to an enzyme concentration of 20 nkat/g of the dispersed phase and in the third series a treatment with no enzyme was studied.

3.3.1.2 Effect of the meal-buffer slurry concentration

The effect of the slurry concentration on the decrease of the CGA concentration was examined in system A. Two different experiments were carried out by changing the concentration of the meal from 2 to 20% (w/v). In the first one, the enzyme concentration was kept proportional to the meal concentration, 3 nkat/g meal treated, while in the second one the enzyme concentration in the liquid phase was kept constant at 3 nkat/mL. As controls, water was used instead of enzyme in both experiments. The enzymatic process was carried out for 30 minutes at 35°C. The pH of the slurry was 4.3.

3.3.1.3 Effect of the enzyme concentration

The effect of the enzyme concentration on the enzymatic decrease of the CGA concentration was tested in system A by changing the enzyme concentration in the system from 0 to 30 nkat/mL of continuous phase. A meal concentration of 10% (w/v)

was used. The pH and the temperature used were 3.4 (buffer) and 35°C, respectively. A control was used for every enzyme concentration studied. The control contained water instead of enzyme. The amount of CGA left in the system was measured after 30 and 120 minutes of treatment.

3.3.1.4 Effect of pH

The effect of pH of the buffer solution on the decrease of the CGA concentration was studied in the system A mentioned in section 3.3.1. The pH of the buffer was varied from 2.2 to 5.6. The process was carried out at 35°C for 30 minutes using 10% (w/v) meal concentration and an enzyme concentration of 3.5 nkat/mL of continuous phase. A control (using de-ionized water instead of enzyme solution) was used for every pH studied in order to determine the effect of the pH of the buffer solution on the CGA extraction.

3.3.1.5 Effect of temperature

The effect of the temperature on the decrease of the CGA concentration in SFM was studied in the system A by measuring the amount of CGA left after the enzymatic treatment when changing the temperature from 25 to 75°C. The enzymatic reaction was carried out for 30 and 120 minutes using a 10% (w/v) meal concentration and an enzyme concentration of 3 nkat/ mL of continuous phase. A buffer pH of 3.4 was used because the buffering capacity of the meal makes the pH of the system to change to 4.3 when a 10% (w/v) meal concentration is used. At each tested temperature, a control run was performed using de-ionized water instead of enzyme.

3.3.1.6 Effects of the particle size and intensity of agitation

System B was used to study the effects of SFM particle size and agitation speed in the enzymatic process. These variables can influence the enzymatic treatment by affecting the mass transfer rate. The effect of the internal diffusion can be investigated by following the decrease of the CGA concentration when using different particle sizes. In order to do so, the enzymatic process was carried out at 55°C using an enzyme and meal concentration of 3 nkat/g meal and 10% (w/v), respectively. Five different fractions of SFM were studied. These fractions were obtained from a sieve analysis.

On the other hand, to examine the effect of the external diffusion, the intensity of agitation was changed during the treatment of a 10% (w/v) meal suspension at 55°C using an enzyme concentration of 3 nkat/g meal.

3.3.2 Thermal stability of the enzyme in the presence of SFM and bovine-albumin

The effects of sunflower meal and sunflower meal proteins on the thermal stability of the enzyme were evaluated in a system containing 2 and 5% (w/v) of meal. The enzyme concentration in the system was 7 nkat/mL continuous phase in both cases. The enzymatic reactions were carried out in 14 mL tubes containing 1.28 mL of enzyme, 8.72 mL citrate phosphate buffer solution and either 0.2 or 0.5 g of sunflower meal. The enzyme was first mixed very well with the buffer solution and then the sunflower meal was added. The tubes were incubated at 55°C in the custom-made temperature controlled air chamber for 48 hours. After a predetermined time, 0.1 mL of the incubated solution was withdrawn from each tube and the activities were measured spectrophotometrically using CGA as the substrate in the model system explained in section 3.2.1. In some tests,

bovine-albumin was used as protein. The results were expressed as percentage of the remaining activity.

3.3.3 Effect of the TCA concentration on the enzymatic reaction

To stop the enzymatic conversion of CGA, it was necessary to investigate the effect of the concentration of thricloroacetic acid (TCA) on the enzymatic conversion of chlorogenic acid. These experiments were carried out in the system A mentioned in section 3.3.1. The system consisted of buffer at pH 3.4, enzyme preparation (3 nkat/mL) and sunflower meal (10% w/v). Eight different TCA concentrations were prepared and 1.25 mL of each concentrated solution was added to the system before adding the enzyme. Once the meal, buffer, TCA and enzyme were mixed, the system was incubated in a water bath shaker at 35°C for 30 minutes. For all the controls, de-ionized water was used instead of enzyme and samples were incubated in the same way.

3.4 Analysis of samples

3.4.1 Moisture

The content of moisture in sunflower meal was measured as a change in the mass of a sample before and after drying at 100 ^{0}C for 12 hours.

3.4.2 Phenolics

3.4.2.1 Extraction of phenolics from sunflower meal

Soluble phenolics: Soluble CGA and quinic acid (QA) were extracted from sunflower meal with water, and with two different aqueous methanol solutions (MtOH-Water: 70-30; and 20-80). The extractions were carried out in 50 mL flasks. The flasks contained 0.1 g SFM in 20 mL of water or methanol-water solution and they were placed in a water bath shaker, Fisher A22, at room temperature. Two to three extractions were

enough to extract all the CGA present in the meal. After each extraction, the suspension was centrifuged for 10 minutes at 3000g. Each extraction was analyzed for free CGA using spectrophotometric and/or HPLC methods, which are explained in section 3.4.2.2 and 3.4.2.3 respectively.

3.4.2.2 Spectrophotometric method

In some cases, to determine the concentration of CGA present in the model and sunflower meal systems, a Beckman DU 640 spectrophotometer equipped with a temperature controller was used. In order to be able to use this method, a standard curve was prepared at a wavelength of 324 nm using different known concentrations of CGA. This standard curve is shown in Appendix A (Figure A-2).

3.4.2.3 High-performance liquid chromatography method

The high-performance liquid chromatography (HPLC) method was used to determine concentrations of CGA with more accuracy. The equipment used was a Waters Associates HPLC system equipped with a 600E System Controller, an U6k Injector and a Water 486 Tunable Absorbance Detector. All the data obtained were analysed using the Millennium Waters Chromatography System Manager, Millennium v. 2.00.

To measure the concentration of CGA, a 250 x 4.6 mm I.D. Supelcosil LC-8 (5 micrometers) column was used. The mobile phase consisted of a mixture of methanol-water-acetic acid (35:65:1) according to Vioque and Millán (1985). The flow rate was set at 1mL/min. The column was kept at room temperature (25°C). CGA was detected at 324 nm, which was the wavelength at which a maximum absorption peak of CGA was found. The size of the sample was 20 microlitres. To determine the concentration of CGA by HPLC, it was necessary to prepare first a standard curve with different known

concentrations of pure CGA (Appendix B, Figure B-1). This standard curve ranged from 0 to 0.1 mM of CGA and it was used as a reference to determine the amount of CGA present in the systems.

3.5 Chemicals

The list of chemicals used during this project and their suppliers are shown in Table 3.1. Poplyphenol oxidase (PPO) was not available commercially, so it was obtained in the laboratory by the procedure described in sections 3.1.1 and 3.1.2.

Table 3.1 List of chemicals used during the course of this research

A	Albumin bovine (Sigma), Acetic acid (Fisher)
B	Boric acid (BDH)
C	Citric acid anhydrous (Sigma), Chlorogenic acid (Sigma), Caffeic acid (Sigma), Cupric sulphate pentahydrate (Baker), Casein (Sigma)
D	Dibasic sodium phosphate anhydrous (Sigma)
E	Ethanol (BDH)
F	Ferrous ammonium sulphate hexahydrate (Sigma), Ferulic acid (Sigma)
G	Glucose (BDH)
H	Hydrochloric acid (BDH)
M	Methanol HPLC and spectrophotometric grade (Merck), Magnesium sulphate monohydrate (Fisher), Malt agar dehydrated (Difco)
N	Nutrient broth (Mikrobiologie-BDH)
P	Potassium hydroxide (Sigma)
S	Sinapic acid (Aldrich), Sodium molybdate (Sigma)
T	Trichloroacetic acid (BDH)
Y	Yeast extract granulated (Mikrobiologie-BDH)
Z	Zinc sulphate heptahydrate (Sigma)

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Model Enzymatic System

4.1.1 Preparation of the enzyme

The characterization of the enzyme preparation used during this project was done in a model system using chlorogenic acid (CGA) as a substrate. Several factors were included in the characterization such as pH, temperature, substrate and enzyme concentrations. Other factors, such as the thermal stability of the enzyme, were also investigated. The results acquired in this part of the project were used as a base to understand the enzymatic transformation of CGA in the sunflower meal system.

4.1.1.1 Enzymatic transformation of chlorogenic acid

To study the enzymatic transformation of CGA several wavelength scans were done from 200 to 800 nm. Figure 4-1 shows the change of absorption in the ultra-violet and visible light when adding the enzyme preparation to a system that contains CGA as a substrate. The loss in absorbency of CGA at 324 nm with time indicated that the enzyme preparation transformed this compound into other by-products, which absorbed at wavelengths of 250 and 400 nm. Figure 4-1 also shows that the enzymatic transformation of CGA occurred in the first 30 minutes of the enzymatic process; after that period of time, a decrease in the absorbency of the by-products obtained from the enzymatic transformation of CGA took place as well.

4.1.1.2 Effect of the substrate concentration on the enzyme activity

The enzyme's component amino acids are arranged to precisely "fit" the substrate molecule in a tiny area of the enzyme, which is called the catalytic site. This molecule is the substance acted upon by the enzyme and changed to a product. The rate of an

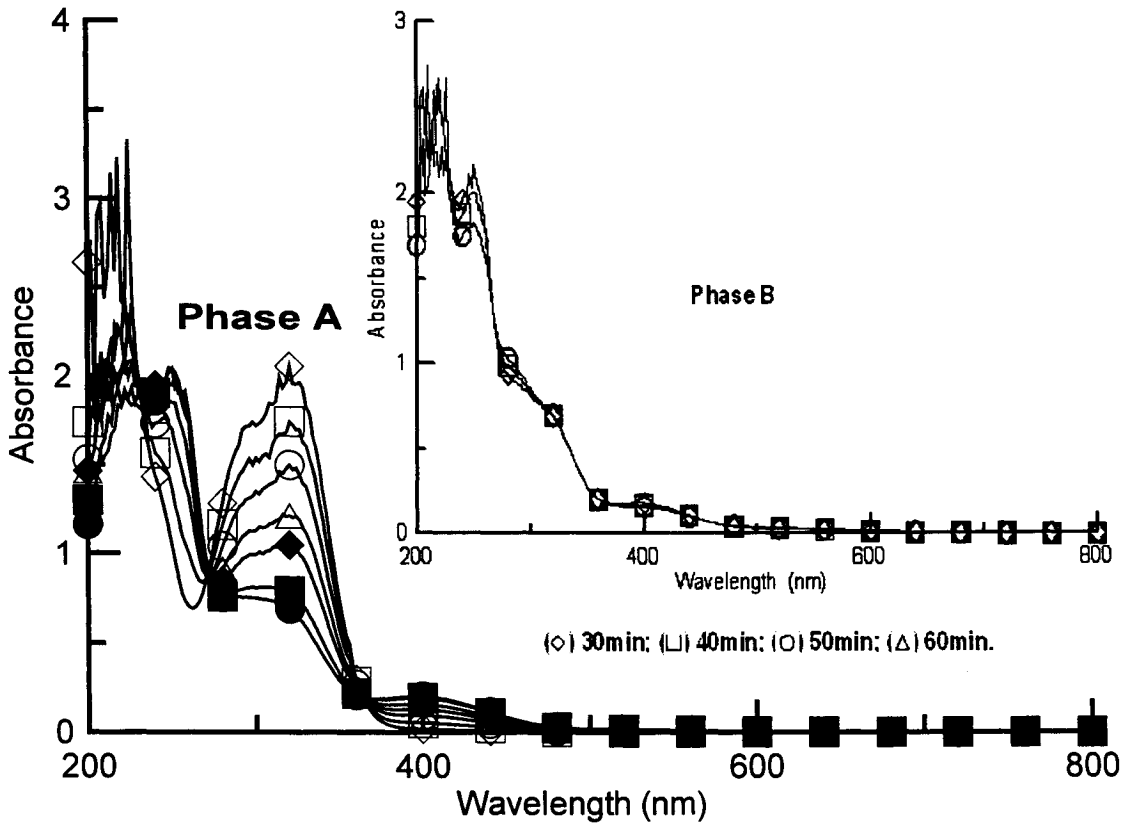


Figure 4-1. Changes in absorbance during enzymatic conversion of CGA. CGA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; temp. 35°C.

enzyme-catalyzed reaction depends partly on how well the enzyme and substrate fit together. Environmental factors such as pH and/or high temperatures might change the shape of either enzyme or substrate and thus alter the rate of reaction. In addition, the concentration of active enzyme and substrate molecules have an important effect on the rate of reaction. Regarding the substrate concentration, at very low substrate concentrations the reaction proceeds slowly due to the fact that collisions between enzyme and substrate molecules are infrequent. As the substrate concentration is increased, collisions between enzyme molecules and reactants become more frequent producing a proportional increase in the reaction rate. The effect of increasing substrate concentration is reduced when the enzymes start approaching the maximum rate at which they can combine with reactants and release products. When enzyme is saturated with substrate, it converts substrate as fast as it can and further increase in the concentration of the substrate has no effect on the reaction rate.

The effect of the substrate concentration on the enzyme activity is shown in Figure 4-2. This figure shows the typical hyperbolic curve described by the Michaelis-Menten kinetics, with a Km value of 0.0423 mM for CGA obtained by using the Lineweaver-Burk plot method. Lacki (1997) found that when using the same enzyme from the same fungus but with different substrates, the Km values were 0.024 mM for sinapic acid (SA), 0.046 mM for sinapaldehyde (SALD) and 0.117 mM for sinapine (SIN). Mazzafera and Robinson (2000) found that when PPO was extracted from leaves (PPO-L) and from fruit endosperm (PPO-E) of coffee, its activity was higher with chlorogenic acid than with any other substrate used and the Km values found were 0.882 mM and 2.27 mM for PPO-L and PPO-E, respectively. A very high activity was

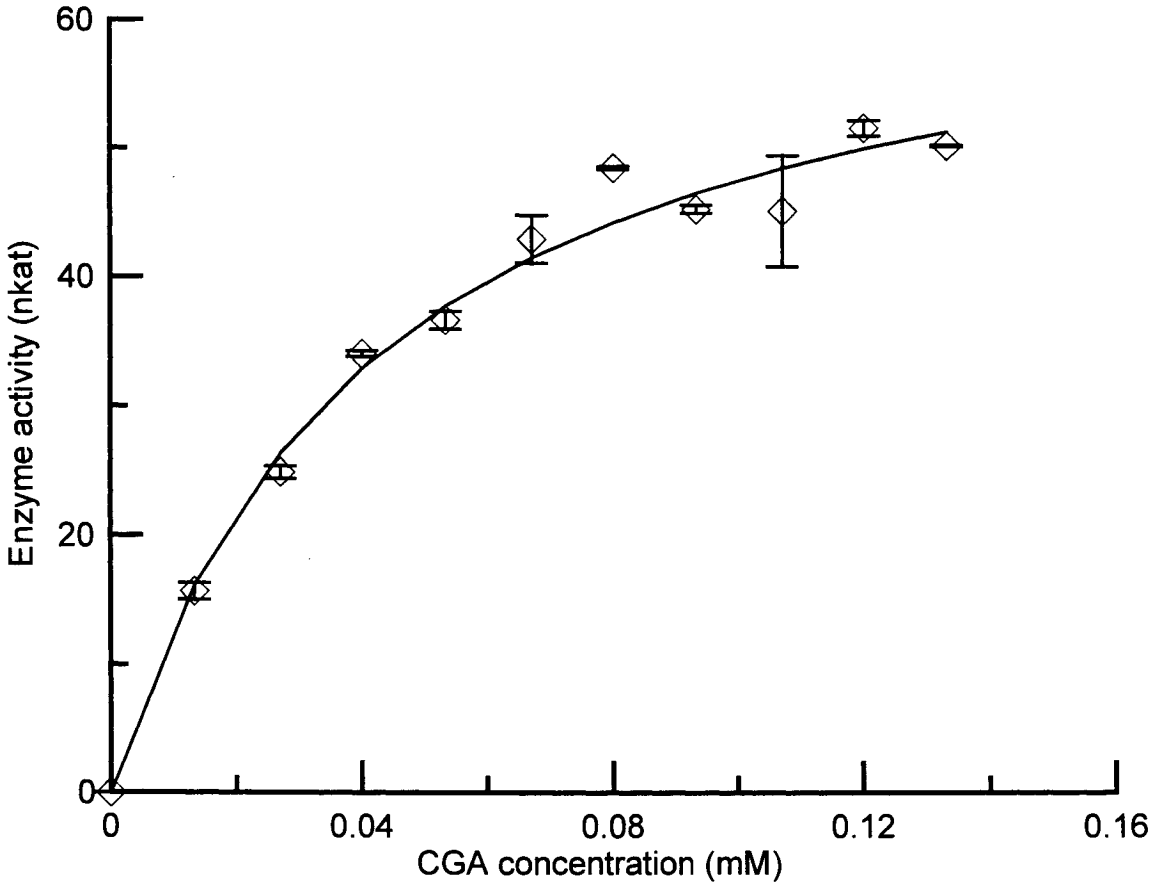


Figure 4-2. Effect of the CGA concentration on enzyme activity. Enzyme concentration 0.73 nkat/mL; pH 4.3; 25°C. Spectrophotometric measurements.

also found with chlorogenic acid as a substrate for an enzyme extracted from loquat fruit (Ding et al., 1998). The Km values in this case were 0.105 and 0.425 mM for chlorogenic and neochlorogenic (a phenol in the hydroxycinnamate family) acids, respectively. The Km value characterizes the affinity between the substrate and the enzyme. A low Km value reflects high affinity of the enzyme for the substrate. Thus according to these results, the affinity of PPO, from *T. versicolor*, for the substrates studied is in the following way (if the conditions are the same): SA > CGA > SALD > SIN.

No inhibition effect of the substrate concentration was noticed during the enzymatic conversion of CGA. However, for high substrate concentrations, the curve in Figure 4-2 eventually will approach a maximum value, V_{max}, because the enzyme is saturated by the substrate and has thus reached the limit of its efficiency. In this region, the initial reaction rate (and therefore the enzyme activity), r_A, is independent of the substrate concentration, (S). This region corresponds to the zero-order kinetics (Segel, 1975). In contrast, for very low substrate concentrations, Figure 4-2 shows that r_A is directly proportional to the concentration of the substrate and thus the reaction is first order with respect to the substrate concentration (first-order kinetics region). In between those two regions, the reaction is of mixed order with respect to the substrate concentration and is best represented by the Michaelis-Menten relationship.

4.1.1.3 Effect of the enzyme concentration

It is known that the concentration of the substrate affects the rate of an enzymatic reaction. The velocity of an enzymatic reaction also depends on the concentration of enzyme. A change in enzyme concentration would alter only the value of Vmax in the Michaelis-Menten relationship. Vmax represents the reaction velocity at very high,

saturating concentrations of substrate. Under these conditions, substrate is attached to an enzyme molecule and the enzyme will be interacting with the substrate to convert it to product as fast as it can. If the number of enzyme molecules were doubled, we would have twice as many enzyme molecules with substrate bound, so it is expected that the overall reaction rate will be doubled as well. Therefore, Vmax is directly proportional to the concentration of the enzyme. On the other hand, the affinity of the enzyme for its substrate is indicated by Km. Changing the number of enzyme molecules doesn't alter their individual chemical characteristics and therefore the enzyme is expected to be able to bind the substrate no better, and no worse, than without a change in the number of enzyme molecules. Consequently, Km in the Michaelis-Menten relationship is independent of the enzyme concentration.

To study the effect of the enzyme concentration on the initial reaction rate, eleven different enzyme concentrations were prepared ranging from 0 to 3 nkat/mL of medium. This effect was investigated at two different temperatures and the conditions used are mentioned in Figure 4-3. This figure shows that, for low enzyme concentrations, an increase in the enzyme concentration produces a linear increase in the reaction rate for both temperatures. However, when the enzyme concentration is above 1 nkat/mL, that linearity disappeared due to several reasons such as: 1) limitations due to substrate concentration and/or other enzyme reactions; 2) involvement of a common form of coenzyme in two enzyme system; 3) presence of competitive inhibitor in an enzyme preparation, etc. (Whitaker, 1972). Figure 4-3 also shows that, at an enzyme concentration of 1 nkat/mL, raising the temperature from 35 to 55°C produces an increase in the reaction rate by 20%; like most chemical reactions, the rate of an enzyme-catalyzed

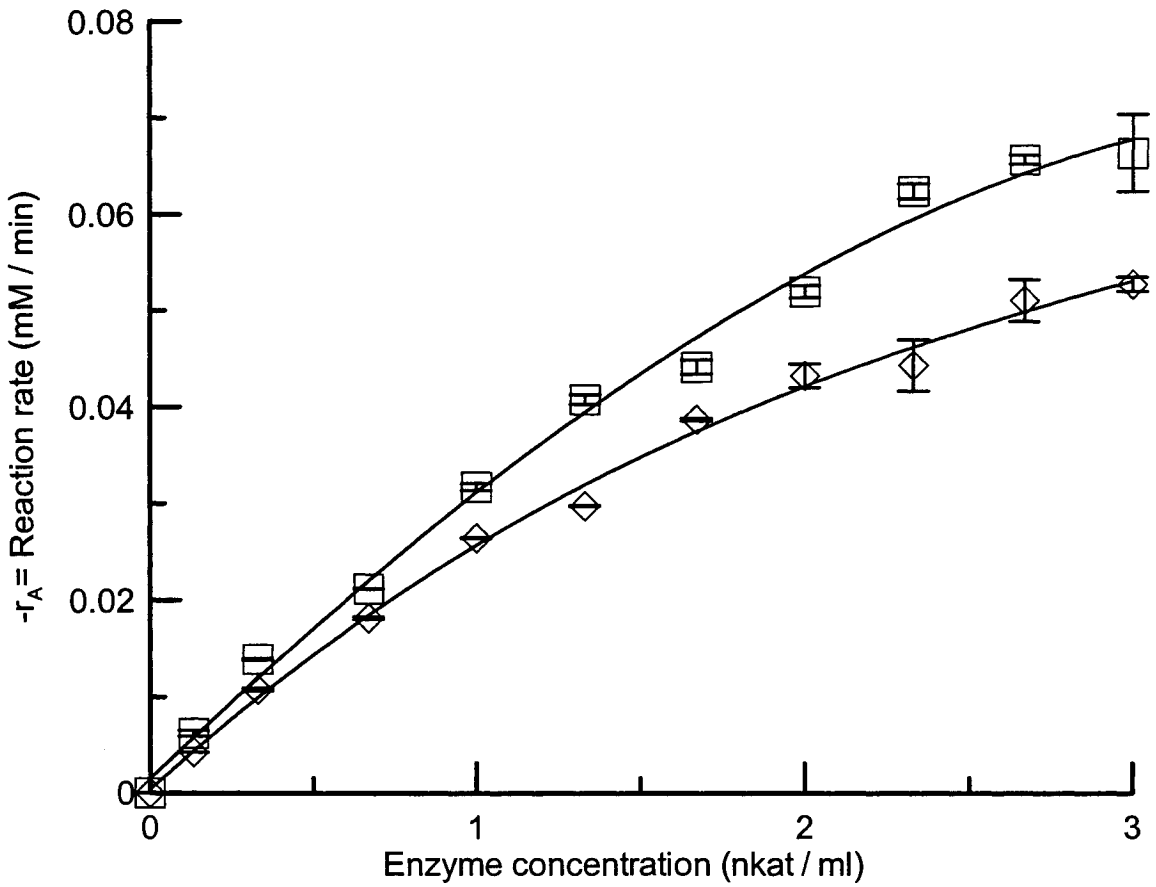


Figure 4-3. Changes in the initial reaction rate with enzyme concentration. CGA concentration: 0.12 mM; pH 4.3. Reaction time: 10 min. (◇) 35°C; (□) 55°C. (Spectrophotometric measurements).

reaction increases as the temperature is raised. The effect of the enzyme concentration was also studied by following the kinetics of the CGA decrease with time (Figure 4-4) at three different enzyme concentrations. In this figure, it is noticed that the rate of conversion of CGA increases as the enzyme concentration is increased due to the fact that more substrate molecules are bound to the enzyme, which speed up the rate of reaction.

4.1.1.4 Effect of the pH on the enzyme activity

The enzyme activity depends on the pH of the solution in the system. The pH of a solution can have numerous effects on the structure and activity of enzymes. For instance, pH can affect the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains while basic amino acids have instead amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can also be altered. This can lead to altered protein recognition (disrupting the normal configuration of the enzyme) and in some cases denaturation of the enzyme.

Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. In general, enzymes have an optimum pH, but the optimum is not the same for each enzyme. In this research work, the optimum pH for the enzymatic system with CGA as a substrate was 4.3 regardless of the temperature used. A similar optimal pH value (pH 4.5) was found by Ding et al. (1998) when using

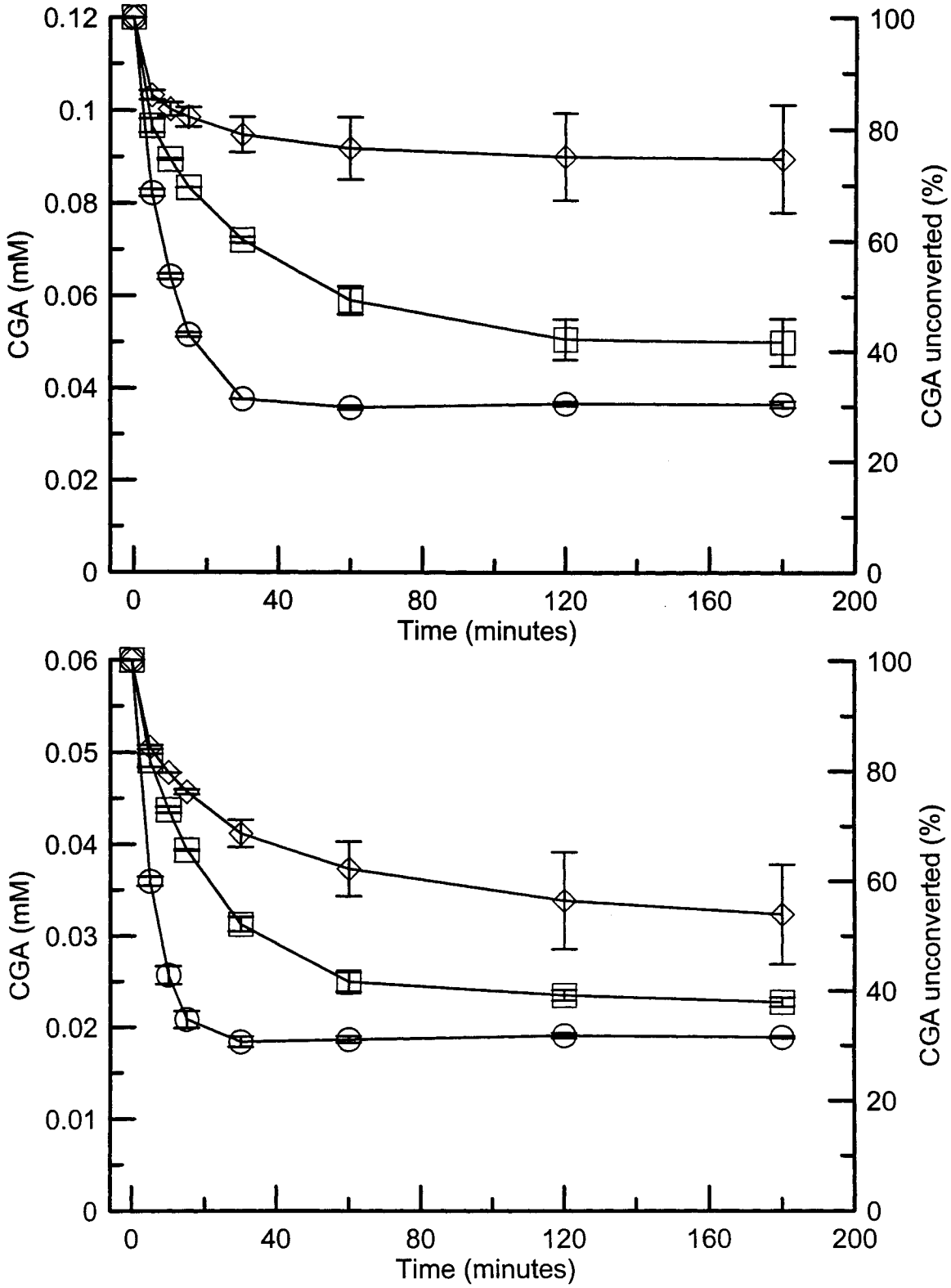


Figure 4-4. Kinetics of the enzymatic conversion of CGA at different enzyme concentrations. CGA concentration 0.12 mM and 0.06 mM; pH 4.3; 35°C. (◇) 0.02 nkat/mL; (□) 0.04 nkat/mL; (○) 0.12 nkat/mL. Spectrophotometric measurements.

chlorogenic acid as a substrate and PPO extracted from loquat (Eriobotrya japonica Lindl. cv. Mogi) fruit. Jiang et al. (1997) found that, when using PPO from litchi fruit peel, the enzyme did not have any activity with chlorogenic acid as a substrate while using 4-methylcathechol, the optimal pH for PPO activity was 7.0. PPO extracted from tobacco leaves exhibited the highest activity at pH 7.0 when catechol was used as the substrate while a low activity was noticed, at the same pH value, with chlorogenic acid as a substrate according to Shi et al. (2001). Lacki (1997) found that, when using the PPO from *Trametes versicolor* with others substrates, the optimum pH became 4.0 for SA, 3.3 for SALD and 4.5 for SIN. These results explain that even though the enzyme used was from the same source as the one used in the enzymatic decrease of CGA in sunflower meal, a change in the pH might have caused a different change in the charge properties of each substrate used obtaining different optima pH values.

Figure 4-5 shows a typical graph obtained when enzyme activity is plotted against pH. An increase in the temperature from 25 to 40°C, at pH 4.3, produces an increase in the enzyme activity by 20% when using substrate and enzyme concentrations of 0.067 mM and 0.73 nkat/mL, respectively. Increasing the reaction temperature from 40 to 55°C would increase the activity by 40% at the same optimum pH value. This means that the rate of chemical reaction increases with temperature as described by the Arrhenius relationship. In other words, the movement of enzyme molecules and substrate molecules increases when the temperature rises causing more collisions between enzyme and substrate and the net result is the formation of more products. If the temperature rises beyond a certain point, however, the enzyme activity eventually levels out and then declines rapidly because the enzyme is denatured by heat. The shape of the enzyme

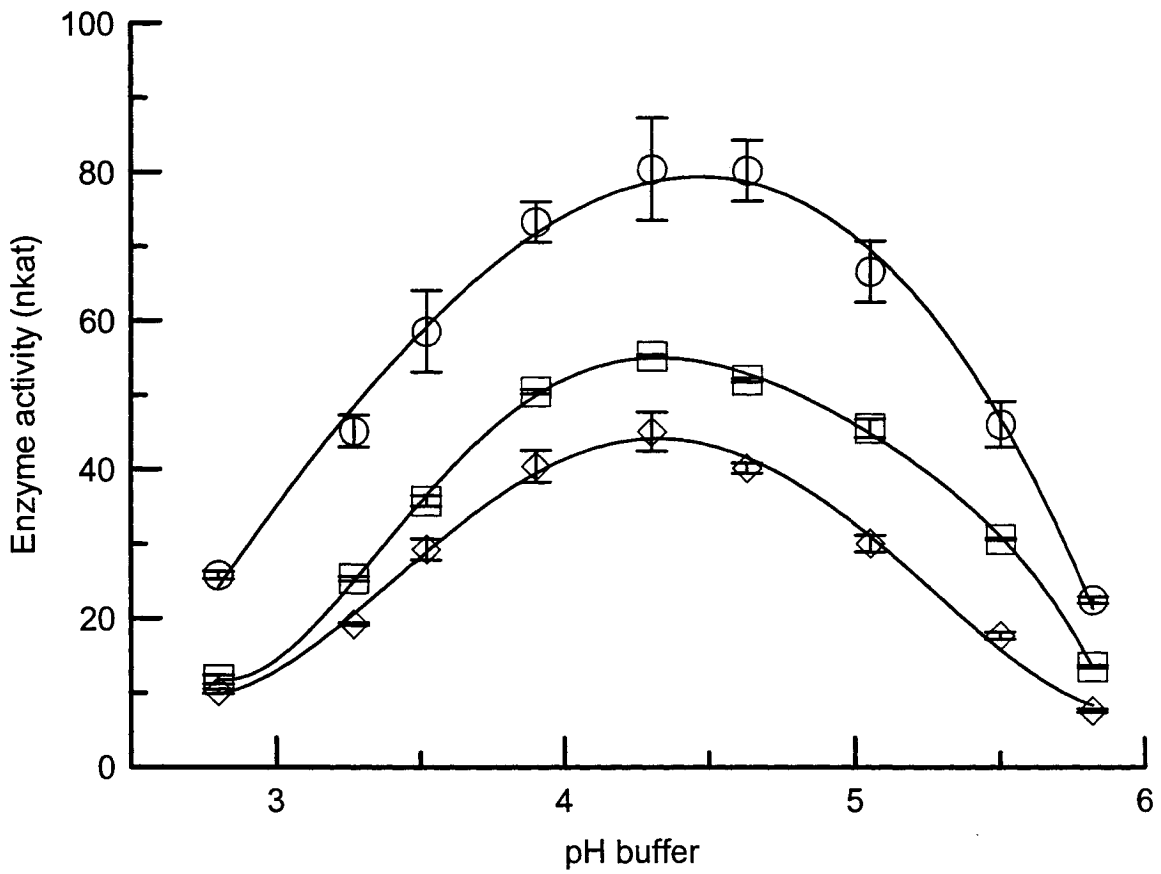


Figure 4-5. Effect of pH and temperature on enzymatic conversion of CGA. CGA concentration: 0.067 mM; Enzyme concentration: 0.73 nkat/mL; Reaction time 10 min. (◇) 25°C; (□) 40°C; (○) 55°C. Spectrophotometric measurements.

molecules changes during denaturation and therefore it cannot catalyze the reaction producing a drop in the rate of the reaction with change in temperature. Figure 4-5 also shows that the curves are bell shape centred around a pH value, which is common in enzyme-catalysed reactions.

When the pH of the system was increased from 2.8 to 4.3 (at 40°C), the enzyme activity at pH 4.3 was almost five times higher than the enzyme activity at pH 2.8. At any temperature studied, the enzyme activity declines above and below pH 4.3. This decline could happen because of the formation of an improper ionic form of the substrate or enzyme (or both), or because of the inactivation of the enzyme, or it may be because of the combination of these effects (Segel, 1975).

The effect of the pH was also studied by following the decrease of the CGA concentration with time at three different pH values as shown in Figure 4-6. In this figure, a faster decrease was seen when the pH of the system was 4.3, which confirmed the results obtained previously. It looks like there was no further increase in the conversion of CGA after 20 minutes of the enzymatic process carried out at pH 4.3. However, at 3.5 and 5.6 reaction rates were much slower and the enzyme continues degrading the substrate even after 20 minutes. From these results, it was found that the presence of by-products, obtained from the enzymatic transformation of CGA, interferes with the spectrophotometer reading. As a result, an overestimation of the CGA concentration was always obtained (after a certain reaction time) with the spectrophotometric technique. This statement was proved when comparing the HPLC and spectrophotometric techniques.

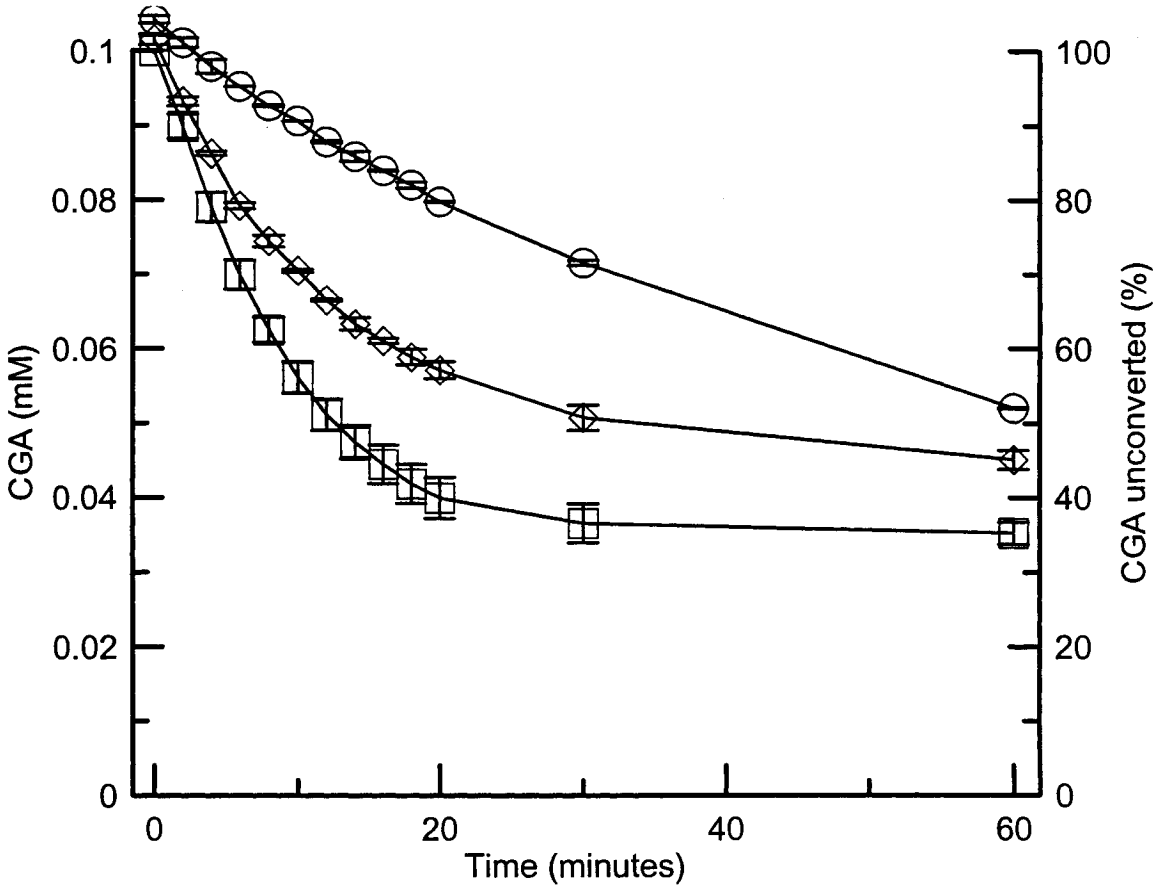


Figure 4-6. Kinetics of enzymatic conversion of CGA at different pH values. Enzyme concentration 0.06 nkat/mL; CGA concentration 0.10 mM; 35°C. pH: (◇) 3.2; (□) 4.3; (O) 5.6. Spectrophotometric measurements.

4.1.1.5 Effect of the temperature on the enzyme activity

It is known that temperature can affect an enzyme in two ways. The first one is a direct influence on the reaction rate constant and the second one is the thermal deactivation of enzymes at high temperatures. When the temperature rises, an increase in the reaction rates is obtained due to the fact that kinetic energy increases; this phenomenon produces an increase in the probability of interaction of molecules, and more molecules will have sufficient activation energy to react. Then at some temperature, reaction rate drops dramatically as temperature becomes too high and enzymes start to denature.

The effect of the temperature on the enzyme activity was studied for six different temperatures, ranging from 25 to 75°C. The enzymatic reactions were carried out at each of these temperatures and the results at two different reaction times are shown in Figure 4-7. These results indicated that at 8 minutes reaction time, the curve of CGA converted did not have a specific optimum value, while an optimum temperature (45°C) was seen when the reaction time was 16 minutes. It should be mentioned that the data plotted in Figure 4-7 were obtained using the spectrophotometric technique. Because of the very low enzyme concentration used in these experiments, it was considered that by-products obtained from the enzymatic degradation of CGA were in very low concentrations and thus these by-products did not affect the reading of the samples. The results obtained at 16 minutes reaction time showed that when the temperature was higher than 45°C, the enzyme started to denature which is typical for most proteins. Several researchers have found different temperature optima when using PPO extracted from several sources. For instance, Jiang et al. (1997) found that when the enzyme, PPO, was extracted from litchi

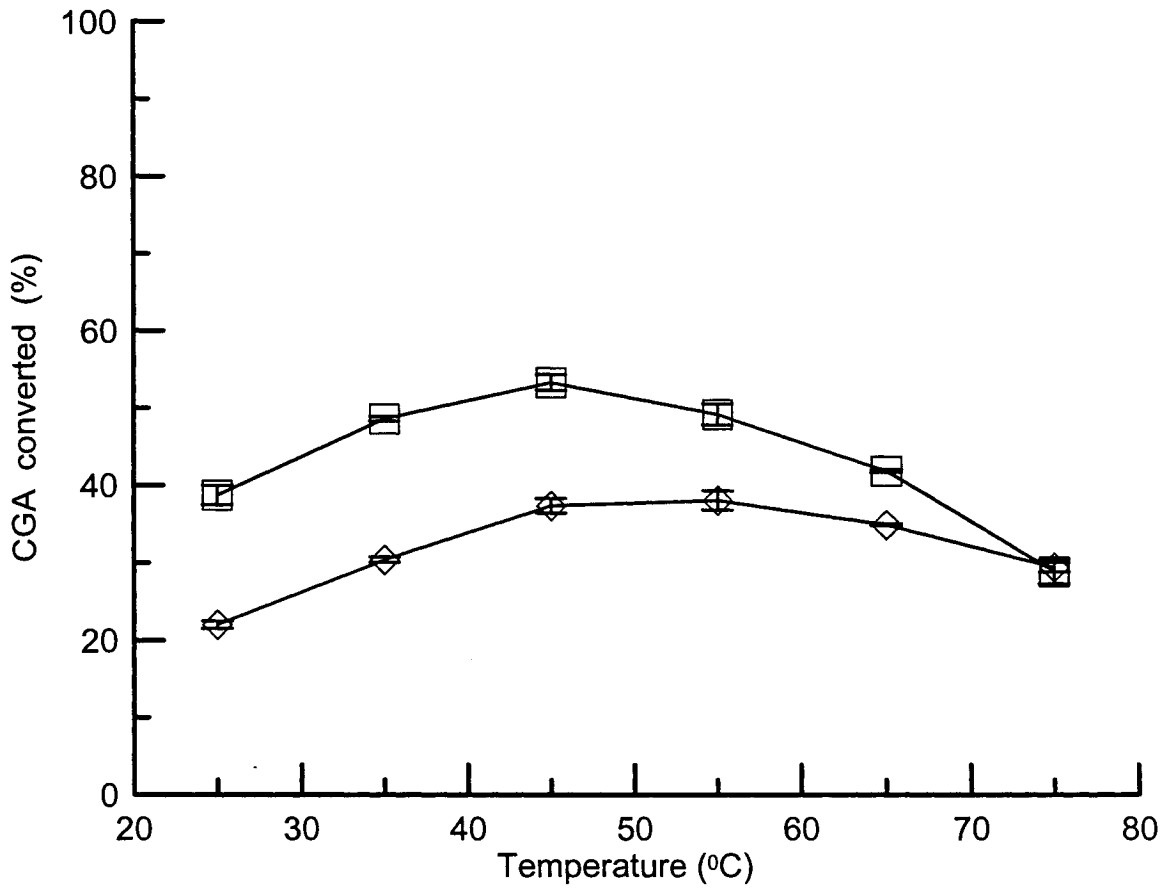


Figure 4-7. Effect of temperature on enzymatic conversion of CGA. CGA concentration: 0.115 mM; enzyme concentration: 0.06 nkat/mL. Conditions: pH 4.3. CGA converted after: (◇) 8 minutes; (□) 16 minutes. Spectrophotometric measurements.

fruit peel and 4-methylcathechol was used as a substrate, the enzyme was relatively temperature stable with a maximum activity at 70°C. On the other hand, Shi et al. (2001) discovered that when the source of the enzyme was tobacco leaves and when using catechol as a substrate, the optimum temperature for the activity of PPO from tobacco was about 40°C. Lacki (1997) found that when the PPO was extracted from *Trametes versicolor*, the optimal temperature for the SA conversion measured at three different pH values was 60°C while for the SALD and SIN transformation was 50°C. Mazzafera and Robinson (2000) and Ding et al. (1998) found that the optimum temperature was 30°C when chlorogenic acid was used as a substrate, but both authors used a totally different source of enzyme (PPO). For instance, Mazzafera and Robinson (2000) used PPO extracted from leaves and from fruit endosperm of coffee (Coffea arabica L.) while Ding et al. (1998) used PPO extracted from loquat (Eriobotrya japonica Lindl. cv. Mogi) fruit.

Figure 4-8 illustrates the decrease of the CGA concentration at six different temperatures, from 25 to 75°C. As shown in this figure, the conversion of CGA is faster at 45°C than at any other temperature studied, which confirms the results shown in Figure 4-7. The results in Figure 4-8 also demonstrate that the denaturation of the enzyme occurs at temperatures higher than 45°C, which is very common for most enzymes. At 65 and 75°C, the enzyme is able to degrade only 42 and 33% of CGA in 10 minutes, respectively, while at 45°C the degradation of CGA is over 50% of its initial value.

4.1.2 Comparison of techniques in the determination of the CGA

Results of the spectrophotometric and HPLC methods were compared to study the reliability of each method. The comparison was done using two different substrate

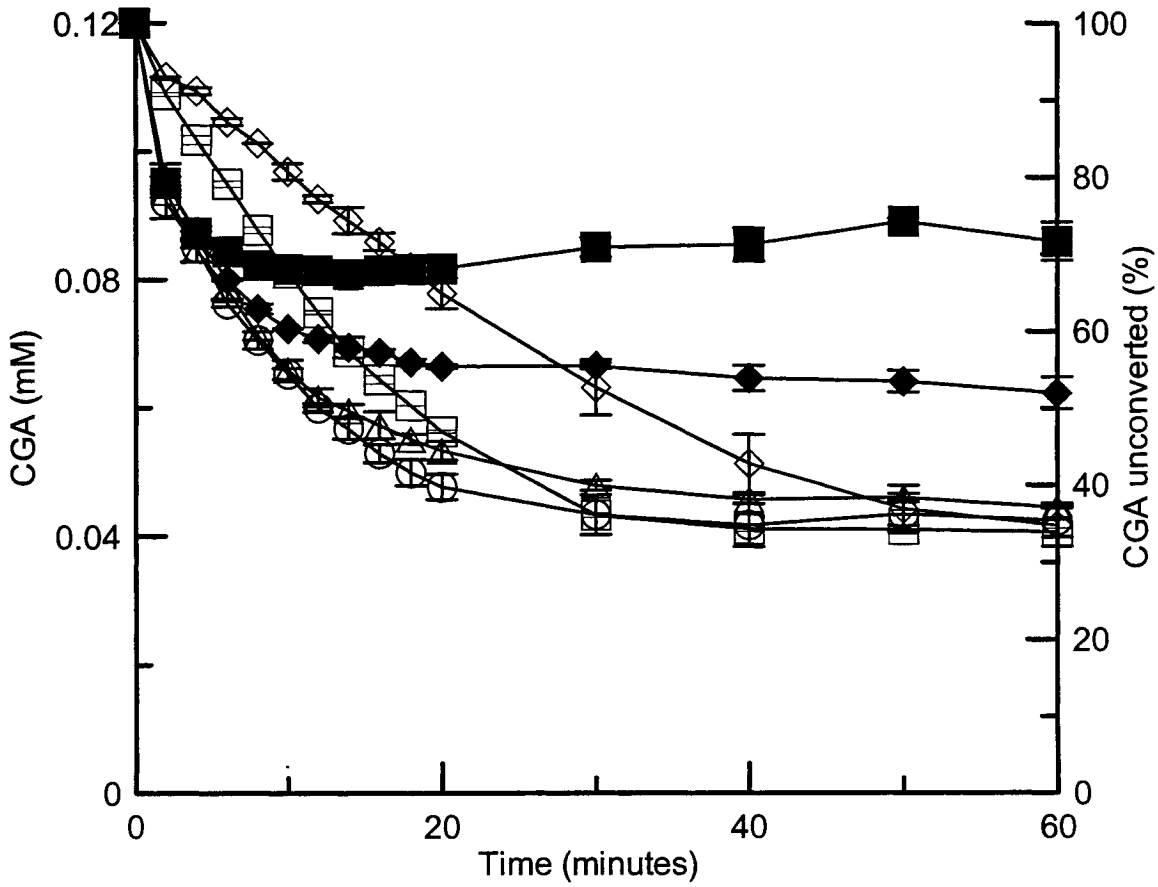


Figure 4-8. Kinetics of enzymatic conversion of CGA at different temperatures; CGA concentration 0.12 mM; enzyme concentration 0.06 nkat/mL; pH 4.3. (◇) 25°C; (□) 35°C; (○) 45°C; (△) 55°C; (◆) 65°C; (■) 75°C. Spectrophotometric measurements.

concentrations (0.13 and 0.07 mM of CGA) and several enzyme concentrations at the conditions specified in Figures 4-9a and 4-9b. The spectrophotometric and HPLC quantification of CGA was based on the standard curves shown in Appendices A and B. Results show that for a substrate concentration of 0.13 mM and low enzyme concentrations, the amounts of CGA quantified are similar in the first eight minutes of the enzymatic reaction regardless of the method used (Figure 4-9a). With an initial concentration of CGA of 0.07 mM, Figure 4-9b, both techniques were in agreement for a period of time a little longer than eight minutes (twelve minutes). This is because of the fact that having lower concentration of CGA means lower reaction rates, as shown previously in Figure 4-2, and therefore longer periods of time to decrease the CGA content in the system. Figures 4-9a (0.13 mM CGA) and 4-9b (0.07 mM CGA) have several differences when measuring the concentration of CGA after eight and twelve minutes respectively. In Figure 4-9a, for instance, the HPLC results showed a reduction in the CGA content of at least 90% in an hour–long enzymatic reaction, while a 65% CGA reduction was obtained in the spectrophotometric results. Lacki (1997) reported similar results to the ones presented here when the SAE content was decreased from canola meal by using the same enzyme. For example, a 78% decrease in the SAE content was measured, in an hour–long enzymatic treatment, using the spectrophotometric method compared to a 93% decrease measured using the HPLC method. Regarding Figure 4-9b, a CGA reduction of 90% was obtained with HPLC measurements while using the spectrophotometric technique, a 70% CGA reduction was seen. Regardless of the initial CGA concentration used, the higher values of the residual CGA measured

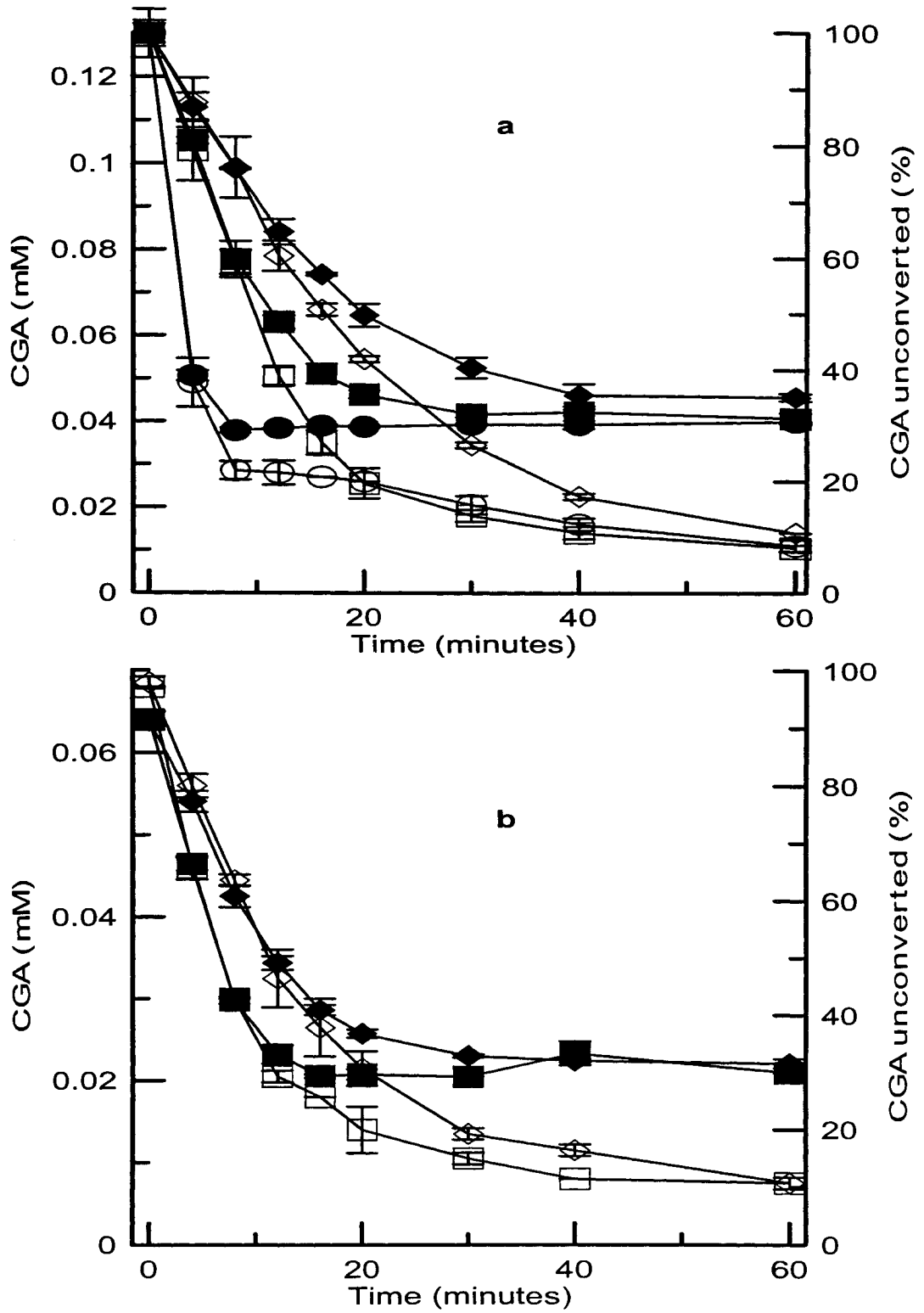


Figure 4-9. Comparison of spectrophotometric and HPLC techniques. CGA concentration: (a) 0.13 mM; (b) 0.07 mM; pH 4.3; 35°C. HPLC: (◇) 0.06 nkat/mL; (□) 0.12 nkat/mL; (○) 0.6 nkat/mL; spectrophotometric: (◆) 0.06 nkat/mL; (■) 0.12 nkat/mL; (●) 0.6 nkat/mL.

when using the spectrophotometric method were a result of the interference that had been caused by by-products of the enzymatic reaction. Because this interference is negligible at early stages of the enzymatic transformation of CGA, the spectrophotometric method was only used when measuring initial reaction rates. On the other hand, the HPLC technique was used as an analytical method to obtain more representative results in further experiments. This is because those experiments were carried out for longer reaction times at which the interference produced by by-products of the enzymatic transformation of CGA was significant.

The comparison of the two techniques was also investigated by several authors such as Dreher and Holm (1983) and Vioque and Millan (1985) when extracting chlorogenic acid from sunflower meal employing aqueous methanol. Both authors were in agreement in finding that the percentage of chlorogenic acid present in the meal was always lower when it was determinate by HPLC measurements than by spectrophotometric measurements.

4.1.3 Dynamics of the CGA decrease in the model system using HPLC

The dynamics of the CGA concentration decrease was studied by following the changes in the concentration of CGA with time in a system with an initial substrate and enzyme concentration of 0.12 mM and 0.06 nkat/mL, respectively. The results in Figure 4-10 show that the enzymatic transformation of CGA is a one-step reaction. In contrast, Lacki (1997) found that the enzymatic transformation of SA was a multi-step system in which more than fourteen by-products were present during the enzymatic process. After one hour of the enzymatic process, some of the by-products of the enzymatic conversion

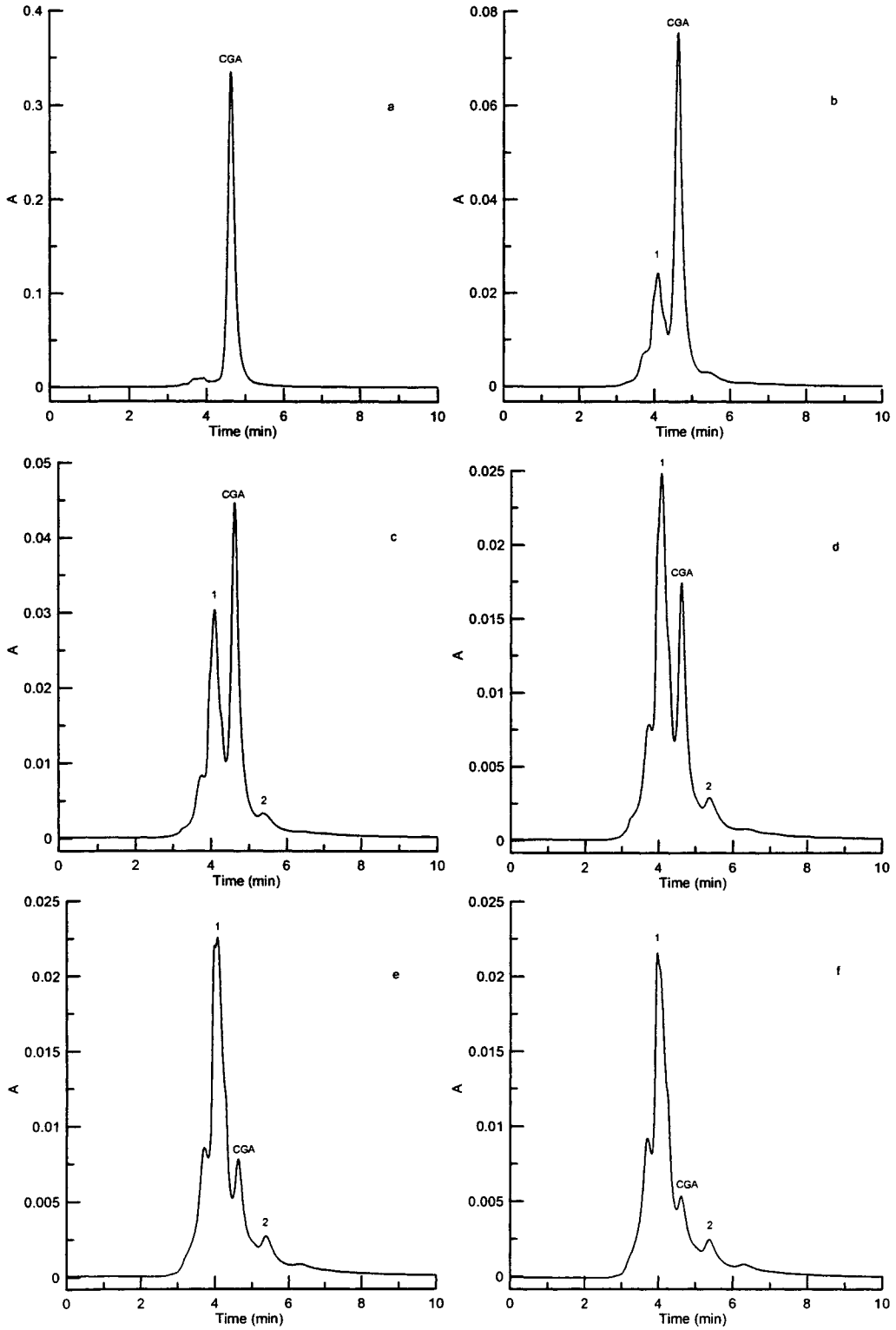


Figure 4-10. Enzymatic transformation of chlorogenic acid at pH 4.3 and 35°C. (a) initial; after: (b) 30 min; (c) 60 min; (d) 120 min; (e) 180 min; (f) 240 min. HPLC measurements.

of SA were in concentrations as high as the initial SA concentration while in the degradation of CGA, the highest concentration found for by-product shown as number one in Figure 4-10 was less than 10% of the initial CGA concentration in the system.

The results in Figure 4-10 also show that the disappearance of the substrate was followed by the formation of by-products, which seem to be more polar than the CGA itself because of their lower retention time. The first chromatogram (a) shows that at 0 minutes only CGA was present; however, after 30 minutes (chromatogram b) a small peak was seen at 4 minutes retention time, which indicated the appearance of by-products absorbing at the same wavelength as the CGA. This peak was rising as the CGA concentration was decreasing (chromatogram c) and afterward, it started to decrease proportionally with the disappearing of CGA (chromatogram d,e) until most of the CGA content had disappeared (chromatogram f). The dynamics of the CGA decrease can also be represented as in Figure 4-11, in which a sharp decline in the CGA concentration was noticed during the first 30 minutes (75% decrease) and then four to five more hours were needed to decrease almost completely all the CGA present in the model system. When comparing the results shown in Figure 4-11 with the ones obtained by Lacki (1997) during the study of the dynamics of the SA transformation, it is noticed that SA is degraded much faster than CGA during the same reaction time and at similar conditions (98% conversion for SA versus 75% conversion for CGA). This statement was also proved in Figure C-7 (Appendix C). In such a figure, the enzymatic conversion of CGA was compared with the SA conversion at the same conditions and it was found that the decrease in SA concentration was much faster than the decrease in CGA concentration. These results confirmed that the affinity of the enzyme for the substrate was higher for

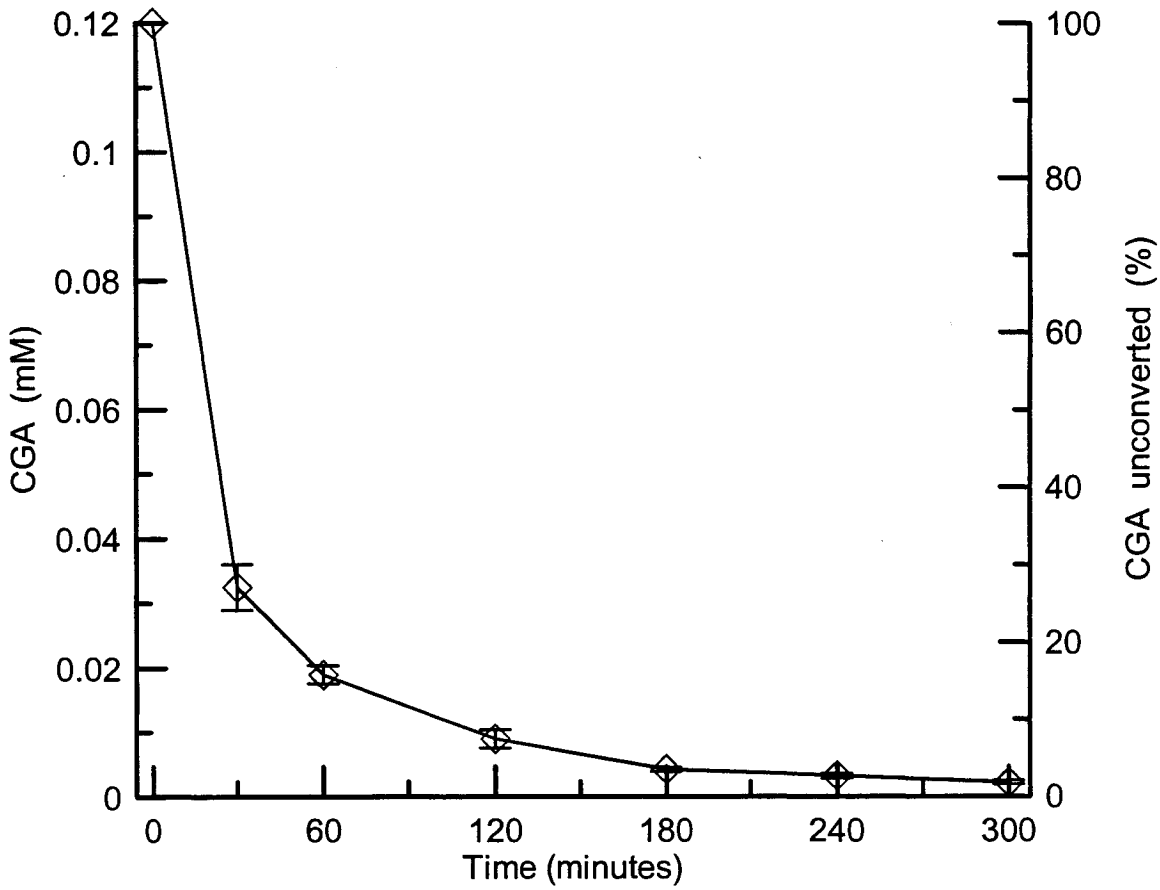


Figure 4-11. Kinetics of the CGA decrease in the model system. CGA concentration 0.12 mM; enzyme concentration 0.06 nkat/mL; pH 4.3; Temp. 35°C. HPLC measurements.

SA than for CGA as mentioned in the study of the effect of the substrate concentration on the enzyme activity (4.1.1.2).

The results displayed in this first part of chapter four confirmed that the enzyme produced by the fungus *Trametes versicolor* could be used to decrease the CGA content in a model system. These results can be used as a base for a better understanding of the enzymatic decrease of the phenolic compounds present in sunflower meal.

4.2 Sunflower meal system (SFM)

4.2.1 Decrease of the chlorogenic acid content in sunflower meal by an enzyme preparation

One of the goals of the project was to use the enzyme preparation in a more complex system (SFM system) to decrease the high concentration of phenolic compounds. It would allow the use of SFM for human and animal consumption. In addition to the phenolic compounds present in the complex system, there are also other compounds such as proteins, sugars and fibers.

The capacity of the enzyme to decrease the CGA content of the SFM system was assessed in the enzymatic process when adding an enzyme preparation to a meal-buffer slurry. Figure 4-12 shows the results of such a treatment for the methanol extracts of untreated, active enzyme treated and deactivated enzyme treated meals. These extracts were obtained after half an hour extraction at room temperature in a temperature-controlled water bath shaker. When the meal was not treated with an active enzyme preparation, almost all the CGA present in the meal was extracted and found in both the continuous phase (Figure 4-13a) and the methanol extracts (Figure 4-12a). In contrast, a very small amount (less than 3%) of CGA was noticed in Figures 4-12c and 4-13c, when

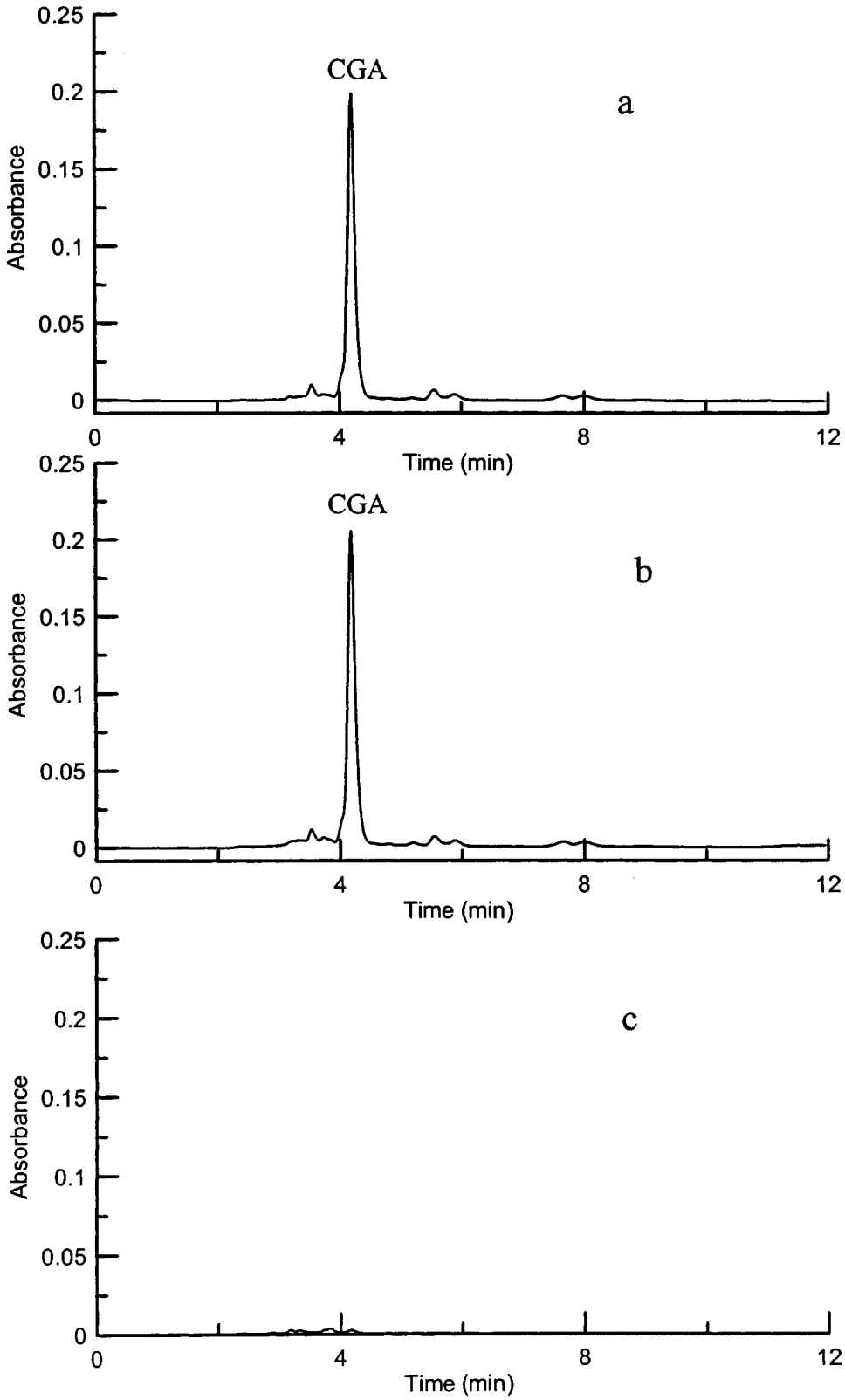


Figure 4-12. Chromatogram of methanol-extract from commercial sunflower meal: (a) untreated; (b) inactive-enzyme treated; (c) activate-enzyme treated. HPLC measurements.

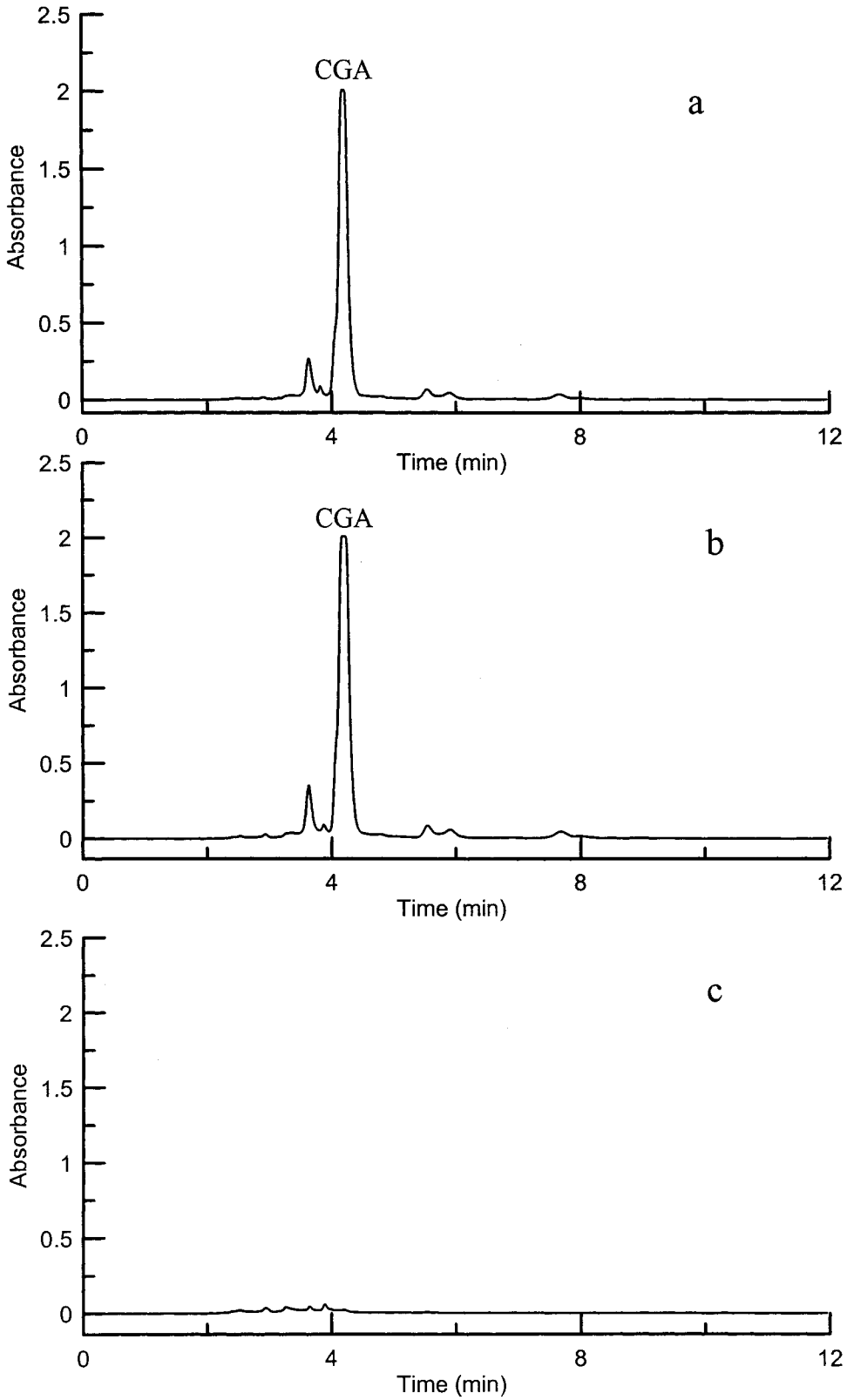


Figure 4-13. Chromatogram of the continuous phase after treatment of sunflower meal with: (a) no enzyme; (b) inactive-enzyme; (c) active-enzyme. HPLC measurements.

the active enzyme was used to treat the meal. The latter results indicated that the enzyme was able to decrease most of the CGA present in the complex system making the enzymatic treatment 97% effective. Moreover the by-products formed during the reduction of CGA with active enzyme were also decreased.

On the other hand, after deactivation of the enzyme for 20 minutes at 95°C, the meal was treated with that deactivated enzyme and the results obtained were similar to those when the meal was not treated with active enzyme. This is because no enzymatic reaction took place in the system and therefore, CGA is removed from the meal not because of the deactivated enzyme preparation but because of the extraction step. Lacki (1997) found that treatment of canola meal with deactivated enzyme resulted in a 50% decrease in the SAE content. According to Lacki (1997), this decrease was also caused by the extraction of phenolics into the continuous phase.

When canola meal was treated and not treated with active enzyme, Lacki (1997) also found similar results to the ones found in this project. In the case of using active enzyme, obtained from *Trametes versicolor,* in the enzymatic treatment of canola meal, it was found that the treatment was 99% effective in the disappearance of SA and SIN. These results also showed a large decrease in the concentration of insoluble-bound phenolic compounds.

4.2.1.1 Dynamics of the CGA decrease in the SFM system

The dynamics of the CGA concentration decrease was carried out in two ways. Case (1) involved a system consisting of 2.5 g sunflower meal, 22.5 mL of citrate phosphate buffer solution, of a chosen pH, and 2.5 mL of an enzyme preparation of a fixed concentration. The decrease of the CGA concentration in the meal was followed

with time as shown in Figure 4-14. The meal and enzyme concentrations used were 10% (w/v) and 3 nkat/mL, respectively. Case (2) to study the kinetics of the process, three series of experiments were carried out at different meal and enzyme concentrations. In each series the meal concentration varied from 6.67 to 20% (w/v). The first series of tests corresponds to an enzyme concentration in the continuous phase of 4 nkat/mL; the second series of tests corresponds to an enzyme concentration per unit of mass of the dispersed phase of 20 nkat/g, and in the third series of tests, a treatment with no enzyme was studied.

In case 1, Figure 4-14 shows the kinetics of the CGA decrease in the sunflower meal system. According to this figure, 98% of the CGA present in the sunflower meal system was converted into by-products in the first four hours of the enzymatic treatment. After that period of time, the substrate was depleted and therefore the curve started levelling off. Regarding case 2, Figure 4-15 illustrates the dynamics of the CGA decrease for different ratios of continuous phase to meal in the first two hours of the enzymatic treatment. The results show that when keeping constant the enzyme concentration in the continuous phase, only a 22% conversion of CGA was noticed in an hour. This small decrease in the CGA concentration was found with the utilization of active enzyme and for the low ratio of continuous phase to meal (ratio 5). The low conversion of CGA in the system, 22%, is due to the fact that a high concentration of meal (20% w/v) is contained in a system with a very small amount of liquid phase making more difficult the extraction of all the CGA into the liquid phase. On the contrary, for the same period of time, at the same ratio and at similar conditions, Lacki (1997) found that 70% of the SAE present in canola meal was decreased when the enzyme concentration was kept constant in the

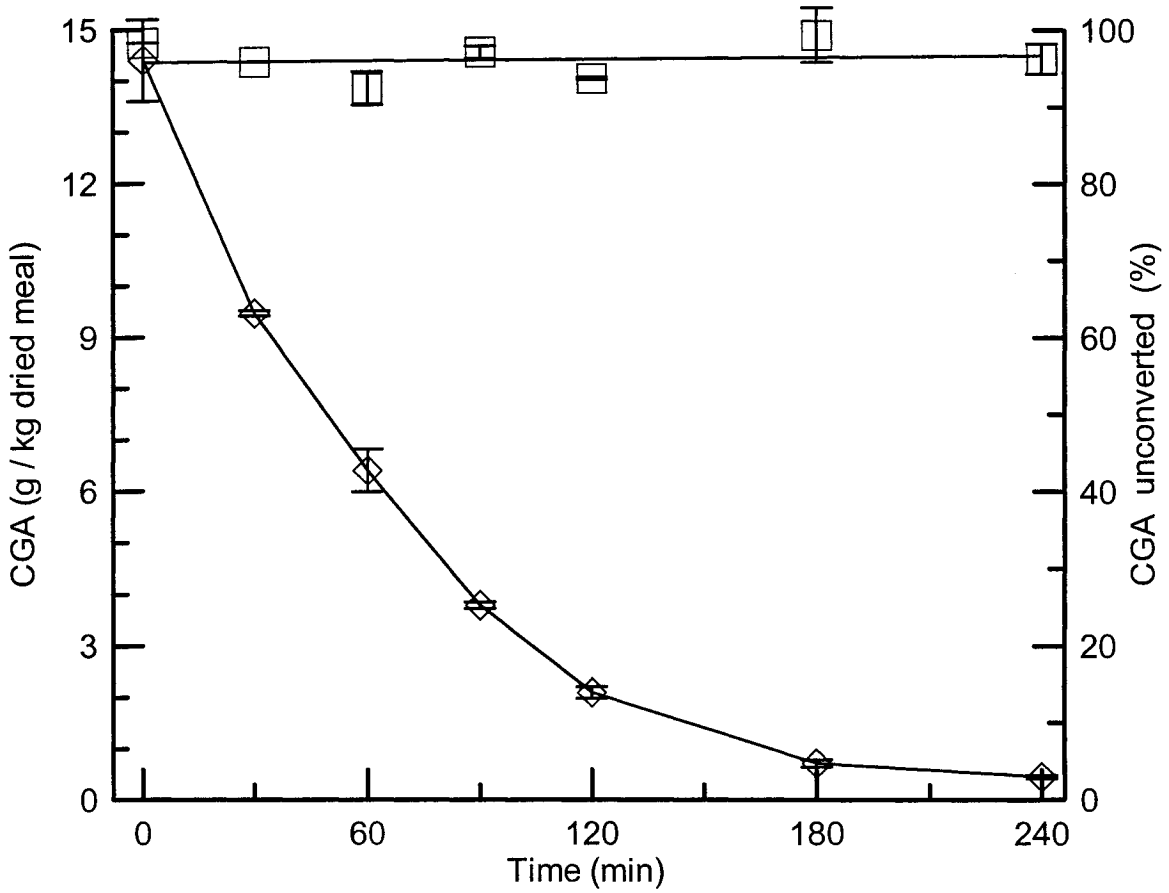


Figure 4-14. Kinetics of CGA decrease in the SFM system. Meal concentration: 10% (w/v); enzyme concentration: 3 nkat/mL continuous phase. Conditions: pH 3.4 (buffer); temp. 35°C. (□) Control; (◇) CGA left after treatment. HPLC measurements.

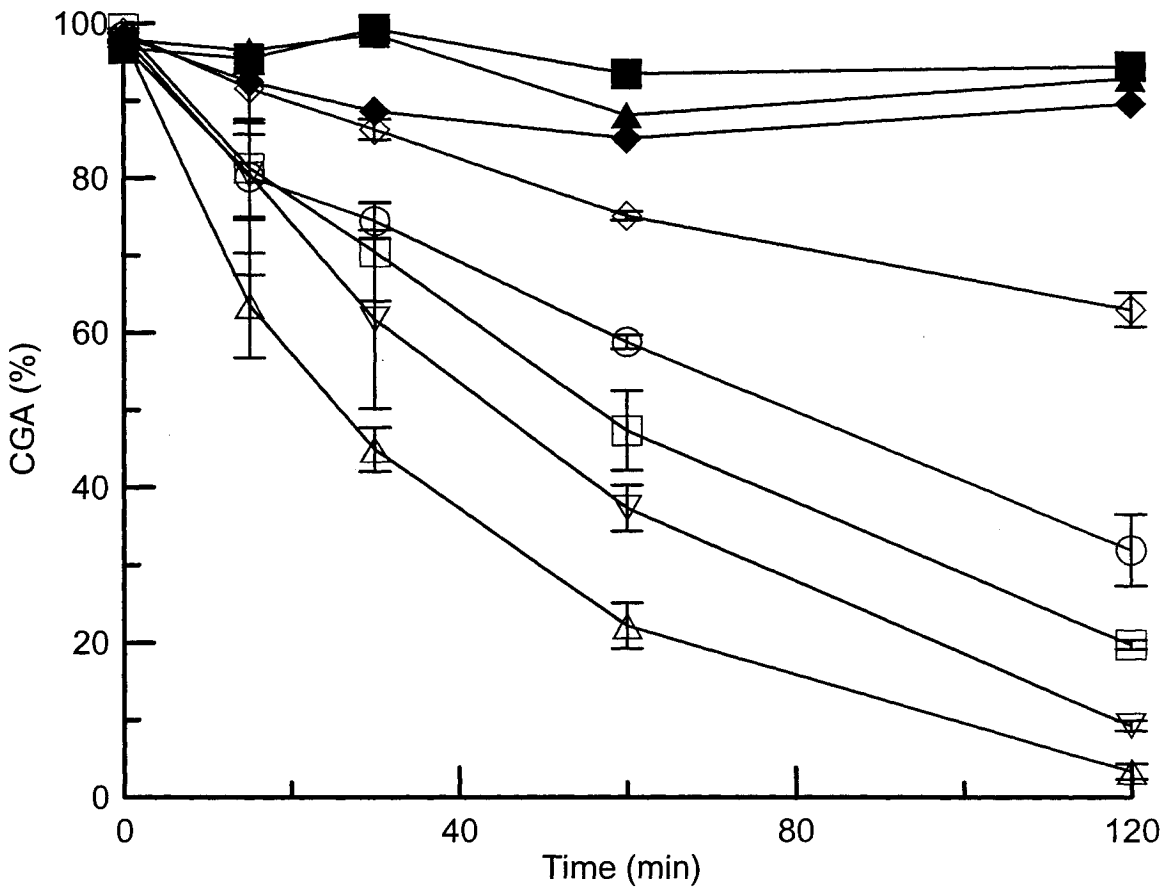


Figure 4-15. Dynamics of CGA content reduction for different ratios of continuos phase to meal (v/w). Ratio 5.0: (◇) 4 nkat/mL; (◆) 0 nkat/mL. Ratio 10: (□) 4 nkat/mL; (○) 2 nkat/mL; (■) 0 nkat/mL. Ratio 15: (△) 4 nkat/mL; (▽) 1.33 nkat/mL; (▲) 0 nkat/mL. Conditions: 35°C; pH 4.3. HPLC measurements.

continuous phase. When having higher ratios of continuous phase to meal (ratio 10 and 15), at least a 50% decrease in the CGA content was noticed after one hour of the enzymatic treatment. This is because the higher the presence of liquid phase in the system, the higher the amount of CGA extracted increasing in this way the ability of the enzyme to decrease this phenolic compound. For the same two ratios, 10 and 15, Lacki (1997) showed that the conversion of SAE became even larger than 70% (86 and 88% for ratio 10 and 15, respectively). The main reasons of such different results could be either the affinity of the enzyme for SAE is higher than for CGA or SAE is extracted much faster into the continuous phase than CGA. It is also possible to have both situations at the same time.

On the other hand, when the enzyme concentration per unit of mass of SFM was kept constant, it was noticed that even though the enzyme concentration (per mL of continuous phase) was higher at 10% (w/v) meal concentration than at 6.67% (w/v), the enzymatic reaction was lower by 25% (in an hour) for the 10% (w/v) meal concentration because of the high content of CGA in the system. Taking into account all these results, it would be good to check whether or not an optimum value exists for the meal concentration in which an addition of a known enzyme concentration will give a maximum conversion of CGA in the SFM system.

4.2.1.2 Effect of the meal-buffer slurry concentration

The effect of the meal concentration was studied in systems with a constant enzyme concentration per millilitre of continuous phase (liquid: enzyme plus buffer) or per gram of dispersed phase (meal). In these systems the sunflower meal concentrations varied from 2% to 20% (w/v). Control samples were used for each meal concentration

studied to determine the amount of CGA converted after 30 minutes of reaction time. The results from Figure 4-16 shows that, despite the fact the ratio of enzyme concentration per mL of continuous phase or per gram of meal was constant, the degradation of CGA content was proportional to the decrease of the meal concentration. In other words, the more meal the system has, the lower amount of CGA that is converted into by- products. The reason is because in the presence of higher meal concentrations, more CGA is extracted into the continuous phase, and thus the system can become saturated by the substrate; this fact can produce a decline in the enzyme capacity to decrease the high amount of CGA present in the system. At higher meal concentrations, the proportionality of CGA converted to meal concentration tends to disappear.

The results from Figure 4-16 also shows that, for meal concentrations lower than 6% (w/v), the concentration of CGA left after the enzymatic process was slightly higher for the systems in which the amount of enzyme per gram of meal was kept constant; this is because working with meal concentrations lower than 6% (w/v), equal or less amount of enzyme was used in order to keep constant the enzyme concentration per gram of meal ratio. In contrast, when the meal concentrations in the systems were higher than 6% (w/v), the enzyme concentration in the continuous phase was always higher in systems in which the amount of enzyme per gram of meal was kept constant. Lacki (1997) found similar results to the latest one when a canola meal concentration above 10% (w/v) was used.

Alternatively, when dealing with sunflower meal concentrations above 20% (w/v), it was found that only one phase (solid phase) was present. When drying that solid phase, part of the CGA content was lost and, therefore, the expected CGA concentrations

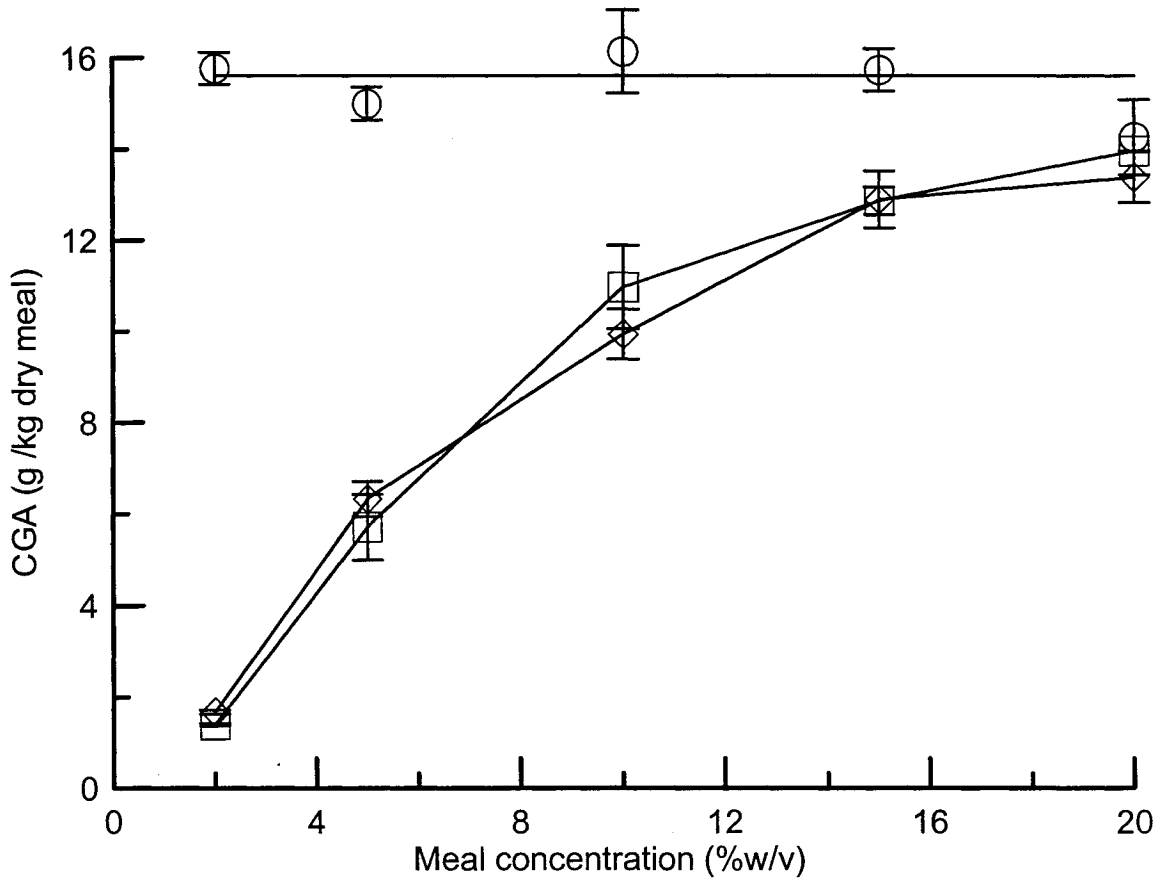


Figure 4-16. Effect of the meal concentration on enzymatic conversion of CGA. pH 4.3 (system); 35°C; 30 min. reaction time. (◇) CGA left with 3 nkat/g; (□) CGA left with 3 nkat/mL; (○) Control. HPLC measurements.

were much lower than the real values. In contrast, when dealing with canola meal, Lacki (1997) found the presence of only one phase when canola meal concentrations were above 49% (w/v).

4.2.1.3 Effect of the enzyme concentration

It was shown previously in the model system that the enzyme concentration has a significant effect on the enzymatic conversion of CGA. That effect can be influenced by the presence of the meal in the system in such a way that the enzyme can be either inhibited or activated by the presence of sunflower meal. In order to investigate so, several experiments were done at 35°C and pH 4.3 using 10% (w/v) meal concentration. In these experiments, the enzyme concentration was changed from 0 to 30 nkat/mL of continuous phase in order to have a wide range of enzyme concentrations.

The results shown in Figure 4-17 illustrate that the longer the reaction time, the higher the conversion of CGA. This is most probably due to the fact that the enzyme has a better contact with the substrate, which helps to speed up the rate of the CGA decrease. For instance, at 30 minutes reaction time, only 13% of the CGA present in the system was converted when using an enzyme concentration equivalent to 20 nkat while at 120 minutes, 55% of the CGA present in the SFM system was converted to by-products. Lorusso et al. (1996) found that using the same enzyme preparation from *T. versicolor*, more than 80% of the tannin content in commercially available canola meal was removed from the meal after 30 minutes of reaction time with an enzyme concentration equivalent to 20 nkat in the system. Figure 4-17 also shows that regardless of the reaction time used (30 or 120 minutes), the rate of decrease in the CGA content is directly proportional to the increase in the enzyme concentration for concentrations up to 2.2 nkat/mL (22 nkat/g

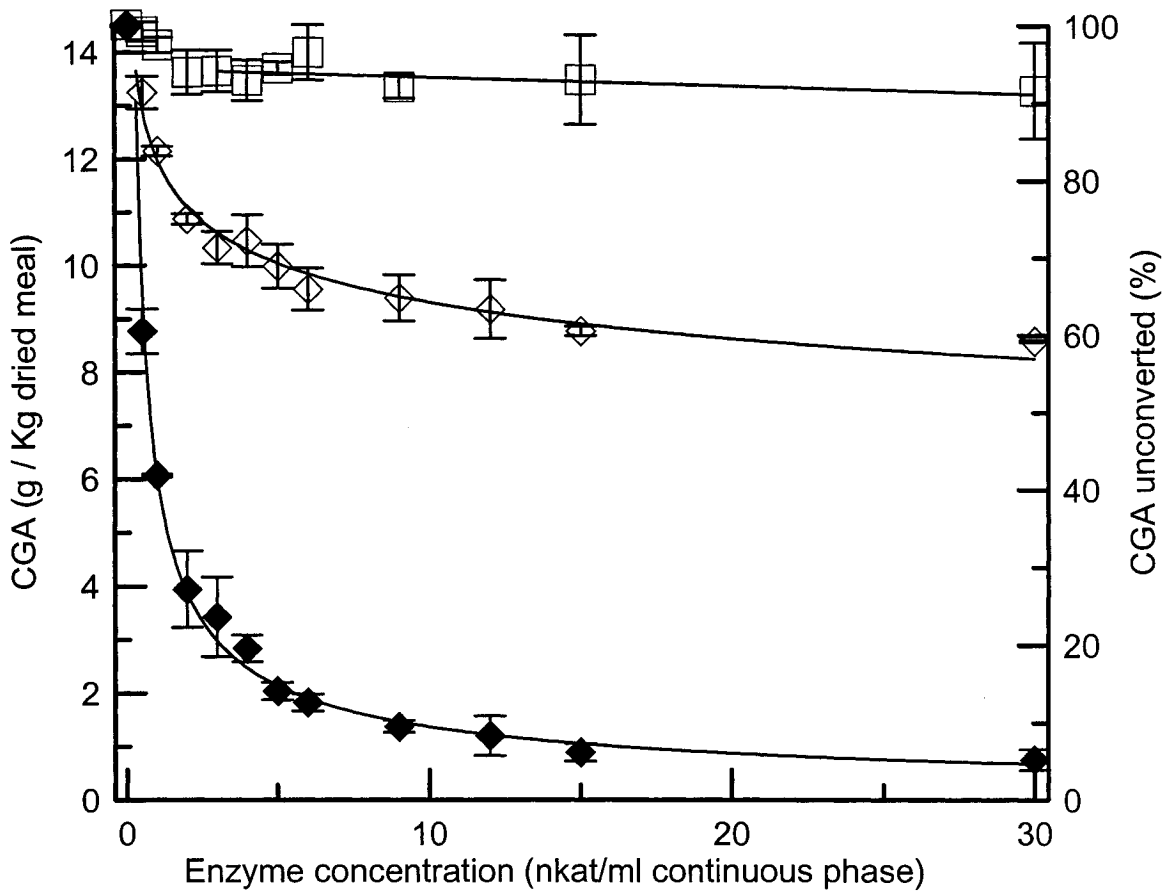


Figure 4-17. Effect of the enzyme concentration on the decrease of CGA content in SFM. Conditions: 35^{0}C; pH 3.4 (buffer); meal concentration 10% w/v SFM. Reaction time: (◇) 30 min; (◆) 2 h; (□) control. HPLC measurements.

meal). Above this concentration, this proportionality disappears and the rate of the decrease of the CGA content becomes independent of the enzyme concentration. A similar behaviour was also found during the decrease of the SAE content in canola meal (Lacki, 1997), but the proportionality between the rate of the SAE decrease and the enzyme concentration was seen up to an enzyme concentration of 7 nkat/g meal compared to 22 nkat/g meal in the results of the CGA decrease. On the other hand, a very small effect on the rate of decrease of CGA content was noticed after a determined enzyme concentration; for instance, an increase of the enzyme concentration from 10 to 30 nkat/mL produced an additional 3 and 4% decrease in the CGA content for reaction times of 30 and 120 minutes, respectively. This small decrease in the CGA content could have been due to the lack of substrate in the system or to the saturation of the system with the enzyme. Taking into consideration these results, in industry it would be better to use higher reaction times and low enzyme concentrations instead of using higher enzyme concentrations and low reaction times.

4.2.1.4 Effect of the pH

The effect of the pH was also considered during the enzymatic treatment of CGA in the SFM system. The meal and enzyme concentrations used were 10% (w/v) and 3.5 nkat/mL, respectively. The enzymatic treatment was carried out at 35°C for a period of 30 minutes and the results obtained are shown in Figure 4-18. These results show a decrease in the enzyme activity by 10% when the pH of the buffer dropped from 4.3 (optimum value in the model system) to 2.2 while a higher decrease in the activity (by 30%) was seen when dropping the pH of the buffer from 3.4 to 2.2. These differences in activities suggested that the pH of the SFM system was not the same as that of the buffer. In reality

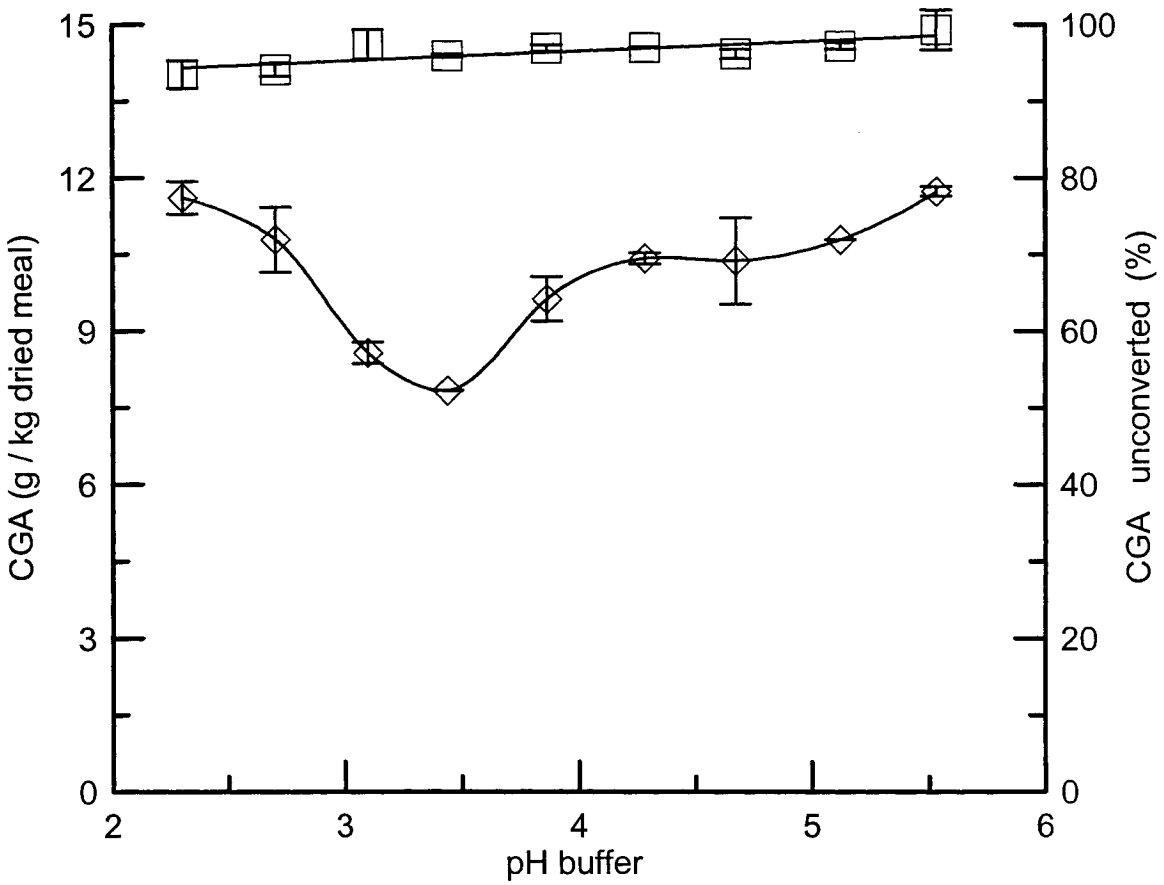


Figure 4-18. Effect of the pH of the buffer on the decrease of CGA content. Meal concentration: 10% (w/v); enzyme concentration: 3.5 nkat/mL continuous phase. Conditions: 35°C; 30 min. reaction time. (□) Control; (◇) CGA left after treatment. HPLC measurements.

due to the buffering capacity of the SFM, the pH of the SFM system depends on the pH of the buffer and on the meal concentration used. This effect is shown in Figure 4-19 for three different meal concentrations (5%, 10%, and 20% w/v) and six different buffer pH values. At 35°C and 20% meal suspension, for instance, the pH of the system fluctuated within 4.5 and 6.0 while the buffer pH was between 3.0 and 6.3 respectively. Lacki (1997) found that when the buffer pH was between 3 and 6.5, the pH of the canola meal system oscillated within a range of 4.5 to 5.5, which is pretty much similar to the results found in this project. The results showed by Lacki (1997) were obtained at 50°C and using a 20% (w/v) meal suspension.

On the other hand, for a 5% meal suspension a change in the pH of the buffer between 3.0 and 6.3 resulted in the change of the pH of the system from 3.8 to 6.4 respectively. A common point was found at buffer pH of 5.6, which corresponds to pH in the system of 5.6, regardless of the meal concentrations used. This common point was in the same range as the one found by Lacki (1997). In general, the results presented in this part of this project were in agreement (with respect to the buffering effect of sunflower meal) with the results found by Lacki (1997), in which the enzymatic decrease of the SAE content in CM was investigated at 50°C. Lorusso et al. (1996) also found the same buffering effect on the pH of the process when the tannin content in CM was decreased enzymatically at 30°C. The buffering capacity of SFM could be related to the extractions of proteins from the meal into the continuous phase as shown by Lacki (1997) in the case of canola meal.

In addition to the results discussed from Figure 4-18, the control samples in this figure indicate that the extraction of CGA into the aqueous phase is independent of the

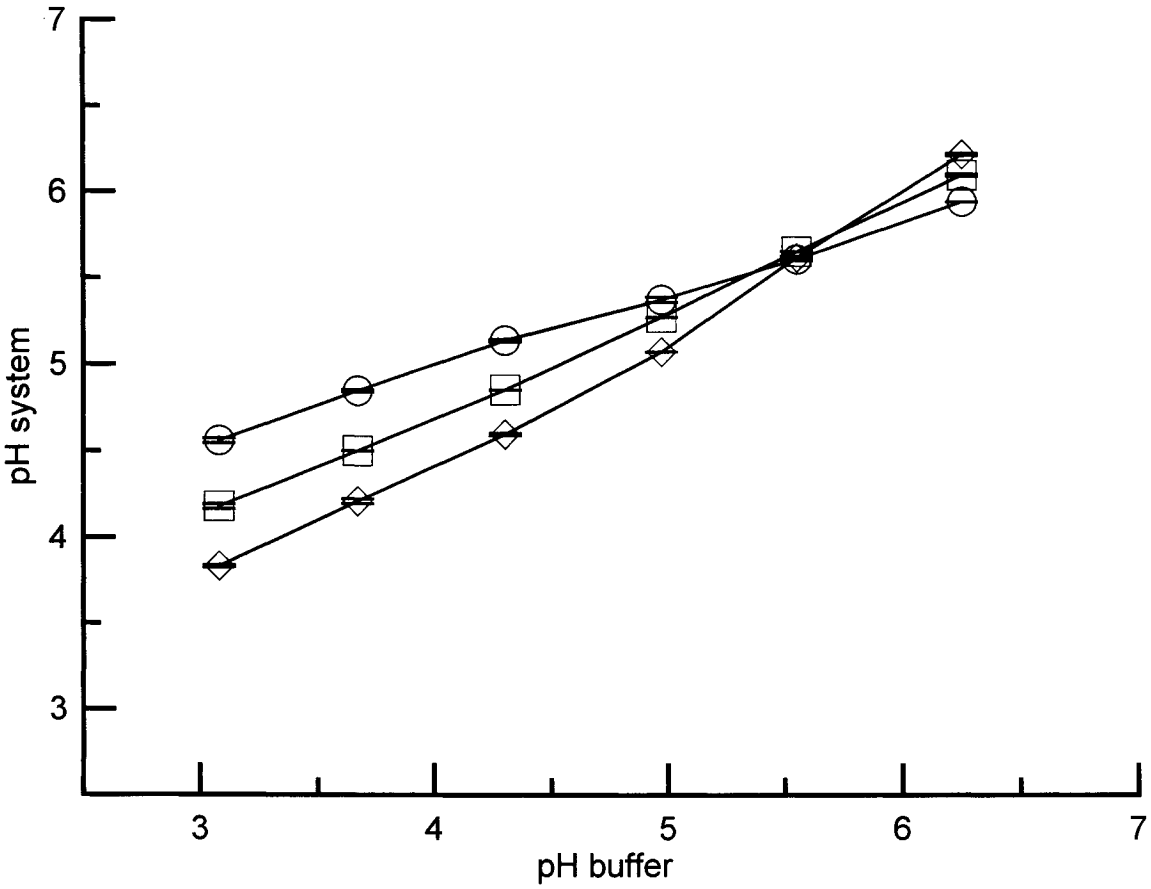


Figure 4-19. Effect of the pH of the buffer and the slurry concentration on the pH of the system. Meal concentration: (◇) 5% (w/v); (□) 10% (w/v); (○) 20% (w/v). Temp. 35°C.

pH of the buffer.

4.2.1.5 Effect of temperature

The temperature plays an important role in the enzymatic conversion of CGA because as most catalyzed chemical reactions, the reaction rate of the enzymatic conversion of CGA increases as the temperature is raised and decreases after a peak temperature has been reached. Previous results on the effect of the temperature on the enzyme activity in the model system (Figure 4-7) indicated that the enzyme possesses thermophilic properties. Therefore, it was necessary to look whether or not the enzyme behaves in the same manner in the SFM system. In order to investigate so, several tests were carried out at buffer pH 3.4, for six different temperatures and for two different reaction times. A meal and enzyme concentration of 10% (w/v) and 3 nkat/mL, respectively were used.

Figure 4-20 shows that the optimum temperature for the enzymatic decrease of CGA was 45°C as it was obtained in the model system. Even though the optimum temperature found was the same in both systems, the results obtained for the SFM system did not show a well-defined bell shape curve as it was noticed for the model system. For example, a change in the temperature from 25°C to 45°C in the model and SFM system produces a difference in activity of 14% and 2% respectively. Similar changes of 22% and 15% were noticed when the temperature was raised from 45 to 75°C. Lacki (1997) found a much higher difference in activities, in both the model system and the CM system, when the temperature was raised from 25 to 45°C and from 45 to 75°C during the enzymatic decrease of the SAE content in canola meal. In the study of the enzymatic treatment of canola meal, it was also found that the highest rate of SAE decrease in

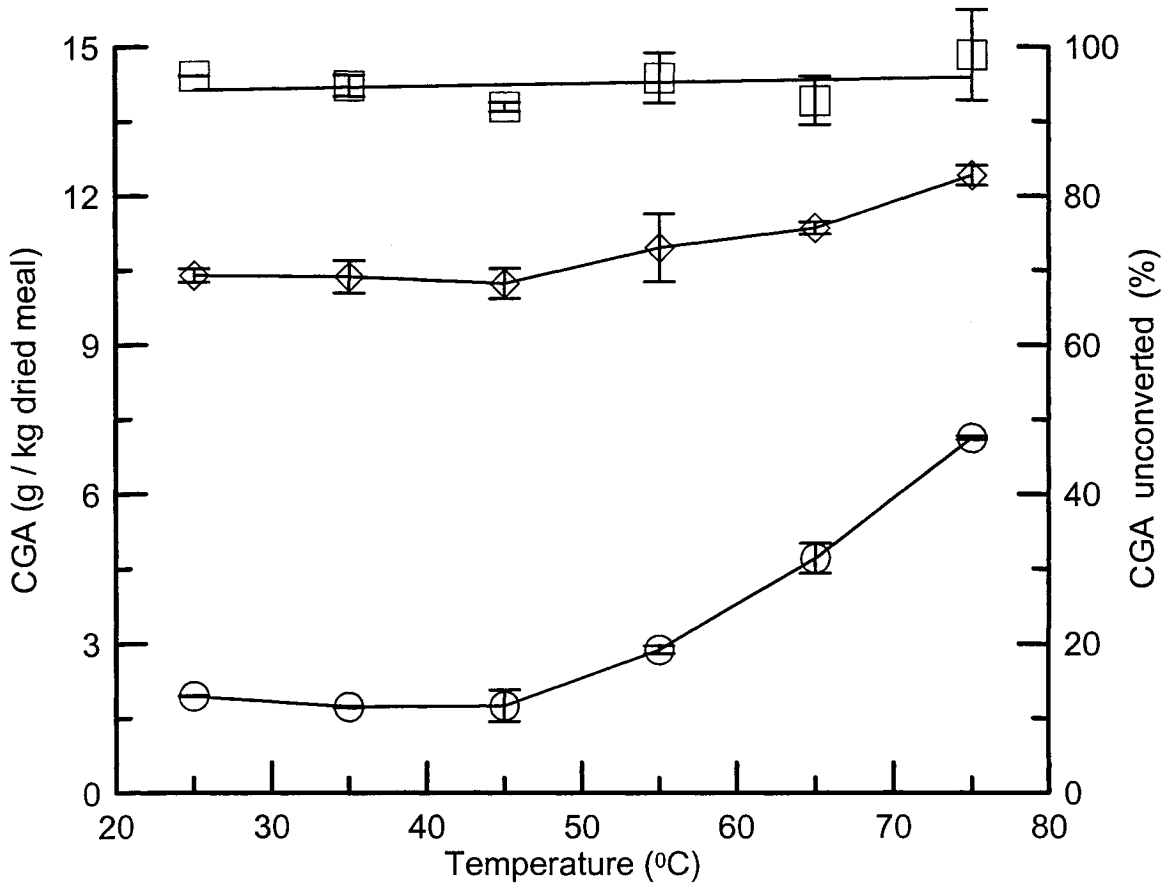


Figure 4-20. Effect of temperature on the decrease of CGA content in SFM. Meal concentration: 10% (w/v); enzyme concentration: 3 nkat/mL continuous phase. Conditions: pH 3.4 (buffer). (□) Control; (◇) CGA after 30 min; (○) CGA after 2 h. HPLC measurements.

canola meal was observed at 50°C, regardless of the enzyme concentration used.

Figure 4-20 also shows that for temperatures lower than 45°C, there is a very small response in the reaction rate of the process to an increase in the temperature, which can be explained by the presence of SFM in the system. The presence of the SFM prevents somehow the enzyme to attain the same stereo-configuration as the one present in the model system. This phenomenon was investigated by Lacki (1997), who found that in the presence of canola meal the enzyme was immobilized. A similar phenomenon of low enzyme response to an increase in the temperature was observed when the inducible form of laccase from *T. versicolor* was immobilized on glass beads (Rogalski et al., 1990).

4.2.1.6 Effect of particle size on the decrease of the CGA content in SFM

The particle size of the meal affects the rate of the enzymatic conversion of CGA. Small particles have more surface area per unit mass or volume than large particles, therefore, it is expected to have a much larger extraction of substrate into the continuous phase when small particles are used. This phenomenon can lead to an increase of the reaction rate.

The effect of the particle size was studied in 15 mL tubes, which contained: 1 g sunflower meal, 9.25 mL of buffer solution of a chosen pH, and 0.75 mL of an enzyme solution of fixed activity (system B mentioned in section 3.2.1). This effect was carried out at 55°C in a custom-made temperature controlled air chamber. The meal and enzyme concentration were 10% (w/v) and 3 nkat/mL respectively. Different fractions of SFM were obtained by a sieving method and each fraction was treated with the enzyme

preparation. The results of these experiments, which are shown in Figure 4-21, were not conclusive. For instance, in the first hour of the enzymatic treatment, the rate of the CGA decrease was higher for particles with sizes between 1 and 2 mm than for smaller particle sizes even though small particles have more surface area per unit mass. In contrast, after two and a half hour of treatment, the decrease of the CGA content in the SFM system was pretty much the same regardless of the particle size used, this means that the particle size did not influence at all the enzymatic conversion of the CGA under these conditions. Lacki (1997) showed that for fractions of canola meal with diameters smaller than one millimetre, the results followed the expected trend: the smaller the particle size the faster the rate of decrease of the SAE content in the canola meal system. However, when the diameters were higher than one millimetre, the opposite pattern was noticed.

The results obtained in this research were not in accordance with that was expected. It can be concluded that particle size does not influence the rate of the enzymatic degradation of CGA. It should also be pointed out that only SFM fractions with a diameter larger than 600 micrometer were considered individually, the remaining fractions were combined since a decrease in the particle diameter produces a decline in the hydrodynamic conditions for the enzymatic process.

4.2.1.7 Effect of agitation speed on the decrease of the CGA content in SFM

Mass transfer steps can affect the enzymatic process by either slowing or increasing reaction rates and thus the conversion of CGA. Since the mass transfer rate can be improved by changing different factors such as the agitation speed of the system, aeration of the system and reactor configuration and mode of operation, a study was done to examine whether or not the enzymatic conversion of CGA was mass transfer limited.

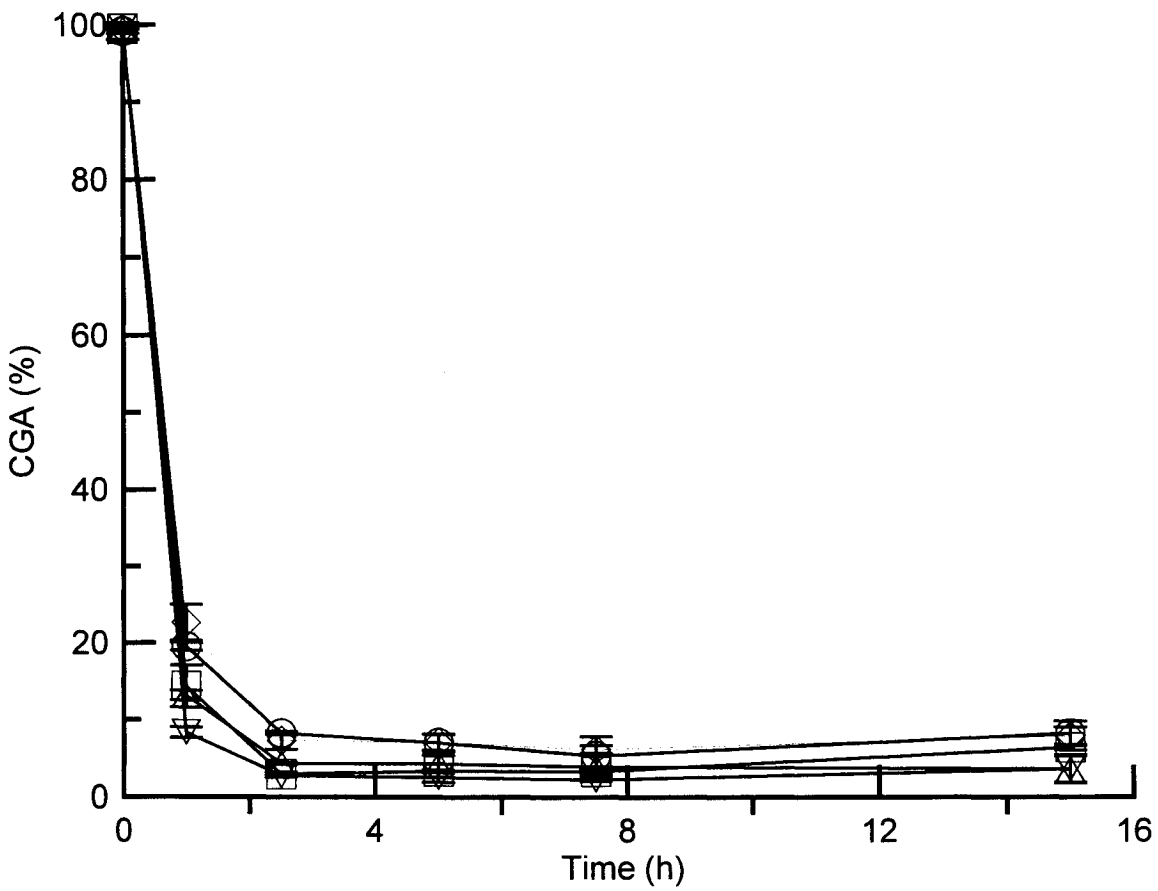


Figure 4-21. Effect of particle size on the decrease of CGA content present in SFM at 55°C, enzyme and meal concentration of 3.0 nkat/g meal and 10% (w/v), respectively: (◇) <600 μm; (□) 600-710 μm; (○) 710-850 μm; (△) 850-1000 μm; (▽) 1.0-2.0 mm. HPLC measurements.

In order to study that, the effect of the agitation speed of the system was investigated at 55°C in a custom-made temperature controlled air chamber. The system for this study consisted of 15 mL tubes, which contained: 1 g sunflower meal, 9.25 mL of buffer solution of a chosen pH, and 0.75 mL of an enzyme solution of fixed activity (system B mentioned in section 3.2.1). A meal and enzyme concentration of 10% (w/v) and 3 nkat/g meal was used, respectively. The results shown in Figure 4-22 indicated that the enzymatic process was mass transfer limited with respect to the agitation speed of the system. For instance, at one hour of the enzymatic treatment of CGA, the reaction rate at 12 rpm was six times greater than when having no agitation at all, while for the enzymatic conversion of SAE in canola meal, at similar conditions, the reaction rate at 12 rpm was only two times greater than when no agitation took place (Lacki, 1997). At the same period of time, an increase in the agitation speed from 12 to 36 rpm, produced an extra three times increase in the reaction rate of the CGA decrease, with respect to the non-agitated system. In contrast, during the decrease of the SAE content in canola meal, no change was noticed in the reaction rate when raising the agitation speed of the system from 12 to 36 rpm (Lacki, 1997). All these results indicated that mixing increased the mass transfer rate by increasing both the turbulence, which decreases the thickness of boundary layers and thus increases mass transfer rates and the rate of movement of molecules through the bulk liquid. Figure 4-22 also shows that after eight hours of the enzymatic treatment of the CGA content in the SFM system, there is no dissimilarity for both agitation speeds.

In the non-agitated system, the SFM particles formed a sediment where most probably CGA was extracted in higher amounts from the top boundary layers of particles

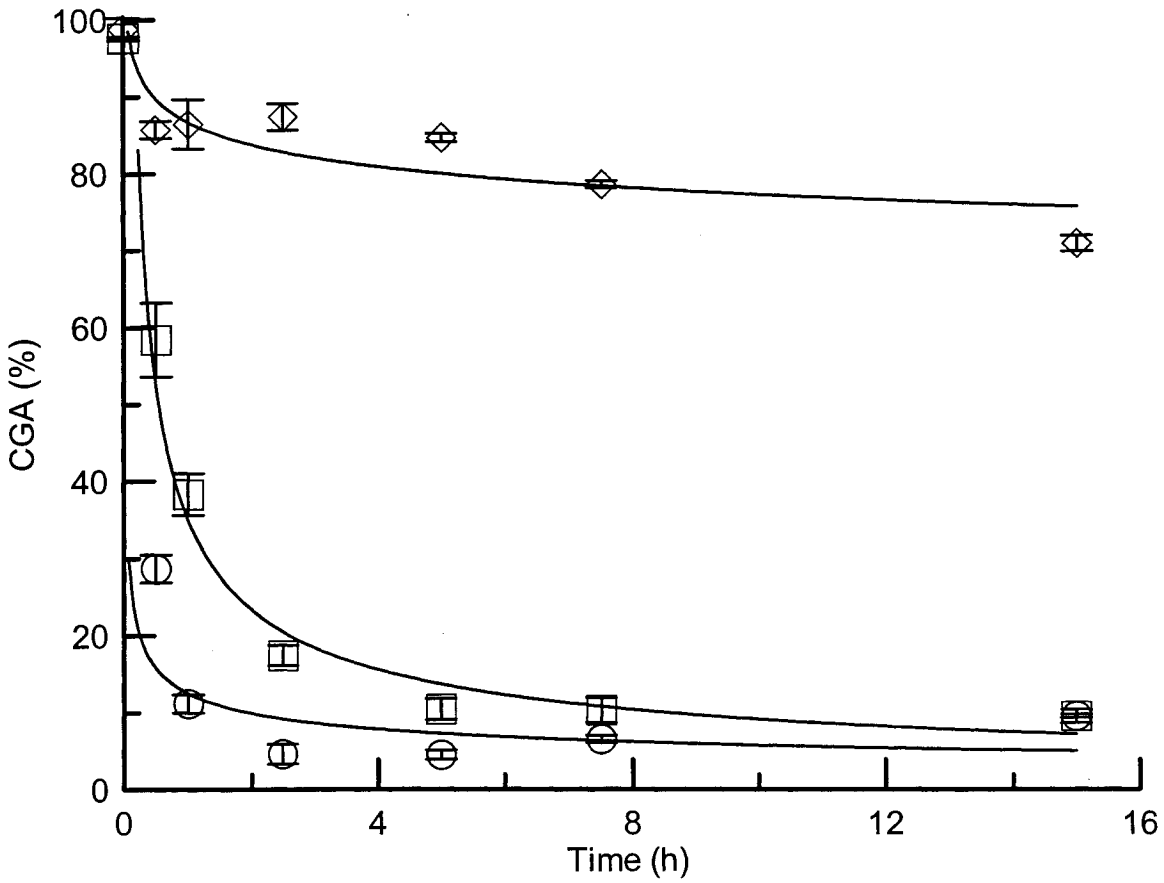


Figure 4-22. Effect of the intensity of agitation on the decrease in CGA content of SFM at 55°C, enzyme and meal concentration of 3 nkat/g and 10% (w/v), respectively: (◇) no agitation; (□) 12 rpm; (○) 36 rpm. HPLC measurements.

by diffusion forces rather than by forced convection. This phenomenon occurs because the diffusion of solutes across a boundary layer is dependent upon the concentration gradient (driving force); therefore, the rate of mass transfer between phases (upper layer of particles and bulk liquid above the sediment) will be higher at a lower concentration of CGA in the bulk liquid. If this is the case, it is expected to find a higher CGA extraction in the top layer of particles. On the other hand, the extraction of CGA in the botton layers of the sediment was governed by a local equilibrium between the concentrations of CGA in the liquid and in the SFM particle. This extraction occurred in a limited volume of liquid phase. Under normal conditions, the enzymatic reaction originates the driving force for the extraction, but at botton layers of the sediment it did not take place once the oxygen concentration in this zone was depleted.

In general, without agitation the thickness of the boundary layer increases leading to the increase of the time required for CGA to diffuse into bulk liquid, and, therefore, a decrease in the mass transfer rates occurs.

4.2.2 Thermal stability of the enzyme in the presence of SFM and bovine-albumin

Before using an enzyme, it is important to know its thermal stability. Enzymes are susceptible to high temperature and their inactivation will start when a certain temperature is reached. In many cases, immobilized enzymes, such as PPO in the presence of CM, show an increase in their thermal stability; therefore, higher temperatures can be applied to them.

The thermal stability of the enzyme in the presence of SFM was studied at 55°C and at two different pH values (3.4 and 4.3). Two meal concentrations, 2 and 5% (w/v), were taken into consideration to carry out these tests. Figure 4-23 shows that for the

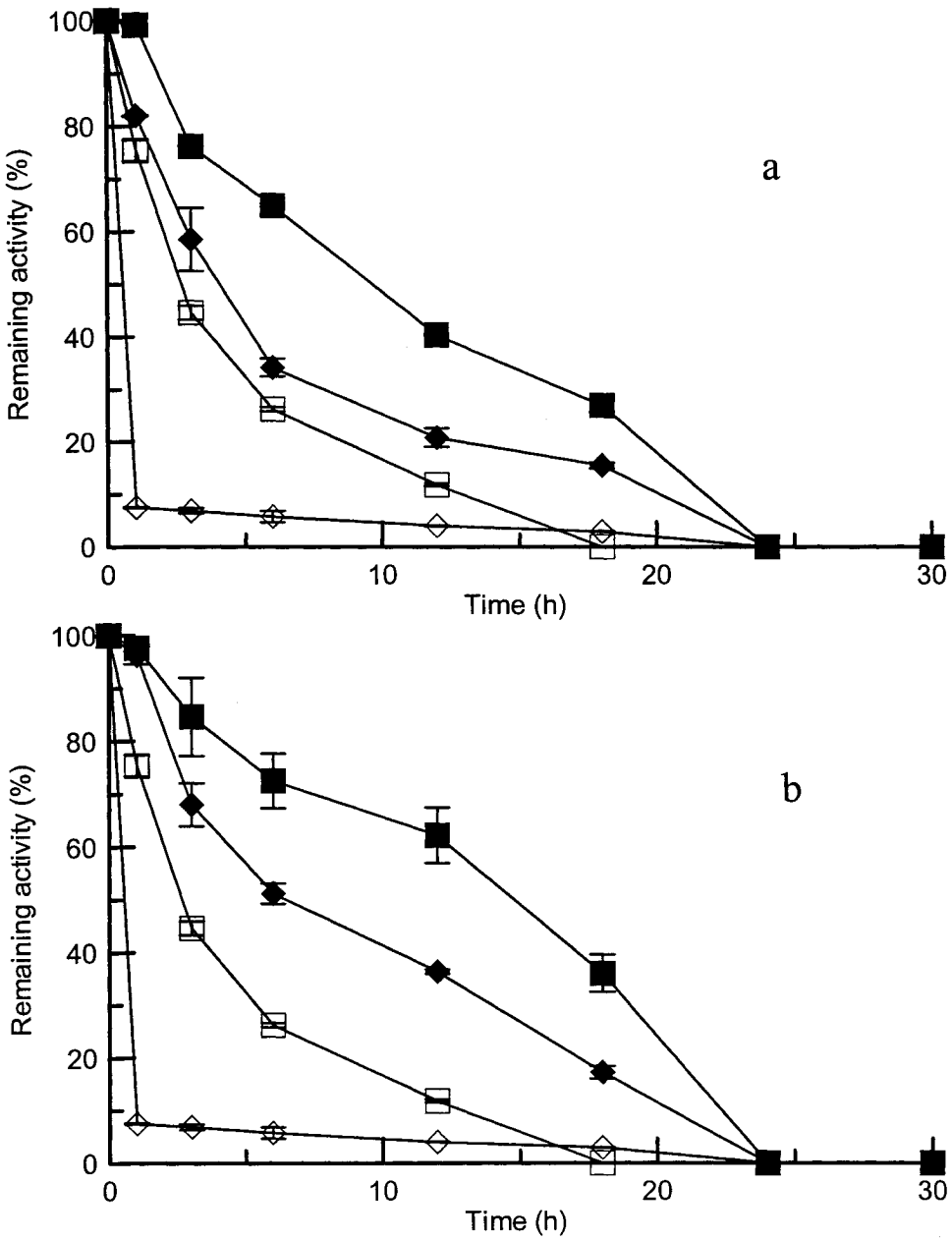


Figure 4-23. Effect of SFM on the stability of the enzyme at 55°C and different pH values. Meal concentration: (a) 2% (w/v); (b) 5% (w/v). Enzyme concentration 7 nkat/mL of continuous phase. pH: (◇) 3.4; (□) 4.3. Open symbols: controls (without SFM); closed symbols: SFM present. Spectrophotometric measurements.

control plots, in the absence of meal, the enzyme is more stable at higher pH values. These results were in agreement with those found by Lacki (1997) during the study of the effect of the pH of the system on the stability of the enzyme in the presence of canola meal at 50°C. Weemaes et al. (1998) found that when using PPO extracted from avocado, the thermal stability of the enzyme increased as the pH was increased up to 6.0. At higher pH values, the thermal stability of the enzyme was approximately constant.

In the presence of 2%(w/v) SFM, the stability of the enzyme increased by 20 and 35% for pH of 3.4 and 4.3 respectively after 10 hours of the enzymatic treatment (Figure 4-23a). Nevertheless, the results were totally different when a higher meal concentration was used. For instance, at 5% (w/v) meal concentration, a 35% increase in the enzyme stability was noticed at pH 3.4 compared to a 20% increase at 2% (w/v) meal concentration. Then again at pH 4.3, a 50% increase was noticed when using 5% (w/v) meal concentration compared to a 35% increase at 2% (w/v). These results showed that in the presence of SFM, the enzyme became more stable than without it, and its thermal stability increased with an increase in the meal concentration in the system. This fact is clearly seen when graphing the plots of both figures, Figure 4-23a and Figure 4-23b, in the presence of meal on one graph (Figure 4-24). Lacki (1997) has also found that the enzyme showed a higher increase in the thermal stability when it was incubated in the presence of canola meal than when incubated in buffer alone or in a solution of canola meal proteins.

The protection of the enzyme from denaturation could have been caused because of the presence of any of the compounds in the SFM. Due to the fact that proteins are the

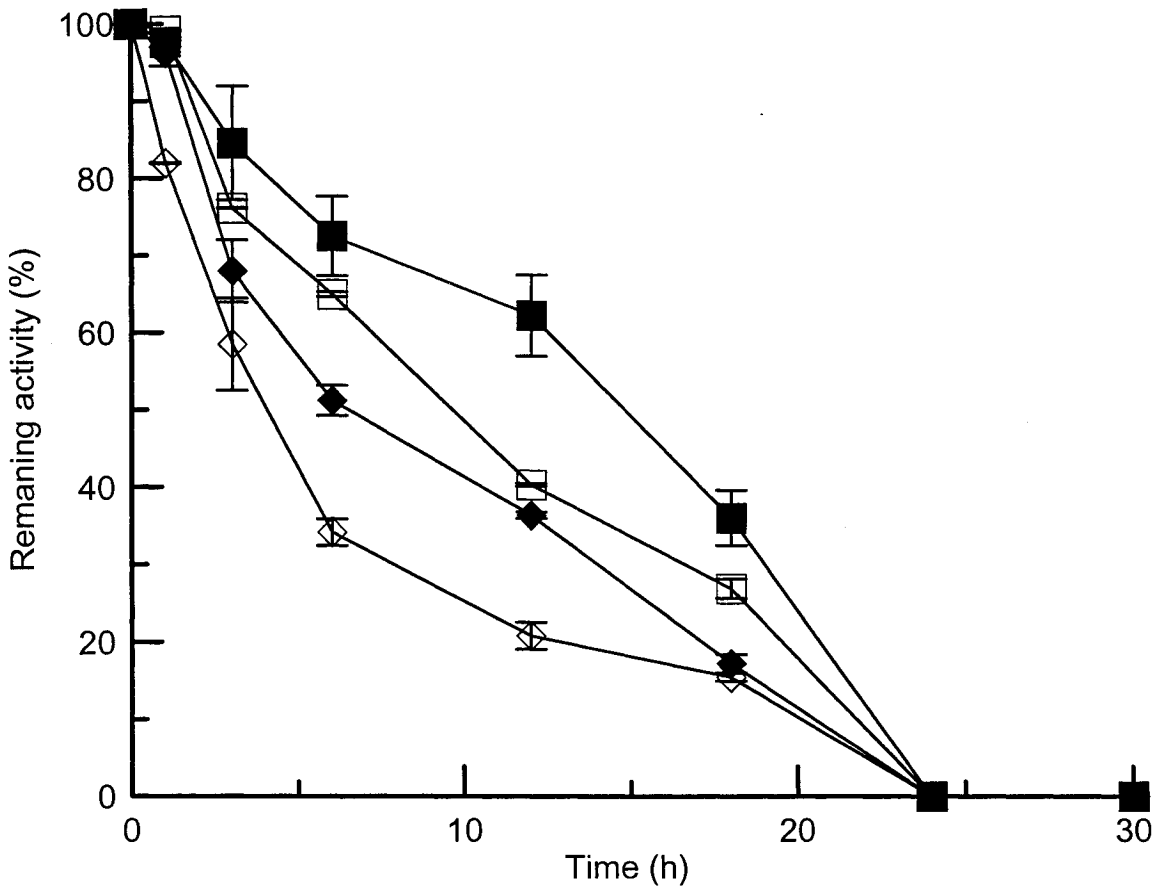


Figure 4-24. Effect of SFM on the stability of the enzyme at 55°C, two meal concentrations and two buffer pH values. Enzyme concentration 7 nkat/mL of continuous phase. pH: (◇) 3.4; (□) 4.3. Open symbols: 2% (w/v) SFM; closed symbols: 5% (w/v) SFM. Spectrophotometric measurements.

main compounds in the meal, several tests were carried out to see whether or not proteins were responsible for that protection. In these tests, bovine-albumin was used as a protein source (The concentration of bovine-albumin used corresponded to the protein concentration present in 2% (w/v) SFM). The results of these tests are presented in Figure 4-25, in which it is clear that regardless of the pH of the system the thermal stability of the enzyme increased in the presence of bovine-albumin. The results lead to a general conclusion, which is that the fact that proteins are present in the meal enhances the thermal stability of the enzyme preparation obtained from the fungus *T. versicolor*.

The control plots in Figure 4-23a and Figure 4-23b also show that the rate of the denaturation of the enzyme follows first-order kinetics, which is typical for most enzymes.

4.2.3 Effect of the TCA concentration on the enzymatic reaction

The fact that enzymes combine with their reactants makes them susceptible to inhibition by unreactive molecules that resemble the substrate. These molecules can combine with the active site of the enzyme and remain bound without change blocking the access to the substrate. As a result, the rate of the reaction slows and if the concentration of the inhibitor becomes high enough, the reaction may stop completely. In some cases, inhibitors interfere with enzyme-catalyzed reactions by combining with enzymes at locations outside the active site and thus instead of reducing accessibility of the active site to the substrate, these inhibitors cause changes in folding conformation that reduce the ability of the enzyme to lower the activation energy.

To stop the enzymatic reaction of the CGA decrease, it was necessary to use a reactant with a specific concentration. Thrichloro acetic acid (TCA) was used as the

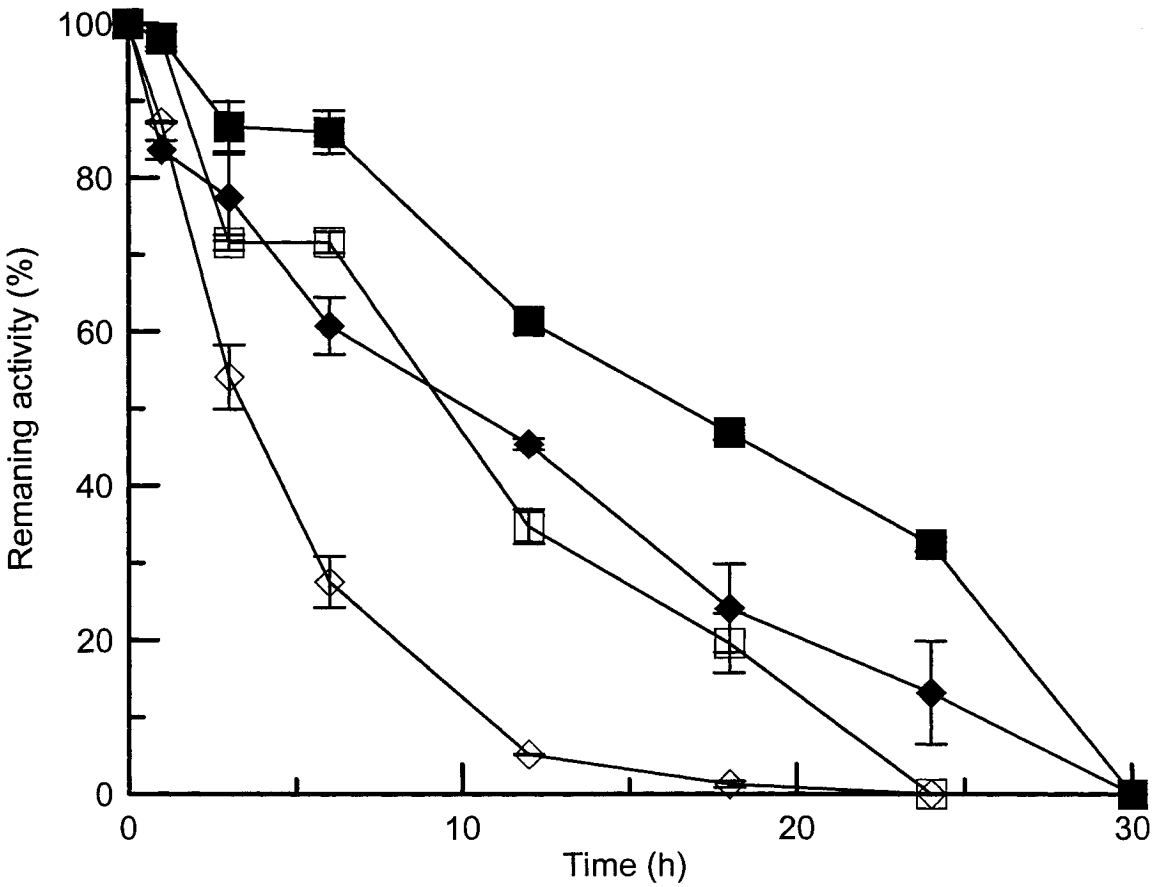


Figure 4-25. Effect of albumin on the stability of the enzyme at 55°C and different pHs. Albumin concentration 0.1 g/10 mL; Enzyme concentration 7 nkat/mL of continuous phase. System pH: (◇) 4.0; (□) 4.6. Open symbols: controls (without albumin); closed symbols: samples with albumin. Spectrophotometric measurements.

reactant and the effect of its concentration on the enzymatic decrease of CGA was investigated. Several concentrations of TCA were studied in a system containing 10% (w/v) SFM and 3 nkat/mL of enzyme. Figure 4-26 shows that the enzymatic reaction is stopped when the TCA concentration in the system was 4.5% (w/v). In this Figure, it is noticed that the CGA conversion is inversely proportional to the TCA concentration. In other words, the less the TCA used, the higher the percentage of CGA converted due to the fact that in the presence of a low concentration of TCA, the enzyme's active sites are not completely filled with TCA molecules. In this case, less inhibition would result using small concentrations of CGA. To inhibit the action of a specific enzyme means to have a molecule whose exact shape and chemistry precisely fit a complex, three-dimensional docking site in the midst of a complex enzyme molecule.

In Figure 4-26, it was also noticed that an increase in the TCA concentration from 1.2% to 4.5% (w/v) results in a 60% of CGA unconverted.

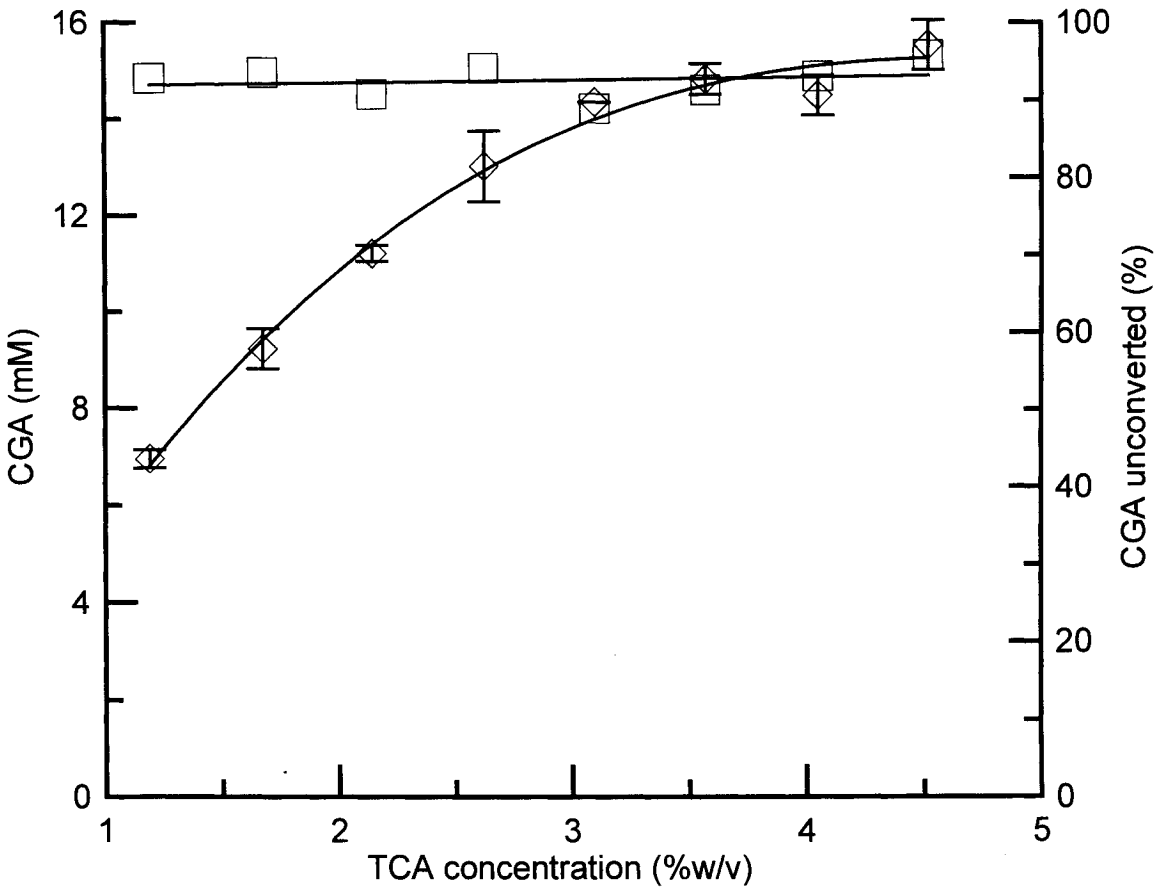


Figure 4-26. Effect of the TCA concentration on the conversion of CGA present in the meal. Meal concentration: 10% (w/v). pH 3.4; temp. 35°C. (◇) CGA unconverted; (□) Control. Reaction time 30 min. HPLC measurements.

CHAPTER 5. MODELLING THE ENZYMATIC TRANSFORMATION OF CGA IN THE MODEL SYSTEM

5.1 Simultaneous effect of pH, temperature, substrate and enzyme concentrations

5.1.1 Development of the model

All the enzyme-catalyzed reactions are reversible (Segel, 1975). A realistic sequence of an enzymatic reaction is given in Figure 5-1:

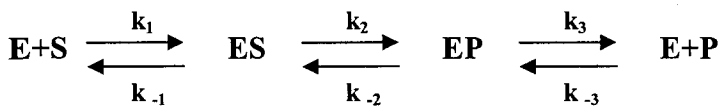


Figure 5-1. Reversible reaction mechanism for the enzymatic transformation of chlorogenic acid by polyphenol oxidase from *T. versicolor.*

where E is the enzyme, S is the substrate (CGA), ES is the enzyme-substrate complex, EP is the enzyme-product complex, P is the product formed from the enzymatic reaction, and k_i (i=1 to 3 and –3 to –1) are rate constants.

To study the enzymatic decrease of chlorogenic acid, different mechanisms were considered but among all of them the one that describes best the enzymatic degradation of CGA is shown in Figure 5-1. In this mechanism there are two intermediates, the enzyme-substrate complex (ES) and the enzyme-product complex (EP). ES is considered the Michaelis-Menten complex, but no assumptions are made as to the nature of EP. Under the usual assay conditions (20-50°C and aqueous buffer of known pH), it is assumed that reaction rates are measured very early in the reaction before any product begins to appear. Therefore, from here on we are going to use only Equation (5.1) to model the enzymatic transformation of CGA; in other words only the forward initial rates

will be treated. The initial velocity for the reaction in each direction can be calculated using the Michaelis-Menten equation:

$$v_f = \frac{V_{\max_f}[S]}{K_{ms} + [S]} \qquad \text{in the absence of P} \qquad \textbf{(5.1)}$$

where $V_{\max_f}$ represents the maximum forward velocity, $V_{\max_f} = \langle e_a^- \rangle k_{cat}$; [S] represents the substrate concentration (CGA); K_{ms} is the Michaelis-Menten constant for substrate S and v_f represents the forward initial rate or activity. Segel (1975) defines K_{ms} and k_{cat} as:

$$K_{ms} = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{k_1(k_2 + k_{-2} + k_3)} \qquad \textbf{(5.2)}$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_{-2} + k_3} \qquad \textbf{(5.3)}$$

In absence of S, v becomes

$$v_r = \frac{V_{\max_r}[P]}{K_{mp} + [P]} \qquad \textbf{(5.4)}$$

where $V_{\max_r}$ is the maximum backward velocity, (P) is the product concentration, K_{mp} is the Michaelis-Menten constant for product P and v_r is the backward initial rate.

Equation (5.1) is valid for any enzyme concentration for which linearity exists between the enzyme concentration and the activity. This equation was used as a base to study the simultaneous effect of temperature, pH, substrate and enzyme concentrations, taking into consideration the following assumptions:

(1) The enzyme is considered a dibasic acid that exists in three forms. E_a^- denotes the active enzyme form, while E^0 and E^{-2} are inactive forms obtained by protonation and

deprotonation of the active site of E, respectively. K_1 and K_2 are equilibrium constants for the indicated reactions (Bailey and Ollis, 1986).

$$E^{-2} \xleftrightarrow{K_2} E_a^{-} \xleftrightarrow{K_1} E^0$$

(2) The thermal denaturation of the active form of an enzyme may be modelled by a first order irreversible deactivation mechanism in which the active enzyme (E_a^-) molecules undergo an irreversible structural or chemical change to an inactive form (E_d^-).

$$E_a^- \xrightarrow{k_d} E_d^-$$

where k_d is the first-order deactivation rate coefficient.

(3) The rate constants are given by the transition state theory

$$k_i = \alpha_i \left[\frac{k_B T}{h}\right] e^{\Delta S_i^* / R} \; e^{-E_{a,i}/RT} = k_{0,i}\, T\, e^{-E_{a,i}/RT}$$

(4) The protonation and deprotonation constants can be expressed by the following equation according to Smith and Van Ness (1987):

$$\ln K_i = -\frac{\Delta G_i^0}{RT}$$

(5) All the enzyme kinetic parameters are independent of pH (Bailey and Ollis, 1986).

The model that was obtained contains the combined effects of pH and temperature. To end up with this model, the effects of pH and temperature were evaluated separately and the relationships obtained were combined to get an overall model.

5.1.2 Effect of pH

The effect of acid H^+ ions or basic OH^- ions on the activity of an enzyme is probably caused by a change in stereo configuration at or around the active sites (Fersht,

1984; Whitaker, 1994). As in almost all protonation reactions, these reactions occur very rapidly and the different configurations are instantaneously in equilibrium.

According to Bailey and Ollis (1986), the concentration of the active form of the enzyme can be given by

$$\left[e_a^{-}\right] = \frac{[e_0]}{1 + \frac{H^+}{K_1} + \frac{K_2}{H^+}} \tag{5.5}$$

Due to the fact that for low enzyme concentrations the initial reaction rates are directly proportional to the enzyme concentration, Equations (5.1) and (5.5) can be used together to evaluate initial reaction rates at one temperature and for any hydrogen ion concentration.

5.1.3 Effect of temperature

Temperature can affect an enzyme in two ways. One way is a direct influence on the reaction rate constant, and the other way is the thermal denaturation of the enzyme at elevated temperatures, which decreases the amount or concentration of the active enzyme. To relate the effect of temperature to the reaction rate constant, the transition state theory was used:

$$k_i = k_{0,i}\, T\, e^{-E_{a,i}/RT} \tag{5.6}$$

where k_i is the rate constant, $k_{0,i}$ is the frequency factor, T is the temperature, R is the gas law constant and $E_{a,i}$ is the activation energy of the enzymatic reaction. On the other hand, deactivation of enzymes can be irreversible or reversible. As mentioned at the

beginning of this chapter, enzyme deactivation was assumed to be irreversible and can be described as

$$e_a^{-}(t) = e_0^{-}\, e^{(-k_d t)} \tag{5.7}$$

According to Lacki (1997), Equation (5.7) cannot be used (in the form shown above) in the model represented by Equation (5.1) because it is not possible to incorporate any transient effects into the evaluation of the initial rate measurements. Therefore, expressing the concentration of the active form of the enzyme as the time-average, an expression can be found for the average enzyme concentration at any reaction time, t_r, at which the initial reaction rate is measured (Lacki, 1997).

$$\left\langle e_a^{-} \right\rangle = \frac{\int_0^{t_r} e_a^{-}\, dt}{\int_0^{t_r} dt} = e_0^{-}\left[\frac{1 - e^{(-k_d t_r)}}{k_d t_r}\right] \tag{5.8}$$

5.1.4 Combining effects of temperature and pH

If Equations (5.5) and (5.8) are combined and substituted into Equation (5.1), the following model that describes the initial reaction rates at a constant temperature and at any pH value is obtained.

$$\upsilon = \frac{[e_o]\, k_{cat}}{1 + \dfrac{H^+}{K_1} + \dfrac{K_2}{H^+}} \frac{1 - e^{(-k_d t_r)}}{k_d t_r} \frac{[CGA]}{K_{ms} + [CGA]} \tag{5.9}$$

In Equation (5.9) it is noticed that to predict the initial reaction rates at any temperature, the parameters k_{cat}, k_d, K_{ms}, K_1, and K_2 need to be expressed as a function of

the temperature. Therefore, using assumptions (3) and (4) these parameters can be expressed as a function of the temperature. Because of the complexity of the model (Equation 5.9) when having K_{ms} and k_{cat} expressed as in equations (5.2) and (5.3), it was necessary to simplify the model by considering four different scenarios:

(a) If it is assume that only initial rate data are being analysed, K_{ms} and k_{cat} are given by the following equations:

$$K_{ms} = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{k_1(k_2 + k_{-2} + k_3)}$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_{-2} + k_3}$$

(b) If it is assume that only initial rate data are being analysed for a system where $k_{-2} << k_1$ and k_{-1}, K_{ms} and k_{cat} are would be given by the following equations:

$$K_{ms} = \frac{(k_{-1} + k_2)k_3}{k_1(k_2 + k_3)}$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3}$$

(c) If it is assume that initial rate data are being analysed for a system where $k_{-2} << k_1$ and k_{-1} and $k_2 << k_3$, K_{ms} and k_{cat} are would be given by the following equations:

$$K_{ms} = \frac{k_{-1} + k_2}{k_1}$$

$$k_{cat} = k_2$$

(d) If it is assume that only initial rate data are being analysed for a system where $k_{-2} << k_1$ and k_{-1} and $k_3 << k_2$, K_{ms} and k_{cat} would be given by the following equations:

$$K_{ms} = \frac{(k_{-1} + k_2)k_3}{k_1 k_2}$$

$$k_{cat} = k_3$$

Having studied these four scenarios, the only one that was found to describe the CGA transformation was scenario c. Using this scenario and assumptions (3) and (4), the overall model that predicts the initial reaction rates at any temperature, pH, enzyme and chlorogenic acid concentration is given by Equation (5.10).

$$\upsilon = \frac{[e_0]}{1 + \frac{K_2}{H^+} + \frac{H^+}{K_1}} \frac{1 - e^{-\left(t_r\, k_{o,d}\, T\, e^{-E_d/RT}\right)}}{t_r\, k_{0,d}\, T\, e^{-E_d/RT}} \frac{[CGA]\{k_{0,2}\, T\, e^{-E_{a,2}/RT}\}}{\left(\frac{\{k_{0,-1}\, T\, e^{-E_{a,-1}/RT}\} + \{k_{0,2}\, T\, e^{-E_{a,2}/RT}\}}{k_{0,1}\, T\, e^{-E_{a,1}/RT}}\right) + [CGA]} \quad \textbf{(5.10)}$$

5.1.5 Parameter estimation

The mathematical technique of fitting that was used was a nonlinear regression analysis based on the Levenberg-Marquardt algorithm. This algorithm is based on a linear combination of the steepest-descent and Gauss-Newton methods and is most commonly used to find a set of parameters satisfying the least-squares criterion (Constantinides, 1987; Johnson and Faunt, 1992). For simulation and parameter estimation, the software "MicroMath Scientist for Windows" and "Datafit" were used.

One of the problems in finding the initial guesses for the parameters is the lack of information about the initial values. This problem has to be solved because, in many cases, the convergence of a nonlinear estimation technique depends upon the right choice of the initial estimates (McLean, 1993). To find the right initial guesses different techniques can be used such as obtaining information from other relevant experiments, carrying out experiments in a laboratory or industrial scale, and using values that were obtained from literature. All three techniques were employed in this work. It should be

mentioned that a parameter transformation of the rate constant with temperature "Transition State Theory" was done by using the Arrhenius equation.

$$k_i = k_i^* \, e^{\left[-\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)\right]} \qquad \textbf{(5.11)}$$

With this transformation if a rate constant value is known at one temperature, then setting T^* equal to that temperature results in the known parameter k_i^*. In this manner, an estimate of the magnitude of the activation energy can be obtained by keeping k_i^* constant.

Another procedure to estimate the parameters in the overall model consisted of two steps. In the first step, one set of data was separately plotted as a function of pH for each temperature. Each set of data were first analysed in a classical manner using nonlinear regression for each temperature separately and the protonation and deprotonation constants were calculated for each of these temperatures using Equation (5.12).

$$\frac{V_{\max}}{V_{\max}^{opt}} = \frac{\upsilon}{\upsilon_{pH}^{opt}} = \frac{1+2\sqrt{K_2/K_1}}{1+\frac{K_2}{H^+}+\frac{H^+}{K_1}} \qquad \textbf{(5.12)}$$

Based on assumption (5), it is known that the ratio of the maximum activity at any pH value to the respective activity at the optimum pH is equal to the ratio of the initial reaction rates at the respective pH values, regardless of the substrate concentration used.

In the second step, the effect of the temperature on the estimation of the parameters was studied using the results obtained during the modelling of the effect of pH on the enzyme activity at constant temperature.

Having experimental data of the effect of pH on the enzyme activity (or initial reaction rate) at constant temperature, the parameters in Equation (5.12), K_1, K_2, and v_{pH}^{opt}, can be calculated by fitting Equation (5.12) to those data and minimizing the sum of squares of the residuals. The parameters obtained for each temperature are showed in Table 5-1.

Table 5-1. Parameter estimates for Equation (5.12)

T (K)	K_1 (M)	std. Dev.	K_2 (M)	std. Dev.	v_{pH}^{opt} (nkat)	std. Dev.
298	3.53E-04	1.64E-05	4.24E-06	2.11E-07	5.99	5.65E-01
303	6.03E-04	1.64E-05	3.41E-06	1.26E-07	7.16	7.95E-01
308	3.87E-04	1.37E-05	1.24E-05	4.46E-07	8.01	5.62E-01
313	1.09E-03	1.64E-05	2.64E-06	7.40E-07	9.80	3.85E-01
318	1.17E-03	3.38E-05	2.75E-06	8.10E-07	10.30	5.55E-01
328	8.88E-04	1.21E-05	2.41E-06	3.51E-07	10.00	2.64E-01
333	7.12E-04	2.21E-05	3.31E-06	1.09E-07	9.20	5.69E-01
338	3.91E-04	1.34E-05	5.10E-06	1.85E-07	7.72	5.39E-01
343	2.75E-04	1.38E-05	4.81E-06	2.56E-07	6.30	6.34E-01

From a mathematical point of view, the data in Table 5-1 could be used to obtain a relationship between the dissociation constants, K_1, K_2, and temperature. The data shown in this table were in the same order as those found by Lacki (1997) during the modelling of the enzymatic transformation of sinapine.

According to the Arrhenius law, when plotting the natural logarithm of the dissociation constants against the reciprocal of temperature, a straight line should be obtained. This phenomenon was not seen in our case as shown in Figure 5-2. Yet, when the standard Gibbs energy of reaction was used to define the dissociation constants (as expressed in assumption 4), the data showed in Figure 5-2 were well described. The standard Gibbs energy of reaction can be given by Equation (5.13) (Lacki, 1997).

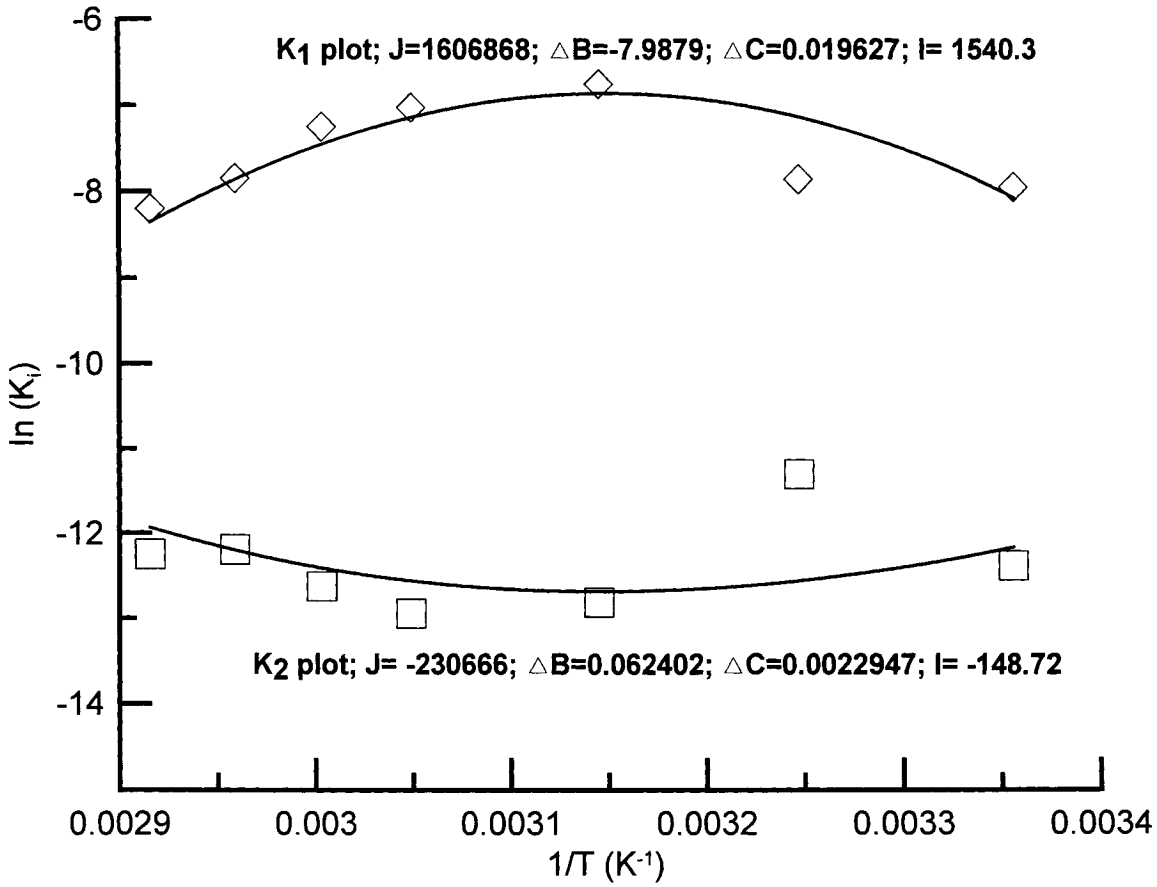


Figure 5-2. Relationship between dissociation contants K_1 (◇) and K_2 (□) and temperature. Solid lines represent the model, Equation (5.14)

$$\Delta G_i^{\,0} = J_i - RT\left(\Delta A_i \ln T + \frac{\Delta B_i}{2}T + \frac{\Delta C_i}{6}T^2 + \frac{\Delta D_i}{2T^2} + I_i\right) \qquad \textbf{(5.13)}$$

Using the data showed in Table 5-1 and Equation (5.13), nonlinear regression analysis was performed with temperature as a variable and it was found that the model (5.13) fits the data with more accuracy when the terms ΔA_i and ΔD_i are equal to zero. This assumption was also found to be necessary in the modelling of the enzymatic transformation of sinapine (Lacki, 1997). As a result Equation (5.14) was used, as a relationship between dissociation constant and temperature, in the overall model.

$$K_i = e^{\left\langle \frac{-J_i}{RT} + \frac{\Delta B_i}{2}T + \frac{\Delta C_i}{6}T^2 + I_i \right\rangle} \qquad \textbf{(5.14)}$$

The parameters obtained using Equation (5.14) and the data in Table 5-1 are given in Table 5-2.

Table 5-2. Parameter estimates for Equation (5.14)

	J	ΔB	ΔC	I
K_1	1606868	-7.9879	0.019627	1540.3
K_2	-230666	0.062402	0.0022947	-148.72

According to Smith and Van Ness (1987), a deviation from the straight line is expected in a graph like Figure 5-2; however, in this figure the deviation is not negligible. This indicated that the model that describes the effect of pH on the enzyme activity is an oversimplification of the actual situation. Nevertheless, Equation (5.14) was used in the overall model. Lacki (1997) also found a similar graph like Figure 5-2, but the expected deviation from the straight line was even larger than the one found in this project.

Regarding the second step to estimate the parameters in the overall model (Equation 5.10), initial estimates were given for the deactivation energy (E_d) and for the frequency factor ($k_{o,d}$). Those values were taken from the literature and from previous results obtained during enzyme deactivation experiments. It should be mentioned that some constraints were given to the model such as:

1) The ones mentioned in scenario c
2) $10^7 < k_1 < 10^{10}\ M^{-1}\ min^{-1}$

   $10^2 < k_{-1} < 10^6\ min^{-1}$

   $50 < k_2 < 10^7\ min^{-1}$

   $10^{-6} < K_{ms} < 10^{-2}\ M$

The initial estimates for the remaining parameters in the overall model were chosen randomly following a literature review and using a data set created from both the estimates obtained in Table 5-1 and Equation (5.12). In this new approach, the created data set contains values for a corrected initial reaction rate, Y_c, which was introduced. This variable can be calculated by combining Equations (5.12) and (5.10); the results of this combination is shown in Equations (5.15) and (5.16), respectively. The corrected initial reaction rate is now expressed as a function of temperature using the parameters of interest, Equation (5.16).

$$Y_c = \upsilon_{pH}^{opt}\left(1+2\sqrt{K_2/K_1}\right) \tag{5.15}$$

$$Y_c = \frac{1-e^{-\left(t_r\, k_{o,d}\, T\, e^{-E_d/RT}\right)}}{t_r\, k_{0,d}\, T\, e^{-E_d/RT}} \frac{\beta[e_0][CGA]\{k_{0,2}\, T\, e^{-E_{a,2}/RT}\}}{\left(\frac{\{k_{0,-1}\, T\, e^{-E_{a,-1}/RT}\}+\{k_{0,2}\, T\, e^{-E_{a,2}/RT}\}}{k_{0,1}\, T\, e^{-E_{a,1}/RT}}\right)+[CGA]} \tag{5.16}$$

The data calculated using Table 5-1 and Equation (5.15) are shown in Figure 5-3. These data can be used to calculate the parameters in Equation (5.16). It can be noticed that due to the fact the effect of temperature was included on the optimum pH for all the data, the estimates found by this method have a better accuracy. Once the parameters in Equation (5.16) were obtained, they were used to simulate the effect of the temperature on Y_c for different chlorogenic acid concentrations (Figure 5-3). In order to validate the model for those substrate concentrations, the enzyme activity was measured for each one of those CGA concentrations at optimum pH (4.3) and 25°C. From these experiments, Y_c was calculated by using Equation (5.15) and the data are shown in Figure 5-3. The estimates obtained for the overall model are given in Table 5-3. Lacki (1997) also found similar behaviour when plotting Y_c vs temperature for several sinapine concentrations.

Table 5.3. Frequency factors and activation energy estimates for the respective rate constants based on the transition state theory*.

Parameter	Value
$k_{0,d}$ (s^{-1})	2.97E+07
E_d (kJ/mol)	79.98
$k_{0,1}$ ($mM^{-1}s^{-1}$)	1.54E+08
$E_{a,1}$ (kJ/mol)	23.997625
$k_{0,2}$ (s^{-1})	1.01E+08
$E_{a,2}$ (kJ/mol)	28.47249
$k_{0,-1}$ (s^{-1})	6.43E+08
$E_{a,-1}$ (kJ/mol)	33.22

*enzyme cocentration given by $e_0 = \beta \times (V_{enz}/V_{sys}) \times dil$; $V_{enz} = 0.1$ mL; $\beta = 1.25 \times 10^{-4}$ mmol/mL of enzyme stock solution; dil = 0.125.

Once the parameters in the overall model were found, the effect of the pH on the enzyme activity can be simulated for three different temperatures (solid lines in Figure 5-4). From this figure, it was noticed that the kinetic model described quite well the enzyme

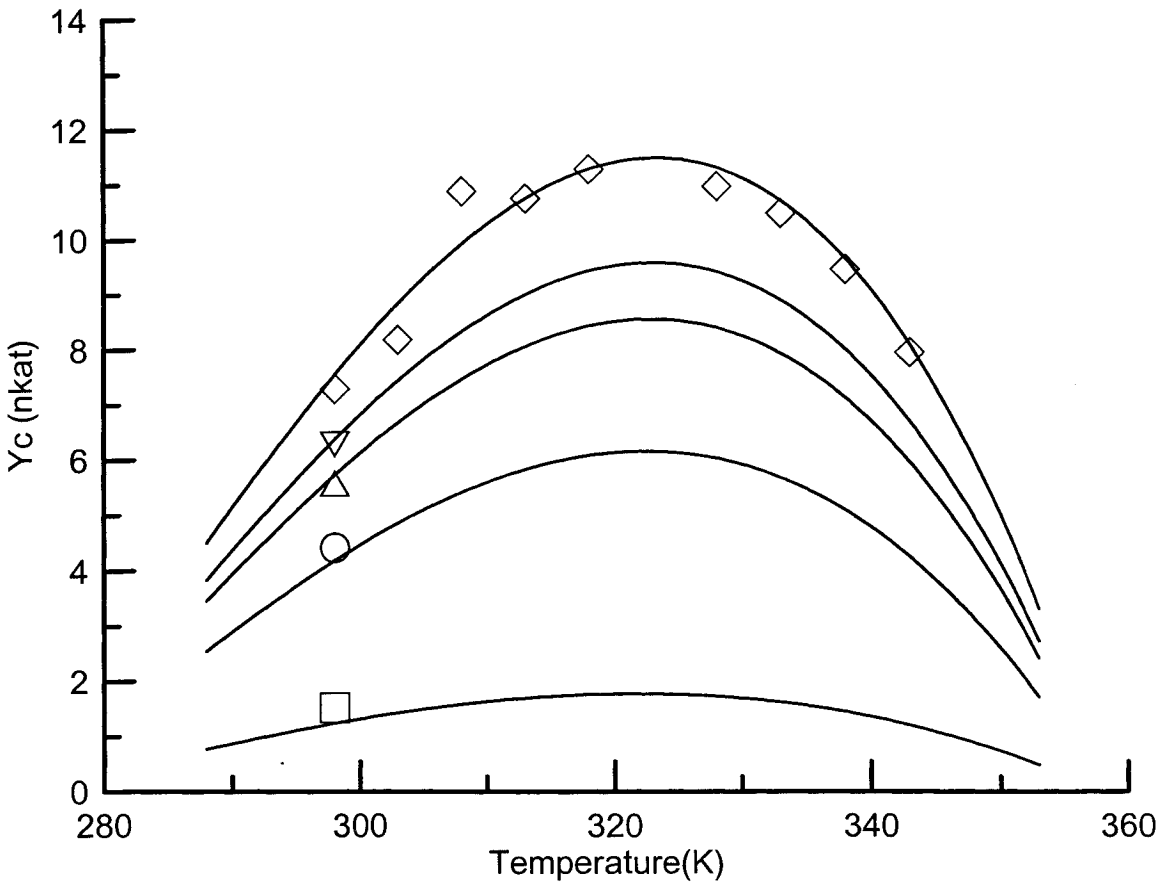


Figure 5-3. Relationship between Yc and temperature. Solid lines as predicted by Equation (5.16). Experimental data for CGA concentration: (◇) 0.12 mM; (▽) 0.093 mM; (△) 0.08 mM; (○) 0.053 mM; (□) 0.0133 mM. Experimental data obtained at pH 4.3.

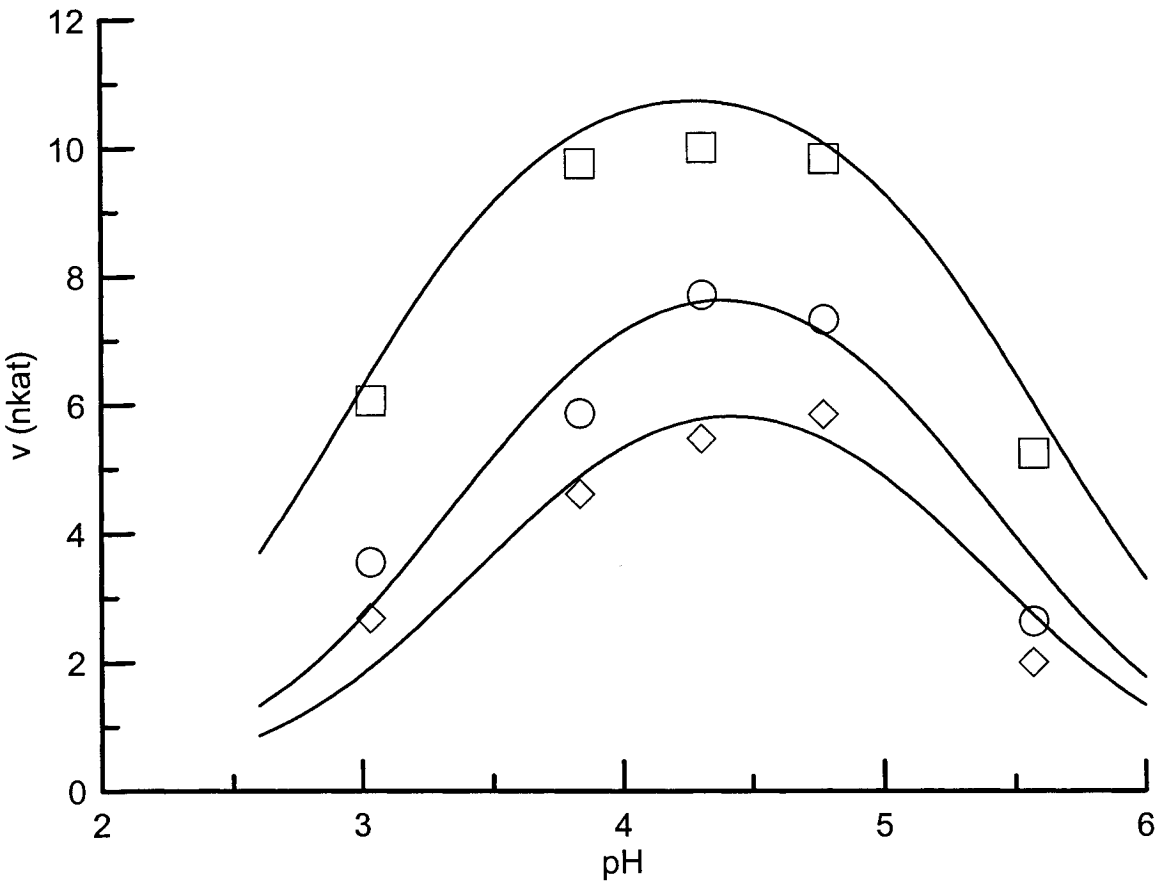


Figure 5-4. Effect of pH and temperature on the enzyme activity in the model system predicted by Equation (5.10) for a CGA concentration of 0.12 mM; Reaction time 10 min. Experimental data at: (◇) 298 K; (□) 318 K; (○) 338 K. Solid lines: Model.

activity as a function of pH and temperature simultaneously over a wide range. In general, the results showed that the proposed method for finding the parameters in the overall model gave realistic values of the respective parameters. Therefore, the parameters given in Tables 5-2 and 5-3 will be used to simulate the simultaneous effects of pH, and temperature on the enzyme activity by using Equation (5.10). This simulation was done at specific substrate and enzyme concentrations. The results from this simulation are presented in the form of an activity-surface plot in Figure 5-5. A similar plot was found when simulating the simultaneous effect of pH and temperature on the enzyme initial activity using a totally different model (Lacki, 1997). This model described the enzymatic transformation of sinapine taking into consideration a different mechanism, Theorrel-Chance Bi-Bi mechanism.

The reliability of the proposed model was checked by doing a scatterplot of the measured (experimental) and simulated (predicted) data as shown in Figure 5-6. Some deviation of the ideal line (45° angle) can be observed for some activities, this could be because of the difficulties while measuring activities either at low or at high pH values. However, the random distribution in this figure confirms that the proposed model predicts the experimental data quite well and its accuracy does not depends on the magnitude of the initial reaction rate. The residuals with respect to pH and temperature (Figure 5-7), reflected roughly the same behaviour indicating that the model is not limited to any particular range of these variables. As in this project, the results found by Lacki (1997) during the modelling of the enzymatic transformation of sinapine showed that the predicted values had some deviation from the real ones in both a scatterplot and a residual plot.

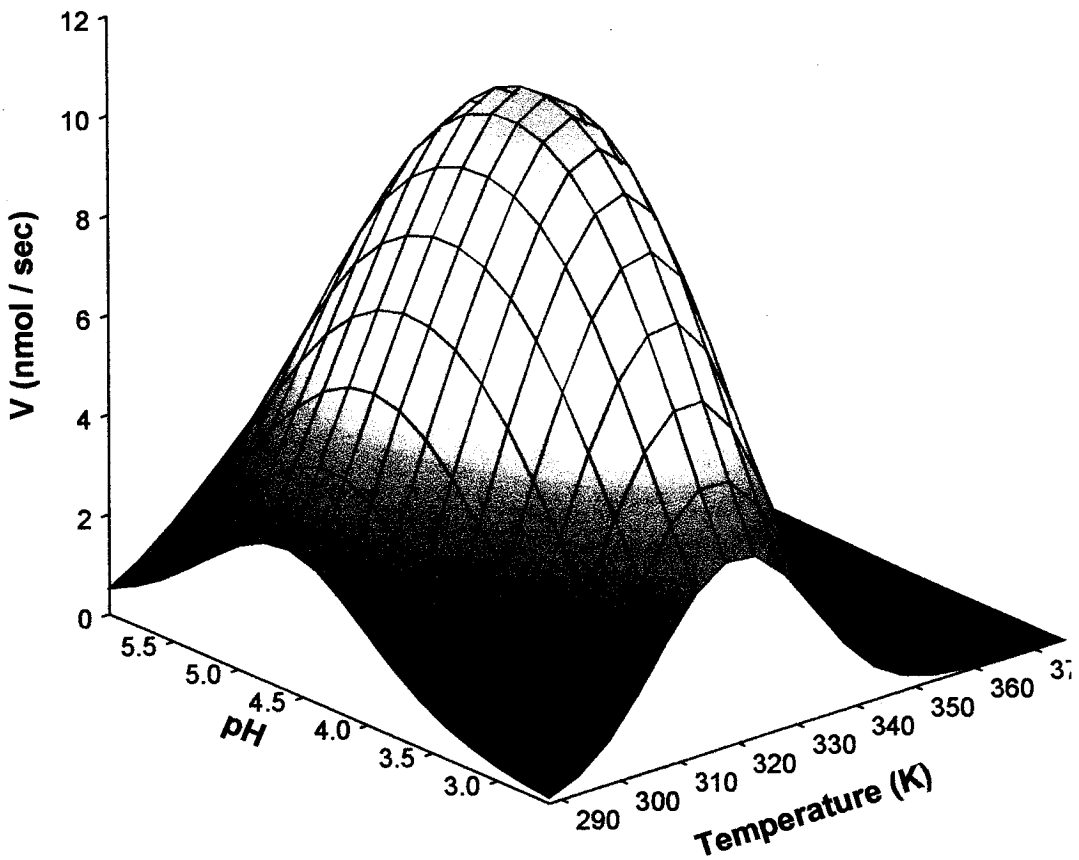


Figure 5-5. Effects of temperature and pH on the CGA oxidase activity in the model system predicted by Equation (5.10) for a CGA concentration of 0.12 mM; enzyme concentration of 0.12 nkat/mL and reaction time 10 min.

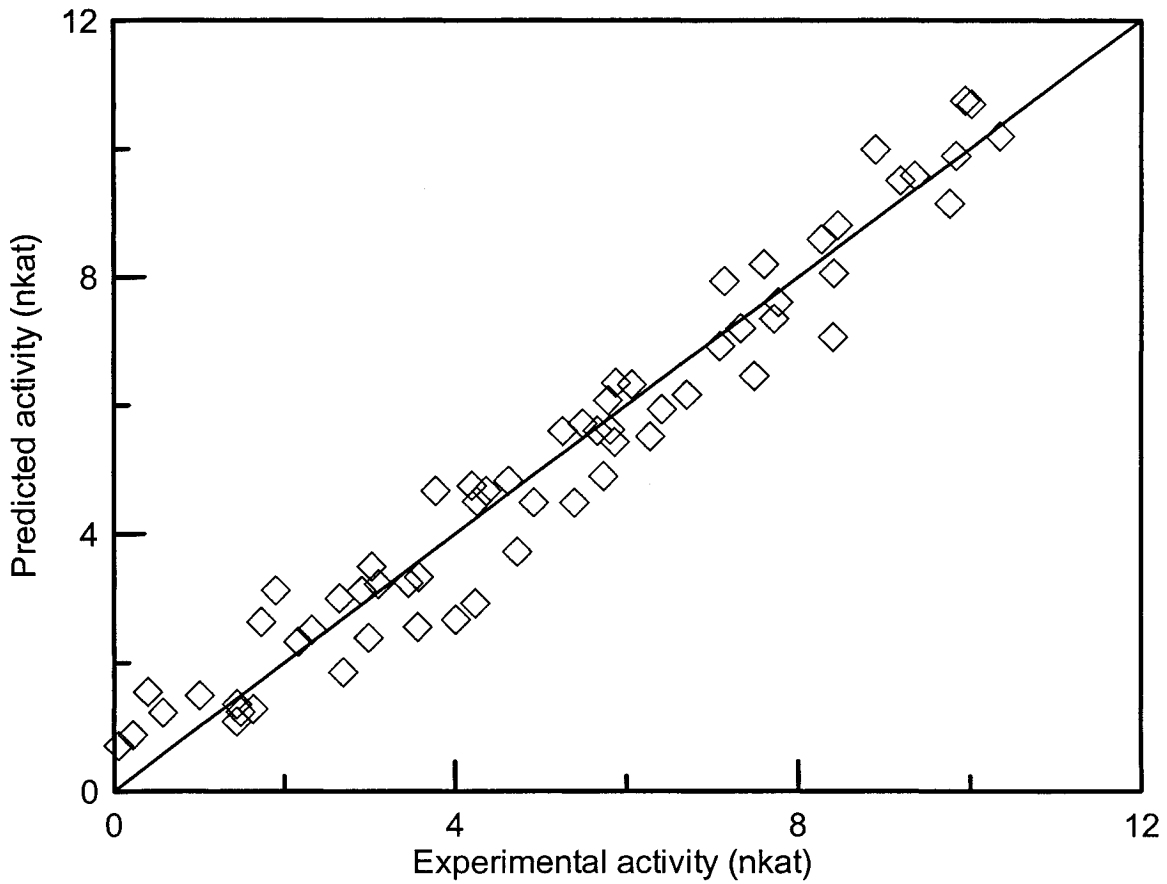


Figure 5-6. Comparison between the experimental activities and those predicted by Equation (5.10).

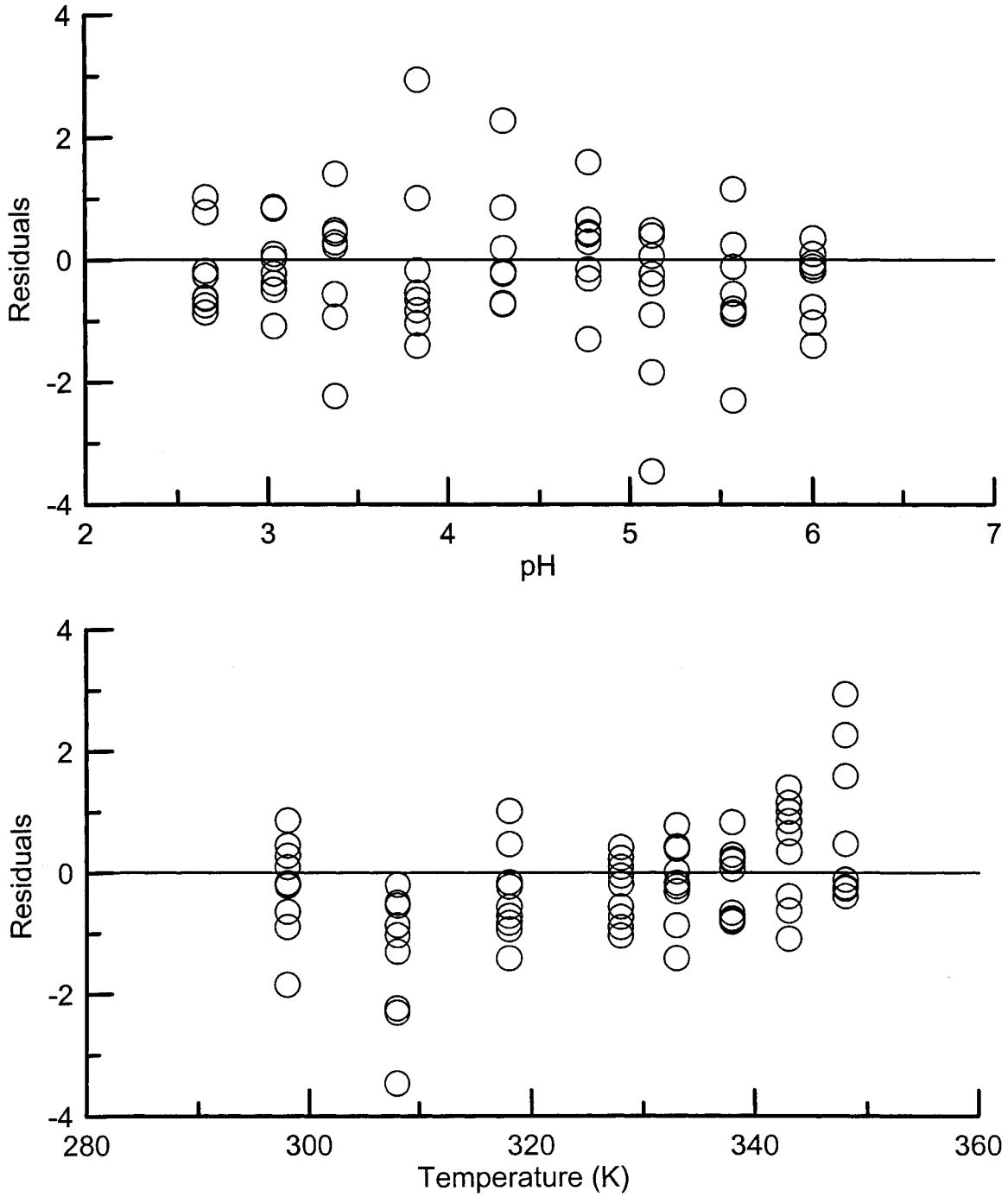


Figure 5-7. Residuals values, with respect to pH and temperature, when comparing results obtained by Equation (5.10) and experimentally.

5.2 Dynamics of the chlorogenic acid decrease

The dynamics of CGA decrease was also investigated using the same mechanism proposed at the beginning of chapter 5. According to that mechanism, the rate of the disappearance of CGA and the formation of products are given by equations (5.17) and (5.18) respectively.

$$\frac{dS}{dt} = k_{-1}[ES] - k_1[E][S] \tag{5.17}$$

$$\frac{dP}{dt} = k_3[EP] - k_{-3}[E][P] \tag{5.18}$$

For the other components in the reaction (Figure 5-1), the following equations can be obtained from material balances:

$$\frac{dES}{dt} = k_1[E][S] + k_{-2}[EP] - (k_2 + k_{-1})[ES] \tag{5.19}$$

$$\frac{dEP}{dt} = k_2[ES] + k_{-3}[E][P] - (k_3 + k_{-2})[EP] \tag{5.20}$$

$$\frac{dE}{dt} = k_{-1}[ES] + k_3[EP] - k_1[E][S] - k_{-3}[E][P] \tag{5.21}$$

To find the parameters in the last five differential equations the software "Scientist for windows" was used. All the equations were written at the main interface of the software. These parameters, reaction rates constants, were calculated based on minimization of the residual sum of squares of the CGA concentrations. This is the sum of the quadratic difference between the measured concentrations and their respective predicted counterparts ($\sum((\text{measured})-(\text{model}))^2$). Using profiles of substrate consumption with time, obtained experimentally for two initial substrate concentrations (Appendix D, Table D-11), the kinetic parameters were estimated by fitting the model to

the experimental data. Initial guesses obtained from literature were used in the determination of the kinetic parameters. It should be mentioned that the same two constraints given in section 5.1.5 for the parameter estimation were also used here. Another constraint that was used is regarding to the V_{max} and K_{ms} values found (by the Lineweaver-Burk plot method) during the study of the effect of the substrate concentration on the initial reaction rates (Appendix D, Table D-10). Those values were made equal to the respective rate constant relationships according to scenario (c). The estimated parameter values were found to be equivalently reproducible for each data set (initial CGA concentrations) within the region of 95% confidence intervals. The parameters found are given in Table 5.4.

Table 5-4. Kinetic parameter values used in the simulation of the model

Parameter	Value
k_1 (mM^{-1} min^{-1})	111200
k_{-1} (min^{-1})	7283
k_2 (min^{-1})	62.3
k_{-2} (mM^{-1} min^{-1})	2202
k_3 (mM^{-1} min^{-1})	2190
k_{-3} (min^{-1})	87.647

Figure 5-8 illustrates the kinetics of the CGA decrease at the two initial substrate concentrations investigated. The solid lines represent the simulations of the model while the experimental data are shown with symbols. Although the mechanistic model describes quite well the dynamics of the enzymatic decrease of the CGA in the model system, more experiments need to be done in order to validate the model.

In general, the results obtained in this chapter can be used as basis to propose a more complex model, which should be able to describe quite well the enzymatic

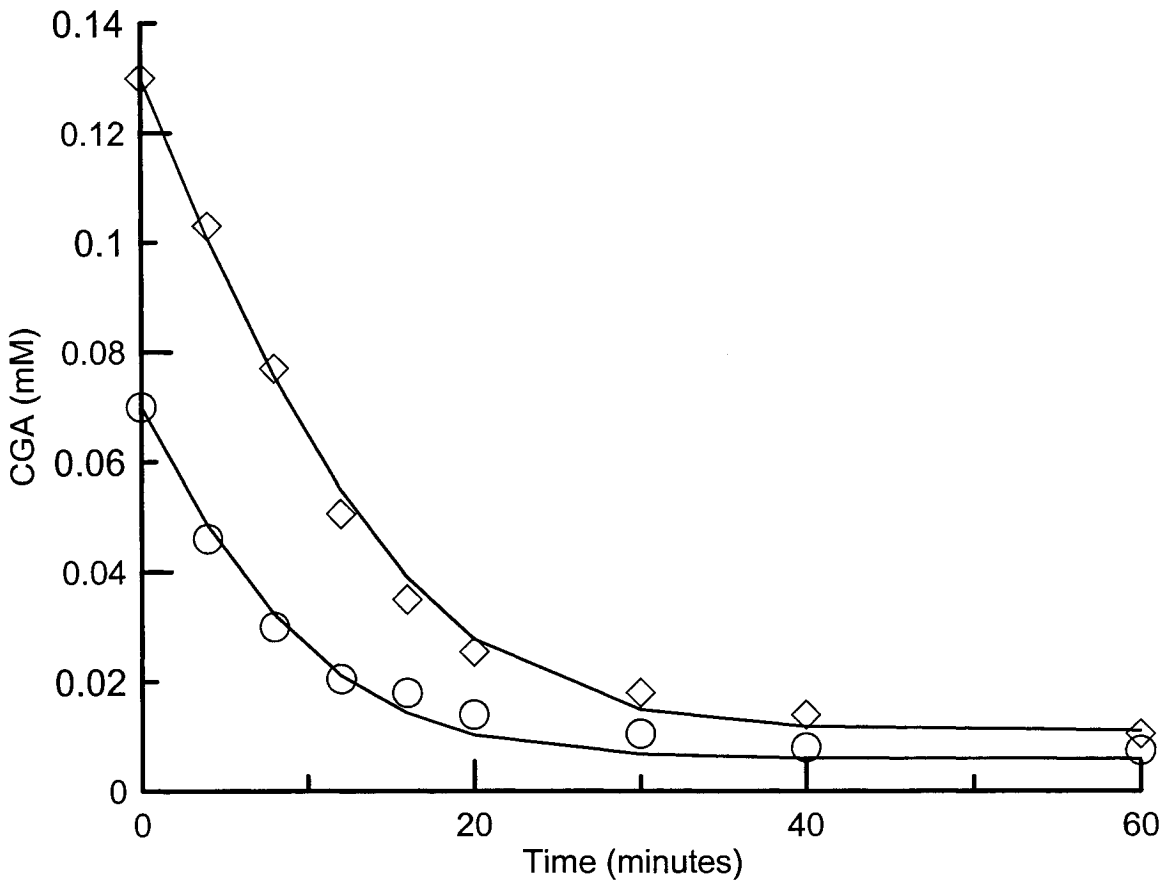


Figure 5-8. Dynamics of the CGA decrease at two different initial substrate concentrations. Solid lines represent the predicted data using Equation (5.17); symbols represent experimental data obtained by HPLC measurements. Conditions: pH 4.3; temp. 35°C. Enzyme concentration 0.12 nkat/mL. CGA concentration: (○) 0.07 mM; (◇) 0.13 mM.

transformation of CGA in the SFM system. This model should take into account the diffusion of CGA through SFM particles, the effects of the intensity of agitation and particle size as well as the thermal stability of the enzyme in the presence of SFM.

CHAPTER 6. CONCLUSIONS

The following conclusions were reached during the execution of this project.

- The polyphenoloxidase produced by the white-rot fungus *Trametes versicolor* decreased the chlorogenic acid (CGA) content in both the model system and the sunflower meal system by 98% in less than four hours of the enzymatic treatment for the established conditions.

6.1 Model enzymatic system

- Optimum values for the parameters studied in the model system were found (pH 4.3; temp. 45°C; CGA conc. 0.12 mM). It was not possible to use higher concentrations of substrate because the results obtained were inconsistent.
- For enzyme concentrations up to 1 nkat/mL, the reaction rate increases proportionally with its concentration. That proportionality disappears for higher enzyme concentrations.
- The chlorogenic acid content can be measured using either spectrophotometric or chromatographic techniques. However, under certain conditions, the spectrophotometric technique overestimates the CGA content in the systems.
- The enzymatic transformation of CGA can be described by a reversible mechanism. Using this mechanism as a base, the predicted values of the enzyme activity were in agreement with the experimental ones when modelling the simultaneous effect of pH, temperature, substrate and enzyme concentrations as well as the dynamic of the CGA decrease.

6.2 Sunflower meal system

- In the sunflower meal (SFM) system, some results differed from those in the model system. For instance, an optimum buffer pH value of 3.4 was found in the SFM system while a 4.3 value was found for the model system. This change in pH was due to the buffering capacity of the meal. A buffer pH of 3.4 corresponds to a pH in the system of 4.3 when a 10% (w/v) meal concentration is present in the SFM system. Even though the optimum temperature was 45°C in both systems, its effect was more noticeable in the model system than in the SFM system due most probably to the presence of meal in the SFM system.
- The presence of larger volumes of continuous phase in the SFM system increases the extraction of CGA, and therefore, the enzymatic decrease of the CGA content.
- Studying the effect of the enzyme concentration in the SFM system, a saturation of the system with the enzyme was seen when the enzyme concentration was 5 nkat/mL of continuous phase.
- An analysis in mass transfer confirmed that the enzymatic process was mass transfer limited with respect to the agitation speed of the system. On the other hand, the results with different particle sizes were not conclusive at all.
- The thermal stability of the enzyme increased in the presence of both SFM and bovine-albumin. The enzyme also became more stable when both the pH of the system and the meal concentration were increased.
- Adding trichloroacetic acid (TCA) in the system in a concentration above 4.5 % (w/v) stops completely the enzymatic degradation of CGA in the SFM system.

CHAPTER 7. RECOMMENDATIONS

The following recommendations should be considered for further research in the enzymatic degradation of chlorogenic acid (CGA) by the white-rot fungus *Trametes versicolor*:

- To investigate the enzymatic degradation of CGA in a solid state fermentation (SSF) system and compare it with the submerged fermentation (SF).
- To identify and quantify by-products obtained from the enzymatic process using analytical and instrumental techniques such as ultraviolet-visible (UV-VIS), infra-red (IR), nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), chromatography, etc.
- It is well known that the enzyme (PPO) uses oxygen as a second substrate; therefore, it will be advisable to study the effect of oxygen on the enzymatic transformation of CGA.
- A pathway should be proposed to describe the transformation of CGA by the enzyme preparation secreted by *T. versicolor*.
- Due to the complexity of the system when SFM is present, it would be interesting to model and simulate the enzymatic decrease of CGA in the SFM system in the presence and in absent of oxygen as a second substrate.
- To investigate the combination of the enzymatic treatment with another process in order to obtain high quality protein isolates. This process could use either organic solvents techniques or membrane separation techniques.
- To perform an evaluation of the quality of the treated meal.

LIST OF REFERENCES

Altschul, Aaron M. "Processed Plant Protein Foodstuffs", Academic Press Inc., New York, (1958).

Archibald, F. and Roy, B. "Production of Manganic Chelates by Laccase from the Lignin-Degrading Fungus *Trametes (Coriolus) versicolor*", Appl. Environ. Microbiol., **58** (5), 1496-1499 (1992).

Bailey, J.E. and Ollis, D.F. "Biochemical Engineering Fundamentals", 2nd edition, McGraw-Hill Book Company, 130-136 (1986).

Bau, H. M.; Mohtadi-Nia, D.J.; Mejean, L. and Debry, G. "Preparation of Colourless Sunflower Protein Products: Effect of Processing on Physicochemical and Nutritional Properties". J. Am. Oil Chem. Soc., **60** (6), 1141-1147 (1983).

Bollag, J.-M.; Liu, S. and Minard, R.D. "Enzymatic Oligomerization of Vanilic Acid", Soil Biol. Biochem., **14**, 157-163 (1982).

Bourbonnais, R.; Paice, M.G.; Reid I.D.; Lanthier P. and Yaguchi M. "Lignin Oxidation by Laccase Isozyme from *Trametes versicolor* and Role of the Mediator 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) in Kraft Lignin Depolymerization", Appl. Environ. Microbiol., **61**, 1876-1880 (1995).

Butt, V.S. "Direct Oxidases and Related Enzymes", The Biochemistry of Plants, ed. Davies, D.D., **2** (3), 81-123 (1980).

Cater, C.M.; Gheyasuddin, S. and Mattil K.F. "The Effect of Chlorogenic Acid, Quinic and Caffeic Acids on the Solubility and Colour of Protein Isolates, Especially from Sunflower Seeds", Cereal Chem., **49**, 508-514 (1972).

Cheeke, P.R. and Shull, L.R. "Tannis and Polyphenolic Compounds". In: Natural Toxicants in Feeds and Poisonous Plants. pp. 332-353. AVI Publishing Company, USA (1985).

Choi, H.T. "Phenoloxidases and Photomorphogenesis in *Coprinus congregatus*", PhD thesis, University of California, Santa Barbara, (1987).

Clandinin, D.R. In Processed Plant Protein Food Stuffs. Altschul, A. M. Ed.: Academic: New York, (1958).

Clutterbuck, A.J. "Absence of Laccase from Yellow-Spored Mutants of *Aspergillus nidulans*", J. Gen. Microbiol. **70**, 423-435, (1972).

Collins, P.J. and Dobson, A.D.W. "Extracellular Lignin and Manganase Peroxidase Production by White-Rot Fungus *Coriolus versicolor*", Biotechnol. Lett., **17** (9), 989-992 (1995).

Constantinides, A. Applied Numerical Methods With Personal Computers. New York: McGraw-Hill, Inc. (1987).

Crueger, W. and Crueger, A. Biotechnology: a Textbook of Industrial Microbiology. Editor of the English edition, Thomas D. Brock. Sunderland, MA: Sinauer Associates; Madison, WI: Science Tech, 161-162 (1984).

Dehorter, B. and Blondeau, R. "Extracellular Enzyme Activities During Humic acid Degradation by the White-Rot Fungi *Phanerochaete chrysosporium* and *Trametes versicolor*", FEMS Microbiol. Lett., **94**, 209-216 (1992).

Dijkstra, L. and Walker J.RL. "Enzymic Browning in Apricots Prunus-Armeniaca", J. Sci. Food & Agric., **54** (2), 229-234 (1991).

Ding, C.K; Chachin, K.; Ueda, Y. and Imahori, Y. "Purification and Properties of Polyphenol Oxidase From Loquat Fruit", J. Agric. & Food Chem., **46** (10), 4144-4149 (1998).

Dominguez, H.; Sineiro, J.; Nuñez, M. J. and Lema, J. M. "Ethanol Extraction of Polyphenols in an Immersion Extractor. Effect of Pulsing Flow", JAOCS, **73** (9), (1996).

Dominguez, H.; Nuñez, M.J. and Lema, J.M. "Aqueous Processing of Sunflower Kernels with Enzymatic Technology", Food Chem., **53**, 427-434 (1995).

Dorrell, D.G. "Chlorogenic Acid Content of Meal from Cultivated and Wild Sunflowers", Crop Sci., **16**, 422-424 (1976).

Dreher, M.L. and Holm, E.T. "A HPLC Method for Chlorogenic Acid Determination in Sunflower Seeds", J. Food Sci., **48**, 264-265 (1983).

D'Souza T.M.; Merritt C.S. and Reddy C.A. "Lignin-Modifying Enzymes of the White Rot Basidiomycete *Ganoderma Lucidum*", Appl. Environ. Microbiol., **65**, 5307-5313 (1999).

Dusterhoft, E,-M.; Engels, F.M. and Voragen, A.G.J. "Parameters Affecting the Enzymatic Hydrolysis of Oil-Seed Meals, Lignocelluloosic By-Products of the Food Industry", Biores. Techn., **44**, 39-46 (1993).

Fahraeus, G. "Formation of Laccase by *Polyporus versicolor* in Different Culture Media", Phys. Plant., **5**, 285-291 (1952).

Fahraeus, G. and Reinhammar, B. "Large Scale Production and Purification of Laccase From Cultures of the Fungus *Polyporus versicolor* and Some Properties of Laccase A", Acta Chem. Scandin., **21** (9), 2367-2378 (1967).

Fan, T. Y.; Sosulski, F. W. and Hamon, N.W. "New Techniques for Preparation of Improved Sunflower Protein Concentrates", Cereal Chem., **53,** 118-125, (1976).

FAO / EBRD Agribusiness Handbooks; Vol. 2 Sunflower / Crude and Refined Oils., pp. 22, (1999).

Felton, G.W. and Duffey S.S. "Protective Action of Midgut Catalase in Lepidopteran Larvae Against Oxidative Plant Defenses", J. Chem. Ecol., **17** (9), 1715-1732 (1991).

Fersht AWH. "Enzyme Structure and Mechanism". New York: WH Freeman & Co. (1984).

Garzillo, A.M.V.; Paola, S.D.; Burla, G. and Buonocore V. "Differentially-Induced Extracellular Phenol Oxidases from *Pleurotus ostreatus*", Phytoch., **31,** 3685-3690 (1992).

Grau, C.R. and Almquist, H.J. "Value of Sunflower-Seed Protein", Proc. Soc. Exptl. Bio. Med., **60**, 373-374 (1945).

Hammel, K. E.; Jensen, K. A., Jr.; Mozuch, M. D.; Landucci, L. L.; Tien, M.and Pease, E. A. "Ligninolysis by a Purified Lignin Peroxidase", J. Biol. Chem., **268:17**, 12274–12281 (1993).

Higuchi, T. "Biodegradation of Lignin: Biochemistry and Potential Applications", Experientia, **38**, 159-166 (1982).

Hurrell, R. F. and Finot, P.A. "Effects of Food Processing on Protein Digestibility and Amino Acid Availability". Chap 8. In Digestibility and Amino Acid Availability in Cereals and Oilseeds, eds. J.W. Finley & D.T. Hopkins, American Association of Cereal Chemists Inc, St. Paul, MN, USA, (1985).

Ishihara, T. and Ishihara, M. "Oxidation of Syringic Acid by Fungal Laccase", J. JPN. Wood Res. Soc., **22**, 371-375 (1976).

Jensen, S. K.; Olsen, H.S. and Sorensen, H. "Aqueous Enzymatic Processing of Rapeseed for Production of High Quality Products", ch. 19 in Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology, ed. Shahidi, F., AVIBook, (1990).

Jiang, Y.M.; Zauberman, G. and Fuchs, Y. "Partial Purification and Some Properties of Polyphenol Oxidase Extracted From Litchi Fruit Pericarp", Posthar. Biol. and Techn., **10**, 221-228 (1997).

Johnson, M.L. and Faunt, L.M. Parameter Estimation by Least-Squares Methods. In: Brand L, Johnson ML, editors. Methods in Enzymology. San Diego: Academic Press, Inc. Vol. 210, p 1-36 (1992).

Joo, H.S.; Lim J.W. and KIM S.S. "Studies on the Peroxidase Activity of Rabbit Hemoglobin", Korean Biochem. J., **24** (6), 564-571 (1991).

Joubert, F.J. "Sunflower Seed Proteins", Biochim. Biophys. Acta., **16**, 520, (1955).

Jung, G. H. and Fahey, C. G., Jr. "Effect of Phenolic Compound Removal on in Vitro Forage Digestibility", J. Agric. Food Chem., **29**, 817-820 (1981).

Katase, T. and Bollag, J.-M. "Transformation of Trans-4-hydroxycinnamic Acid by Laccase of Fungus *Trametes versicolor*: Its Significance in Humification", Soil Sci., **151** (4), 291-296 (1991).

Kertesz, D. and Zito, R. "Mushroom Polyphenol Oxidase. I. Purification and General Properties", Biochim. Biophys. Acta, **96**, 447-462, (1965).

Khan, W.A. and Overend, R.P. "An Oxygenase Enzyme System from *Trametes versicolor*", FEMS Microbiol. Lett., **66**, 215-220 (1990).

Khindaria, A.; Grover, T.A. and Aust, S.D. "Oxalate-Dependent Reductive Activity of Manganese Peroxidase from *Phanerochaete chrysosporium*", Arch. Biochem. Biophys., **314:2**, 301–306 (1994).

Kirk, T.K. and Farrell, R.L. "Enzymatic "Combustion": The Microbial Degradation of Lignin", Ann. Rev. Microbiol., **41**, 465-505 (1987).

Lacki, K. and Duvnjak, Z. "Transformation of 3,5-Dimethoxy, 4-Hydroxy Cinnamic Acid and Its Derivatives Using Enzyme From White-Rot Fungus *Trametes versicolor*: Enzyme Characteristics and Its Application", J. Chem. Tech. Biotechnol., **65**, 211-220 (1996a).

Lacki, K. and Duvnjak, Z. "Comparison of Three Methods for the Determination of Sinapic Acid Esters Content in Enzymatically Treated Canola Meal", J. Appl. Microbiol. Biotechnol., **45**, 530-537 (1996b).

Lacki, K. and Duvnjak, Z. "Modelling of the Enzymatic Transformation of 3,5-dimethoxy, 4-hydroxy Cinnamic Acid by an Enzyme of White-Rot Fungus *Trametes versicolor*", Biotechnol. Bioeng., **51**, 249-259 (1996c).

Lacki, K. and Duvnjak, Z. "Enzymatic Transformation of Sinapine Using Polyphenol Oxidase from *Trametes versicolor*: Effect of pH and Temperature and Model Development, Chem. Eng. J., **65**, 27-36 (1997).

Lacki, K. M. A Novel Method For the Decrease of Phenolic Content in Commercial Canola Meal Using an Enzyme Preparation Secreted by the White-Rot Fungus *Trametes versicolor*. PhD. Thesis, Department of Chemical Engineering, University of Ottawa, Ottawa, Canada (1997).

Leonowicz, A.; Edgehill, R.U. and Bollag, J.-M. "The Effect of pH on the Transformation of Syringic and Vanilic Acids by the Laccase of *Rhizoctonia Practicola* and *Trametes versicolor*", Arch. Microbiol. 137, 89-96 (1984).

Leonowicz, A. and Trojanowski, J. "Induction of Laccase by Ferulic Acid in *Basidiomycetes*", Acta Biochem. Polonica, **22** (4), 291-295 (1975).

Leonowicz, A.; Trojanowski, J. and Orlicz, B. "Induction of Laccase in Basidiomycetes: Apparent Activity of the Inducible and Constitutive Forms of the Enzyme With Phenolic Substrates", Acta Biochem. Polonica, **25** (4), 122-129 (1978).

Loomis, W.D. and Battaile, J. "Plant Phenolic Compounds and the Isolation of Plant Enzymes", J. Phytochem.., **5**, 423, (1966).

Lorusso L.; Lacki K.; and Duvnjak Z. "Decrease of Tannin Content in Canola Meal by an Enzyme Preparation from *Trametes versicolor*", Biotechn. Lett., **18** (3), 309-314 (1996).

Lundquist, K. and Kristerrson, P. "Exhaustive Laccase-Catalyzed Oxidation of a Lignin Model Compound (Vanillyl Glycol) Produces Methanol and Polymeric Quinoid Products", Biochem. J., **229**, 277-279 (1985).

Mann, J. "Secondary Metabolism". Oxford Chemistry Series, Clarendon Press, Oxford, (1978).

Mason, H.S. "Structures and Functions of the Phenolase Complex", Nature, **177**, 79-81, (1956).

Mazzafera, P. and Robinson, S.P. "Characterization of Polyphenol Oxidase in Coffee", Phytochem., **55** (4), 285-296 (2000).

McCurdy, S.M. and March, B.E. "Processing of Canola Meal for Incorporation in Trout and Salmon Diets", JAOCS, **69** (3), 213-220 (1992).

McLean, D.D. "CHG 8185 Course Notes", Internal Communication: Department of Chemical Engineering, University of Ottawa (1993).

Mester, T. and Tien M. "Oxidation Mechanism of Ligninolytic Enzymes Involved in the Degradation of Environmental Pollutants", Intern. Biodeter. & Biodegrad., **46** (1 Special Issue SI), 51-59 (2000 Jul).

Mester T. and Field J.A. "Characterization of a Novel Manganese Peroxidase-Lignin Peroxidase Hybrid Isozyme Produced by *Bjerkandera* Species Strain BOS55 in the Absence of Manganese", J. Biol. Chem., 273:25, 15412-15417 (1998).

Milic, B.; Stojanovic, S.; Vacureivic, N. and Turcic, M. "Chlorogenic and Quinic Acids in Sunflower Meal", J Sci. Food Agric., **19**, 108, (1968).

Mikolajczak, K.L; Smith, C.R. Jr. and Wolf, I.A. "Phenolic and Sugar Components of Armavirec Variety Sunflower (*Helianthus annuus*) Seed Meal", J. Agric. Food Chem., **18**, 27-32 (1970).

Morohoshi, N. and Haraguchi, T. "Degradation of Lignin by the Extracellular Enzymes of *Coriolus versicolor*, VI", Mokuzai Gakkaishi, **33** (6), 495-502 (1987).

Morohoshi, N.; Wariishi, H.; Muraiso, C.; Nagai, T. and Haraguchi, T. "Degradation of Lignin by the Extracellular Enzymes of *Coriolus versicolor*, IV", Mokuzai Gakkaishi, **33** (3), 218-225 (1987).

Nikolaiczuk, N.; Brisson, G.J. and Mani, K.V. Official Rept. World's Poultry Congr., **8**, 181 (1948).

Palmieri, G.; Giardina, P.; Bianco, C.; Scaloni, A.; Capasso, A. and Sannia, G. "A Novel White Laccase from *Pleurotus ostreatus*", J. Biol. Chem., **272**, 31301-31307 (1997).

Parrado, J.; Bautista, J. and Machado, A. "Production of Soluble Enzymatic Protein Hydrolysate from Industrially Defatted Nondehulled Sunflower Meal", J. Agric. Food Chem., **39**, 447-450 (1991).

Paszczynski, A. and Lobarzewski, J. "Modification of Peroxidase Polymorphism in Trametes versicolor Fungus after Short Time Incubation Under Starvation Conditions", Biochem. Physiol. Pflanzen, **179**, 749-757 (1984).

Perry, C.R.; Smith, M.; Britnell, C.H.; Wood, D.A. and Thurston, C.F. "Identification of Two Laccase Genes in the Cultivated Mushroom *Agaricus bisporus*", J. Gen. Microbiol., **139**, 1209-1218 (1993).

Piacquadio P.; De Stefano G. and Sciancalepore V. "Phenolic Composition of Apple Juice Treated by Laccase Immobilized on Copper-Chelate Regenerable Carrier", Italian J. Food Sci., **14** (3), 291-294 (2002).

Pomenta, J.V. and Burns, E.E. "Factors Affecting Chlorogenic, Quinic and Caffeic Acid Levels in Sunflower Kernels", J. Food Sci., **36**, 490, (1971).

Ponting, J.D. "The Control of Enzymatic Browning in Fruits". In "Food Enzymes" (H.W. Schultza, ed.), pp.105-124. Avi Publ., Westport, Connecticut. (1960).

Putnam, D.H.; Oplinger, E.S.; Hicks, D.R.; Durgan, B.R.; Noetzel, D.M.; Meronuck, R.A.; Doll, J.D. and Schulte, E.E. Departments of Agronomy and Plant Genetics, Entomology and Plant Pathology. University of Minnesota, St. Paul, MN 55108 (November 1990).

Reed, G. "Enzymes in Food Processing". (Academic Press Inc.), pp 187-189. Berkeley Square House, London W.1, (1966).

Robb, D.A. "Copper Protein and Copper Enzyme. Vol 2. In: Contie R. (ed). pp 207-241. CRC Press. Boca Raton, Fl. (1984).

Rodriguez, E.; Pickard, M.A. and Vazquez-Duhalt, R. "Industrial Dye Decolorization by Laccases from Lignolytic Fungi", Curr. Microbiol., **38**, 27-32, (1999).

Rogalski, J.; Dawidowicz, A.L. and Leonowicz, A. "Purification and Immobilization of the Inducible Form of Extracellular Laccase of the Fungus *Trametes versicolor*", Acta Biotechnol., **10** (3), 261-269 (1990).

Ross, I.K. "The Role of Laccase in Carpophore Initiation in *Coprinus congregatus*", J. Gen. Microbiol. **128**, 2763-2770, (1982).

Roy-Arcand, L. and Archibald, F.S. "Direct dechlorination of Chlorophenolic Compounds by Laccases from *Trametes* (*Coriolus*) *versicolor*", Enzyme Microb. Technol. **13**, 194-203, (1991).

Sabir, M.A.; Sosulski, F.W. and Finlayson, A.J. "Polyphenols-Protein Interactions in Sunflower", J Agric. Food Chem., **22:4**, 575-578 (1974).

Sabir, M.A.; Sosulski, F.W. and Kernan J.A. "Phenolic Constituents in Sunflower Flour", J Agric. Food Chem., **22**, 572-574 (1974a).

Sabir, M.A.; Sosulski, F. W. and Mackenzie, S.L. "Gel Chromatography of Sunflower Proteins", J. Agric. Food Chem., **21**, 988, (1973).

Salas, S.D.; Bennett, J.E.; Kwon-Chung, K.J.; Perfect, J.R. and Williamson, P.R. "Effect of the Laccase Gene, CNLAC1, on virulence of *Cryptococcus neoformans*", J. Exp. Med., **184**, 377-386, (1996).

Sanchez-Amat A. and Solano F. "A Pluripotent Polyphenol Oxidase from the Melanogenic Marine *alteromonas* Shares Catalytic Capabilities", Biochem. Biophys. Res. Comm., **240**, 787-792, (1997).

Sariaslani, F.S. "Microbial Enzymes for Oxidation of Organic Molecules", Critical Reviews in Biotechn., **9** (3) (1989).

Sastry, M.C.S. Ph.D. Thesis, University of Mysore, (1984).

Segel, I.H. "Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems", ed. A Willey-Interscience Publication, John Wiley & sons, Inc, (1975).

Shamanthaka S.M.C. and Sastry R. N.M.S. "Binding of Chlorogenic Acid by the Isolated Polyphenol-free 11S Protein of Sunflower (*Helianthus annuus*) Seed", J. Agric. Food Chem., **38** (12), 2103-10 (1990).

Shi, C.; Dai, Y.; Xia, B.; Xu, X.; Xie, Y. and Liu Q. "The Purification and Spectral Properties of Polyphenol Oxidase I From *Nicotiana tabacum*", Plant Mol. Biol. Reporter, **19**, 381a-381h (2001).

Smith, A.K. and Johnsen, V.L. "Sunflower Seed Protein", Cereal Chem., **25**, 399 (1948).

Smith, J.M. and Van Ness, H.C. "Introduction to Chemical Engineering Thermodynamics", 4th edition, McGraw-Hill International, 507-508 (1987).

Sodini, G. and Canella, M. "Acidic Butanol Removal of Colour-Forming Phenols from Sunflower Meal", J. Agric.Food Chem., **25** (4), 822-825 (1977).

Sosulski, F.W. "Organoleptic and Nutritional Effects of Phenolic Compounds on Oilseed Protein Products: a Review", J. Am. Oil Chem. Soc., **56** (8), 711-715 (1979).

Sosulski, F. W.; McCleary, C. W. and Soliman, F. S. "Diffusion Extraction of Chlorogenic Acid from Sunflower Kernels", J. Food Sci., **37**, 352-356 (1972).

Sosulski, F. W.; Sabir, M.A. and Fleming, S. E. "Continuous Diffusion of Chlorogenic Acid from Sunflower Kernels", J. Food Sci., **38**, 468-470 (1973).

Sosulski, F.W. and Fleming, S.E. "Chemical, Functional, and Nutritional Properties of Sunflower Protein Products", J. Am. Oil Chem. Soc., **54**, 100A-104A (1977).

Sosulski, K. and Sosulski, F.W. "Enzyme Pretreatment to Enhance Oil Extractability in Canola", ch. 16 in Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology, ed. Shahidi, F., AVIBook, (1990).

Sripad, G. and Narasinga, Rao M.S. "Effects of Methods to Remove Polyphenols from Sunflower Meal on the Physicochemical Properties of the Proteins", J. Agric. Food Chem., **35**, 962-967 (1987).

Sripad G, Prakash V., and Narasinga Rao M.S. "Extractability of Polyphenols of Sunflower Seed in Various Solvents", J. Biosci., **4** (2), 145-152 (1982).

Sundaramoorthy, M.; Kishi, K.; Gold, M. H. and Poulos, T. L. "The Crystal Structure of Manganese Peroxidase from Phanerochaete Chrysosporium at 2.06-A Resolution", J. Biol. Chem., **269:52,** 32759–32767 (1994).

Sundaramoorthy, M.; Kishi, K.; Gold, M. H. and Poulos, T. L. "Crystal Structures of Substrate Binding Site Mutants of Manganese Peroxidase", J. Biol. Chem. **272:28,** 17574–17580 (1997).

Szklarz, D.G.; Antibus, R.K.; Sinsabaugh, R.L. and Linkins, A.E. "Production of Phenol Oxidase and Peroxidases by Wood-Rotting Fungi", Mycologia, **81** (2), 234-240 (1989).

Vioque, E and Millán F. "Determinación Cuantitativa de los Acidos Clorogénico y Cafeico por Cromatografía Líquida de Alta Eficacia", Grasas y Aceites: Revista Bimestral de Información Cientifica y Técnica, **36** (516), 325-327 (1985).

Vogel, H.J. "A Convenient Growth Medium for *Neurospora* (Medium N)", Microbial. Gen. Bull., **13**, 42-43 (1959).

Wariishi, H.; Valli, K. and Gold, M.H. "Maganese (II) oxidation by Manganese Peroxidase from the Basidiomycete *Phanerochaete chrysosporium*", J. Biol. Chem., **267**, 23688-23695 (1992).

Weemaes, C.A.; Ludikhuyze L.R.; Vandenbroeck, I. and Hendrickx, M.E. "Effect of pH on Pressure and Thermal Inactivation of Avocado Polyphenol Oxidase – A Kinetic Study", J. Agric. & Food Chem., **46** (7), 2785-2792 (1998).

Whitaker, John R. "Principles of Enzymology For The Food Sciences". New York: M. Dekker (1972).

Whitaker, JR. "Principles of Enzymology For The Food Sciences". 2nd edition. New York: Marcel Dekker. (1994).

Whitehurst, R. J and Law, B.A. Enzymes in Food Technology. Sheffield Academic Press, Mansion House, 19 Kingfield Road, Sheffield, UK, ch. 10, pp.229-244 (2002).

Xu, F. "Oxidation of Phenols, Anilines, and Benzenethiols by Fungal Laccases: Correlation Between Activity and Redox Potentials as well as Halide Inhibition", Biochem., **35**, 7608-7614, (1996).

Yaver, D.S.; Xu, F.; Golightly, E.J.; Brown, K.M.; Brown, S.H.; Rey, M.W.; Schneider, P.; Halkier, T.; Mondorf, K. and Dalboge, H. "Purification, Characterization, Molecular Cloning, and Expression of two Laccase Genes from the White Rot Basidiomycete *Trametes villosa*", Appl. Environ. Microbiol., **62**, 834-841 (1996).

Yelena, G. S. and Brudvig G.W. "Isolation and Characterization of Spinach Photosystem II Membrane-Associated Catalase and Polyphenol Oxidase", Biochem., **35**, 16255-16263, (1996).

Youn H.D.; Hah Y.C. and Kang S.O. "Role of Laccase in Lignin Degradation by White-Rot Fungi", FEMS Microbiol. Lett. **132**, 183-188, (1995).

Appendix A

Determination of the CGA concentration by spectrophotometer

Beer's Law states that the absorbance, A(w), of a species at a particular wavelength of electromagnetic radiation, w, is proportional to the concentration, c, of the absorbing species and to the length of the path, l, of the electromagnetic radiation through the sample containing the absorbing species. This can be written in the form:

$$A(w) = e(w)\, l\, c$$

The proportionality constant e(w) is called the absorptivity of the species at the wavelength, w. e(w) is also called the molar absorptivity if the concentration is measured in *moles/liter*. The way in which e(w) depends on wavelength defines the spectrum of the substance in question. Most substances show a maximum in e(w) over a sufficiently broad wavelength range. The wavelength at that maximum value is called the analytical wavelength of the substance. Normally, Beer's law is applied at the analytical wavelength of a given material. The sensitivity to concentration differences should be largest at that wavelength. The experimental procedure for using Beer's Law to measure concentrations generally involves the following:

1) Determination of the analytical wavelength of the substance whose concentration is desired, w_{anal} (Figure A-1).
2) Preparation of a series of samples of known concentration of the substance.
3) Measuring of the absorbance of each of the solutions of known concentration at the analytical wavelength (Table A-1).
4) Plotting of the values of the absorbance as a function of the concentration (Figure A-2).

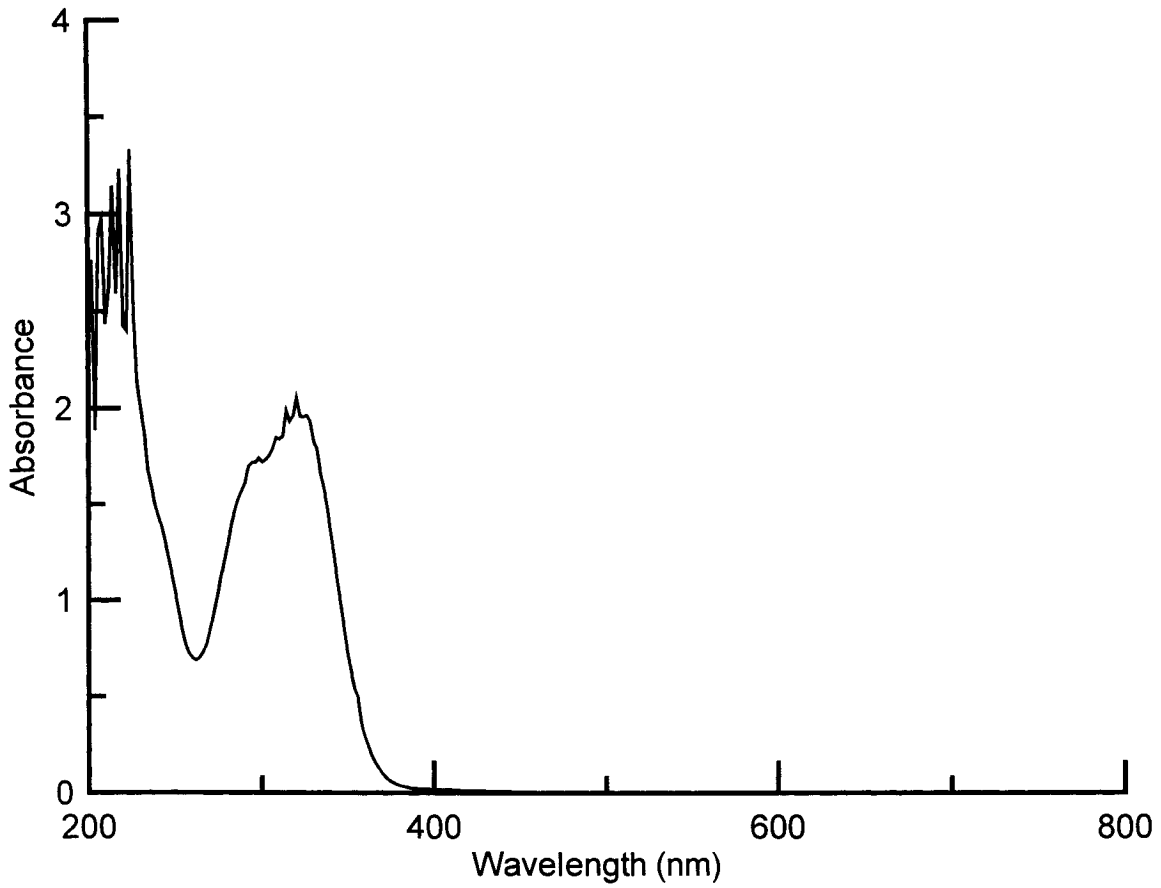


Figure A-1. Wavelength scan of CGA in the presence of buffer. CGA concentration 0.12 mM; pH 4.3; temp. 25°C.

Table A-1. Absorbance data of CGA concentrations in the standard curve

Concentration (mM)	Absorbance
0	0.0020
0.02	0.3731
0.04	0.748
0.06	1.1232
0.08	1.4921
0.10	1.8717

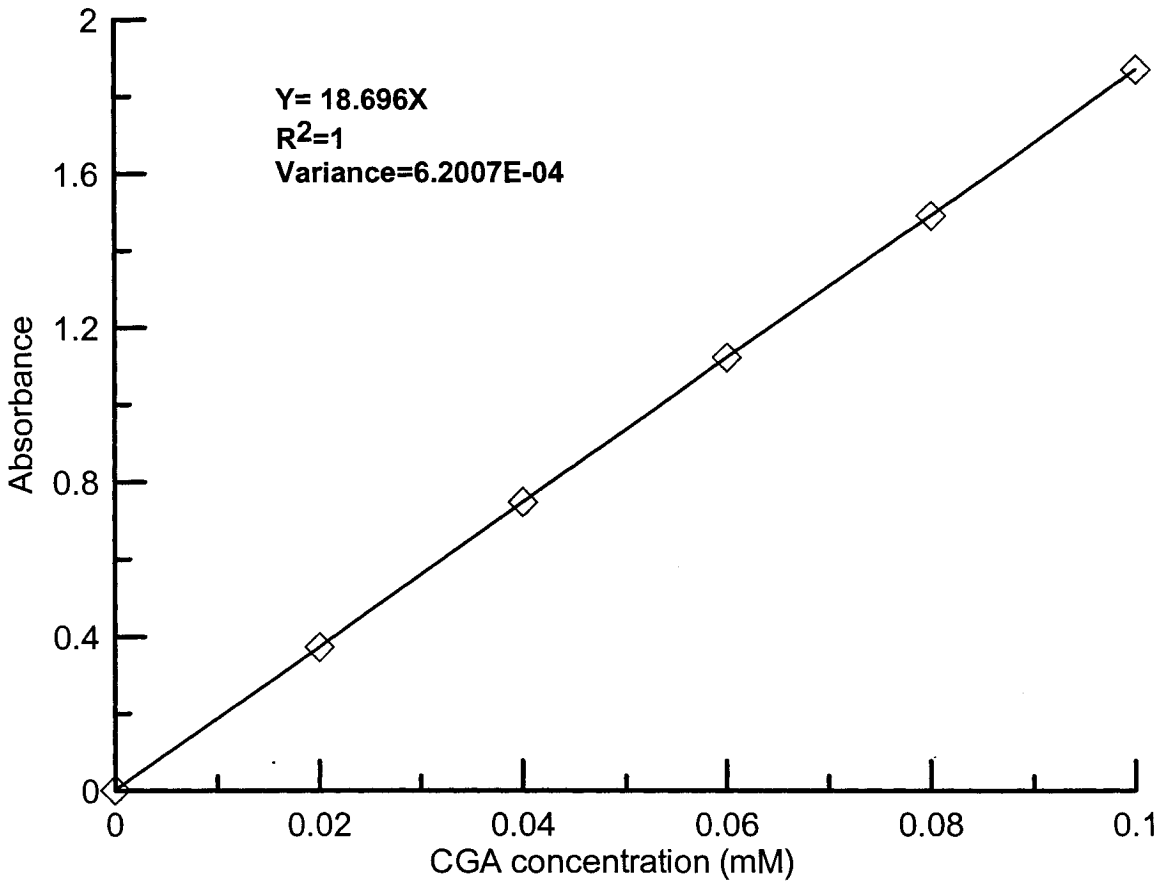


Figure A-2. Standard curve (Beer's law plot) for the determination of the CGA concentration by spectrophotometer. Analytical wavelength 324 nm; temp. 25°C.

5) Verification of that, within experimental error, the absorbance is a linear function of the concentration. In the above graph, the value R^2 is a measure of the "goodness of fit" of the data to a straight line. A value of 1 indicates a perfect fit.

6) If linearity is confirmed, determination of the slope of the best straight line through the experimental points in the absorbance *vs* concentration plot. Call this the Beer's Law slope. The Beer's law slope has the value $e(w_{anal})$ l, where l is the path length through the sample. In the above plot, the Beer's law slope is the value of the coefficient in the equation y = 18.696 x. In this case the slope is 18.696. The equation can also be written as A = 18.696 c, where c is the concentration in mM.

7) If the same experimental arrangement (same spectrometer, same cell) will be used for subsequent absorbance measurements, the value of the slope can be used to determine the concentration corresponding to a given absorbance by samples of unknown concentration. Example, suppose in the above example, we measure the absorbance of a solution of unknown concentration to be 1.84. The concentration of that solution will be 1.84 / 18.696 ≅ 0.09842.

If a different experimental arrangement were used (e.g., a different cell), the Beer's Law slope would need to be adjusted by the pathlength through the cell used in the Beer's Law determination. This will provide the value of the absorptivity, $e(w_{anal})$, which should be characteristic only of the substance and the wavelength, and independent of the experimental arrangement used to determine it.

Appendix B

Determination of the CGA concentration by High Performance Liquid Chromatography (HPLC)

To determine the CGA concentration by HPLC, a standard curve was prepared by using known concentrations of pure CGA. The specifications of the HPLC equipment, the type of column and size of the sample injected were explained in section 3.4.2.3. The response of each of the solutions of known concentration at the analytical wavelength with the percentage of deviation is given in Table B-1. The plotting values of the responses as a function of the concentration are illustrated Figure B-1.

Table B-1. Data for the calibration curve obtained by HPLC measurements

Amount (mM)	Response	Calculated amount (mM)	% Deviation
0.02	393323.5594	0.019166	-4.168
0.02	395397.7617	0.019268	-3.662
0.04	802856.538	0.039123	-2.193
0.04	829784.2769	0.0404	1.087
0.06	1240008.3695	0.060425	0.708
0.06	1254492.998	0.061131	1.885
0.08	1618760.773	0.07888	-1.398
0.08	1639666.9571	0.0799	-0.125
0.10	2023134.7829	0.098586	-1.414
0.10	2092057.6875	0.101945	1.945

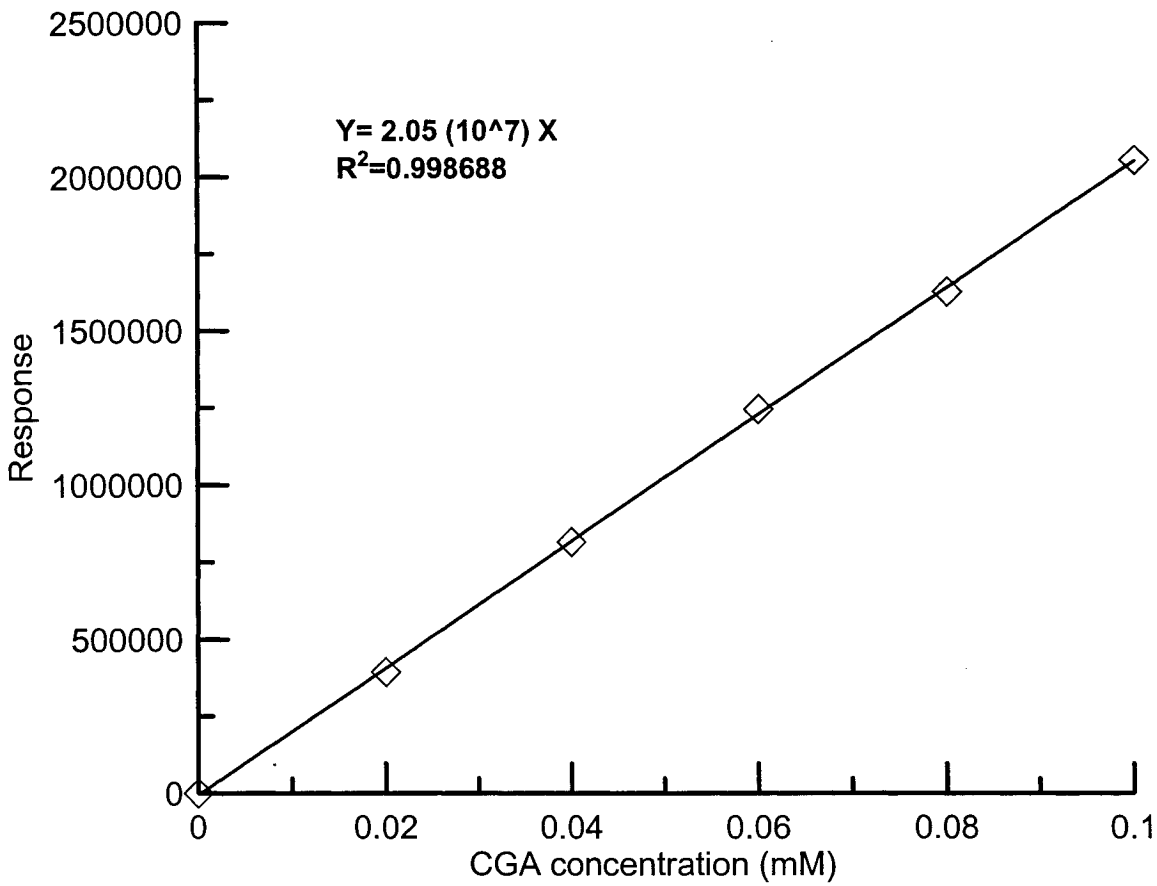


Figure B-1. Standard curve for the determination of the CGA concentration by HPLC. Analytical wavelength 324 nm; temp. 25°C.

Appendix C

Enzyme denaturalization studies

Several experiments were done using active and denatured enzyme. The denaturalization of the enzyme was carried out by placing a tube, which contained the enzyme, in a hot water bath. The temperature of this bath was kept constant at 70°C and 95°C for 20 minutes. After the enzyme was denatured, it was added to a system containing buffer and either CGA or SA as a substrate. The total volume of the system was 50 mL. Samples were withdrawn from the system after certain periods of time and the changes in the absorbance during the enzymatic conversion of the substrates were obtained by wavelength scans. The results of these experiments are shown in Figures C-1 and C-2 for CGA and at 70 and 95°C enzyme denaturation temperatures respectively. On the other hand, Figures C-3 and C-4 show the wavelength scans for the case of SA and at the same two enzyme denaturation temperatures. The wavelength scans with active enzyme are shown in Figures C-5 and C-6 for CGA and SA, respectively. When comparing the last two graphs, it was noticed that the degradation process was faster when SA was used as a substrate. This behaviour was also found when the enzyme was denatured at 70°C for 20 minutes before adding it to the system. The comparison of these results is shown in a kinetics graph as the one showed in Figure C-7. From this graph it looks like there is an equilibrium during the CGA decrease with active enzyme after a 15 minutes of reaction time, but as it was mentioned in chapter 4 the 30% unconverted CGA was due to an overestimation, of the real CGA concentrations, obtained by the spectrophotometer.

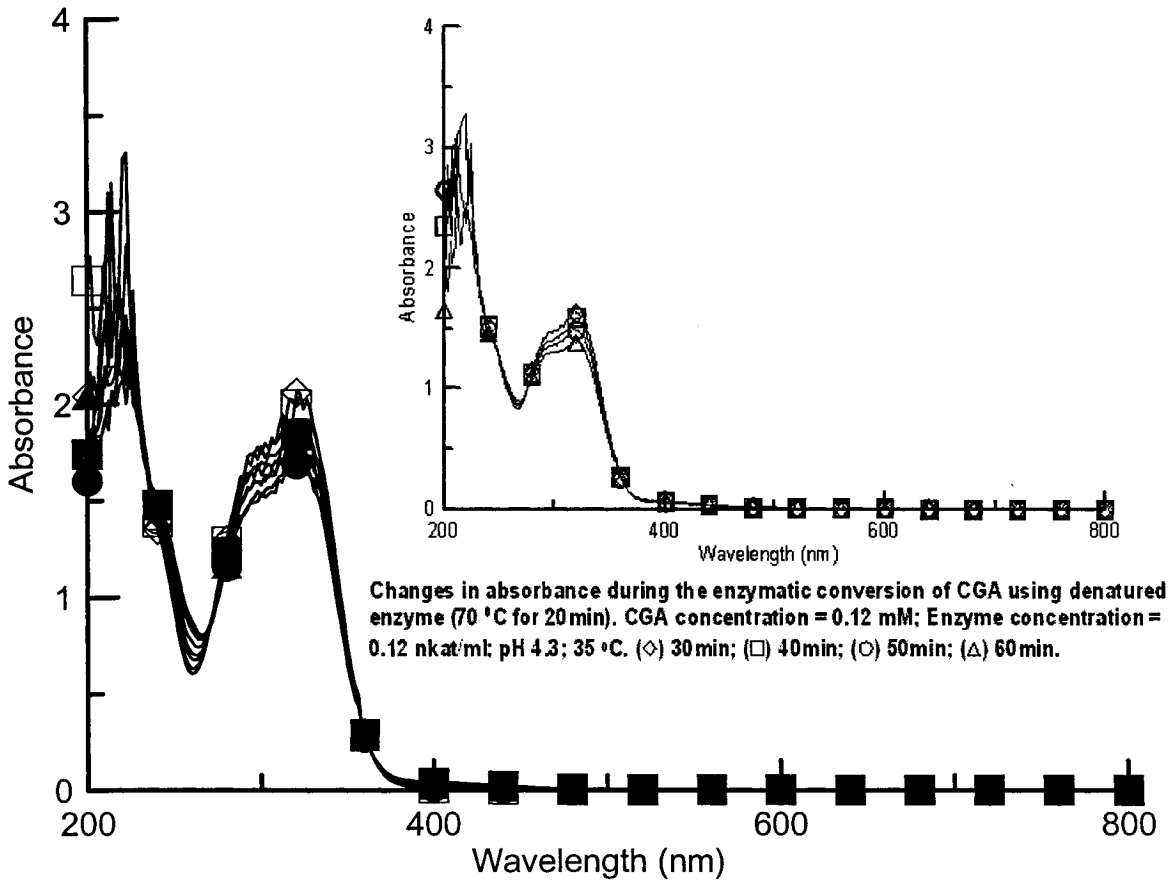


Figure C-1. Changes in absorbance during the enzymatic conversion of CGA using denatured enzyme (70°C for 20 min). CGA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

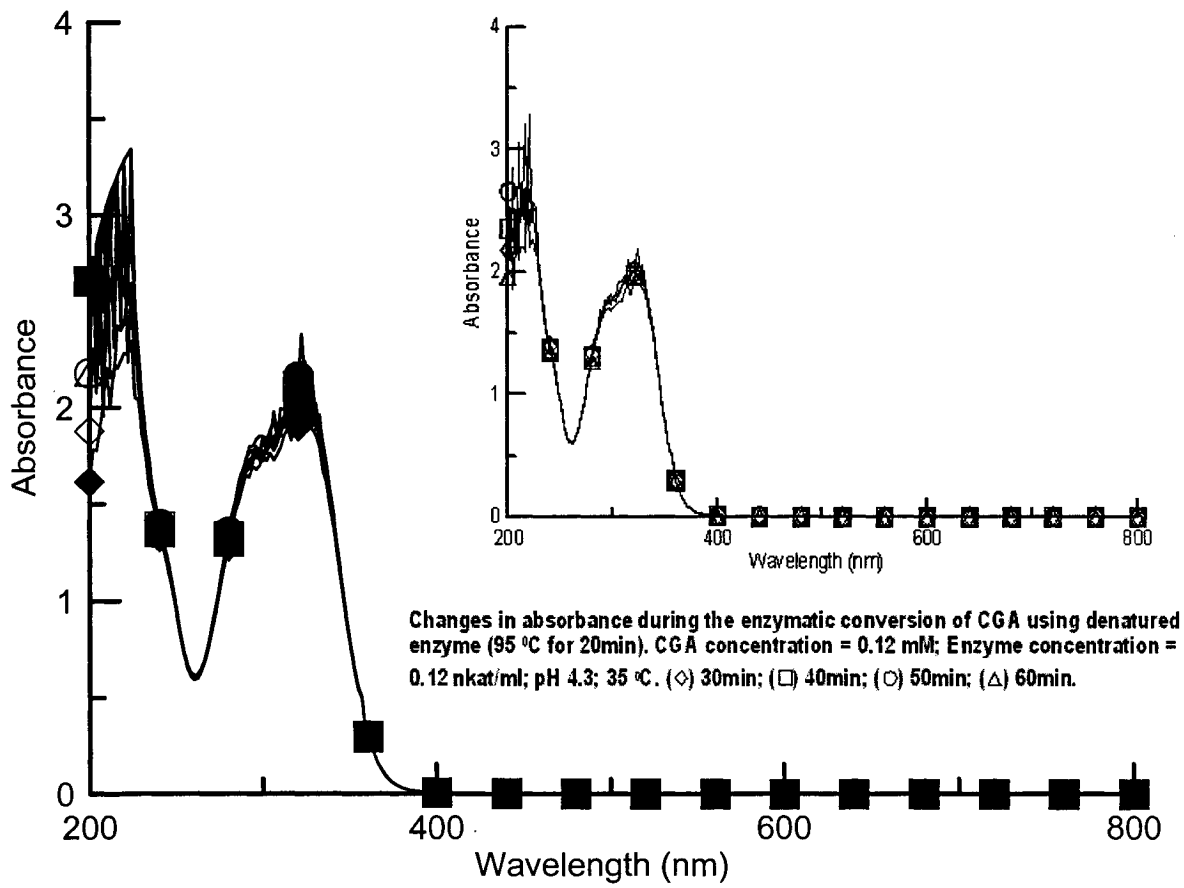


Figure C-2. Changes in absorbance during the enzymatic conversion of CGA using denatured enzyme (95°C for 20 min). CGA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

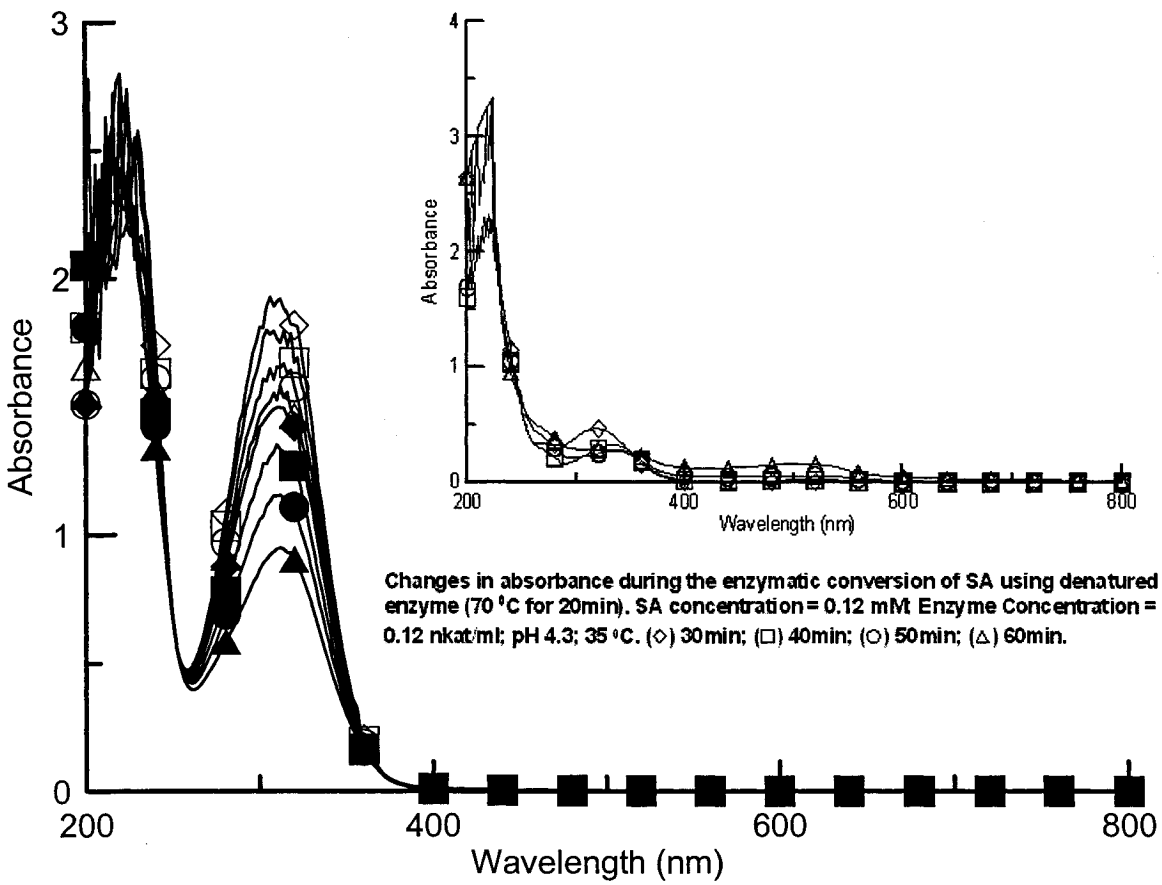


Figure C-3. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (70°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

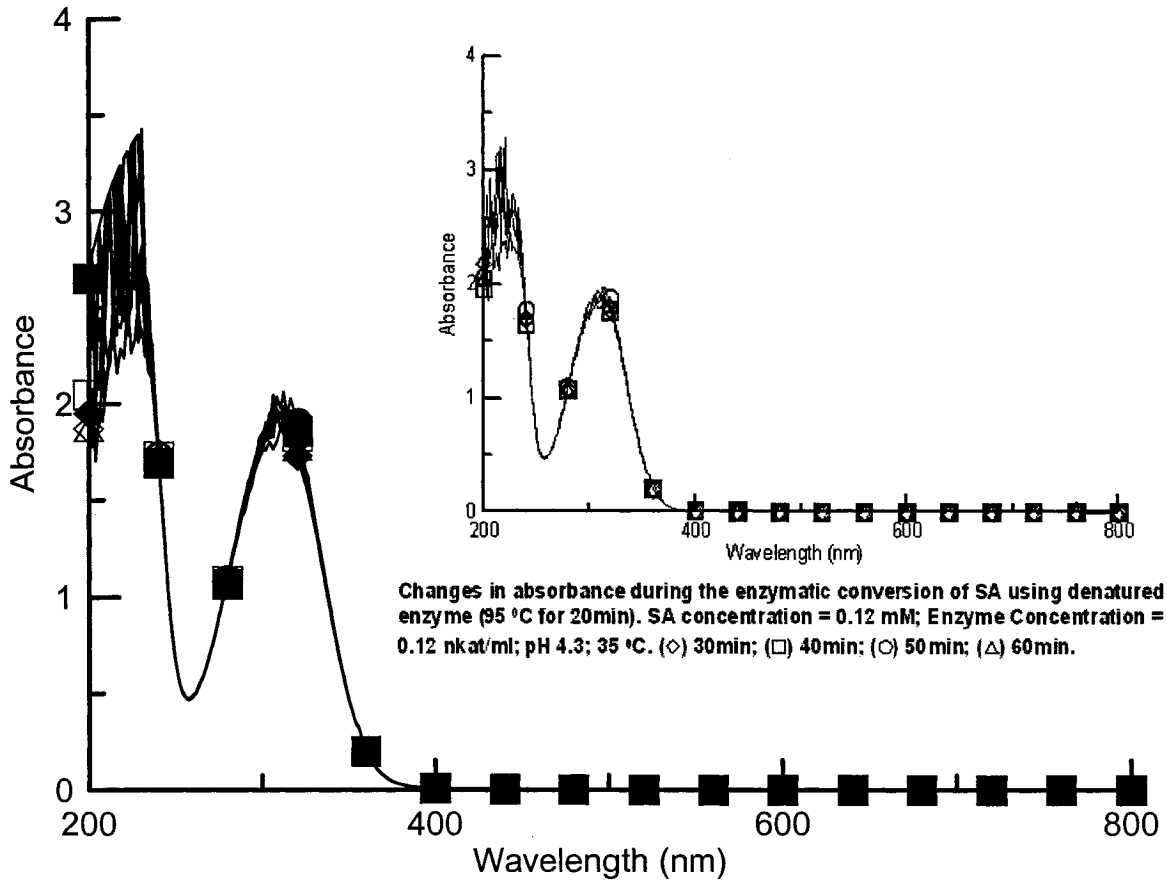


Figure C-4. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (95°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

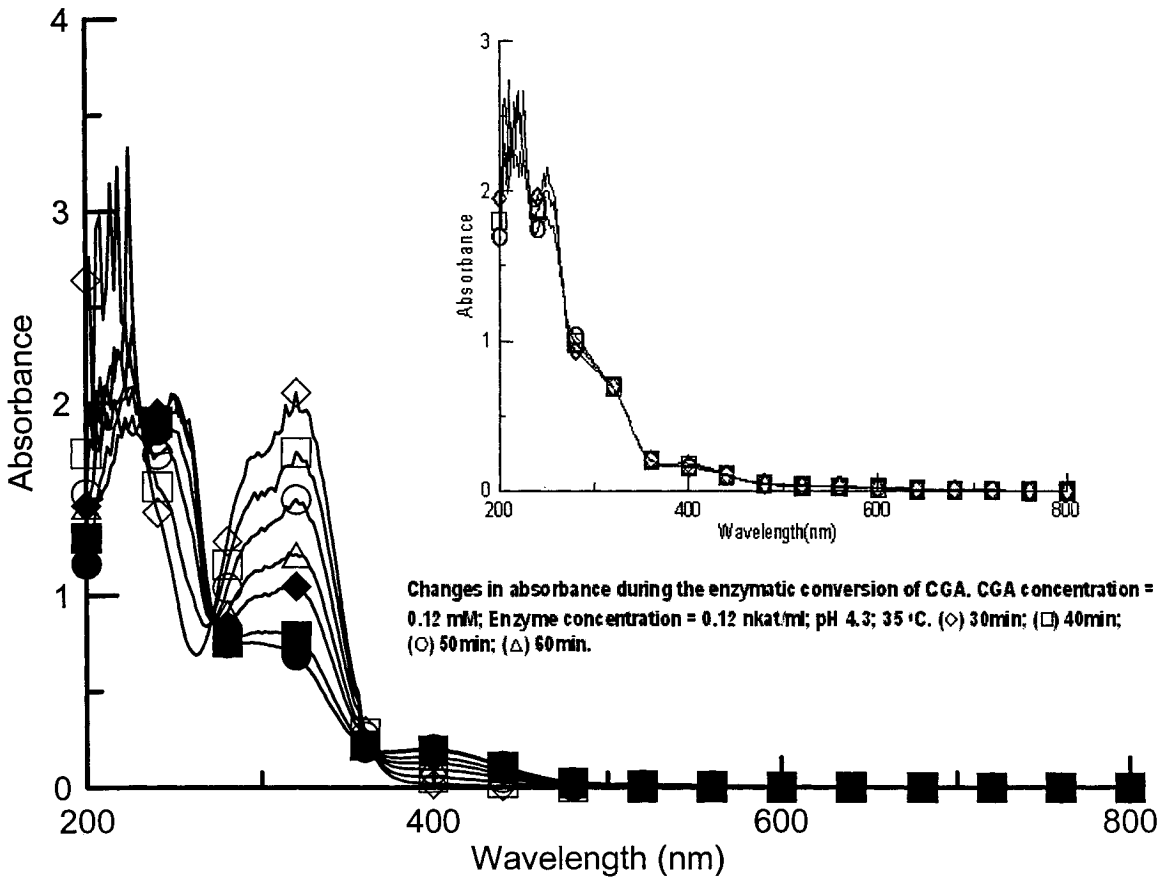


Figure C-5. Changes in absorbance during the enzymatic conversion of CGA using active enzyme. CGA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

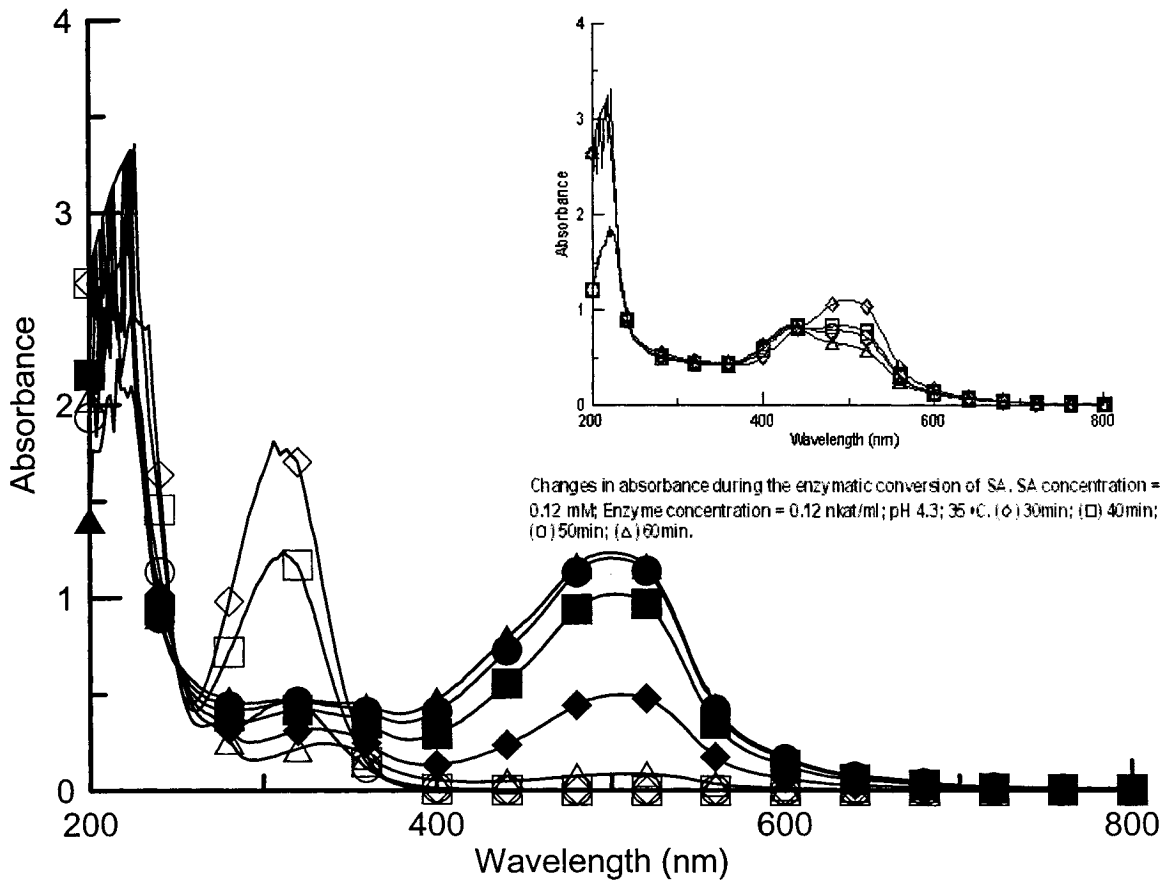


Figure C-6. Changes in absorbance during the enzymatic conversion of SA using active enzyme. SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

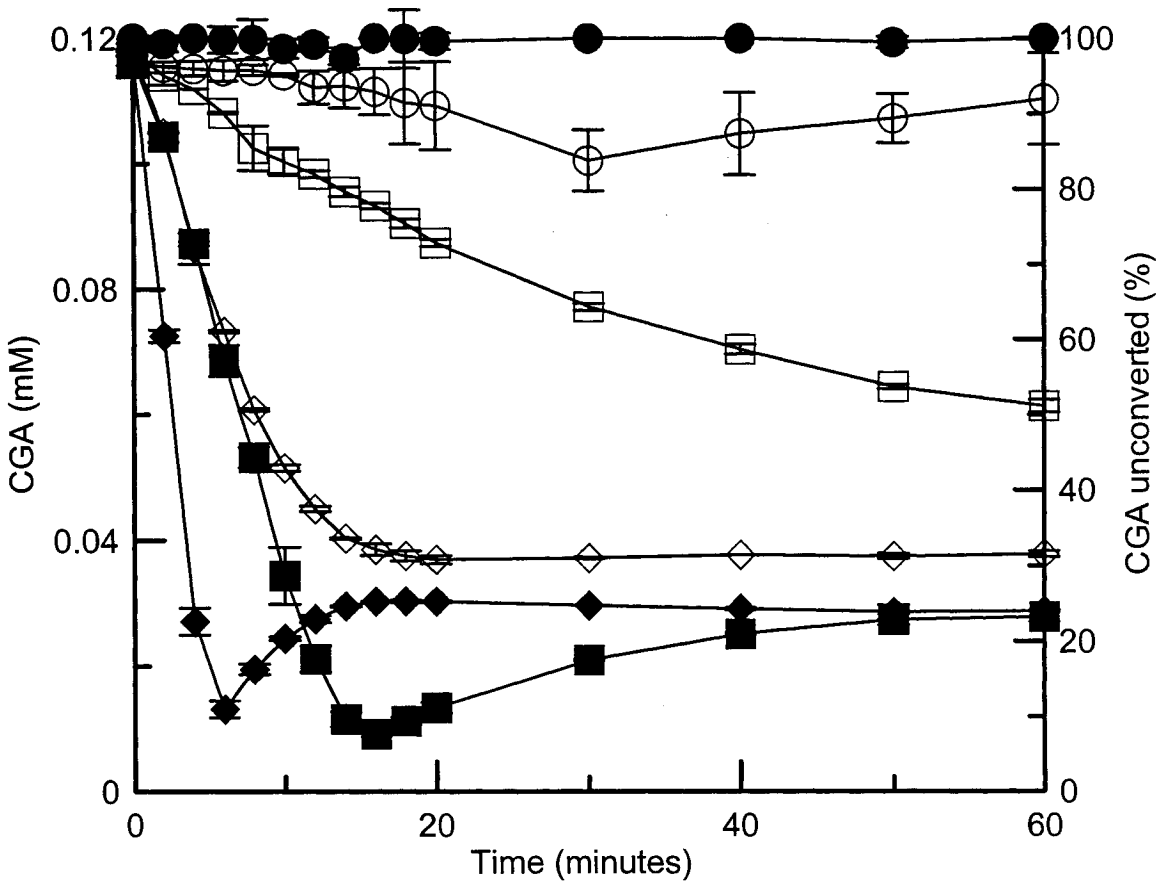


Figure C-7. Enzymatic conversion of CGA (opened symbols) and SA (closed symbols) with and without denatured enzyme. CGA and SA concentrations 0.12 mM; pH 4.3; 35°C. (◇◆) 0.12 nkat/mL active; (□■) 0.12 nkat/mL denatured enzyme at 70°C for 20 min. (○●) 0.12 nkat/mL denatured enzyme at 95°C for 20 min.

When comparing Figure C-6 with Figures C-3 and C-4, it seems to be that two enzymes were present in the system. One enzyme is degrading sinapic acid and the other its by-products. The second one became denatured when the enzyme was placed at high temperature (70°C) while the first one was still active at this temperature as shown in Figure C-3. This change was not noticed in the case of using CGA as a substrate. Due that temperature plays an important role in the denaturation of enzymes; a study was conducted to see at which temperatures these two enzymes were denatured. In order to investigate so, a system was prepared using buffer at pH 4.3, an enzyme preparation, which was placed to different temperatures for 20 minutes in a water bath, and SA as the substrate for the enzymatic system. The results of this study are shown from Figures C-8 to C-13. In these figures it is clear that no denaturation effect was seen for neither of the enzymes when dealing with temperatures below 65°C; however when the enzyme denaturation temperature was raised up to 75°C, the enzyme that degrade the by-products of the enzymatic conversion of SA was denatured completely while the one that degrades SA was still active at this temperature. The enzyme that degrades SA became completely inactive when the enzyme denaturalization temperature was raised up to 85°C confirming in this way the results discussed previously (when comparing Figure C-6 with Figures C-3 and C-4).

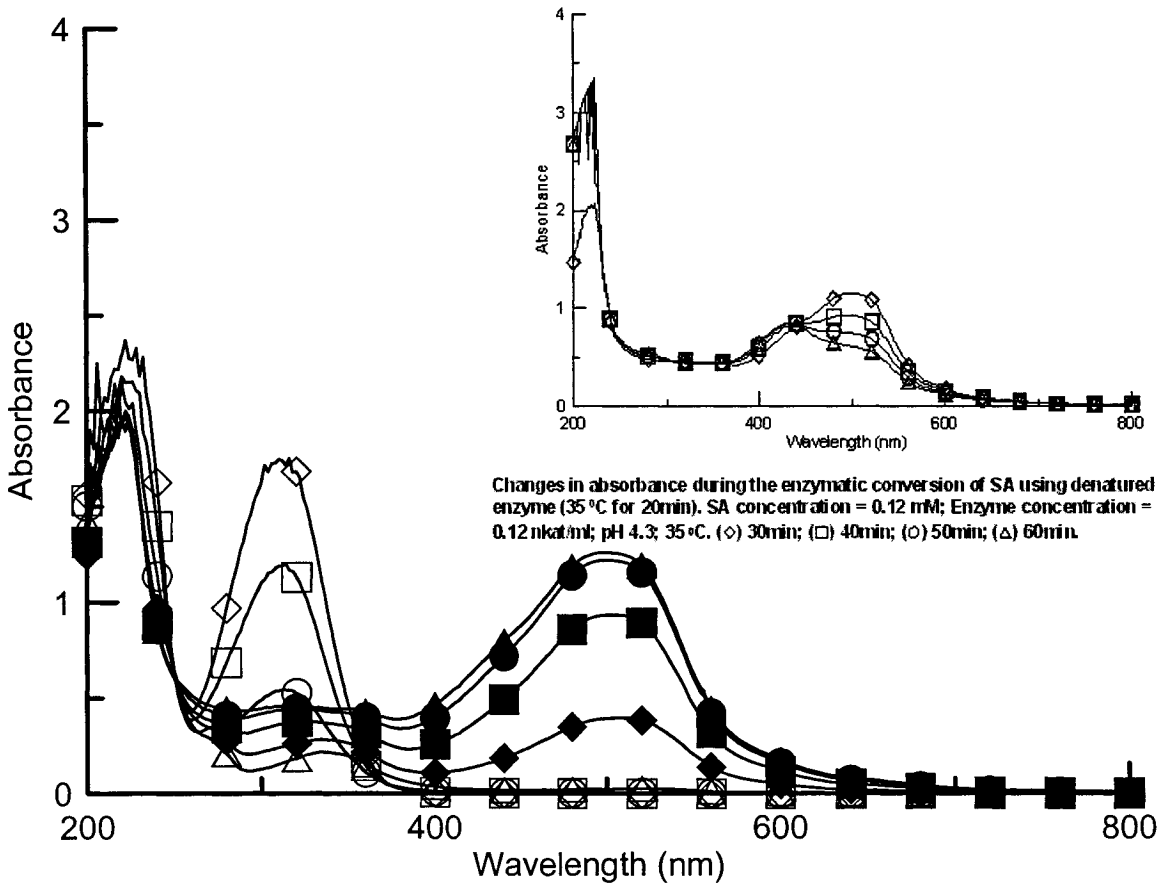


Figure C-8. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (35°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

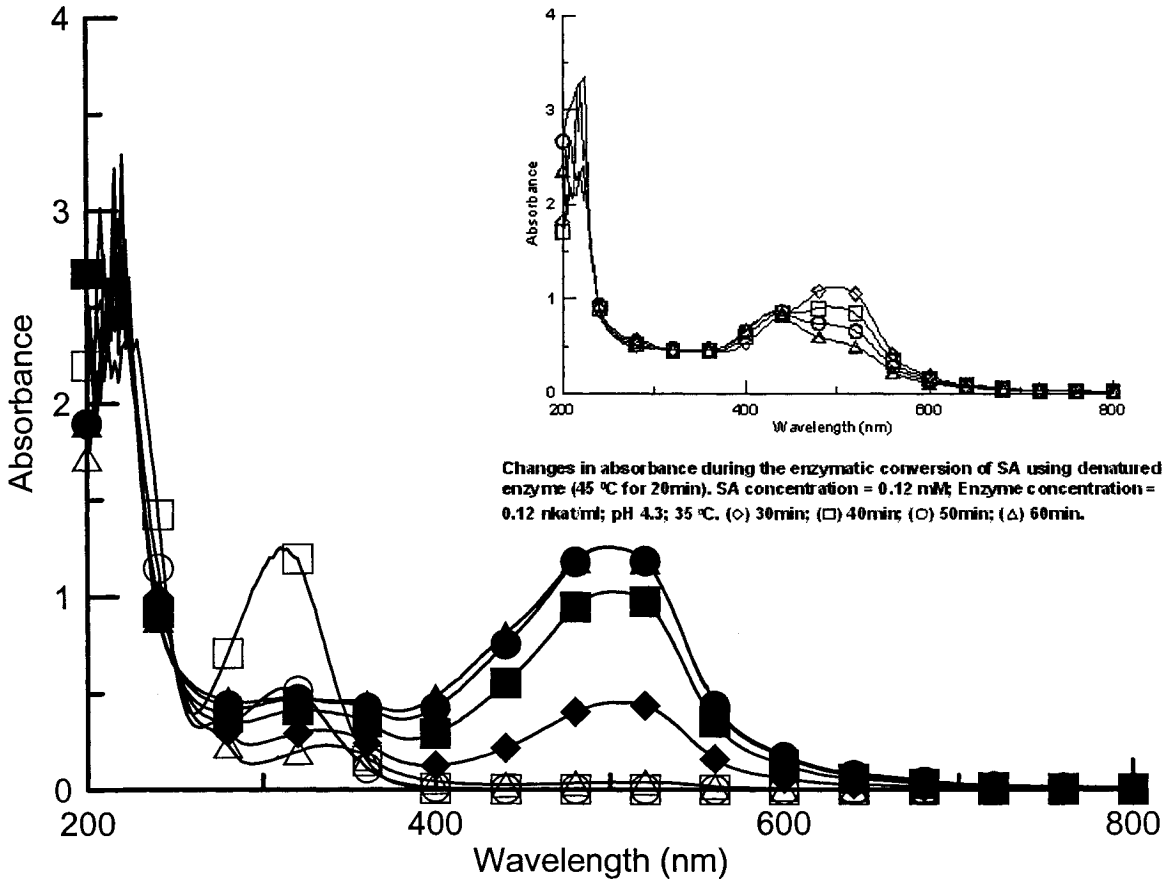


Figure C-9. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (45°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

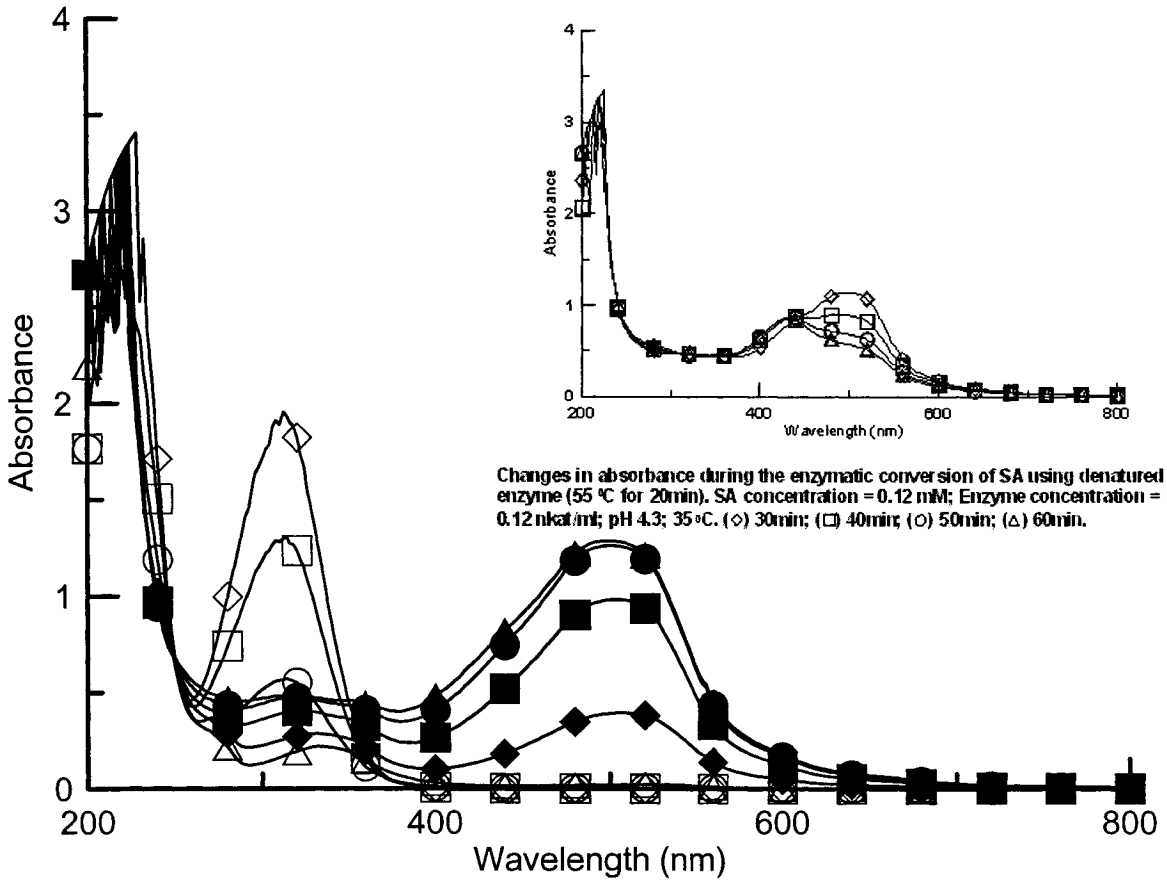


Figure C-10. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (55°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

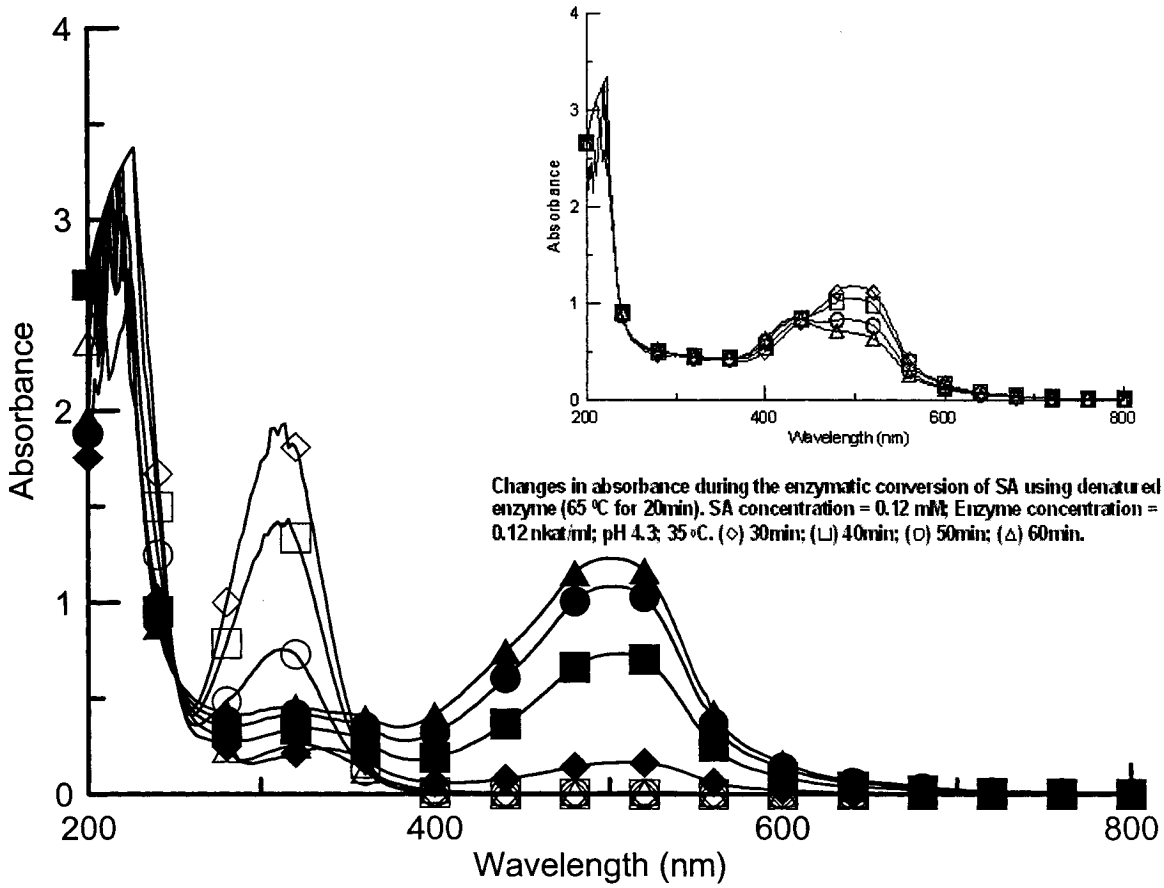


Figure C-11. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (65°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

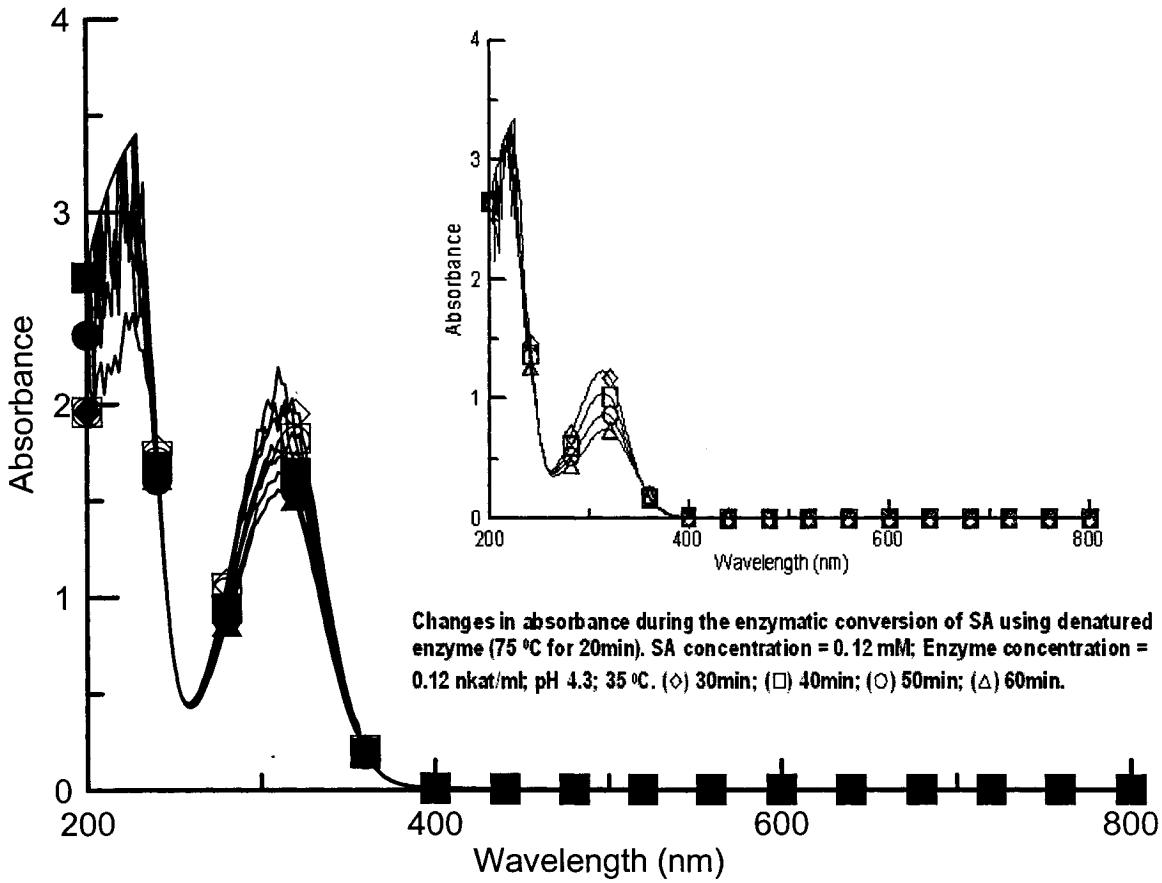


Figure C-12. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (75°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

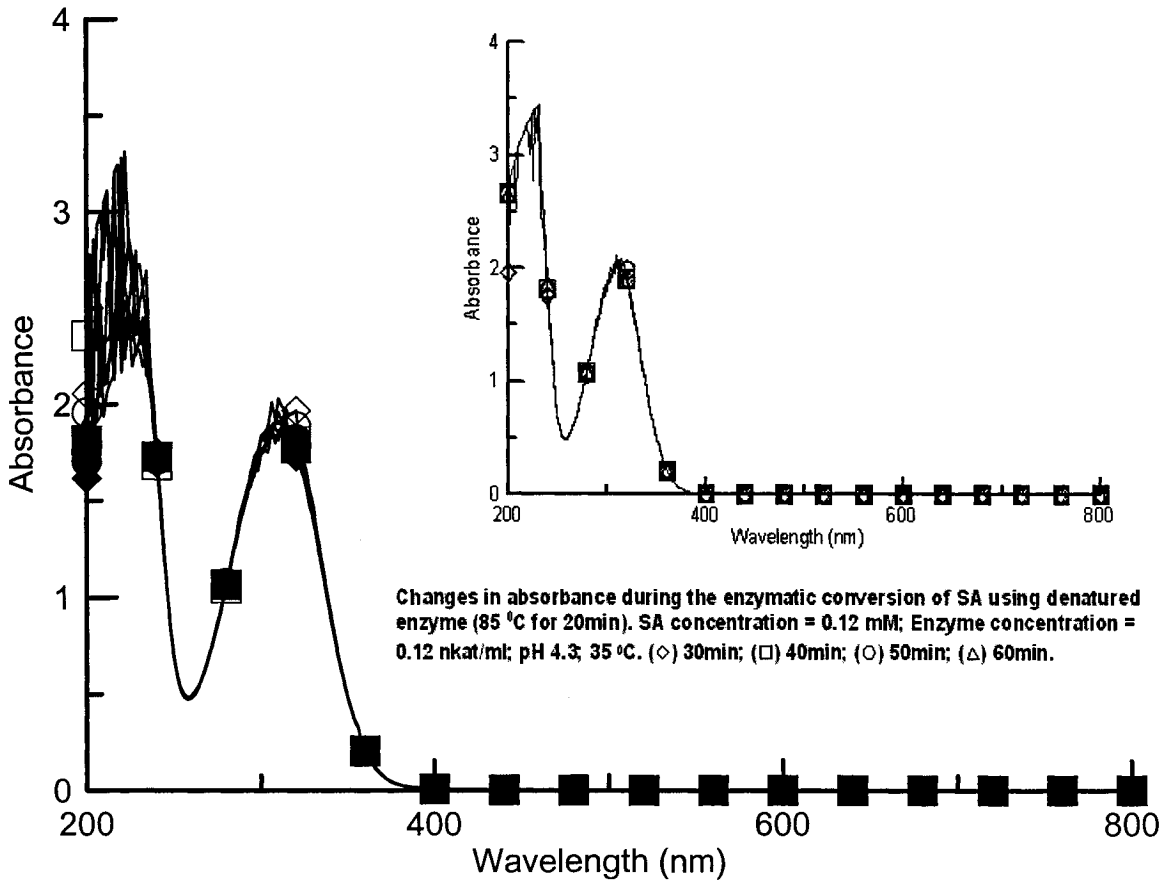


Figure C-13. Changes in absorbance during the enzymatic conversion of SA using denatured enzyme (85°C for 20 min). SA concentration 0.12 mM; enzyme concentration 0.12 nkat/mL; pH 4.3; 35°C. (◇) 0 min; (□) 2 min; (○) 4 min; (△) 6 min; (◆) 8 min; (■) 12 min; (●) 16 min; (▲) 20 min.

Appendix D

Experimental data used during the modelling of the enzymatic transformation of CGA

To model the enzymatic transformation of CGA, several experimental data were used in both the modelling of the simultaneous effect of pH, temperature, substrate and enzyme concentrations, and in the modelling of the dynamic of the CGA decrease. These experimental data are given from tables D-1 to D-11.

Table D-1 Effect of pH on enzyme activity at 25°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.072954	0.0267484353187247
3.03	0.895713	0.128010369025325
3.37	1.190231	0.124189163979793
3.83	1.54014	0.0267484353187135
4.3	1.827903	0.0897983185700068
4.77	1.953546	0.17577543209448
5.12	1.910314	0.0267484353187467
5.57	0.5764717	0.0510130873578556
6	0.479605	0.0248378327959588

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 25°C

Table D-2 Effect of pH on enzyme activity at 35°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.544453	0.00955301261382662
3.03	1.336139	0.185328444708304
3.37	1.455027	0.01337421765939
3.83	2.496648	0.141384586684694
4.3	2.803325	0.0210166277503354
4.77	2.237256	0.0955301261382964
5.12	1.032164	0.015284820182136
5.57	0.720083	0.0019106025227107
6.00	0.479605	0.0248378327959588

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 35°C

Table D-3 Effect of pH on enzyme activity at 45°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	1.575266	0.0420332555008504
3.03	2.021096	0.0687816908195739
3.37	2.588516	0.0649604857740233
3.83	3.12081	0.198702662367679
4.3	3.339672	0.076424100910613
4.77	3.280228	0.0878877160472289
5.12	3.25591	0.0802453059561628
5.57	1.750896	0.042033255500861
6.00	0.632268	0.0267484353187239

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 45°C

Table D-4 Effect of pH on enzyme activity at 55°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.965965	0.0248378327959633
3.03	1.885996	0.0191060252276649
3.37	2.534476	0.0840665110017009
3.83	2.970849	0.212076880027029
4.3	3.315354	0.240735917868525
4.77	3.453156	0.0534968706374603
5.12	2.758742	0.0534968706374769
5.57	1.927877	0.051586268114674
6.00	1.005144	0.122278561457026

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 55°C

Table D-5 Effect of pH on enzyme activity at 60°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.995687	0.0401226529780869
3.03	1.417199	0.0745134983878719
3.37	2.36425	0.00764241009107524
3.83	2.383164	0.0917089210927673
4.3	3.1470719	0.059228678205775
4.77	3.092439	0.059228678205745
5.12	2.807378	0.194881457322138
5.57	1.25643	0.118457356411494
6.00	0.772772	0.061139280728513

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 60°C

Table D-6 Effect of pH on enzyme activity at 65°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.191842	0.0191060252276605
3.03	1.187529	0.246467725436819
3.37	1.640114	0.141384586684685
3.83	1.95895	0.133742176593624
4.3	2.302104	0.152848201821284
4.77	2.443959	0.0897983185700068
5.12	2.139984	0.0573180756829636
5.57	1.750896	0.042033255500861
6.00	0.632268	0.0267484353187239

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 65°C

Table D-7 Effect of pH on enzyme activity at 70°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.017563	0.00573180756829817
3.03	0.132398	0.0191060252276605
3.37	1.6553803	0.00248378327955341
3.83	1.527981	0.0668710882968104
4.3	2.09405	0.0191060252276184
4.77	1.935983	0.0745134983878838
5.12	1.398285	0.0821559084789386
5.57	1.149701	0.128010369025327
6.00	0.494466	0.019106025227662

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 70°C

Table D-8 Effect of pH on enzyme activity at 75°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.018914	0.00382120504553207
3.03	0.1059184	0.0183417842185542
3.37	1.38143803	0.190811873948646
3.83	1.923824	0.1069937412749
4.3	2.172408	0.0496756655919265
4.77	1.699558	0.0764241009106362
5.12	0.940296	0.0802453059561738
5.57	0.426916	0.137563381639156
6.00	0.182385	0.00191060252276881

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 75°C

Table D-9 Effect of pH on enzyme activity at 80°C*

pH	Average enzyme activity (nkat/mL)	Standard deviation
2.65	0.017563	0.00573180756829817
3.03	0.132398	0.0191060252276605
3.37	1.6553803	0.00248378327955341
3.83	0.24318	0.00764241009106526
4.3	0.320187	0.00573180756830159
4.77	0.683606	0.110814946320432
5.12	0.335048	0.00764241009106072
5.57	0.036477	0.0133742176593624
6.00	0.494466	0.019106025227662

*CGA concentration 0.12 mM; enzyme concentration 0.18 nkat/mL; temp. 80°C

Table D-10 Effect of substrate concentration on the initial reaction rates*

CGA concentration (mM)	Initial reaction rates x 10^{-3} (mM/min)
0	0
0.0133	0.823705605477107
0.027	1.48694908001712
0.04	2.06461275139067
0.053	2.39088575096277
0.06702	3.03273427471117
0.08	3.02203679931536
0.093	3.40179717586649
0.1072	3.02738553701326
0.12	3.7280701754386
0.14	3.55691056910569

*Enzyme concentration 0.12 nkat/mL; pH 4.3; temp. 35°C

Table D-11 Dynamics of the CGA decrease at two initial CGA concentrations (0.13 and 0.07 mM)*

Time (min)	CGA concentration (mM)	CGA concentration (mM)
0	0.13	0.07
4	0.103	0.046
8	0.077	0.03
12	0.0505	0.0205
16	0.035	0.018
20	0.0255	0.014
30	0.018	0.0105
40	0.014	0.008
60	0.0105	0.0075

* Enzyme concentration 0.12 nkat/mL; pH 4.3; temp. 35°C