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INHIBITION OF TYROSINASE ACTIVITY

BY METALLOTHIONEIN FROM *Aspergillus niger*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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Montreal, Québec

June, 1999

SHORT TITLE

INHIBITION OF TYROSINASE BY COPPER METALLOTHIONEIN

ABSTRACT

Copper metallothionein (Cu-MT) was extracted from the induced biomass of *Aspergillus niger*. The crude extract (FI), obtained by cell homogenization, was partially purified by heat treatment (FII) and ultrafiltration (FIII). Further purification of the Cu-MT extract by affinity chromatography resulted in three major fractions, FIVa, FIVb and FIVc, of which fraction FIVc was considered to be the Cu-MT extract fraction. Fraction FIVc was re-chromatography on affinity chromatography and the eluted fraction showed a single peak (FIVc'). Spectrophotometric analysis of fraction FIVc' demonstrated a maximum absorption peak at 268 nm. Native and denatured electrophoretic analysis of fraction FIVc' showed the presence of a single band with an estimated molecular weight of 9.5 and 10.0 kDa, respectively. Inhibition of mushroom tyrosinase (PPO) by the Cu-MT extracts was investigated, using selected phenolic substrates, including catechin, chlorogenic acid, catechol, 4-methylcatechol, caffeic acid, L-3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, 3-(*p*-hydroxyphenyl) propionic acid, *p*-hydroxyphenylpyruvic acid, *p*- and *m*-cresol. The results showed that the inhibitory effect of the Cu-MT extract increased with the degree of purification. The results revealed that the Cu-MT extracts were effective inhibitors of PPO activity and the best inhibitory effect was demonstrated with catechin as substrate; however, PPO activity was not inhibited by the Cu-MT extract when *p*-hydroxyphenylpyruvic acid and *p*- and *m*-cresol were used as substrates. The results also showed that the Cu-MT extracts exhibited different types of inhibition, including mixed, competitive and uncompetitive on PPO activity. In addition, the experimental findings indicated that the nature and degree of enzymatic inhibitions by the Cu-MT extracts were dependent upon the structural nature of the substrates as well as the methods including, spectrophotometer and polarograph, used for the detection of enzyme activity.

RÉSUMÉ

Une metallothionéine contenant du cuivre (Cu-MT) a été extraite d'une biomasse induite d'*Aspergillus niger*. L'extrait brut (FI), obtenu par broyage des cellules, a été partiellement purifié par traitement thermique (FII) et ultrafiltration (FIII). La purification de l'extrait de Cu-MT par chromatographie d'affinité a donné trois fraction majeures FIVa, FIVb et FIVc, parmi lesquelles la fraction FIVc a été considérée comme étant la fraction de Cu-MT. La fraction FIVc a été resoumise à une chromatograhie d'affinité et la fraction purifiée n'a montré qu'un seul pic (FIVc'). Une analyse spectrophotométrique de la fraction FIVc' a démontré un pic d'absorption maximale à 268 nm. Des analyses d'electrophorèses native et en condition dénaturante ont montré la presence d'une seule bande avec un poids moleculaire estimé à 9,5 et 10,0 kDa, respectivement. L'inhibition de la tyrosinase de champignon (PPO) par les extraits de Cu-MT a été étudiée, en utilisant des substrats phénoliques sélectionnés, incluant la catéchine, l'acide chlorogénique, le catéchol, le 4-méthylcatéchol, l'acide caféique, la L-3,4-dihydroxyphénylalanine, l'acide 3,4-dihydroxyphénylacétique, l'acide 3-(*p*-hydroxyphényl)propionique, l'acide *p*-hydroxyphénylpyruvique et le *p*- et *m*-crésol. Les résultats ont aussi montré que l'effet inhibiteur de l'extrait de Cu-MT augmentait avec le degré de purification. Les résultats ont révélé que les extraits de Cu-MT étaient des inhibiteurs effectifs de l' activité PPO et le meilleur effet inhibiteur a été observé avec la catechine comme substrat; cependant, l'activité de la tyrosinase n'a pas été inhibée par les extraits de Cu-MT lorsque l'acide *p*-hydroxyphénylpyruvique, et le *p*- et *m*-crésol ont été utilisés comme substrat. Les résultats ont aussi indiqué que les extraits de Cu-MT ont montré différents types d'inhibition incluant des inhibitions mixte, compétitive et incompétitive de l'activité PPO. De plus, les résultats expérimentaux ont indiqué que la nature et le degré d'inhibition enzymatique était dépendant de la nature structurale des substrats aussi bien que des méthodes utilisées, comprenant le spectrophotomètre et le polarographe pour la détection de l'activité enzymatique.

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1. INTRODUCTION

Enzymatic browning in food is often undesirable phenomenon and represents a major problem in the food industry (Vamos-Vigyazo, 1981). Tyrosinase (PPO) is an enzyme responsible for the browning reaction in processed and injured fruits and vegetables (Whitaker, 1985). PPO catalyzes the hydroxylation of monophenols to diphenols and their subsequent oxidation to *o*-quinones which upon further oxidation, lead to polymerization and transformation into dark brown pigments (Walker, 1977; Rouet-Mayer *et al.*, 1993). Several approaches have been commonly used for the inhibition of enzymatic browning, particularly sulfiting agents (McEvily and Iyengar, 1992). However, the adverse health effects and lack of consumer acceptance of chemical additives as well as increased regulatory scrutiny (Mayer, 1987) have created the need for natural ingredients as alternatives to chemical agents.

PPO is a copper containing mono-oxygenase (EC 1.14.18.1) that contains one copper prosthetic group per subunit (Richardson and Hyslop, 1985). PPO activity can be inhibited by chelating agents which either bind to the copper located at the enzymatic active site or reduce the level of copper available for incorporation into the holoenzyme (McEvily and Iyengar, 1992). In addition, PPO activity may be inhibited by reducing agents such as cysteine which reduce the enzymatically formed quinones to stable, less colored *cis*-quinone adducts (Richard *et al.*, 1991).

Metallothioneins (MTs) are ubiquitous low molecular weight proteins (Nordberg and Kojima, 1979), which selectively bind certain metal ions including zinc, copper, mercury and cadmium (Byrd *et al.*, 1988). In addition, MTs exhibit strong reductive activities (Bremner, 1991). MTs have been reported in a broad range of organisms, including vertebrates, invertebrates, plants and microorganisms (Cherian and Chen, 1993). In particular, Cu-MT from fungi was reported to possess four copper atoms and eight cysteine residues in the form of Cu(I)-S4 centers (Bordas *et al.*, 1983). By chelating a copper at the active site of a copper-containing enzyme such as PPO, Cu-MT can act as an inhibitor. Lerch (1980) reported that a Cu-MT extract from *Neurospora crassa* acted as a chelating agent by incorporated copper ion into copper containing enzymes, such as

PPO. A low molecular weight copper-chelating peptide extract from *Dactylium dendroides* was also reported to inhibit mushroom PPO activity (Harel *et al.*, 1967). Goetghebeur and Kermasha (1996) demonstrated that a Cu-MT extract from *A. niger* inhibited the activity of mushroom tyrosinase with chlorogenic acid and catechin as substrates.

In previous research work in our laboratory, Kermasha *et al.* (1993c) established a procedure for the optimization of a biomass production of *A. niger* as well as the partial purification of a Cu-MT extract from *A. niger*. In addition, Goetghebeur *et al.* (1995) further purified and characterized a Cu-MT extract from *A. niger*.

This work is a part of ongoing research aimed at the development of a biotechnological approach for the inhibition of enzymatic browning. The specific objectives of this work were:

(1) To optimize the biomass production of *A. niger* and to promote the induction of Cu-MT.

(2) To purify and characterize the purified Cu-MT fractions in terms of the copper/protein mass ratio, molecular weight and electrophoretic profile.

(3) To investigate the nature and degree of inhibition of mushroom tyrosinase activity by the Cu-MT fractions, using a wide range of substrates.

(4) To characterize, spectrophotometrically, the tyrosinase-catalyzed end products, using selected substrates.

2. LITERATURE REVIEW

2.1. Enzymatic Browning

The occurrence of browning in raw fruits, vegetables and beverages is a major problem in the food industry and major cause of quality loss during post harvesting and processing (Mathew and Parpia, 1971). Browning can be enzymatic and nonenzymatic in origin (Richardson and Hyslop, 1985). Nonenzymatic browning results from the polymerization of endogenous phenolic compounds as well as from the Maillard reaction which occurs upon heating mixtures of amino acids and reducing sugars are heated (Walker, 1977). Enzymatic browning occurs as a result of the formation of brown or sometimes yellow, black or pink pigments by the action of a family of enzymes generally known as polyphenol oxidases (PPOs). PPOs catalyze the oxidation of phenolic compounds in the presence of oxygen to produce a brown color on cut surfaces of fruits and vegetables (Mayer, 1987; Mayer and Harel, 1991). However, in intact tissue, the endogenous phenolic substances are separated from PPO and browning does not occur (Richardson and Hyslop, 1985; Langdon, 1987).

So far, browning has been a problem for the food processor. However, not all cases of enzymatically-catalyzed browning are undesirable. In certain instances such as in the manufacture of tea, coffee, or cocoa, these reactions are essential to the manufacturing processes (Zawistoski *et al.*, 1991).

2.2.1. Chemistry of Enzymatic Browning

Enzymatic browning is dependent on the level of oxygen accessibility, reducing compounds and the type of phenolic substrates present in fruits and vegetables. In the process of enzymatic browning, PPOs hydroxylate monophenols to *o*-diphenols, which are then further oxidized to *o*-quinones (Fig. 1) (McEvily and Iyengar, 1992). The yellowish *o*-quinones are highly reactive and can polymerize spontaneously in the presence of O_2 to form complex high molecular weight compounds that are further converted into the pigment melanin (Vamos-Vigyazo, 1981). The *o*-quinones can also

react with endogenous aromatic amines and thiol compounds, including those found in proteins, to give a great variety of products, including high molecular weight protein polymers that enhance the brown colors produced (McEvily and Iyengar, 1992).

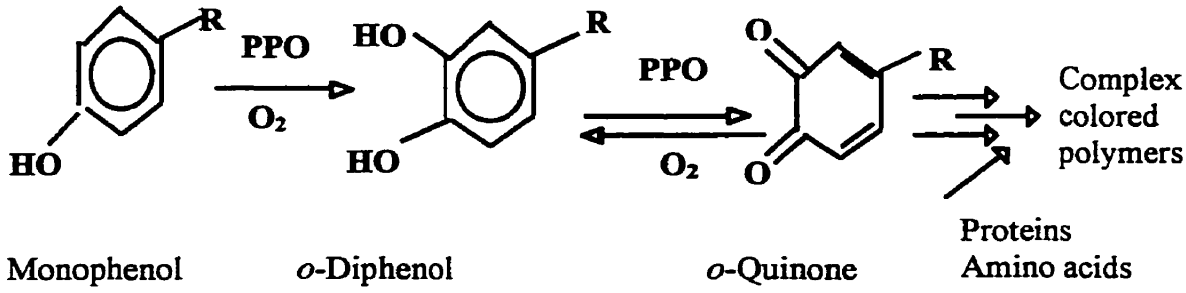


Figure 1. Schematic representation of enzymatic browning biocatalysis

2.1.2. Enzymes Involved in Enzymatic Browning

It is now generally accepted that enzymatic browning in plants and fruits is brought about by the action of an enzyme system variously known as catecholase, diphenol oxidase, phenolase, polyphenoloxidase or tyrosinase. PPOs is found in animals, plants and microorganisms. The role of PPO in animals is largely for protection, (for example, against pigmentation of skin), while the role of PPO in higher plants and microorganisms is not yet known with certainty. Intensive efforts to show that it is involved in photosynthesis and/or energy induction have not been obtained with much success to date (Whitaker, 1985).

PPO enzymes isolated from food sources are characterized as being oligomers and contain one copper prosthetic group per subunit (Walker, 1977; Richardson and Hyslop, 1985). The molecular weight of PPOs ranges from 30.0 to 130.0 kDa and the enzyme is found to be relatively heat liable. The optimum pH for PPO activity is between pH 5.0 and 7.0 (Sapers, 1993). Based on the substrate specificity, the Enzyme Commission has designated the PPO that include monophenol tyrosinase or cresolase (EC 1.14.18.1) and *o*-diphenolase or catecholase (EC. 1. 10.3.1) which catalyze monophenols and diphenols respectively, and laccase or *p*-diphenol oxidoreductase (EC 1.10.3.2) which catalyses the oxidation of *p*-diphenols into their corresponding quinones.

The presence of laccase has been mainly reported in many fungi and species of the genus *Rhus* (Japanese lacquer tree).

2.1.3. Substrate Specificity of Polyphenol Oxidase

Many workers have investigated the substrate specificity of PPO from different plant sources. Among the wide variety of phenolic compounds found in fruits and vegetables, only a small number serve as direct substrates for PPO. The most common natural substrates for the PPO reaction are caffeic acid derivatives and monomeric flavan-3-ols catechin and epicatechin, which often appear to be the best substrates. With respect to enzymatic-browning, the so-called 'catecholase' activity, the common natural substrate is chlorogenic acid (CHL) (Rivas and Whitaker, 1973). However, in the vast majority of cases CHL, caffeic acid (CF), CT, 3,4-dihydroxyphenyl propionic acid (HOPPA) and 4-methylcatechol (4-MCT) were found to be readily oxidized by PPO (Siddiq *et al.*, 1994).

2.1.4. Reaction Mechanism of Polyphenol Oxidase

PPO contains one copper pair, which is the site of interaction for both molecular O_2 and the organic substrate. To act on its substrates, the Cu^{2+} of the enzyme must first be reduced to Cu^{+} so that the enzyme can bind to O_2. The phenolic substrates bind only to the oxy-diphenol oxidase moiety and, as a result of this binding, hydroxylation of the monophenol or oxidation of the diphenol occurs (Zawistoski *et al.*, 1991).

Based on the information of the electronic and geometric structure of the binuclear copper complex and on the results regarding how different anion and organic ligands interact with the site, the following reaction mechanism (Fig. 2) for tyrosinase was proposed by Winkler *et al.* (1981). In Figure 2, both the hydroxylation reaction pathway (cresolase activity) and the oxidation reaction pathway (catecholase activity) are shown.

The catecholase activity involves the oxidation of two *o*-diphenols to two *o*-quinones with the concomitant 4e' reduction of molecular oxygen to two molecules of water. For this reaction, the binuclear copper site in met- and oxytyrosinase is

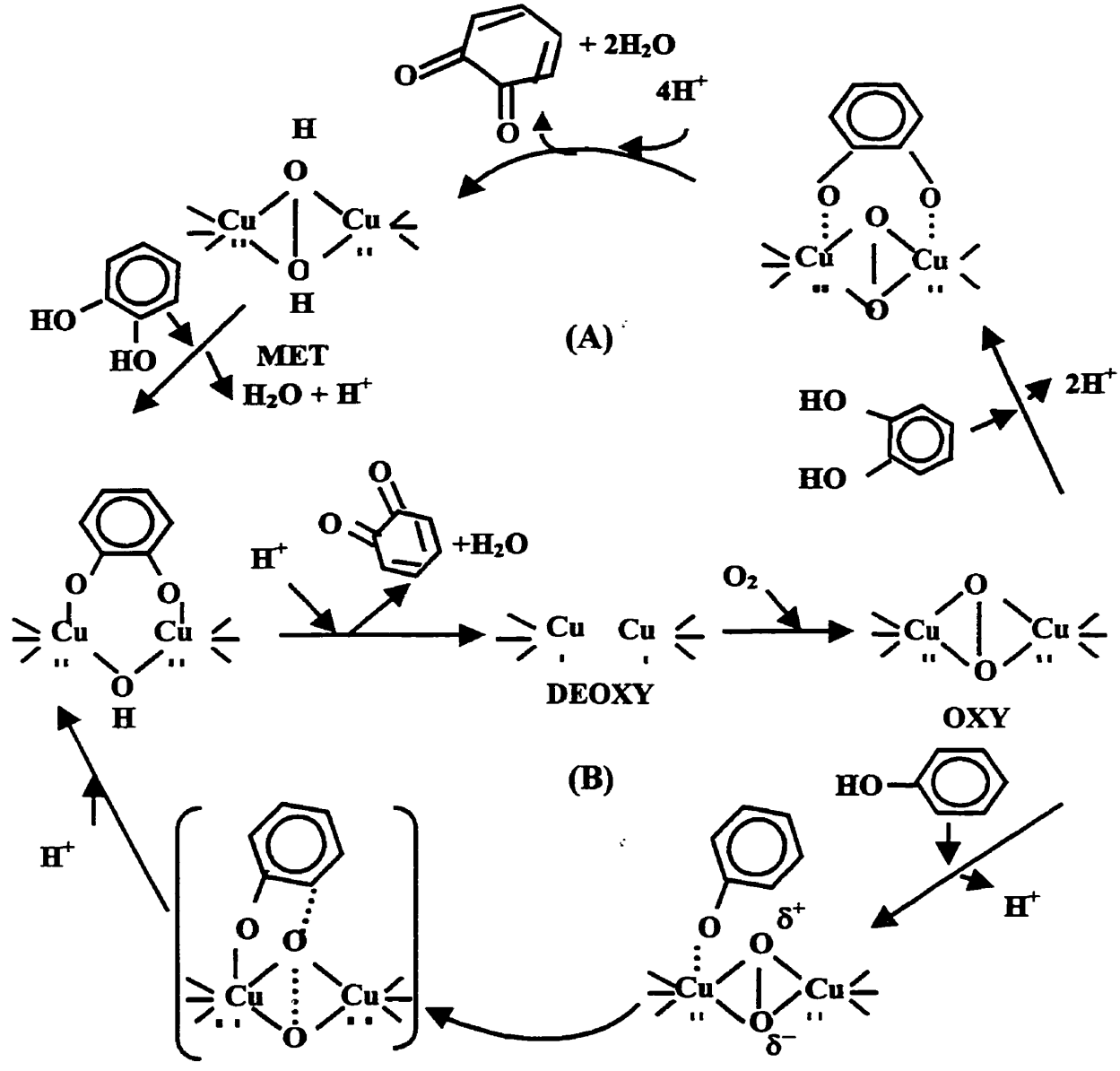


Figure 2. Reaction mechanism scheme of catecholase (A) and cresolase (B) activity of tyrosinase (Lerch, 1995).

geometrically corrected for axial coordination of both *o*-phenolic oxygen to the Cu(II) ions with a copper-copper distance of 3.6 Å. This interaction then allows for an efficient 2e' transfer from the *o*-diphenolic substance to the binuclear site. As shown in Figure 2A, the binding of an *o*-diphenol to mettyrosinase and the subsequent reduction of the bicupric cluster leads to deoxytyrosinase which becomes oxytyrosinase upon binding with molecular oxygen. The binding with a second *o*-diphenol to oxytyrosinase and subsequent reduction of the peroxide to water closes the cycle (Lerch, 1995).

For the cresolase activity (Fig. 2B), the coordination of a monophenol to one copper of the binuclear site of oxytyrosinase is followed by a rearrangement to a trigonal bipyramidal intermediate (shown in parentheses) as generally observed with square planar and tetragonal associate substitute chemistry. Coordination of the monophenol results in a deprotonated, polarized peroxidase, which is bound to only one copper. Such a species is expected to be highly reactive, hydroxylating the correctly positioned monophenol most likely via an electrophilic attack on the aromatic ring. The resulting *o*-diphenol is then oxidized from the equatorial position with the formation of an *o*-quinone and deoxytyrosinase (Lerch, 1987).

2.1.5. Assay of Tyrosinase Activity

There are several methods that have been developed for determining the rates of the enzymatic reaction. The most common methods are the polarographic (Mayer and Harel, 1991) and spectrophotometric (Cabanes *et al.*, 1987) methods.

The spectrophotometric method is based on the detection of colored end-products, generated from *o*-quinones or other intermediates of the melanin biosynthesis pathway as a results of the enzymatic reaction, measured at definite absorbance (Duckworth and Coleman, 1970). The advantage of this method is that it is simple and rapid but it also requires optically clear solutions. The diphenolase activity of pear PPO was assayed on several substrates such as CT, 4-MCT or CHL, by measuring in increase on absorbance at 420 nm (Vamos-Vigyazo, 1981; Zhou and Feng, 1991). Espin *et al.* (1995 and 1996) also

reported the use of the spectrophotometric method for the determination of mono- and diphenolase activities of mushroom PPO.

The polarographic or oxygen electrode method measures the oxygen uptake during the enzymatic reaction in the presence of enzyme and substrates. It measures the reaction continually from the beginning and the whole reaction may be completed in a few minutes. This method is especially suitable for kinetic studies using the pure enzyme as well as the crude extract (Wisseman and Montgomery, 1985) and does not require optically clear substrate solution for measuring oxygen uptake. The diphenolase activity of pear PPO was assayed, using catechol or chlorogenic acid as substrates by measuring oxygen consumption (Smith and Montgomery, 1985). Kermasha *et al.* (1993b) and Goetghebeur and Kermasha (1995) demonstrated the use of both spectrophotometric and polarographic methods in the determination of the inhibitory effect on tyrosinase activity with various substrates.

2.1.6. Principles for the Prevention of Enzymatic Browning

The principles involved in the production of browning in model system are essentially the same as those used to inhibit the enzyme activity in tissue. These include (a) inhibition/inactivation of the involved enzymes, (b) elimination/transformation of the substrate(s), and (c) a combination of (a) and (b).

In light of recent findings, it is, however, not easy to classify an inhibitor or an inhibitory process belonging exclusively to one of the three categories. Most of the inhibitors act on both the enzyme and its substrates. In general, the methodology for the prevention of enzymatic browning is based on the elimination of one or more essential components such as oxygen, enzyme, copper, or substrate from the reaction medium (Richardson and Hyslop, 1985). Moreover, in all the cases, the procedures employed require compatiblity with food safety and marketability.

2.1.7. Inhibition of Enzymatic Browning

The prevention of enzymatic browning has always been a challenge to food scientists owing to the loss of quality that it causes in many food products, e.g. fruits and vegetables during either storage or processing. A considerable effort has been devoted towards finding the means of preventing or reducing such enzymatic browning. In practice, three approaches have been adopted for the prevention of enzymatic browning. The approaches are physical methods, chemical inhibitors and inhibitors of microbial origin.

2.1.7.1. Use of Physical Methods

A marked decrease in enzymatic browning is induced by a reduction in temperature. However, as color change is still rapid at 0°C, a method of rapid freezing has been adopted. Once frozen, color change is slowed down drastically at the temperature of commercial storage (-18°C). Heat treatments that denature the enzyme are the most direct method of enzyme inactivation and are often employed to decrease the PPO activity responsible for browning. Usually a short exposure to temperatures between 70 and 90°C is sufficient to inactivate PPO activity; this consists of a brief immersion of the food product in water, boiling syrup or steam at approximately 100°C. However, steam blanching, which can effectively inhibit PPO, is used primarily for frozen and canned foods (McEvily and Iyengar, 1992) as it has some adverse effects on the flavor and texture of fresh produce (Langdon, 1987).

2.1.7.2. Use of Chemicals

Anti-browning agents such as acidifiers, chelators, reducing agents, and other chemical compounds can inhibit or control enzymatic browning by sterilely hindering the enzyme or binding to its active site thereby rendering it incapable of catalyzing the enzymatic reaction. Anti-browning agents may be also act on the enzymatically-catalyzed products to control browning. The primary reaction products of PPO catalysis are *o*-quinones, which are highly reactive. Using chemical means, they can either be reduced back to less reactive *o*-diphenols or trapped as colorless compounds thereby preventing

the occurrence nonenzymatic condensations to perceptible pigments (Sapers and Miller, 1992).

2.1.7.3. Use of Reducing Agents

Probably the most common method of controlling enzymatic browning, both in industry and the laboratory, is by the addition of reducing agents such as sulfur dioxide, sulfites, bisulfates, metabisulfates and/or ascorbic acid (Friedman and Bautista, 1985). These compounds prevent browning by reducing the enzymatically formed quinones back to their parent *o*-diphenols. In addition to acting as a reducing agent, metabisulfites can also react with the quinone intermediates to form sulfoquinones, which may irreversibly inhibit PPO activity (Zawistoski *et al*, 1991). Although sulfites are very effective in the inhibition of browning reactions, there are several negative attributes associated with their use in food. Sulfites are known to cause adverse health effects such as the cause of some forms of asthma (Iyengar and McEvily, 1992) and have been restricted or banned in several countries (Taylor, 1993).

2.1.7.4. Use of Thiol Compounds

Compared to other reducing agents, few studies have been carried out on sulfhydryl-containing reducing agents. In this category, the use of sulfur-containing amino acids such as L-cystine, L-cysteine and D,L-methionine (Cherry and Singh, 1990) and reduced glutathione (Molnar-Perl and Friedman, 1990) have been reported in controlling browning. The primary mode of action of sufhydral compounds in the prevention of enzymatic browning is to react with enzymatically-catalyzed *o*-quinones to produce stable, colorless addition compounds (Pierpoint, 1966), the structures of which have been elucidated (Sanada *et al.*, 1972; Richard *et al.*, 1991). However, some authors have indicated that cysteine reduces the *o*-quinones to their phenol precursors (Cilliers *et al.*, 1990). Kahn (1985) and Kermasha *et al.* (1993c) have also proposed the direct inhibition of PPO activity by cysteine through the formation of stable complexes with copper. In addition, the literature has reported on two major mechanisms for the inhibition of PPO activity by cysteine, the first as a direct inhibitor of PPO (Kahn, 1985)

and the second consisting of the formation of colorless *cis*-quinone products (Walker, 1977).

2.1.7.4. Use of Chelating Agents

As mentioned previously, PPO contains copper at its active site. In the context of PPO catalyzed browning reaction, chelating agents such as EDTA, phosphates, and citric acid are reported to either bind to the copper at the active site of PPO or reduce the level of copper available for incorporation into the holoenzyme (McEvily and Iyengar, 1992). However, chelating agents can only slow down the enzymatic reaction but do not completely inhibit it.

2.1.7.5. Use of Acidulants

Since optimum enzyme activity depends on pH, changing the pH of the environment can control the activity of PPOs. The pH optimum of PPO activity varies depending on the source of enzyme and the particular substrate, but in most cases, PPO has an optimum pH in the range of pH 6.0 to 7.0 (Aylward and Haisman, 1969). Acidifiers such as citric, malic, and phosphoric acids lower the pH of the system to below 3.0 where PPO is effectively inactive (Richardson and Hyslop, 1985). However, it is not always possible to lower the pH of fresh foods due to the significant acid flavor that prevails. Citric acid is widely used in the food industry for browning prevention. It may have a dual inhibitory effect on PPO by reducing the pH of the environment and by chelating the copper at the enzyme-active site.

2.1.7.6. Use of Enzyme Inhibitors

There are numerous reports on specific PPO inhibitors. Different modes of action have been proposed for the inhibitors, which react directly with PPO. Mayer and Harel (1991) described two types; the first group consists of compounds that interact directly with copper while the second is composed of those affecting the active site of interaction used by the phenolic substrates.

In the first group, inhibition by metal ion chelators such as azide (Zawistowski *et al.*, 1988), cyanide (Duckworth and Coleman, 1970), carbon monoxide and sodium diethyldithiocarbamate (Healey and Strothkamp, 1981) which are more or less copper specific, is well documented for PPOs from different sources. Similarly inorganic halides have also been reported to be inhibitors of PPO activity (McEvily and Iyenga, 1992). The order of decreasing inhibitory power of halides has been reported to be F>Cl>Br>I (Sharon and Mayer, 1967).

Among inhibitors belonging to the second group, aromatic carboxylic acids of the benzoic and cinnamic series have been widely studied (Kuttner and Wagreich, 1953). These compounds act as competitive inhibitors of PPO activity because of their structural similarities with phenolic substrates. However, some authors indicated that this type of inhibition was dependent on the substrate used for the assay and could be either competitive, noncompetitive or mixed (Menon *et al.*, 1990).

2.1.7.7. Use of Complexing Agent

Compounds that bind or complex PPO substrates may also have potential value as browning inhibitors. Polyvinylpolyprollidone (PVPP) can bind PPOs and prevent their participation in enzymatic browning reactions (Van Buren *et al.*, 1976). Similarly, cyclodextrins can form inclusion complexes with polyphenol substrates of PPO. Addition of soluble cyclodextrins to juices or filtration of juices using insoluble cyclodextrin columns can prevent browning (Sapers and Miller, 1992). Borates have also been shown to inhibit apple browning by forming complexes with polyphenols at the *o*-dihydroxy groups, thereby preventing their enzymatic oxidation (Bedrosian *et al.*, 1960).

2.1.7.9. Use of Inhibitors of Microbial Origin

Some studies have been devoted to the use of natural inhibitors for prevention of enzymatic browning. Proteins, peptides or amino acids can affect PPO-catalyzed browning by direct inhibition of the enzyme and/or by reaction with the quinone products of PPO catalysis. Khan (1985) studied the effect of proteins, protein hydrolyzates and

amino acids on the activity of mushroom, avocado and banana PPOs using L-DOPA or 4-methylcatechol as substrate.

Kojic acid (5-hydroxy-2-hydroxymethyl-*r*-pyrone), a metabolite produced by several species of *Aspergillus* and *Penicillium* (McEvily and Iyengar, 1992), is a reducing agent and antioxidant which has also been shown to be a fungal PPO inhibitor (Saruno *et al.*, 1979). Kojic acid was found to inhibit browning by interfering with oxygen uptake and by reducing *o*-quinones to diphenols thereby preventing pigment formation (Chen *et al.*, 1991a). It was reported to be a competitive inhibitor for the oxidation of chlorogenic acid and catechol by apple PPO activity and also showed a mixed type inhibition of PPO activity in oxygen uptake studies (Chen *et al.*, 1991b).

A low molecular weight, copper-chelating peptide from *Dactylium dendroides* was reported to inhibit apple PPO and mushroom tyrosinase activity (Harel *et al.*, 1967). Under conditions of high exposure, induced metallothioneins were also reported to be highly effective de-metallizing agents because of their strong avidity for the metal ion (Hamer, 1986). Since PPO contains copper at it active site, Goetghebeur *et al.* (1995) reported that a Cu-MT extract isolated from *A. niger* can be used as a perspective inhibitor for this enzyme activity; however, these authors also reported that the degree of inhibition of mushroom PPO activity by the purified Cu-MT extract varied with respect to the phenolic substrates used.

2.2. Metallothionein

Metallothioneins (MTs) are ubiquitous low molecular weight cystosolic proteins that are usually characterized by a high cysteine content and absence of aromatic acids, and are reported to bind both essential (e.g., $Zn^{2+}$and Cu^{2+}) and non essential metals (e.g., Cd^{2+} and Hg^{2+}). Recently, these proteins have gained special importance in diverse scientific disciplines like toxicology, environmental health, medicine, pathology, biochemistry, nutrition and lately in molecular biology, radio-immunology, and molecular genetics due to their diverse biological role (Nordberg and Kojima, 1979).

2.2.1. Definition of Metallothionein

Historically, the term MT was introduced to designate a cadmium-, zinc-, and copper-containing sulfur-rich protein obtained from equine renal cortex (Kägi and Vallee, 1960). However, due to increase interest in this field, confusion arose with the use of different terminology by various authors. At the first international meeting on the MT and other low molecular weight protein Kägi and Kojima (1979) proposed that the term "metallothionein" should be used in a broader sense to include low molecular weight proteins possessing an extraordinarily high content of metals and cysteine. In addition, the terms "multiple forms of MT or iso-MTs" should be used as broad terms covering all MTs occurring naturally in a single species. These terms should apply only to forms of MT arising from genetically determined differences in primary structure and not to forms that differ only in metal composition or are derived by modification of the same primary sequence. In general, the nomenclature of MTs genes reflect the multiple forms of MTs in which the isolation and sequence of the iso-MTs encoding genes, precede the identification, isolation, and sequencing of the proteins.

2.2.2. Sources of Metallothionein

MTs are widely distributed in a large variety of organisms including vertebrates, invertebrates, plants and microorganisms (Nordberg and Kojima, 1979). The presence of MTs was reported in the liver and kidneys, primary sites of heavy metal accumulation, of sheep (Bremner *et al.*, 1977), monkeys (Koizumi *et al.*, 1985), rats (Winge *et al.*, 1975), cows (Hartmann and Weser, 1985), and california sea lion (Lee *et al.*, 1977). Apart from mammalian species, similar proteins have been isolated from lower vertebrates such as carp (Kito *et al.*, 1982), rainbow trout (Price-Huaghey *et al.*, 1987), plaice (Overnell *et al.*, 1981), and winter flounder (Shears and Fletcher, 1985). Microbial sources of MTs have also been reported such as *Neurospora crassa* (Lerch, 1980), *Agaricus bisporus* (Münger and Lerch, 1985), *Saccharomyces cervisie* (Butt and Ecker, 1987) and *Aspergillus niger* (Kermasha *et al.*, 1993c).

2.2.3. Classes of Metallothionein

MTs isolated from different sources have frequently been shown to consist of a number of iso-proteins called iso-MTs, which can be separated from each other due to differences in their charge. With the exception of charge, iso-MTs have similar properties (Webb, 1979). The international nomenclature committee on MT recommended that MTs be divided into three classes (Flower, 1987).

Class I of MTs is found in animal sources and consists of 59-63 amino acids, 20 of which are cysteines, held in invariant positions within a β-domain protein sequence. Class II represents the MTs which exist in yeast (Winge *et al.*, 1985) and are also rich in cysteines, but have a distant homology in their amino acid sequence in comparison to those of Class I. Class III are metal-thiolate polypeptides occurring exclusively in plants and little is known about the genetics of this class (Lin *et al.*, 1990).

Class I and II MTs have been studied extensively at the generic and protein levels. A close examination reveals that either a single gene or two isogenes, depending on the species or organism, codes for them. In fungi including *N. crassa* (Lerch, 1980), *S. cerevisiae* (Butt and Ecker, 1987) and *A. bisporus* (Münger and Lerch, 1985), only unique sequences can be detected; these sequences are either re-arranged or truncated when compared to those found in mammals. Although the sequence in fungi is unique, it may be present in single or multiple copies in response to genetic manipulations.

2.2.4. Function of Metallothionein

The biological functions or roles of MT have been focused on the regulation of heavy metals in relation to the detoxification of harmful heavy metal ions and the homeostatic regulation of essential metal ions (Ravi *et al.*, 1984). Recently, attention has been devoted to its radical scavenging ability and also on its possible participation in cell differentiation and/or proliferation in relation to its localization in the nucleus (Thornally and Vasak, 1985). It has been reported that the level of resistance cells to heavy metal toxicity is proportional to the cellular concentration of hepatoma MT (Freedman and Peisach 1989). Huibregtse *et al.* (1989) also indicated that the yeast *S. cerevisiae* encodes

a MT-like protein to consume copper in order to avoid copper-induced damage. In addition, Bremner (1991) indicated that an increase in tissue copper concentration suggested that MTs have a role in the cellular detoxification or storage of metals; the same author also suggested that the elevated concentration of MTs in the liver of fetal and neonatal animals might indicate that these proteins are a storage reserve for copper. On the other hand, Lerch (1980) suggested that copper MT might serve as a metal donor to the active site of copper-containing enzymes. However, Hamer (1986) state that a detoxification role for MTs might be triggered by the presence of a highly toxic metals while a storage or metal donor role might be induced when the metal is likely to be used in important or essential enzymatic reactions.

2.2.5. Characteristics of Metallothionein

2.2.5.1. Amino Acid Composition

One of the most important characteristic features of MTs is its amino acid composition. Analyses, carried out so far on different animal species, reveal that MTs are characterized by high contents of cysteine (22-30%), serine (7-17.5%), lysine (10-15%) and a complete lack of aromatic amino acids such as tyrosine and tryptophan (Nath *et al.*, 1988).

2.2.5.2 Molecular Weight

The molecular weight of mammalian MTs has been found to be approximately 10,000 to 12,000 Dalton using globular protein as standard. Under denaturing conditions, the molecular weight of the apoprotein was reported to be around 6000 Dalton (Kägi and Nordberg, 1979). Invertebrate MTs were reported to have a lower molecular weight than those reported from mammalian sources. A zinc-binding protein from sea urchin eggs, possessing most of the characteristics of MTs, was reported to have a molecular weight of 3655 (Ohtake *et al.*, 1983) while a copper-binding MT like protein from *N. crassa* showed a molecular weight of 2220 Dalton (Lerch, 1980).

2.2.5.3 Structure

The structure of MTs from different sources is shown in Figure 2. The amino acid sequence analysis of MTs from various sources established that all the mammalian MTs have a chain length of a total of 60 to 62 amino acids of which 20 were cysteine (cys) residues. All these cysteine residues are involved in collectively binding seven bivalent metal ions in two separate metal-thiolate oligo-nuclear clusters (M_3Cys_9 and M_4Cys_{11}) buried as mineral cores in the interior of the two globular domains formed by the carboxyl-(β-domain) and the amino-terminal (α-domain) halves of the polypeptide chain (Vasak, 1991). Two other structural motifs have been identified for mono-valent metals ($M_{12}Cys_{20}$ and $M_{18}Cys_{20}$). Mammalian MTs occur in two principal iso-forms (conventionally labeled as MT-I and MT-II based on their order of elusion from a DEAE-Sephadex column) although several sub-isoforms have also been reported which have been separated by HPLC (Vasak, 1991; Kägi, 1991; Suzuki, 1991).

The Cu-MT extracts isolated from the mold *N. crassa* and the common mushroom *A. bisporus*, are both built up of 25 amino acid residues and reveal a striking homology in their primary structure to the amino terminal part of mammalian MTs; they both contain 6 atoms of copper/molecule, which are bound to the 7-cysteinyl residues in the form of a single Cu(I)-thiolate cluster (Beltramini and Lerch, 1983; Münger and Lerch, 1985).

2.2.5.4. Spectroscopic Properties

Spectroscopic studies have established that MTs have a well-defined tertiary structure. The involvement of sulfhydryl groups in metal binding of MTs is long known, but the coordination geometry of the binding sites has been only recently worked out. The analysis of the amino acid composition of MTs from various sources has revealed that it lacks aromatic acids, demonstrated by a minimal UV absorption at 280 nm. However, binding of metal ions to the apoprotein-thionein leads to a UV spectrum profile that is characteristic for that particular metal. Cadmium-thionein (Cd-thionein) has an absorption maximum at 254 nm, while zinc-thionein (Zn-thionein) exhibits a maximum absorption at 215 nm (Kägi and Vallee, 1961).

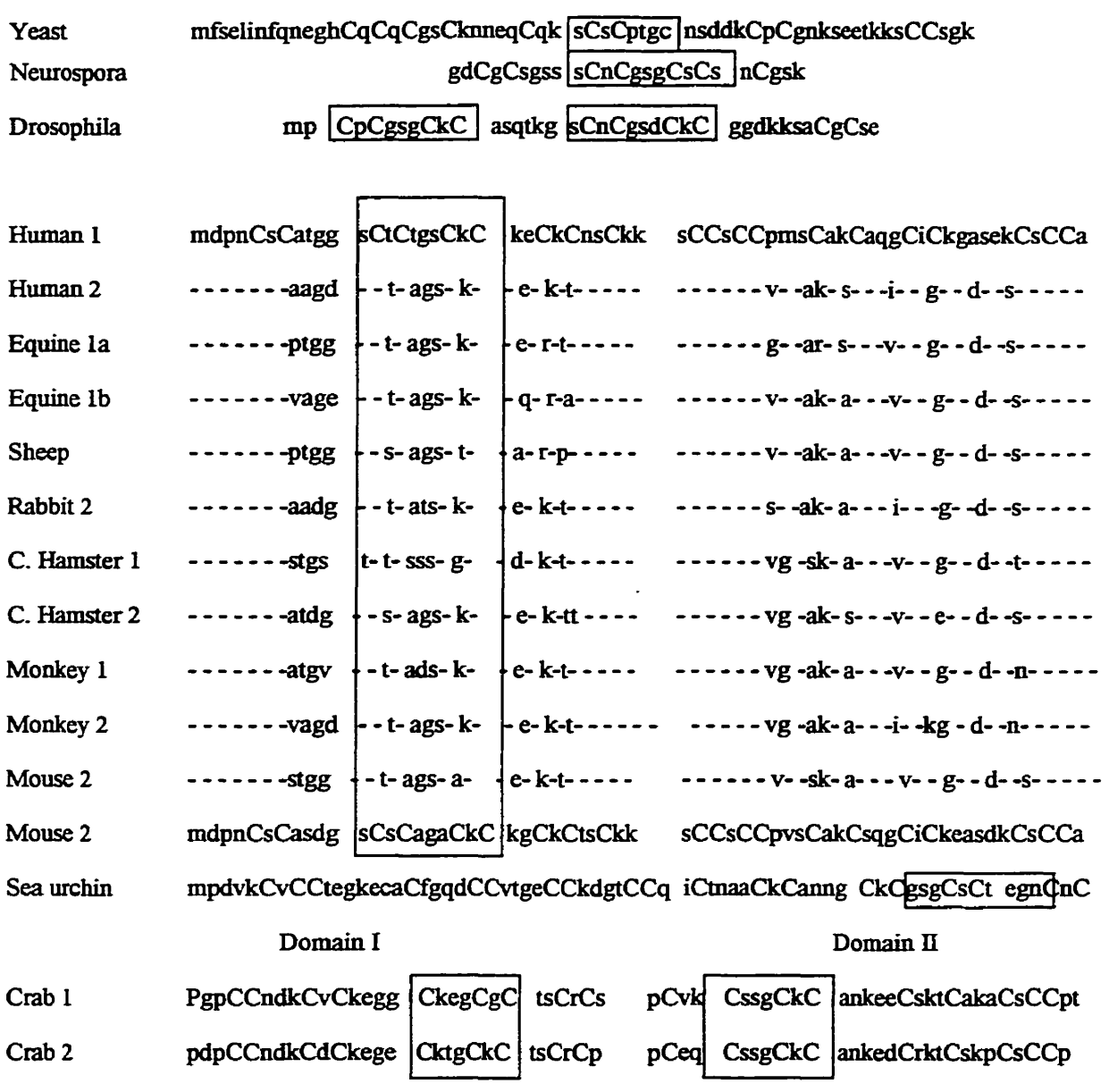


Figure 3. Conservation of the central segment, a model for evolution of the MT gene. Other sequences are: Neurospora MT, Yeast MT, and Drosophila MT. Human MT1 and MT2, Equine MT1a and MT1b, Sheep MT1, Rabbit MT2, Chinese hamster MT1 and MT2. Monkey MT1 and MT2, Mouse MT1 and MT2, Sea urchin, crab MT1 and MT2. The central segment, described in the text, is enclosed in a box. One-letter code a, alanine; C, cysteine; d, aspertic acid; e, glutamic acid; f, phenylalanine; g, glycine; h, histidine; i, isoleucine; k, lysine; l, leucine; m, methionine; n, asparagine; p, proline; q, glutamine; r, arginine; s, serine; t, threonine; v, valine (note only cysteine is represented by an uppercase letter for emphasis). (Nemer *et al.*, 1985).

Mercury-thionein (Hg-thionein) from rat liver showed a shoulder peak at 270 nm (Sokolowski and Weser, 1974), while copper-thionein (Cu-thionein) from yeast exhibited a shoulder peak at 275 nm, which tailed off into the visible region (Prinze and Weser, 1975). Similar spectra were observed for Cu-Zn-thionein from pig liver (Bremner, 1991) and monomeric fetal mitochondrocupein (Hartmann and Weser, 1985). However, it was also observed by Law and Stillman (1980) and later by Paliwal *et al.* (1986) that the acidification of Cd-thionein gradually results in a concomitant decrease in the UV absorption of rat and monkey liver MT at 250 and 253 nm, respectively. The absorption spectra of native and fully protonated *Neurospora* Cu-MT showed that binding of copper increases the absorption of MT in the near and far UV regions and introduces the characteristic shoulder peak at 250 nm.

2.2.6. Purification of Metallothionein

MT continues to generate a great deal of interest among investigators because of the many unique characteristics exhibited by this family of metal-binding proteins including the putative role of MT in the intracellular metabolism of heavy metals. Consequently, there is an on-going need for new analytical techniques applicable for the isolation and quantification of MT that are both rapid and sensitive.

A two-step, low-pressure, column chromatographic procedure, generally involving a combination of gel-permeation and anion-exchange modes, has been widely used to isolate MTs. In a typical chromatographic separation procedure, the tissue is homogenized in a suitable buffer and the recovered cytosolic fraction, obtained by centrifugation, is separated on a Sephadex G-75 column to give metal containing fractions (Bremner and Davies, 1975; Lin *et al.*, 1990). Weser and Hartmann (1991) reported the use of gel chromatography (Sephadex G-50) for the purification of copper-MT from the yeast *S. cerevisiae*. Lerch (1991) investigated a reverse phase high performance liquid chromatography procedure to purify of a *N. crassa* Cu-MT extract. Reversed-phase high performance liquid chromatography in direct combination with atomic absorbance spectrometry was also used by Beek and Baars (1988) to separate and quantified Zn-, Cd-, and Cu-MT from horse kidney and rabbit liver. McCormic and Lin

(1991) employed reverse-phase liquid chromatography and electrospray ionization mass spectrometry to identify different proteins including copper-MT directly from a sample mixture. Copper-MT was also purified from an *A. niger* extract by successive size-exclusion and ion-exchange chromatography (Kermasha *et al.*, 1993c). Goetghebeur *et al.* (1996) reported that affinity chromatography was a rapid and efficient method for the purification of copper-MT.

2.2.7. Copper-Metallothionein

In contrast to mammalian MTs, which binds various metals fungal MTs most often contain copper only (Lerch, 1980). Copper-metallothioneins have been isolated from the fungal strains of *S. cerevisiae* (Winge *et al.*, 1985), *N. crassa* (Lerch, 1991) and *A. bisporus* (Münger and Lerch, 1985). El-Meleigy (1992) reported the presence of a low molecular mass copper-chelator in the mycelia of both *A. fumigatus* and *Penicillium chrysogenum*. A Cu-MT extract was also recently isolated from *A. niger* (Kermasha *et al.*, 1993c, 1995).

The structure of the mammalian Cd/Zn-MT has been elucidated, whereas structural information on the copper molecules is less complete. Cu-MT is composed of only seven different amino acids with a strikingly high content of cysteine (28%) serine (28%), and glycine (24%). The amino acids composition consists of asparagine, 2.9; serine 6.9; glycine 6.0; alanine 1.0; cysteine 6.9 (determined as carboxymethylcysteine); lysine, 1.0. The MT protein binds a total of 6.0 g atoms of copper per molecular weight of 2,200 Dalton (Lerch, 1980). Friedman *et al.* (1970) determined that the primary structure of *Neurospora* copper-MT showed an amino acid sequence that was strikingly similar to those of Cd/Zn-MT from man, horse, and mice (Kojima *et al.*, 1979). Moreover, the same authors reported that the cysteinyl residues of the *Neurospora* copper-binding protein occupied exactly the same position as the first seven-cysteinyl residues in the amino-terminal sequence region of the Zn/Cd-MTs; in addition, the serine residue 12 was invariant in all four MT protein sources and there was a complete lack of aromatic amino acids (Kägi and Kojima, 1979).

2.2.8. Induction of Cu-metallothionein

Piscator (1964) first developed the concept that MT is an inducible protein and demonstrated that metals could be used to induce it. Klaassen and Lehman-Mckeeman (1989) established that various organic and inorganic chemical compounds and physiological conditions can also be used to induce MT synthesis but heavy metals remain the most effective inducers. It was also observed that labeled amino acids were incorporated in MT when protein synthesis of Cu-MT was induced by cadmium, zinc, and copper (Webb, 1979). Copper-induced thionein was initially named copper-cheletin (Winge *et al.*, 1975) and later renamed copper-thionein (Bremner, 1983).

Alonso and Martin-Mateo (1996) showed that MT has a greater affinity for copper compared to zinc. The same authors also observed that copper induces protein synthesis better than zinc. In addition, they also found that the gonad organ, which was treated with both metals, showed a lower total protein concentration in the zinc treated organ than that observed in the copper treated organ. In fact, the affinity for metals for the binding sites of mammalian MTs follows the order of: Zn(II) < Cd(II) < Cu(I), Ag(I) < Hg(II), Bi(III) (Kägi and Kojima, 1987). The difference in affinity could explain the occurrence of a faster transfer rate of copper compare to zinc in the presence of moderate MT mucosa levels (Oestreicher and Cousins, 1985).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Magnesium chloride ($MnCl_2.H_2O$) cupric sulfate ($CuSO_4.5H_2O$), sodium hydroxide (NaOH), hydrochloric acid (HCl), and citric acid were purchased from Fisher Scientific (Fair Lawn, NJ) while zinc sulfate ($ZnSO_4.7H_2O$) was obtained from BDH Inc. (Darmstadt, Germany). Phenolic reagent, calcium chloride ($CaCl_2.4H_2O$), ferrous sulfate ($FeSO_4.7H_2O$), magnesium sulfate ($MgSO_4.7H_2O$), ammonium nitrate (NH_4NO_3), Tween 20 (polyoxyethylene-sorbitan monolaurate), sodium phosphate monobasic and dibasic, citric acid and D-glucose were obtained from ACP Chemicals Inc (Montreal Qc). Filter membranes and chromatography columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

3.1.2. Enzymes and Substrates

Commercially purified mushroom tyrosinase and bovine serum albumin were obtained from Sigma Chemical Co. (St-Louis, MO). Substrates catechol (CT), 4-methyl catechol (4-MCT), caffeic acid (CF), catechin (CH), chlorogenic acid (CHL), 3,4-dihydroxyphenylpyruvic acid (HOPA), 3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAA), 3-(*p*-hydroxyphenyl)-propionic acid (HOPPA) were also purchased from Sigma Chemical Co.; *m*-cresol was obtained from Aldrich Chemical Co. (Milwaukee, WI) and *p*-cresol from ICN Biomedicals Inc. (Aurora, OH).

3.2. Methods

3.2.1. Culture Media

3.2.1.1. Potato Dextrose Medium

Potato dextrose agar (Fisher Scientific Co.) was prepared by dissolving 50 g in 1 liter of distilled water, followed by autoclaving at 115°C for 20 min; the agar medium was subsequently cooled to approximately 60°C, and poured into petri dishes and test tubes (to make slants) and then stored at 4°C until required.

3.2.1.2. Modified Watson and Smith Medium

The biomass culture of *A. niger* was grown for three days on modified Watson and Smith medium according to the procedure described by Kermasha *et al.* (1993c). The medium consisted of a mixture of D-glucose (50.0 g/l), NH_4NO_3 (3.0 g/l), K_2HPO_4 (2.0 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $FeSO_4.7H_2O$ (0.012 g/l), $ZnSO_4.7H_2O$ (0.016 g/l), $CuSO_4.5H_2O$ (0.002 g/l), $MnCl_2.H_2O$ (0.005 g/l), and $CaCl_2.4H_2O$ (0.005 g/l) which was dissolved in 1liter of distilled water. The pH of the medium was adjusted to 4.5 with 1N HCl prior to sterilization by autoclaving at 115°C for 20 min.

3.3. Microbial Source

The fungal strain *A. niger* (CBS 132.52) used in this study was obtained from the Central bureau voor Schimmelcultures, Baarn, Netherlands. The culture was maintained at 4°C on potato dextrose agar slants and sub-cultured every three to four weeks.

3.4. Biomass Production

3.4.1. Preparation of Inoculum

Cultures of *A. niger* were incubated on potato dextrose agar slants for 3 days at 28°C and were always freshly prepared from refrigerated stock. A suspension of spores was made from the slant and $1x10^6$ spores/ml were aseptically transferred into 100 ml of the modified Watson and Smith medium contained in a 250 ml Erlenmeyer flask. The flasks were then incubated at 28°C for 60 hours on a rotatory shaker at 110 rpm.

3.4.2. Shake Flask Culture

The shake flask culture was carried out in a 2 liters conical flask containing 600 ml of the Watson and Smith medium and 5 % (v/v) inoculum. Flasks were incubated at 30°C on an orbital shaker (110 rpm) for 72 hours.

3.4.3. Large Scale Biomass Production

The large-scale biomass production was performed according to a modification of the procedure described by Kermasha *et al.* (1993c). The fungus was grown in a 150 liter fermentor (Bioengineering AG, Switzerland) equipped with an agitation and aeration system and a working volume of 100 liter. The modified Watson and Smith medium was used as the culture incubation medium. A constant agitation of 110 rpm was used and sterile air (5 % air/min) was passed through a sterile millipore filter to the fermentor. The culture was maintained at a temperature of 28°C and allowed to grow for 72 hours to reach the stationary phase.

3.4.4. Induction of Culture and Harvesting

The culture of *A. niger* was induced with copper according to the procedure described by Kermasha *et al.* (1993c). The 72-hour incubated mycelia were collected by filtration through a cheesecloth and transferred into fresh Watson and Smith medium containing 6.0 and 25.0 g/l of $CuSo_4.5H_20$ and D-glucose, respectively. The same culture conditions and procedure were maintained as those used for biomass production. To complete induction, the mycelia were allowed to incubate for 5 hours under these conditions. The mycelia were then separated from the culture medium by filtration through a cheesecloth. The collected biomass was washed twice with deionized water followed by Tris-HCl buffer solution (10 mM, pH 8.0), freeze-dried and stored at -80°C.

3.5. Extraction of Copper-Metallothionein

The extraction of copper-metallothionein (Cu-MT) from the biomass was performed according to the procedure developed by Kermasha *et al.* (1993c). The lyophilized mycelia were suspended in Tris-HCl buffer solution (10 mM, pH 8.0) and homogenized (1:4, w/v) for 2 min by a three-cycle treatment using a MSK cell homogenizer (Braun, Melsungen, Germany). The temperature of the homogenization medium was maintained at 4°C by a gentle flow of liquid CO_2. The homogenized suspension was then centrifuged at 20,000 x *g* for 30 min. The pellet was discarded while the resulting supernatant, considered to be the crude extract (FI), was collected and lyophilized.

3.6. Partial Purification of Copper-Metallothionein

3.6.1. Heat Treatment

Partial purification of the crude Cu-MT extract (FI) was performed according to the procedure outlined by Kermasha *et al.* (1993c). The crude extract was diluted with Tris-HCl buffer solution (10 mM, pH 8.0) to a protein concentration of 113 mg/ml. The suspension was heated for 10 min at 60°C and then allowed to cool at room temperature. The precipitate, consisting of heat-labile high-molecular weight proteins, was removed by centrifugation at 50,000 x *g* for 10 min at 4°C using an Avanti-J25I centrifuge (Beckman Instrument, San Ramon, CA) while the supernatant was lyophilized for further purification and characterization. The lyophilized supernatant was referred to as the partially purified fraction (FII).

3.6.2. Ultrafiltration

In this purification process, the free copper was removed from the partially purified fraction FII by the procedure described by Goetghebeur *et al.* (1995). The partially purified fraction FII was re-dissolved in sodium phosphate buffer solution (10 mM, pH 7.2) and filtered through a 0.20 μm membrane (Acrodise, VWR Scientific, Plainfield, NJ). The filtrate was further subjected to diafiltration using an ultrafiltration cell unit (Amicon, Denver, MA), equipped with a Diaflo membrane YM3 (3 kDa cut-off) and nitrogen pressure of 42 psi. The recovered protein fraction FIII was freeze-dried and stored at -80°C.

3.7. Purification by Affinity Chromatography

Affinity chromatography was performed using a 1.6 ml chelating Superose HR 10/2 column (Pharmacia) which contained copper ions. An automated fast protein liquid chromatography system (FPLC, Pharmacia) was also used. According to the purification process described by Goetghebeur *et al.* (1995), the partially purified fraction FIII was re-dissolved in sodium phosphate buffer solution (20 mM, pH 7.2) at a protein concentration of 2.0 mg protein/ml, and filtered through a 0.20 μm membrane. The column was equilibrated with buffer A consisting of sodium phosphate buffer (20 mM, pH 7.2)

containing 1 M NaCl. A sample volume of 200 µl was injected into the column through a superloop using a Pharmacia Peristaltic Pump P-1. Elution was performed at a flow rate of 1.0 ml/min for 30 min using a linear gradient buffer system ranging from 0 to 100% of buffer B which consisted of sodium phosphate buffer (20 mM, pH 7.2), containing 1 M NH_4Cl. Fractions of 1 ml were collected by an automatic fraction collector (Pharmacia). The elusion profile was monitored by the absorption of protein at 280 nm using an UV detector. The collected fractions FIV (a-c) were desalted with the ultrafiltration system described above, freeze-dried and stored at -80°C.

3.8. Copper Measurements

The copper content of the purified samples was measured by an atomic absorption spectrometer (ASS) (GBC-903: GBC Scientific Equipment PTY Ltd. Victoria, Australia), equipped with a conventional 10 cm length burner containing an air-acetylene flame and a copper lamp with a 3.0 mA current and a 0.5 cm wide slit. An injection volume of 100 µl was used and absorbance was measured at 324.7 nm. A range of $CuSO_4$ (AAS grade) solutions of 0.025, 0.05, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ppm was prepared in 0.5% (v/v) nitric acid and used as standards for calibration. The lyophilized samples were also dissolved in 0.5 % (v/v) nitric acid prior to injection.

3.9. Protein Determination

The protein content of the samples was determined according to a modification of the Lowry method (Hartree, 1972). Bovine serum albumin was used as a standard for calibration.

3.10. Spectroscopic properties

The spectroscopic properties of Cu-MT from *A. niger* were determined using a Beckman model DU 650 spectrophotometer (Beckman, Fullerton, CA). The purified samples (10.8 µg protein/ml), obtained from affinity chromatography, were dissolved in sodium phosphate buffer solution (20 mM, pH 7.2). Absorption spectra were recorded after the samples were scanned at a wavelength range of 200 to 600 nm at 25°C.

3.11. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure outlined in the Pharmacia manual (1992). A PhastSystem Unit (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used.

Low molecular weight standards (Pharmacia) were run in tandem with the samples and consisted of phosphorylase *b* (M_r 94.0 kDa), bovine serum albumin (M_r 67.0 kDa), ovalbumin (M_r 43.0 kDa), carbonic anhydrase (M_r 30.0 kDa), soybean trypsin inhibitor (M_r 20.0 kDa), and α-lactabumin (M_r 14.0 kDa).

3.11.1. Native PAGE

Native PAGE mini gels (5 x 4 cm; 0.45 mm thickness) of 10.0 % polyacrylamide were prepared in accordance to the procedure outlined in the manual (Pharmacia, 1992). The gels were run on the PhastSystem for 30 min at a constant current of 10 mA, using the Phast gel native buffer strips (Pharmacia); the buffer strips consisted of 0.88 M L-alanine and 0.25 M Tris, pH 8.8 in 2.0 % agarose. After separation of the proteins, the gels were transferred to the development section of the PhastSystem unit. Silver staining was performed on the gels as outlined in the manual (Pharmacia, 1992) and consisted of fixing and removing buffer ions, sensitizing the proteins in a glutardialdehyde solution, reacting the proteins with silver ions using a silver nitrate solution, developing in a basic formaldehyde solution and stopping the development with the addition of acetic acid. A final rinsing step in 10.0 % acetic acid and 5.0 % glycerol was used to prevent the gels from curling or cracking after being dried.

3.11.2. SDS PAGE

The molecular mass of the selected purified MT fractions (FIVc and FIVc') were estimated by sodium dodecyl sulfate (SDS PAGE) according to the procedure described by Laemmli (1970), using mini-gels (5.0 cm x 4.0 cm) whose total monomer concentration was 12.5 % and crosslinker concentration was 3.0 %. The freeze-dried sample (2.0 mg protein/ml) was dissolved in the stacking buffer and diluted twice with

Laemmli buffer. The protein standards and the purified fractions (FIVc and FIVc') were then incubated in Laemmli reducing buffer at 90°C for 10 min. A portion (15.0 µl; 2.0 mg) of the denatured protein were applied to the gel. Electrophoretic separation was performed for 30 min at a constant current of 10 mA, with the PhastSystem (Pharmacia). The separated proteins were then stained with silver nitrate according to the method described in the Pharmacia manual (1992). The molecular mass of the MT was estimated from the standard curve established with a mixture of molecular-mass standards.

3.12. Mass Spectrometry (MS) Analysis

A SCIEX API- triple-quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization (API) source (Sciex, Inc. Thornhill. ONT) was used to sample ions produced from an ion spray interface (Alli *et al.*, 1993 and 1994). Liquid samples were introduced to the electro-spray source (ES) by continuous infusion with a syringe pump (Model 22, Harvard Apparatus, South Natick, MA), through a fused silica capillary (100 µm i.d.) at flow rate of 1-2 µl per min. The ion were detected by scanning the first quadrupole of the mass spectrometer; scans were monitored for *m/z* between 700 to 1200 in 5 s. Every 10 to 15 scans were averaged to obtain the representative spectra. The abundance of ions was reported as relative percentage to that of the peak with the most intensity. The detector was a Chevron type multi-channel plate multiplier (3025, Galieo Electro-optics, Strurbidge, MA) used in pulse-counting mode. Signals were recorded with a 100-MH_2 pulse counter (SR430, Stanford Research System. Sunny-vale, CA) which incorporated internal amplitude discrimination. All data was transferred to a Macintosh computer using Apple File Exchange software (Apple computer, Inc., Cupertino, CA). Post acquisition analyses were conducted on the Macintosh using the Passages Software package (VersionF, Passage Software, Inc. Ft. Collios, CO).

The instrument *m/z* scale was calibrated with singly-charged ammonium adduct ions of polypropylene glycol ($1x10^{-4}$ M) prepared 50% methanol solution (1 methanol: 1 water, v/v) containing 2 mM ammonium formate) under unit *m/z* resolution. Before molecular weight determination of the FPLC Cu-MT fractions, the mass spectrometer accuracy was first verified with 1mg/ml lysozyme in 10 % acetic acid (Sigma Chemical

Co.). Figure 11 showed the interpreted mass spectra of the lysozyme standard solution; the expected MW of 14,308 + 1.23 kDa was confirmed. The potential on the sampling orifice of the instrument was set at +30 V during calibration and raised to +100 to enhance ion signal for protein.

3.13. Enzyme Assay

3.13.1. Substrate Preparation

Stock solutions of CF (10 mM), CH (10mM), CHL (10mM), CT (10 mM), 4-MCT (10 mM), HOPA (5 mM), L-DOPA (10 mM), DOPAA (10 mM), HOPPA (5 mM), *m*-cresol (10 mM) and *p*-cresol (10 mM) were prepared in phosphate-citrate buffer solution (10 mM, pH 6.0).

3.13.2. Source of Enzyme

Mushroom tyrosinase (Sigma Chemical Co.), with an activity of 3400 units/mg, was used throughout this study. One unit of enzyme activity was defined as an increase in absorbance, at 280 nm, of 0.001/min at pH 6.5 and 25°C in a reaction mixture containing L-tyrosine.

3.13.3. Spectrophotometric Assay

The spectrophotometric assay for enzyme activity was performed according to a modification of the method described by Kermasha *et al.* (1993c). The spectrophotometric assay was carried out at 25°C, using a Beckman DU-65 Spectrophotometer, where the enzymatic activity was followed by increase in absorbance at 420 nm (CF), 372 nm (CT), 420 nm (CHL), 420 nm (CT), 398 nm (4-MCT), 520 nm (HOPA), 393 nm (L-DOPA), 410 nm (DOPAA), 423 nm (HOPPA), 325 nm (*m*-cresol) and 340 nm (*p*-cresol). The specific activity of the enzyme was expressed as mmol products at the selected wavelength per μg enzyme protein per min.

3.13.4. Polarographic Assay

The polarographic assay was carried out at 25°C, using a Gilson oxygraph equipped with a Clark electrode (Kermasha and Metche, 1986), where the enzymatic activities were followed by the rate of oxygen uptake. The specific activity of the enzyme was expressed as μmol O_2 uptake per μg enzyme protein per min.

3.13.5. Tyrosinase Assays

The tyrosinase activity was assayed in phosphate citrate buffer solution (10 mM, pH 6.0) using the selected substrates; 500 μl of the substrate stock solution was added to the 1.0 and 1.5 ml buffer solution and the enzymatic reaction was initiated by the addition of 20 μl (3.5 μg protein) and 35 μl (6.2 μg protein) of PPO suspension for the spectrophotometric and polarographic methods, respectively. The enzymatic reactions were incubated at 25°C and vigorously shaken for 3 sec to 5 min. A blank trial, containing all components except the enzyme, was also performed concurrently. The enzyme activity was determined both spectrophotometrically and polarographically as described previously.

3.13.6. Inhibition Assays

The inhibitory effect of the Cu-MT extract on tyrosinase activity was investigated, using a number of the selected substrates. The Cu-MT concentrations used varied from 0.02 to 7.0 mg protein/ml. The reaction mixture consisted of a final volume of 1.0 and 1.5 ml for the spectrophotometric and polarographic methods, respectively. The substrate concentrations ranged from 0.25 to 5.0 mM for CF, CT, CH, CHL, 4-MCT, L-DOPA, DOPAA, and *p*- and *m*-cresol and 0.13 to 2.5 mM for HOPA and HOPPA in phosphate-citrate buffer solution (10 mM, pH 6.0). The enzymatic reaction was initiated by the addition of the PPO suspension ranging from 3.5 to 6.2 μg protein to the temperature-equilibrated 25^0C reaction mixture. The reaction mixtures were vigorously shaken for 3 sec to 5 min. A blank trial, containing all the components except the PPO suspension, was run in tandem with the inhibitory assay. The enzymatic assays were performed by using both the spectrophotometric and polarographic methods as described earlier.

3.14. Characterization of Tyrosinase-Catalyzed End Products

The formation of the brown colored compounds during the enzymatic reactions was followed spectrophotometrically from 300 to 700 nm for 12 min, at periodic intervals of 1 min. Substrate stock solutions were prepared at a concentration of 5.0 mM for CF, CT, 4-MCT, L-DOPA, DOPAA, and HOPPA. The substrate solution (1.0 ml) was then added to the reaction mixture containing 3.5 μg protein of the PPO suspension. End product characterization was carried out in the absence and presence of Cu-MT at concentrations of 0.2, 0.3, 0.4, 1.4, 1.8 and 3.3 μg protein with the respective substrate CF, CT, 4-MCT, L-DOPA, DOPAA and HOPPA in the reaction mixture.

4. RESULTS AND DISCUSSION

4.1. Biomass Production

4.1.1. Effect of Agitation on Biomass Production

Figure 4 shows the effect of different agitation rates ranging from 60 to 130 rpm under shake flask culture conditions. The results show that the biomass production of *A. niger* was directly related to the agitation rate of the culture medium. The results also show that the biomass production was found to increase with an increase in agitation rate and reached a maximum of 0.28 g/l dry weight at an agitation rate between 110 to 120 rpm; however, above and below of this level, biomass production was lower. These experimental findings suggest that between 110 to 120 rpm, the smallest mycelial pellets were formed which ensured sufficient aeration to the cells, necessary for maximum growth. In contrast, the results suggest that at higher agitation conditions, the fusion between mycelia became disrupted thereby resulting in less biomass production.

Figure 5 shows the effect of time on the biomass production of *A. niger* under shake flask cultures using the optimum agitation rate of 110 rpm. The results show that maximum biomass production of 12.8 and 0.28 g/l on a fresh and dry weight basis, respectively, was found at 72 hours of culture incubation after which biomass production decreased. Maximum biomass production was obtained at the stationary phase of growth, which occurred at 72 hours.

Figure 6 shows the effect of time on the pH and optical density of the culture medium during biomass production of *A. niger*. The experimental results show that the optical density increased with time and reached a maximum of 0.45 at 72 hours. These results suggest that *A. niger* released metabolites into the culture medium during its stationary phase of growth. Figure 6 also shows that the pH of the culture medium was found to decrease to 2.5 at the early stage of culture incubation until 60 hours after which it increased to pH 4.5 at 72 hours of incubation. These findings suggest that the culture of *A. niger* preferred acidic conditions for maximum biomass production.

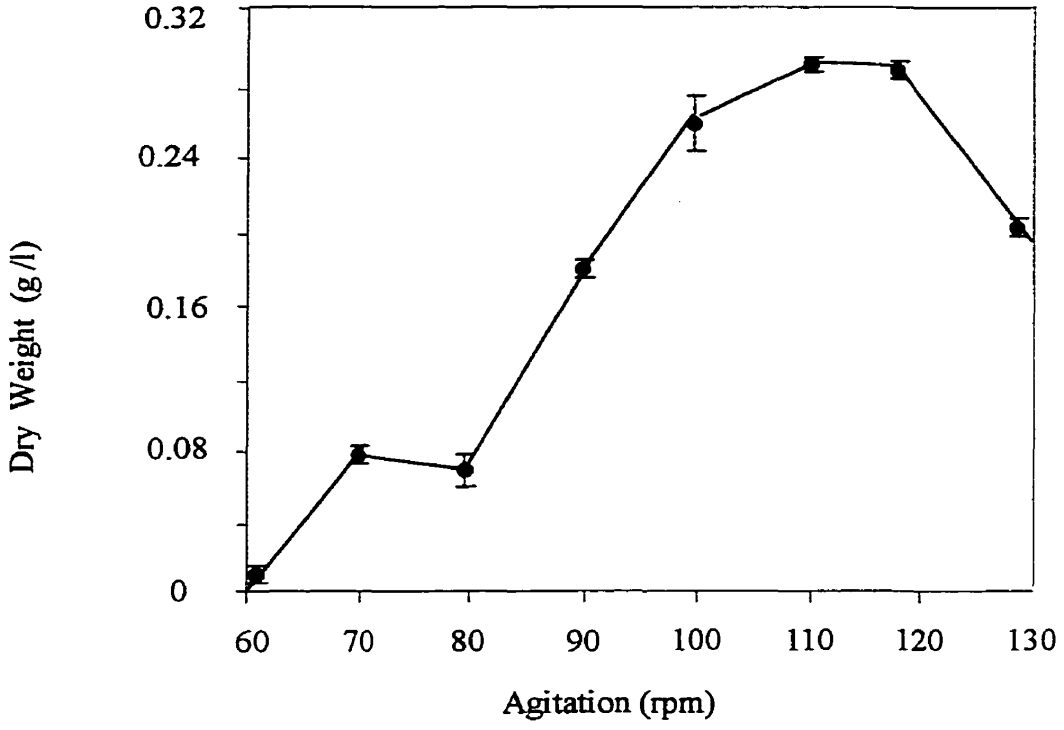


Figure 4 . Effect of agitation on the biomass production of *Aspergillus niger.*

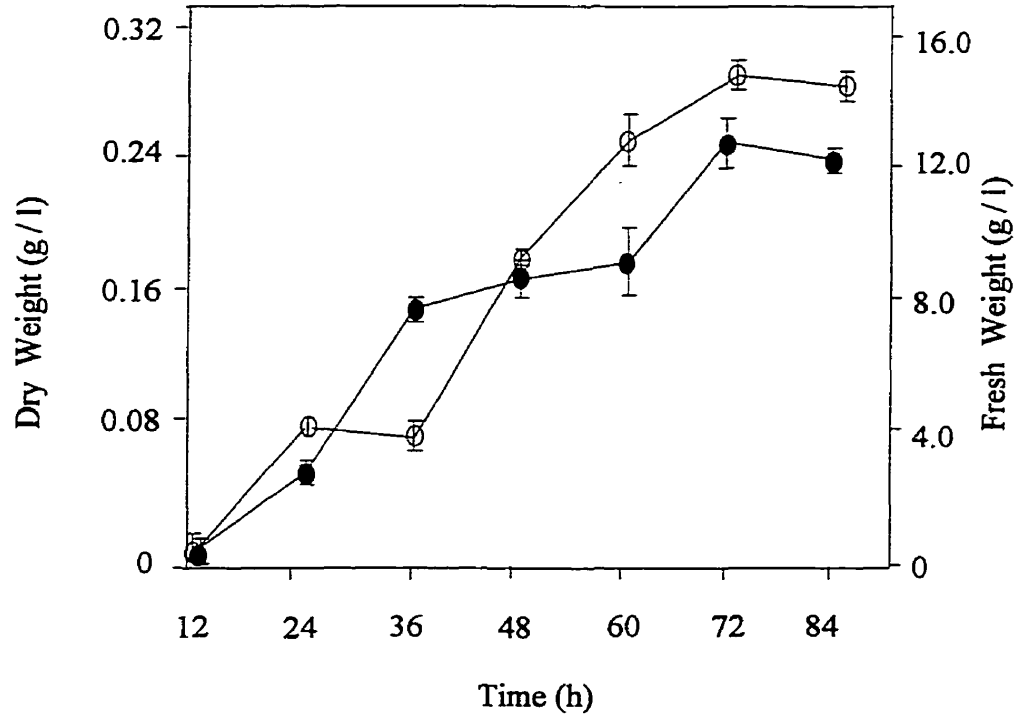


Figure 5. Effect of time on the fresh (●) and dry (○) weight of *Aspergillus niger* during biomass production.

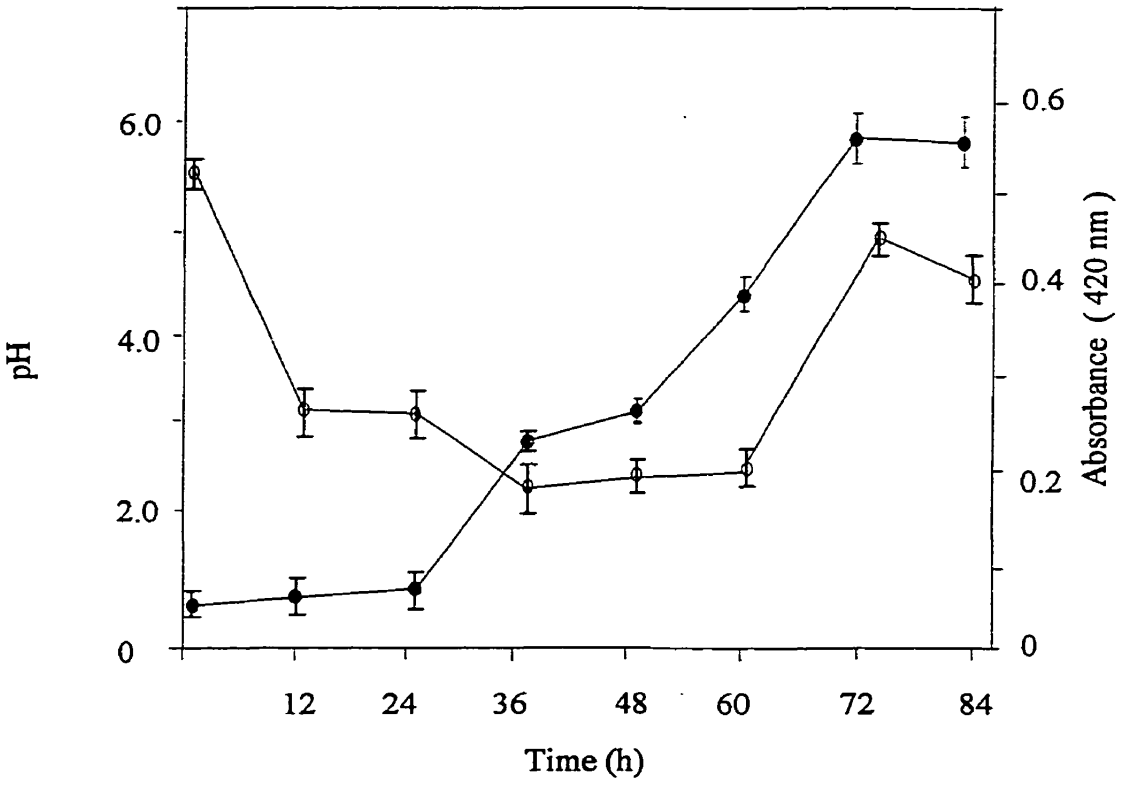


Figure 6. Effect of time on the pH (○) and absorbance (●) of the culture medium during biomass production of *Aspergillus niger*.

4.2. Induction of Copper-Metallothionein

The induction of Cu-MT synthesis in the culture of *A. niger* was performed during the stationary phase of growth, obtained after 72 hours of incubation. The experimental results (not shown) indicate that 0.15 % (w/v) copper was necessary in the incubation medium for induction of Cu-MT. Munger *et al.* (1987) reported that 0.25 mM $CuSO_4$ was necessary for maximum induction, as higher levels of $CuSO_4$ remained unused. These authors also suggested that the amount of Cu-MT synthesis was strongly dependent on the quantity of copper ions present in the culture medium. El-Meleigy (1992) reported that the presence of 0.16 % (w/v) copper in the culture medium of *A. fumigatus* and *P. chrysogenum* induced the biosynthesis of low molecular weight copper-chelators. However, Lerch (1991) stated that during the exponential growth phase of *N. crassa*, most of the intracellular copper was associated with the high-molecular-mass protein fractions whereas during the stationary phase of growth, copper uptake accumulated in the low- molecular-mass proteins

4.3. Partial Purification of Copper-Metallothionein

After induction of Cu-MT synthesis by *A. niger*, the mycelia were subsequently recovered by filtration through a cheesecloth and subjected to homogenization. The homogenized suspension was then centrifuged and the debris was discarded whereas the supernatant, considered to be the crude extract (FI), was considered for further purification. A number of purification methods including heat treatment, ultrafiltration and affinity chromatography were performed successively to obtain a purify Cu-MT extract from *A. niger*.

4.3.1. Heat Treatment

The results (Table 1) show that heat treatment of the crude cell extract (FI) yielded a partially purified Cu-MT extract (FII) with a decrease in total protein content of 73.3%, from 390.0 to 104.0 mg. These results suggest that heat-treatment removed heat-liable high molecular weight proteins in the crude extract. Turanek *et al.* (1987) suggested that heat treatment could be used for the removal of high molecular mass proteins from crude cell extracts. Similar levels of reduction of 72.3 and 69.8 % in total

Table 1. Purification scheme of copper-metallothionein from *Aspergillus niger*.

Fraction	Sample mass (mg)	Protein conc. (μg/mg)	Total Protein (mg)	Total copper (mg)	Copper/ protein mass ratio	Recovery[a] (%)	Purification[b] (fold)
Crude extract (FI)	1987.9	196.2	390.0	201.0	0.52	100.00	1.00
Heat treatment (FII)	905.9	114.8	104.0	155.7	1.49	26.67	2.87
Ultrafiltration (FIII)	439.4	141.1	62.0	3.4	0.06	15.90	0.12
AF (FIVc)[c]	190.0	2.0	0.38	0.065	0.17	0.09	0.33
Re-AF (FIVc')[d]	78.9	3.8	0.30	0.081	0.27	0.08	0.52

[a]Percentage of recovery was calculated on the basis of total protein with reference to the crude extract, multiplied by 100.

[b]purification was calculated as the copper to protein mass ratio with respect to the crude extract.

[c]Affinity chromatography was performed using a Chelating Superose HR 10/2 column.

[d]Affinity re-chromatography was performed using a Chelating Superose HR 10/2 column.

protein content were reported during the purification of a Cu-MT extract from *A. niger* (Goetghebeur *et al.*, 1995) and *N. crassa* (Lerch, 1991), respectively. Table 1 also indicates that a 2.87 fold increase in purification of the Cu-MT extract was obtained after heat treatment. Kermasha *et al.* (1993c) and Goetghebeur *et al.* (1995) also obtained a 1.1 and 2.29 fold increase in purification, respectively, after heat treatment of the Cu-MT extract from *A. niger*.

4.3.2. Ultrafiltration

The heat-treatment fraction (FII) was subjected to diafiltration using a 3.0 kDa molecular weight cut-off membrane. The results (Table 1) show that the protein concentration in the ultrafiltration fraction (FIII) increased from 114.8 to 141.1 μg/mg sample with respect to that obtained in the heat treatment fraction (FII). The results also show that the total copper content was 46 times lower in the ultrafiltration fraction (FIII) than in the heat-treatment fraction (FII). Moreover, the degree of purification was much lower for the ultrafiltration fraction (FIII) compared to that obtained for the heat treatment fraction (FII). These findings suggest that the excess unbound copper present in the heat treatment fraction (FII) was removed in the ultrafiltration Cu-MT extract (FIII).

4.4. Purification of Copper-Metallothionein

4.4.1. Affinity Chromatography

Further purification of Cu-MT from the ultrafiltration fraction (FIII) was performed by affinity chromatography. Figure 7 shows that the Cu-MT extract (FIII) was separated into three major fractions, FIVa, FIVb and FIVc by affinity chromatography. However, the results (not shown) also indicate that the analysis of the copper and protein contents of the three fractions demonstrated that only fraction FIVc contained Cu-MT. The results (Table 1) also show that the degree of purification of fraction FIVc was 2.8 times higher than that of the ultrafiltration fraction (FIII). Similar findings were obtained by Goetghebeur *et al.* (1995) using a similar type of purification process to obtain a

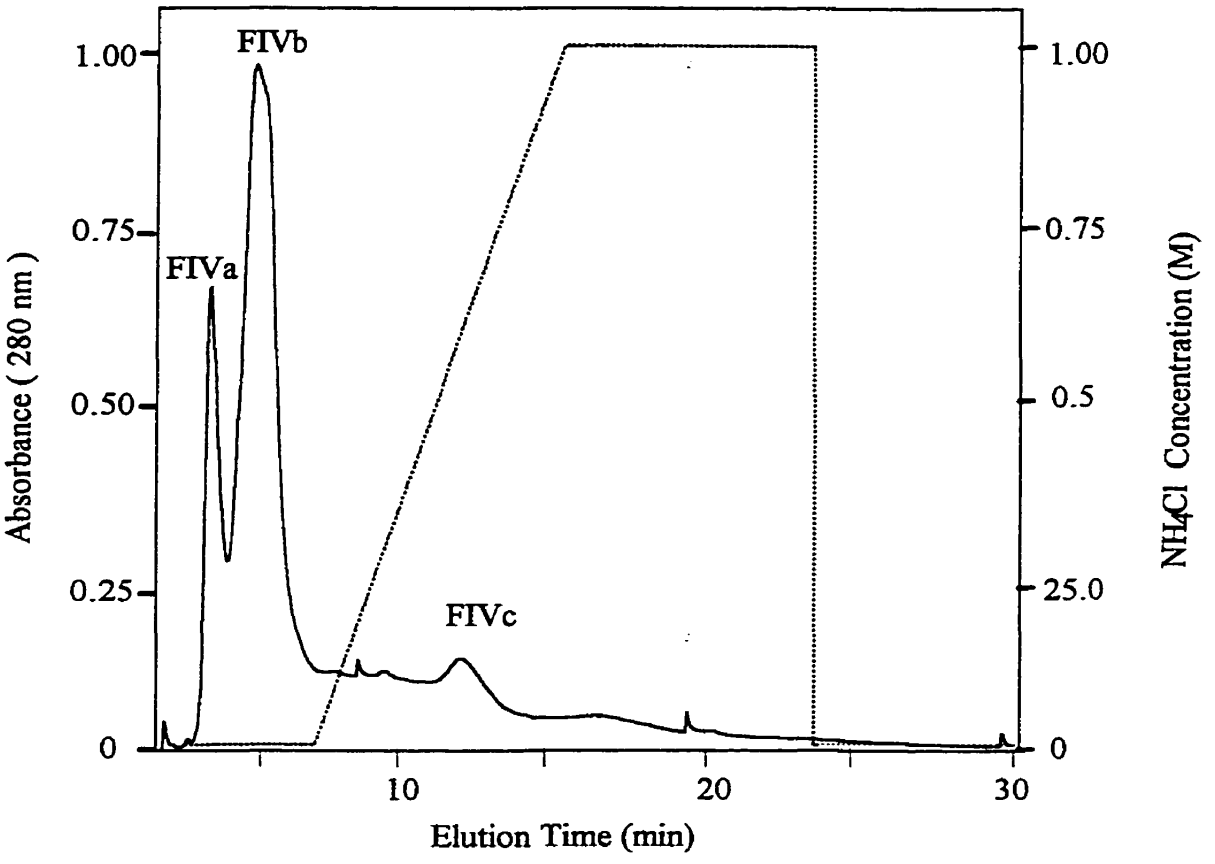


Figure 7. Fast-protein liquid chromatography (FPLC) elution profile of the copper-metallothionein fraction (FIII), obtained by ultrafiltration, on an affinity chromatography Chelating Superose HR 10/2 column: absorbance (——) and NH_4Cl concentration (——).

Cu-MT extract from *A. niger*. However, the results (Table 1) also demonstrate that a low recovery of Cu-MT (0.09%) was obtained using affinity chromatography. Lerch (1991) also reported a similar recovery of 0.098 % for the purification of a Cu-MT extract from *N. crassa*. Carlsso *et al.* (1977) reported that metal chelating affinity chromatography is an efficient procedure for the purification of metal binding proteins. These findings suggest that Cu-MT from both *A. niger* and *N. crassa* are minor constituents of the total protein content.

4.4.2. Affinity Re-Chromatography

The selected fraction FIVc was subjected to further purification using the same affinity chromatography column. Figure 8 shows that re-chromatography of fraction FIVc showed only one major peak (FIVc'). Table 1 also shows that the affinity re-chromatography fraction FIVc' resulted in a 4.3 and 1.6 fold increase in purification compared to fractions (FIII) and (FIVc), respectively. Table 1 also shows that a similar recovery of the Cu-MT extract FIVc' (0.08%) was obtained as that for fraction FIVc. Fraction FIVc' was therefore selected as the purified extract of *A. niger* and subjected to further characterization.

4.5. Characterization of Copper-Metallothionein

4.5.1. Spectroscopic Properties

Figure 9 indicates that the scanning of the purified Cu-MT fraction FIVc' showed a maximum absorption shoulder peak at 268 nm in the UV spectrum. However, previous research in our laboratory showed that maximum absorption of the Cu-MT extract was obtained at 259 nm (Goetghebeur *et al.*, 1995). In addition, Winge *et al.* (1985) reported that a Cu-MT extract from *S. cerevisiae* exhibited an absorption shoulder near 260 nm. However, the UV spectrum of a purified canine Cu-MT extract showed higher absorption at 250 nm than at 280 nm (Richards *et al.*, 1991). These finding suggest that Cu-MT will exhibit maximum absorption between 250 and 268 nm depending upon its source of origin as well as degree of purification.

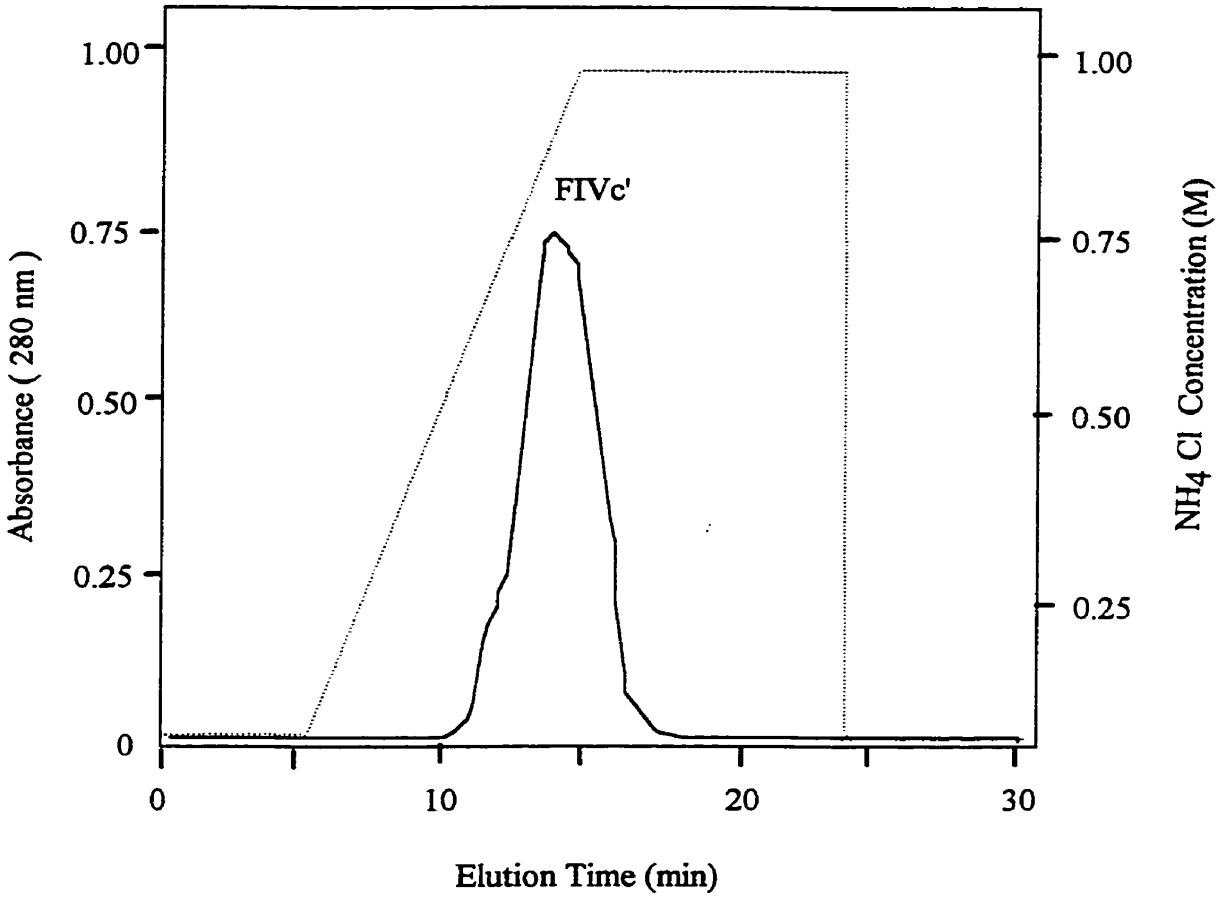


Figure 8. Fast-protein liquid chromatography (FPLC) elution profile of the copper-metallothionein fraction FIVc, obtained by affinity chromatography, on an affinity chromatography Chelating Superose HR 10/2 column: absorbance (——) and NH_4Cl concentration (– – –).

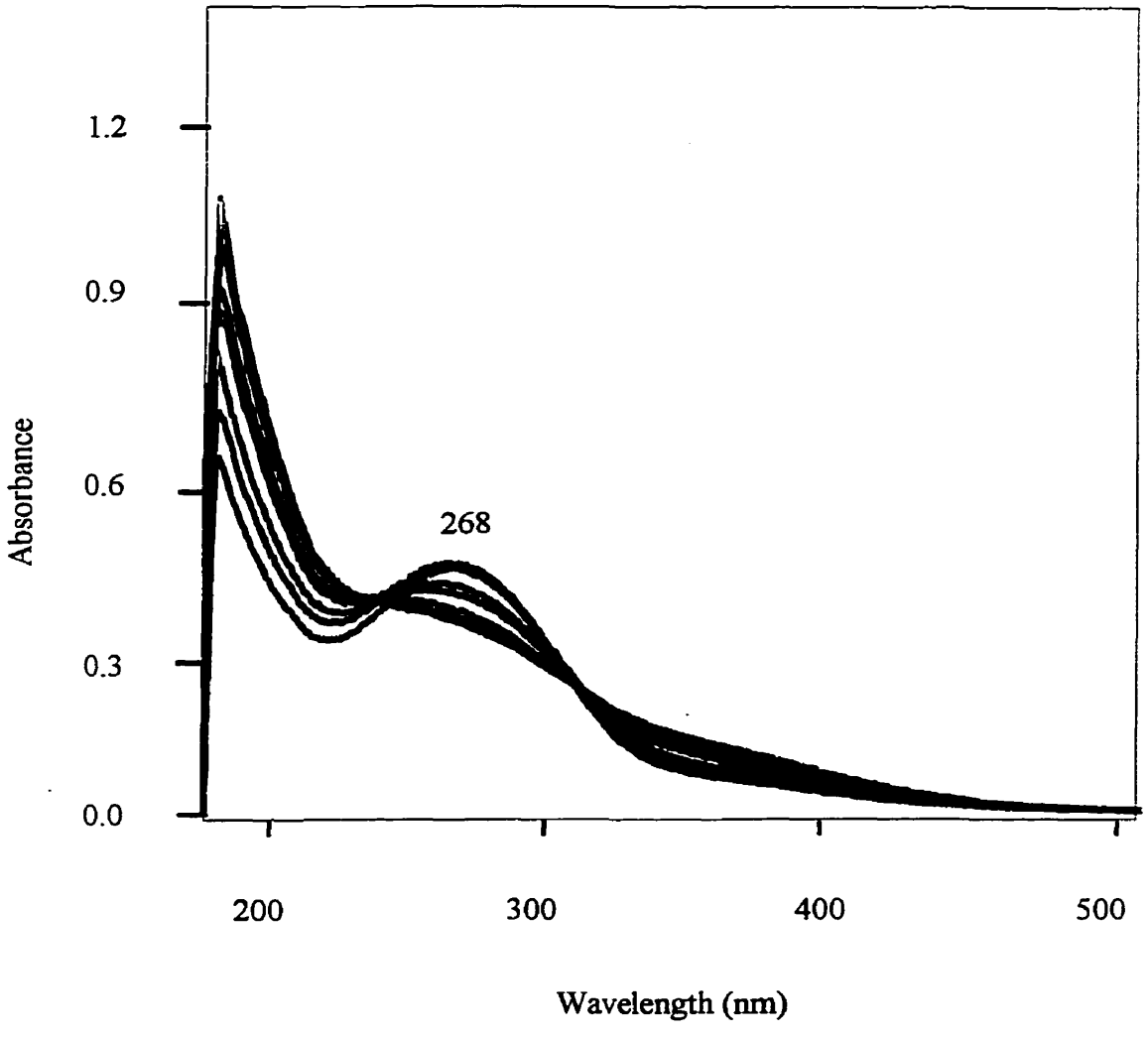


Figure 9. Spectroscopic absorption spectrum of purified copper-metallothionein from *Aspergillus niger*.

4.5.2. Electrophoretic Analyses

4.5.2.1. Native PAGE

Native PAGE was carried out on the Cu-MT fractions FIVc and FIVc' in order to estimate the molecular weight of Cu-MT as well as to assess the degree of purity of the fractions. Figure 10 shows that native PAGE of fractions FIVc and FIVc' demonstrated the presence of only one protein band on the gel. The results also show that the estimated molecular mass of both fractions was approximately 9.5 kDa.

4.5.2.2. SDS PAGE

Figure 11 shows the electrophoretic profile of the SDS-PAGE of the purified Cu-MT fractions FIVc and FIVc'. The results show the presence of only one band with an approximate molecular weight of 10.0 kDa. These results (Fig. 11) indicate that the molecular mass of the purified Cu-MT fractions FIVc and FIVc' from *A. niger* was lower than that (21.0 kDa) reported previously by Goetghebeur *et al.* (1995). However, similar findings were reported earlier by Kermasha *et al.* (1993c), who observed a molecular mass of 11.0 kDa for the *A. niger* Cu-MT, purified by successive size-exclusion and ion-exchange chromatography. Our experimental findings are also in agreement with the molecular weight reported for the Cu-MT extract from the yeast *S. cerevisiae* (Winge *et al.*, 1985). In contrast, Cu-MT from the fungi *N. crassa* (Lerch, 1991) and *A. bisporus* (Munger and Lerch, 1985) showed a much lower molecular mass of 2.2 and 2.6 kDa, respectively. These findings suggest that the discrepancies in the molecular mass of Cu-MT from *A. niger*, obtained using different purification procedures, could be due to an association of protein molecules, which can produce aggregates of higher molecular weight.

4.5.3. Molar Ratio of Copper to Protein

The ratio of copper to moles of protein was calculated by multiplication of the copper/protein mass ratio (Table 1) with the estimated molecular weight of Cu-MT obtained by electrophoresis (Fig. 11). The results show that the copper/protein ratio of the Cu-MT fraction FIVc' was 42.5 (M_r 10.0 kDa). Kermasha *et al.* (1993c) and

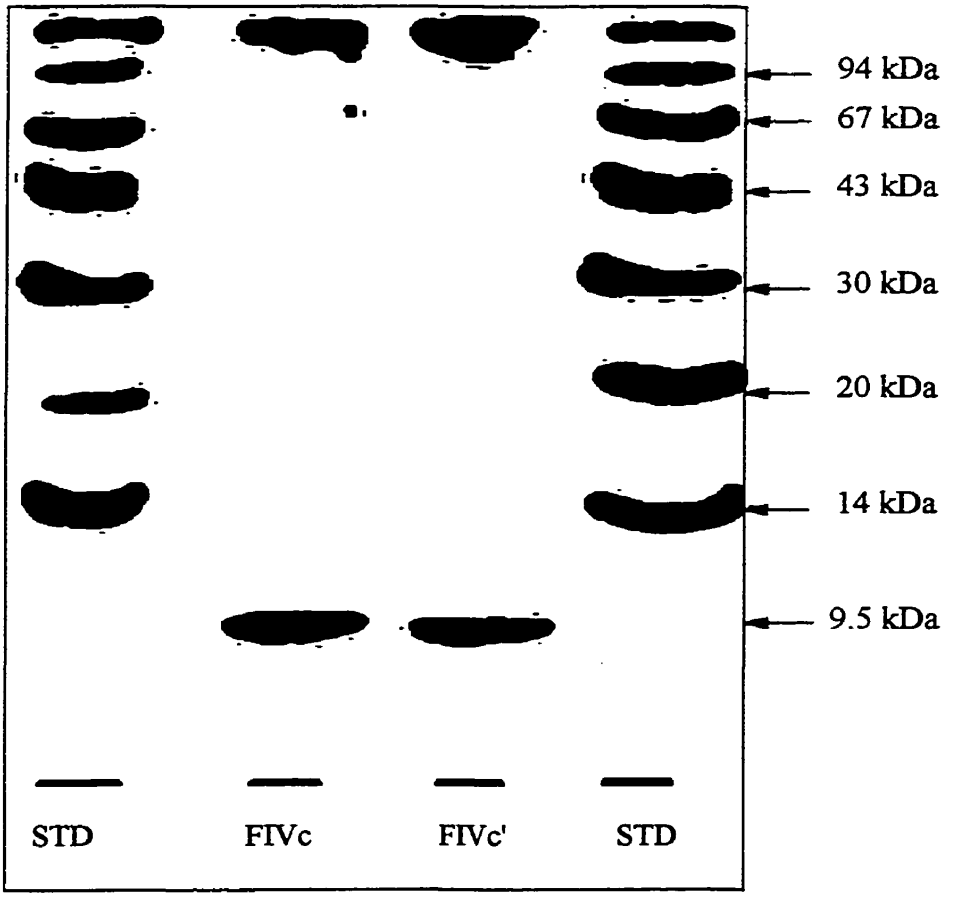


Figure 10. Electropherogram of the native polyacrylamide gel of the purified copper-metallothionein fractions FIVc and FIVc' from *Aspergillus niger*.

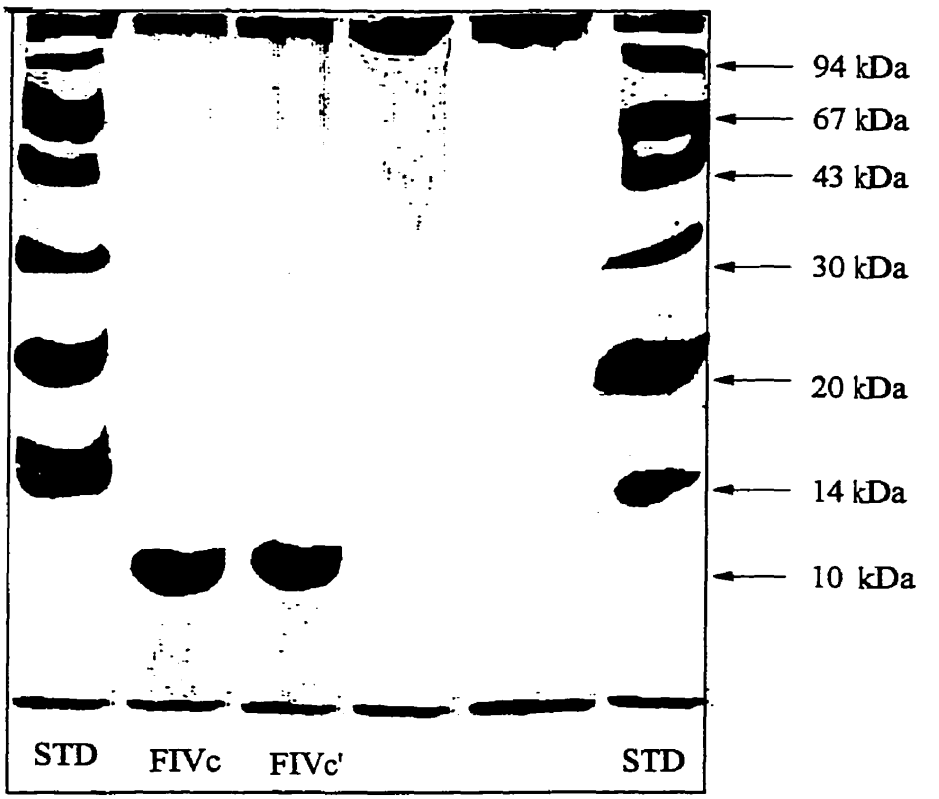
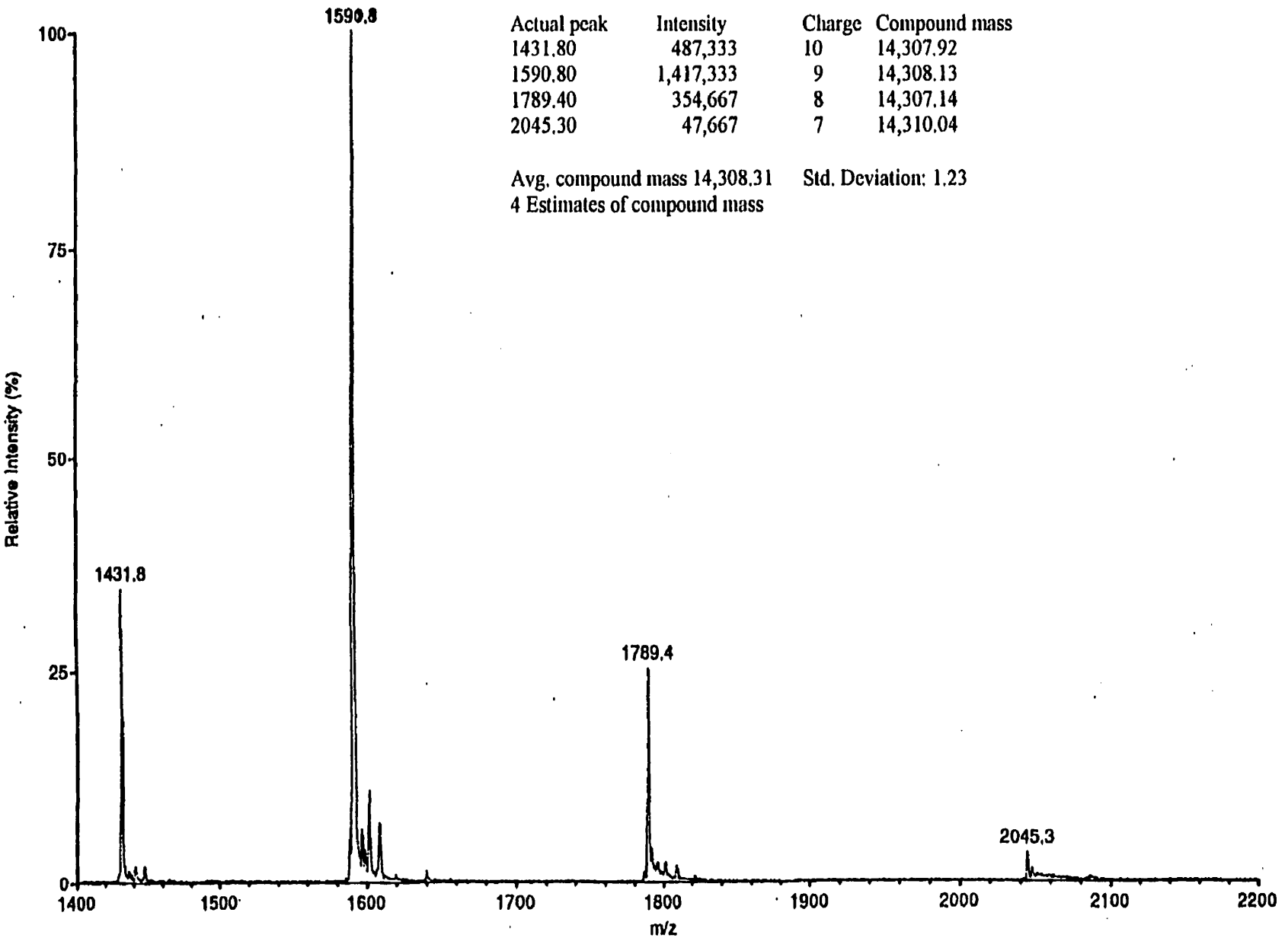


Figure 11. Electropherogram of the sodium dodecyl sulfate polyacrylamide gel of the purified copper-metallothionein fractions FIVc and FIVc' from *Aspergillus niger*.

Goetghebeur *et al.* (1995) reported a copper/protein ratio of 33.0 (M_r 11.0 kDa) and 78.0 (M_r 21.0 kDa), for the Cu-MT extract of *A. niger* purified by a combination of ion-exchange and size-exclusion chromatography, and by affinity chromatography, respectively. In contrast, Lerch (1991) and Munger and Lerch (1985) reported a lower copper/protein ratio of 6.0 and 5.8 for a copper-binding protein extract isolated from *N. crassa* (M_r 2.2 kDa) and *A. bisporus* (M_r 2.6 kDa), respectively.

4.6. Analysis of Cu-MT by Electro-Spray Quadrupole Mass Spectrometry

Electro-spray mass spectrometry (ESMS) was used to determine the molecular weight of the Cu-MT fraction FIVc' purified by affinity chromatography. An excellent electrospray signal was generated when the purified protein sample was dissolved at a final concentration of ~10 μM in diluted acetic acid at pH 4. Figure 12 shows a typical ESMS spectrum of the lysozyme standard where average molecular weight was 14 kDa; lysozyme was used as a protein standard for the ESMS analysis. Our preliminary results (Fig. 13) show that the molecular weight of the apo-Cu-MT fraction FIVc' was 10.2 ±1.6 kDa. The results also indicate that formation of re-constituent Cu-MT from the apo-Cu-MT was enhanced at a higher repelled voltage (data not shown). Figure 13 also shows the presence of other minor peaks, observed in the spectrum, which might be due to the interference of free copper and/or protein residues during the de-metalization and formation of re-constituents of Cu-MT; these findings suggest that this fragmentation could be attributed to the electrospray process, since the intensity of these peaks varied with respective to their charge state (greater intensity was observed at higher charge states). The overall results indicate that the molecular mass of the purified FIVc' fraction of the reconstitute Cu-MT from *A. niger*, obtained by ESMS, was similar to those obtained in the native and SDS PAGE electrophoretic analyses. Yu *et al.* (1993) reported a molecular weight of 6126 Dalton for rabbit liver apo-metallothionein, determined by ESMS. Knudsen *et al.* (1998) also determined the molecular weight of rabbit, sheep and yeast (*S. cerevisiae*) metallothionein to be 6157, 6091 and 6149 Dalton, respectively, using capillary electrophoresis ESMS.



Actual peak	Intensity	Charge	Compound mass
1431.80	487,333	10	14,307.92
1590.80	1,417,333	9	14,308.13
1789.40	354,667	8	14,307.14
2045.30	47,667	7	14,310.04

Avg. compound mass 14,308.31 Std. Deviation: 1.23
4 Estimates of compound mass

Figure 12. Electro-Spray Mass Spectrometric Spectrum of Lysozyme

Actual peak	Intensity	Charge	Compound mass
787.60	92,056	13	10,225.70
853.00	25,227	12	10,223.91
930.60	8,904	11	10,225.51
1023.80	5,899	9	10,225.92
1137.00	2,775	9	10,223.93

Avg. compound mass 10,225.39 Std. Deviation: 1.65
5 Estimates of compound mass

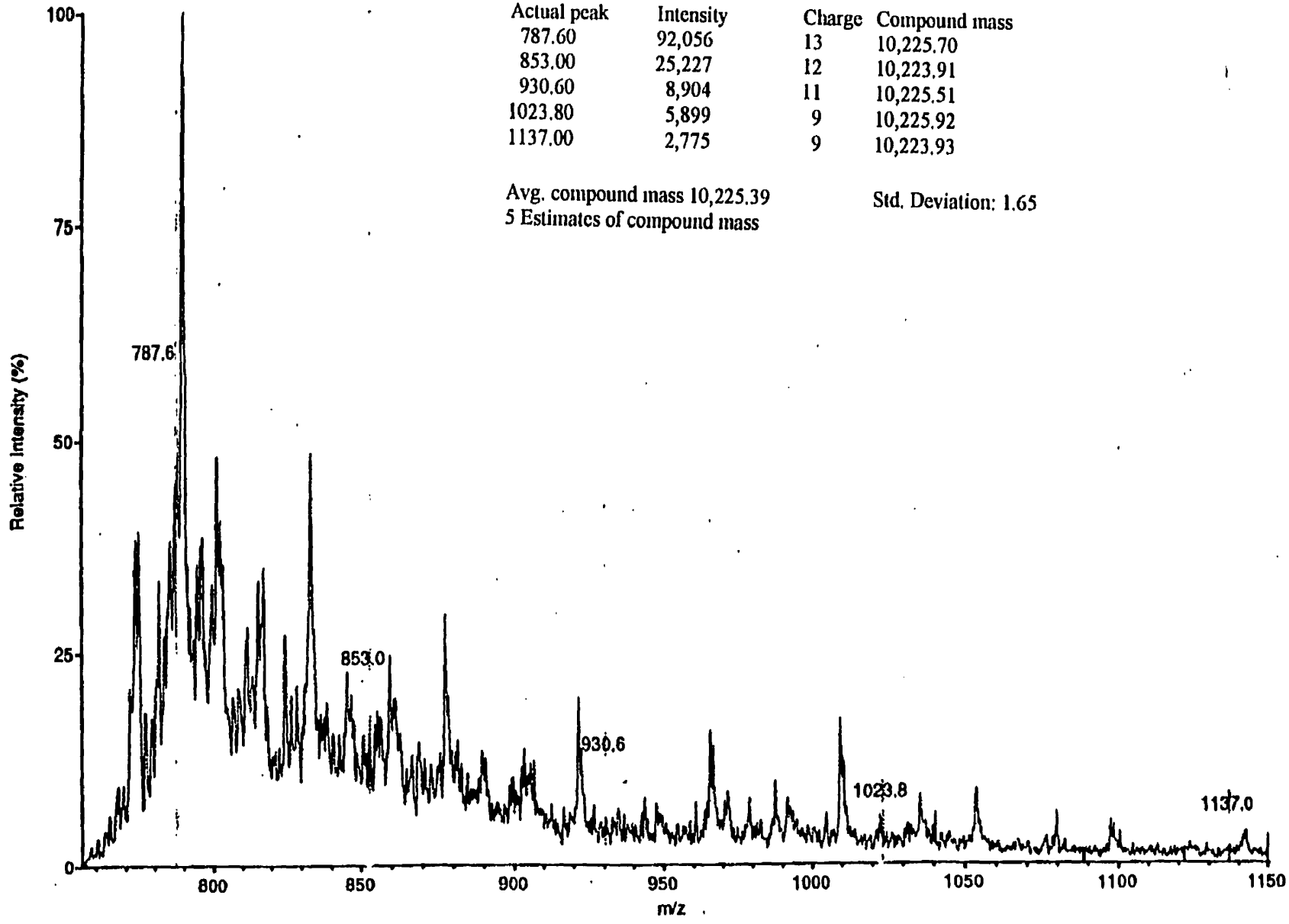


Figure 13. Electro-Spray Mass Spectrometric Spectrum of Cu-MT from *A. niger*

4.7. Inhibition of Tyrosinase Activity by Cu-MT

The extent of inhibition of tyrosinase activity by Cu-MT was investigated with respect to the degree of purity of the Cu-MT extracts. Four different Cu-MT fractions (FII, FIII, FIVc and FIVc') possessing different degrees of purity were obtained during the purification process and used to investigate their inhibitory effect on tyrosinase activity. The inhibitory effect of the ultrafiltration Cu-MT fraction FIII on tyrosinase activity was investigated using eleven selected substrates. In addition, the inhibitory effect of the three other Cu-MT fractions FII, FIVc, and FIVc' on tyrosinase activity was determined using the two substrates CH and CHL. The inhibition of enzyme activity by the Cu-MT extracts was determined by two different methods, spectrophotometric and polarographic.

4.7.1. Determination of Purity of Tyrosinase

A commercially purified mushroom tyrosinase extract was used in the inhibition studies. The commercially purified tyrosinase extract was investigated for the presence of contaminating oxidizing enzymatic activities, particularly laccase, using two specific substrates including 3,5-dimethoxy-4-hydroxybenzaldehyde azine 'syringaldazine' and 2,6-dimethoxyphenol. The results (not shown) indicated that the commercially purified tyrosinase extract, used throughout this study, contained negligible laccase activity (0.03-0.40%).

4.7.2. Inhibition of Tyrosinase Activity by the Ultrafiltration Cu-MT Extract

The inhibitory effect of the ultrafiltration Cu-MT fraction (FIII) on mushroom tyrosinase activity was investigated by spectrophotometer and polarograph, using a number of selected mono- and di-phenolic substrates including CF, CT, 4-MCT, L-DOPA, DOPAA and HOPPA, CHL, CH, HOPA, *p*- and *m*-cresol.

4.7.2.1. Spectrophotometric Determination

Figure 14 shows that an inhibitor concentration range of 0.02 to 1.7 mg protein/ml was used in this study. The results show that the effectiveness of the Cu-MT extract in

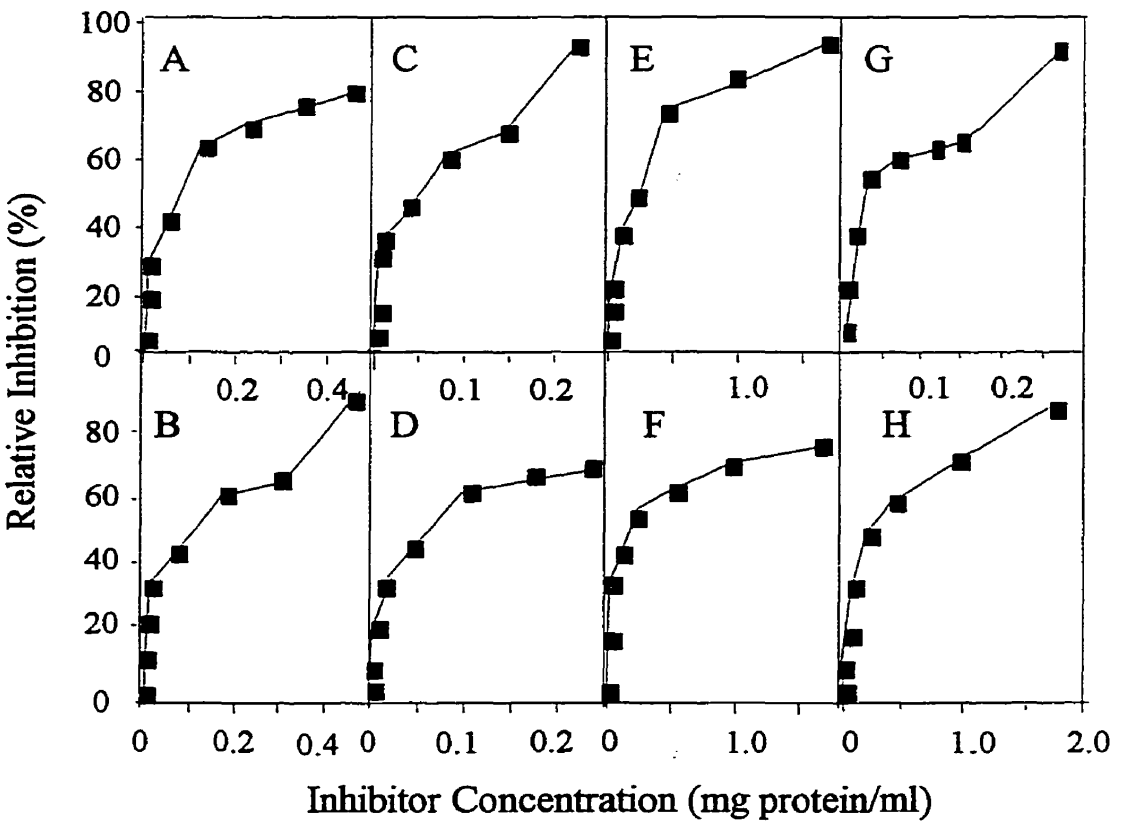


Figure 14. Inhibitory effect of the ultrafiltration copper-metallothionein fraction FIII on the mushroom tyrosinase activity, using as substrate caffeic acid (A), catechol (B), chlorogenic acid (C), L-3,4-dihydroxyphenyl alanine (D), 4-methyl catechol (E) , 3,4-dihydroxyphenyl acetic acid (F), catechin (G), and 3-(*p*-hydroxyphenyl)-propionic acid (H), determined by spectrophotometric method.

inhibiting the onset of color formation in the reaction mixture was in the order of CH > CHL > L-DOPA > CF > HOPPA > CT > DOPAA >4-MCT. The results also indicate that the addition of initial concentrations of the Cu-MT extract produced a dramatic decrease in tyrosinase activity with all the selected substrates. In addition, the results show that the percentage of enzymatic inhibition increased with a concomitant increase in Cu-MT concentration, thereby indicating that PPO biocatalysis was inhibited by the Cu-MT extract.

Table 2 shows the inhibition effect of the Cu-MT fraction (FIII) on tyrosinase activity as determined by the I_{50} values, i.e. the concentration of inhibitor required to inhibit enzyme activity by 50%. The results show that the degree of inhibition varied with the substrate used. The range of inhibitor concentrations that yielded 50% of inhibition of PPO activity varied from 25.0 to 185.0 μg protein/ml for the spectrophotometric assays. The results show that 86.5, 72.9 and 68.1% less of the Cu-MT extract was required to obtain 50% of inhibition of tyrosinase activity with the substrates CH, CHL and L-DOPA, respectively, in comparison to that required with 4-MCT as substrate. The data (Table 2) also indicates that the Cu-MT extract did not show any inhibitory effect on mushroom PPO activity with the monophenolic substrates such as HOPA and *p*-and *m*-cresol. However, the Cu-MT extract showed a relatively moderate inhibitory effect on tyrosinase activity with CT, CF and the monophenolic HOPPA as substrates.

The inhibition dissociation constants, K_i values, obtained from the Dixon (1953) plots of inverse rates of oxidation ($1/v$) versus the concentration of inhibitor (I), are also shown in Table 2. The results show that depending on the substrates used, the K_i values varied from 0.08 to 0.40 μg protein/ml of Cu-MT extract for the spectrophotometric analyses. Table 2 also indicates that the Cu-MT extract showed the best inhibitory effect on PPO activity with CH (0.08) as substrate while the least inhibitory was found when 4-MCT (0.40) was used as substrate. These findings are analogous with the trend observed with the I_{50} values (Table 2).

Table 2. Inhibition of tyrosinase activity by a partially purified copper-metallothionein extract (FIII) obtained from *Aspergillus niger*, determined by spectrophotometer.

Substrate	I_{50}[a]	K_i[b]
Caffeic acid	80	0.28
Catechol	130	0.25
4-Methyl catechol	185	0.40
L-3,4-Dihydroxyphenyl alanine	59	0.21
3,4-Dihydroxyphenyl acetic acid	175	0.35
3-(*p*-Hydroxyphenyl)-propionic acid	159	0.12
Chlorogenic acid	50	0.25
Catechin	25	0.08
3,4-Dihydroxyphenylpyruvic acid	-[c]	-[c]
P-Cresol	-[c]	-[c]
m-Cresol	-[c]	-[c]

[a]The concentration of copper-metallothionein (μg protein/ml) required to inhibit tyrosinase activity by 50%.

[b]Inhibition dissociation constant (μg protein/ml).

[c]No inhibition effect.

4.7.2.2. Polarographic Determination

Figure 15 shows that the maximum inhibitory effect on tyrosinase activity by the Cu-MT extract increased in the reaction medium containing the selected substrate in the order of CH > CHL > HOPPA > L-DOPA > 4-MCT > CT > DOPAA > CF, measured polarographically. The results also indicate that lower concentrations of inhibitor were needed to obtain inhibition of tyrosinase activity determined with the spectrophotometer in comparison to those required for the polarograph as demonstrated by a decrease in PPO activity of 95% for the former assay in comparison to only a 60% decrease for the latter in the presence of 1.0 mg protein/ml Cu-MT extract with CT as substrate. A similar PPO inhibitory trend was reported by Kermasha *et al.* (1993b) with the inhibitors L-cysteine and DL-cysteine using CH as substrate, determined by polarograph. Previously, Golan-Goldhirsh and Whitaker (1984) also reported the inhibition of PPO activity by the reducing agents ascorbic acid, sodium bisulfite and thiol-compounds, using CF, L-DOPA and DL-DOPA as substrates; moreover, the same authors reported that less amounts of inhibitor were needed to produce a decrease in PPO activity for the spectrophotometric method than the polarographic one.

The results (Table 3) show that the range of inhibitor concentrations that yielded 50% of inhibition of PPO activity (I_{50}), varied from 55.0 to 750.0 μg protein/ml for the polarographic assays. The results also show that the I_{50} values for the inhibitory effect of the Cu-MT extract on PPO biocatalysis were approximately 13.6, 5.4 and 4.7 times lower with the substrates CH, CHL, and HOPPA, respectively, compared to that obtained with CF as substrate for the polarographic assays. In comparison to the spectrophotometric assays, the overall I_{50} values for tyrosinase biocatalysis were found to be higher in the polarographic assays. In addition, both spectrophotometric and polarographic assays showed that tyrosinase activity was inhibited by the lowest concentration of Cu-MT using CH as substrate and that no inhibitory effect on PPO activity was observed with the substrates HOPA and *p*-and *m*- cresol.

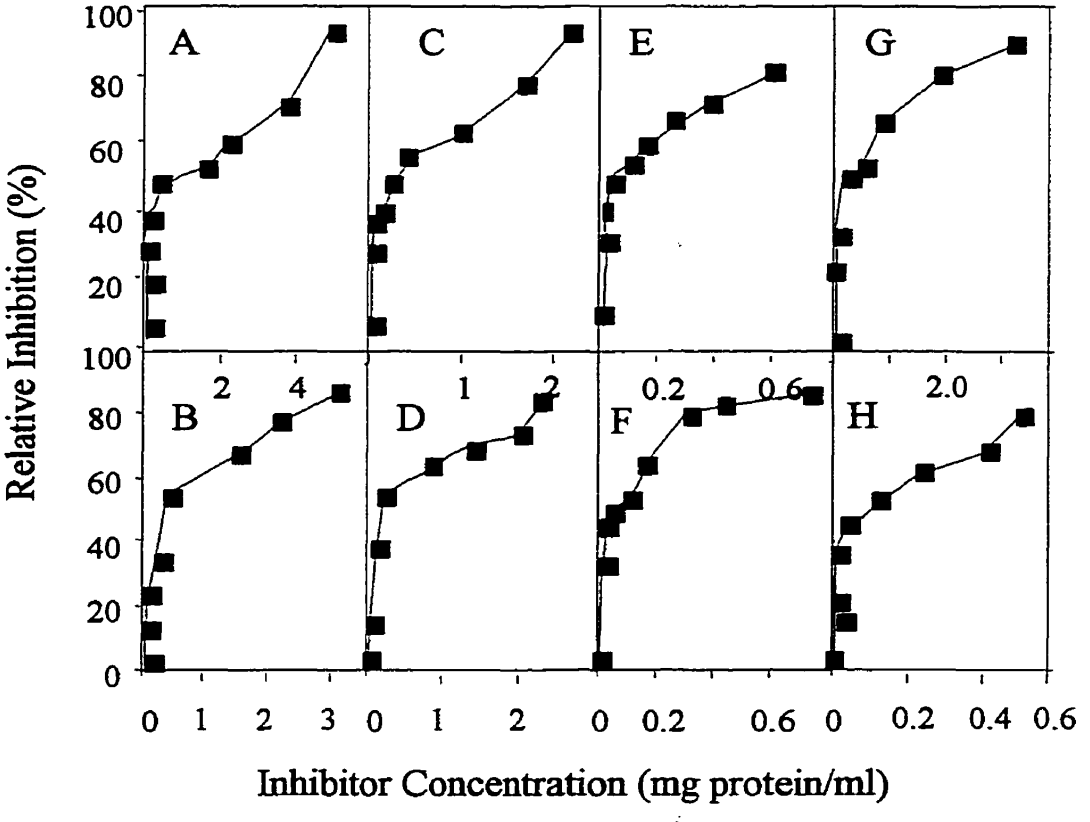


Figure 15. Inhibitory effect of the ultrafiltration fraction copper-metallothionein FIII on mushroom tyrosinase activity, using as substrate caffeic acid (A), catechol (B) , 4-methyl catechol (C), 3,4-dihydroxyphenyl alanine (D), catechin (E), chlorogenic acid (F), 3,4-dihydroxyphenyl acetic acid (G), and 3-(*p*-hydroxyphenyl)-propionic acid (H), determined by the polarographic method.

Table 3. Inhibition of tyrosinase activity by a partially purified copper-metallothionein extract (FIII) obtained from *Aspergillus niger*, determined by polarograph.

Substrate	I_{50}[a]	K_i[b]
Caffeic acid	750	2.69
Catechol	450	4.31
4-Methyl catechol	420	6.19
L-3,4-Dihydroxyphenyl alanine	350	3.51
3,4-Dihydroxyphenyl acetic acid	510	2.25
3-(*p*-Hydroxyphenyl)-propionic acid	159	5.30
Chlorogenic acid	139	3.20
Catechin	55	0.70
3,4-Dihydroxyphenyl pyruvic acid	-[c]	-[c]
P-Cresol	-[c]	-[c]
m-Cresol	-[c]	-[c]

[a]The concentration of copper-metallothionein (μg protein/ml) required to inhibit tyrosinase activity by 50%.

[b]Inhibition dissociation constant (μg protein/ml).

[c]No inhibition effect.

Table 3 shows that the K_i values ranged from 0.70 to 6.19 μg protein/ml for the Cu-MT extract with the selected substrates in the polarographic analyses. The results also indicate that in the polarographic assays, the Cu-MT extract showed the best inhibitory effect with CH as measured by the K_i values which decreased in the order of: CH<DOPAA < CF<CHL < L-DOPA < CT < HOPPA < 4-MCT.

The overall findings suggest that the observed differences in the degree of inhibition of PPO activity by the Cu-MT extract, with respect to the methods used, may be related to the complex structure of the PPO molecule. Lerch (1987) reported that PPO enzymes contain a copper pair, one at the active site for interaction with molecular oxygen and the other for organic substrates. Pifferi *et al.* (1974) also indicated that PPO might contain certain inhibitory sites, which were distinct from the active sites. These authors suggested that the different degrees of enzymatic inhibition obtained by these two methods may be due to the occurrence of a complex kinetics phenomenon, involving various interactions between the active and inhibitory sites of the enzyme with the selected substrates, in the presence of the Cu-MT extract.

The overall findings also confirmed that the spectrophotometric assay had a lower limit of detection than the polarographic one for the determination of the inhibitory effect of the Cu-MT extract on PPO activity. Previously in our laboratory, Goetgherbeur and Kermasha (1996) reported similar findings in the use of the spectrophotometer in the determination of the inhibitory effect of the Cu-MT extract on mushroom PPO activity, using CHL and CH as substrates. Espin *et al.* (1995) also reported a lower limit of detection for the spectrophotometric assays in comparison to the polarographic assays in the determination of the catalytic efficiency of PPO using HOPPA as substrate.

4.7.3. Kinetic Studies of Tyrosinase Activity in the Presence of the UltrafiltrationCu-MT Fraction as Inhibitor

The Lineweaver and Burk plot (1934) of $1/v$ versus $1/S$ was used to determine the kinetic parameters of tyrosinase biocatalysis in the presence and absence of the Cu-MT extract measured by both the spectrophotometric and polarographic methods. In all the

cases, the Michaelis constant K_m and K_{mapp}, i.e. the equilibrium constant of the reversible combination of an enzyme and substrate with and without inhibitor, respectively, were calculated. Furthermore, the V_{max} and V_{maxapp}, values, i.e. the maximum velocity of the enzymatic reaction with and without inhibitor, respectively, were also determined from the experimental findings obtained using the spectrophotometric and polarographic methods.

4.7.3.1. Spectrophotometric Method

The results presented in Table 4 show that in the presence of the Cu-MT extract, the PPO biocatalysis exhibited higher K_{mapp} (0.32-2.00 mM) and lower V_{maxapp} (1.00-5.41 mmol products/μg protein/ml) values than the K_m (0.28-1.68 mM) and V_{max} (1.46-6.29 mmol products/μg protein/ml) values for the spectrophotometric analyses. The experimental findings (Fig. 16 and Fig. 17) show that in terms of patterns of inhibition, the spectrophotometric analyses demonstrated that a mixed type inhibitory effect of the Cu-MT extract on PPO activity was obtained with all the selected substrates. Chen *et al.* (1991a) reported that Kojic acid showed a mixed type inhibitory effect on PPO activity with DL-DOPA as substrate as well as a competitive inhibitory effect with L-tyrosine as substrate, determined spectrophotometrically. The overall findings suggest that in the spectrophotometric analyses, the Cu-MT extract inhibited the formation of brownish pigments by PPO activity with the selected substrates by either interacting directly with the enzyme and affecting its affinity for the substrates and/or by reducing the enzymatically-catalyzed *o*-quinones to stable colorless end-products.

4.7.3.2. Polarographic Method

Kinetic studies of mushroom PPO activity with a number of selected substrates in the presence and absence of the Cu-MT extract, determined polarographically, are shown in Figures 18 and 19. The results show that the Cu-MT extract showed different types of inhibitory effects on PPO biocatalysis with respect to the different substrates used. A mixed type of inhibition was observed with the substrates L-DOPA and CHL while with the substrates CT and DOPAA, the Cu-MT extract showed a competitive type of inhibitory effect on PPO activity. The Cu-MT extract also showed an uncompetitive type

Table 4. Kinetic parameters of the inhibitory effect of a partially purified coppe-metallothionein extract FIII on tyrosinase activity, using the spectrophotometric method.

	Without Cu-MT			With Cu-MT			
Substrate	K_m[a]	V_{max}[b]	Enzyme efficiency[c]	K_{mapp}[d]	V_{maxapp}[e]	Enzyme efficiency[c]	Type of Inhibition
CF[f]	1.16	3.15	2.7	1.43	2.28	1.6	mixed
CT[f]	0.44	1.69	3.8	0.53	1.62	3.1	mixed
4-MCT[f]	0.33	5.41	16.4	0.36	3.45	9.6	mixed
L-DOPA[f]	0.42	3.33	7.9	0.49	2.43	5.0	mixed
DOPAA[f]	1.68	6.29	3.7	2.00	5.41	2.7	mixed
HOPPA[f]	0.28	1.46	5.2	0.32	1.36	4.2	mixed
CH[f]	0.35	5.13	14.7	0.50	3.87	7.7	mixed
CHL[f]	0.32	1.53	4.8	0.43	1.00	2.3	mixed

[a] K_m values for polyphenol oxidase in the absence of copper-metallothionein (μg protein /ml).

[b] V_{max} values for polyphenol oxidase in the absence of copper-metallothionein (mmol product/μg protein /ml) $x10^{-2}$.

[c] Enzyme efficiency V_{max}/K_m or V_{maxapp}/ K_{mapp} ($x10^{-2}$).

[d] K_{mapp} values for polyphenol oxidase in the presence of copper-metallothionein (μg protein/ml).

[e] V_{maxapp} values for polyphenol oxidase in the presence of copper-metallothionein (mmol product /μg protein /ml) $x10^{-2}$.

[f] Caffeic acid (CF); catechol (CT); 4-methyl catechol (4-MCT); L-3,4-dihydroxyphenyl alanine (L-DOPA); 3,4-dihydroxyphenyl acetic acid (DOPAA); 3-(*p*-hydroxyphenyl)-propionic acid (HOPPA); catechin (CH); chlorogenic acid (CH).

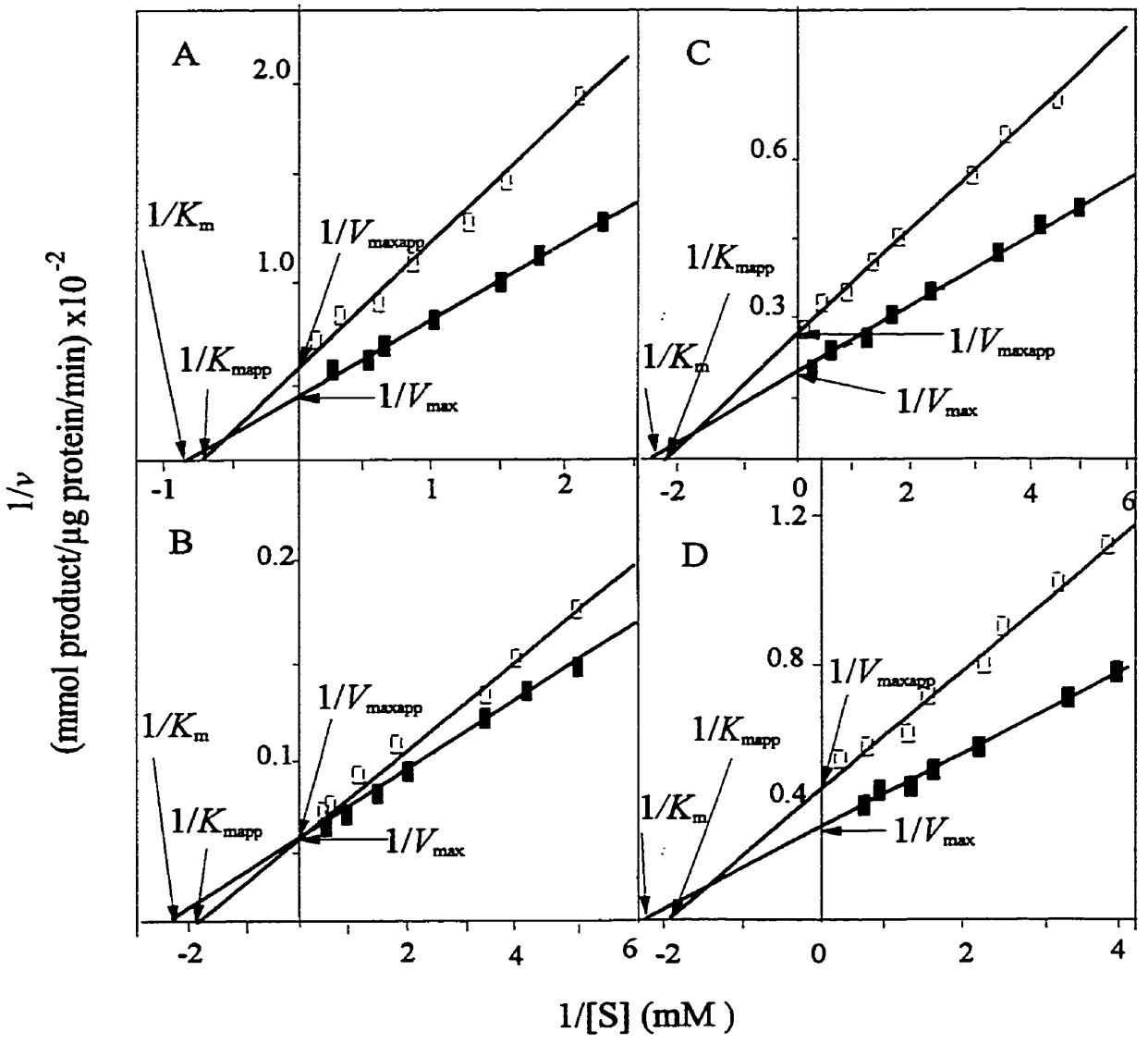


Figure 16. Spectrophotometric measurement of mushroom tyrosinase activity using the selected phenolic substrates including caffeic acid (A), catechol (B), 4-methlyl catechol (C), and 3,4-dihydroxyphenyl- alanine (D) with (□) and without (■) the partially purified copper-metallothionein fraction FIII used as inhibitor.

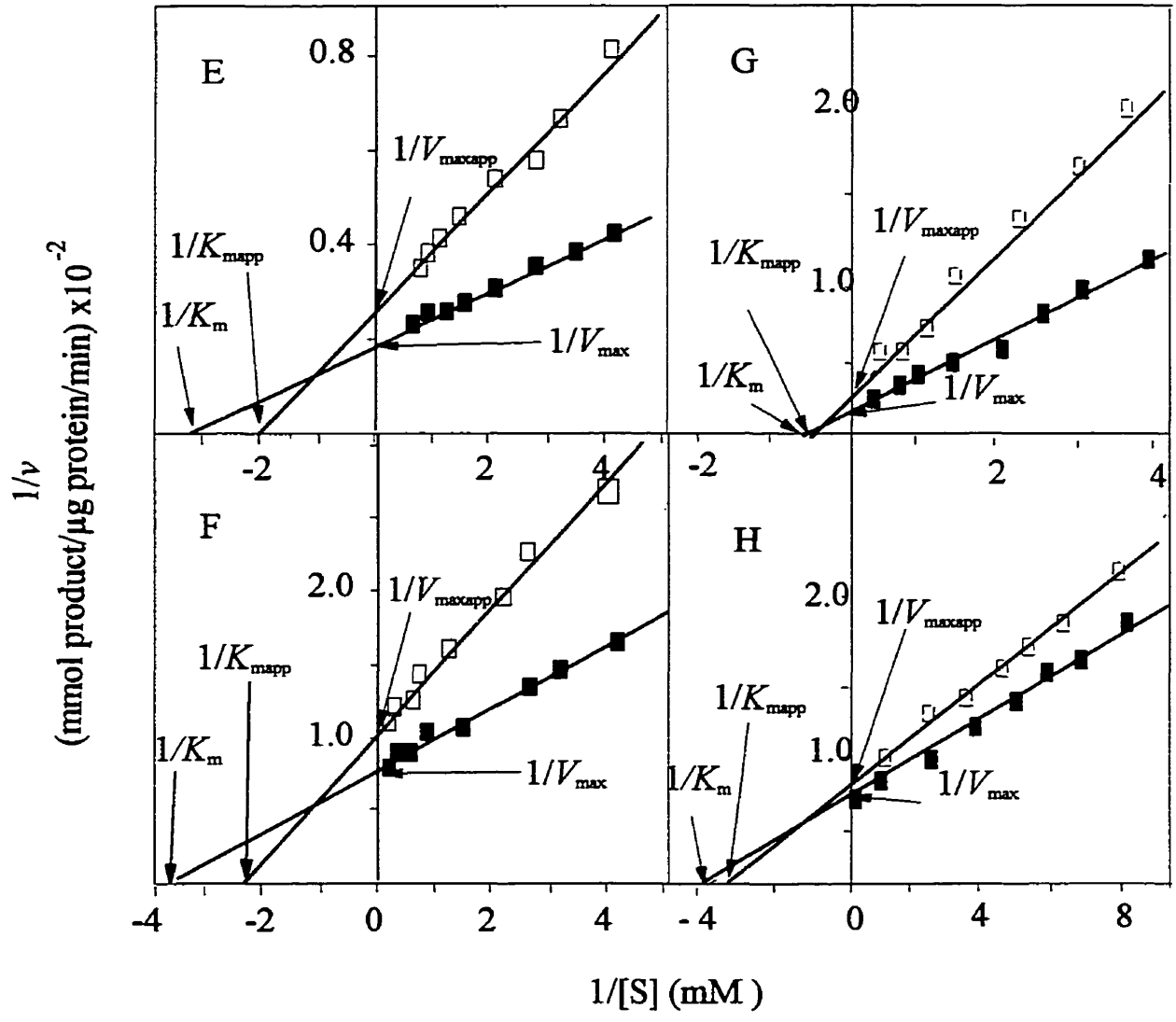


Figure 17. Spectrophotometric measurement of mushroom tyrosinase activity using the selected phenolic substrates including catechin (E), chlorogenic acid (F), 3,4-dihydroxyphenyl acetic acid (E), and 3-(*p*-hydroxyphenyl) propionic acid (F), with (□) and without (■) the partially purified copper-metallothionein fraction FIII used as inhibitor.

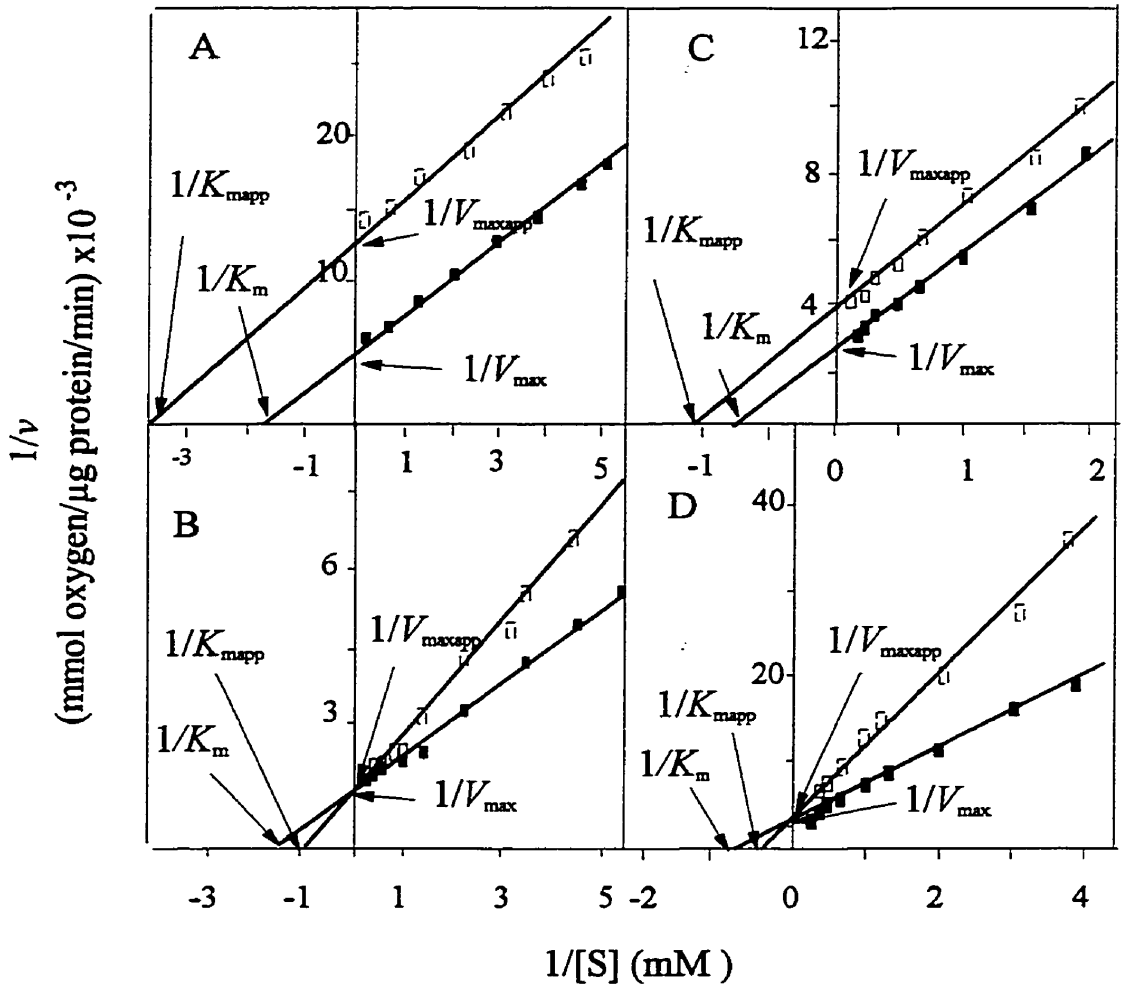


Figure 18. Polarographic measurement of mushroom tyrosinase activity using the selected phenolic substrates including caffeic acid (A), catechol (B), 4-methlylcatechol (C), and 3,4-dihydroxyphenylalanine (D) with (□) and without (■) the partially purified copper metallothionein fraction FIII used as inhibitor.

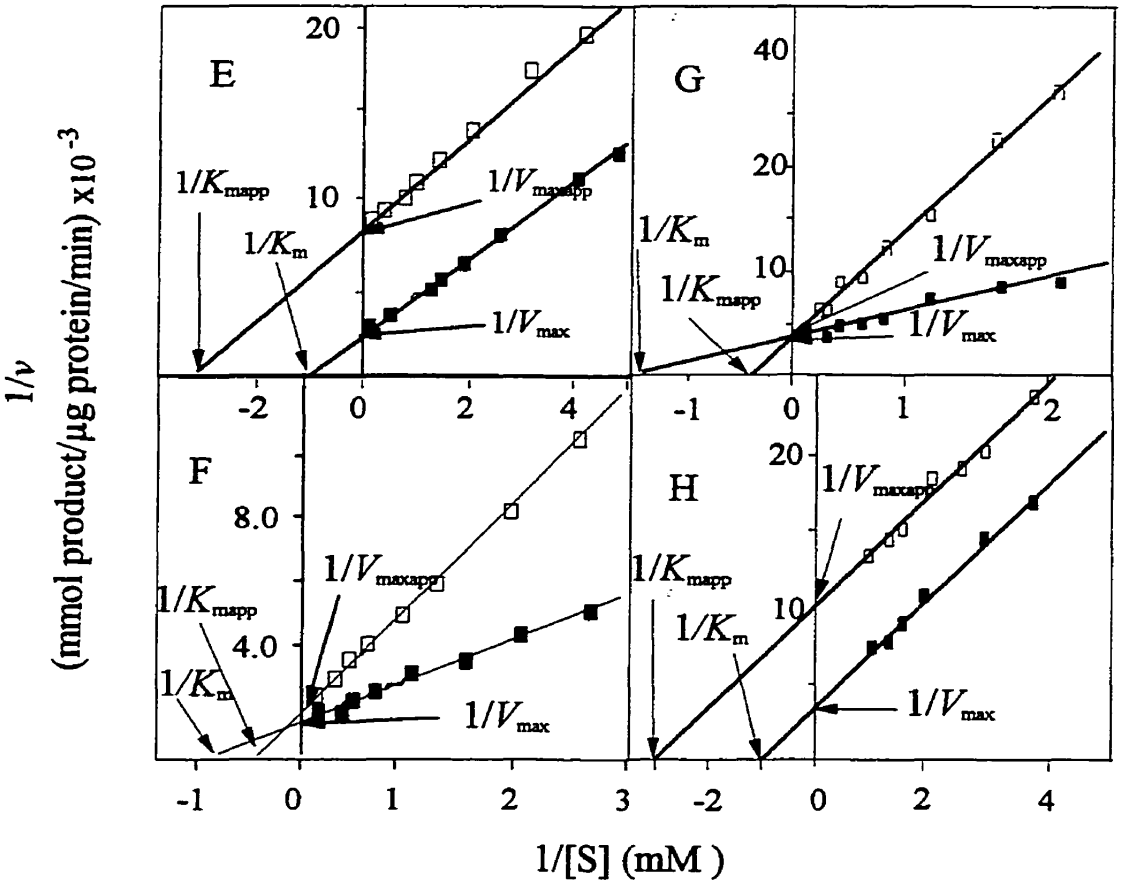


Figure 19. Polarographic measurement of mushroom tyrosinase activity using the selected phenolic substrates including catechin (E), chlorogenic acid (F), 3,4-dihydroxyphenyl acetic acid (G), and 3-(*p*-hydroxyphenyl) propionic acid (H), with (■) and without (□) the partially purified copper-metallothionein fraction FIII used as inhibitor.

of inhibition on tyrosinase activity with the substrates CF, 4-MCT, HOPPA and CH. These findings suggest that the inhibitory effect of the Cu-MT extract depended on the presence of the substrates used for the polarographic assays.

Table 5 shows that the kinetic values obtained in the polarographic analyses indicated that a competitive type of inhibition by the Cu-MT extract on PPO activity was obtained using CT and DOPAA as indicated by the higher K_{mapp} values and similar V_{max} and V_{maxapp} values. These findings suggest that the Cu-MT extract inhibited enzymatic activity by combining with the free PPO in such a manner that decreased the substrate binding capacity of the enzyme, so that the inhibitor and the substrate were mutually exclusive due to true competition for the same active sites (Segel, 1976).

The results (Table 5) also indicate that an uncompetitive type of inhibition by the Cu-MT on PPO activity was exhibited with CF, 4-MCT, HOPPA, and CH as indicated by the higher V_{max} and K_m values in comparison to the V_{maxapp} and K_{mapp}. These experimental findings suggest that CF, 4-MCT, HOPPA, and CH may have bound independently at different active sites on the enzyme molecules thereby forming enzyme substrate inhibition complexes (Segel, 1976).

The collective results, obtained in this study, demonstrate that the spectrophotometric and polarographic methods have basically different principles in measuring the enzyme activity, since the first is a determination of the absorbance of end products, whereas the second is a measurement of oxygen uptake. The enzymatic rates of the hydroxylation of monophenols to diphenols and the dehydroxylation of diphenols to quinones may be measured by oxygen uptake with the polarograph whereas the latter rate is only measured spectrophotometrically at a wavelength where maximal formation of the brown pigment polymers is observed. Moreover, the overall findings suggest that differences in the inhibitory effect of the Cu-MT extract on PPO biocatalysis may occur due to the presence of reducing agents, such as cysteine residues in the Cu-MT extract, which may reduce *o*-quinones to less reactive *o*-diphenols and monophenols, thereby preventing the occurrence of secondary reactions that produce brown polymers

Table 5. Kinetic parameters of the inhibitory effect of a partially purified copper-metallothionein extract FIII on tyrosinase activity, using the polarographic method.

	Without Cu-MT			With Cu-MT			
Substrate	K_m[a]	V_{max}[b]	Enzyme efficiency[c]	K_{mapp}[d]	V_{maxapp}[e]	Enzyme efficiency[c]	Type of Inhibition
CF[f]	0.48	8.0	16.6	0.22	2.46	11.2	uncompetitive
CT[f]	0.61	6.70	10.9	1.02	6.70	6.6	competitive
4-MCT[f]	1.27	4.00	3.1	1.06	2.86	2.7	uncompetitive
L-DOPA[f]	1.67	3.51	2.1	2.00	2.50	1.3	mixed
DOPAA[f]	0.38	0.24	0.6	2.42	0.24	0.1	competitive
HOPPA[f]	0.92	0.35	0.4	0.32	0.10	0.3	uncompetitive
CH[f]	0.82	0.64	0.8	0.31	0.13	0.4	uncompetitive
CHL[f]	0.84	0.81	0.4	0.28	0.10	0.3	mixed

[a] K_m values for polyphenol oxidase in the absence of copper-metallothionein (μg protein /ml).

[b] V_{max} values for polyphenol oxidase in the absence of copper-metallothionein (mmol products/μg protein /ml) $x10^{-2}$.

[c] Enzyme efficiency V_{max}/K_m or K_{mapp}/V_{maxapp} ($x10^{-2}$).

[d] K_{mapp} values for polyphenol oxidase in the presence of copper-metallothionein (μg protein/ml).

[e] V_{maxapp} values for polyphenol oxidase in the presence of copper-metallothionein (mmol product /μg protein /ml) $x10^{-2}$.

[f] Caffeic acid (CF); catechol (CT); 4-methyl catechol (4-MCT); L-3,4-dihydroxyphenyl alanine (L-DOPA);3,4-dihydroxyphenyl acetic acid (DOPAA); 3-(*p*-hydroxyphenyl)-propionic acid (HOPPA); catechin (CH); and chlorogenic acid (CHL).

characteristics of the browning reaction; no browning will therefore occur and no activity will be measured spectrophotometrically, whereas, oxygen uptake measurements will confirm that a reaction has taken place (Whitaker, 1985).

Different types of inhibitory effects on PPO activity, depending on the substrates as well as the analytical methods used, were also reported by other researchers. Studies carried out by Goetgherbeur and Kermasha (1996) and Kermasha *et al.* (1993b) on the inhibitory effect of the Cu-MT extract on mushroom PPO activity showed different types of inhibition using L- and DL-cysteine and CH as substrate, respectively. Azelaic acid and curcumin were also reported to be competitive inhibitors of mushroom tyrosinase activity with L-tyrosine as substrate (Schallreuter and Wood, 1990; Shirota *et al.*, 1994). In addition, the tyrosinase activity from mushroom (Cabanes *et al.*, 1987) was competitively inhibited by mimosine with L-tyrosine as substrate as well as the PPO activity from grape (Valrero *et al.*, 1991) by tropolon with L-DOPA as substrate. Moreover, oxyresveratrol was reported to be a noncompetitive type of inhibitor of mushroom tyrosinase activity with L-DOPA as substrate (Shin *et al.*, 1998).

Differences in the degree and nature of inhibition of PPO activity reported in the literature may be explained by variations in the enzymatic assays used; these include the effects of pH, temperature, nature and concentration of buffer, ionic strength, extract source and concentration of enzymes, substrate and inhibitor as well as time of reaction (Whitaker, 1985). In addition, measurement of activity may be complicated by many factors including the rapid inactivation of the enzyme during the reaction, further polymerization of quinones formed with a resultant change in absorbance and additional uptake of oxygen, and inhibition of activity at high substrate concentrations (Whitaker, 1985).

4.8. Comparison of the Inhibitory Effect of Cu-MT Extracts of Different Degrees of Purity on Tyrosinase Activity

The influence of the purity of the Cu-MT extract on the inhibition of tyrosinase was investigated using fractions FII, FIVc and FIVc'; Fraction FII was partially purified

by heat treatment, Fraction FIII was further subjected to ultrafiltration while fractions FIVc and FIVc' were further purified by affinity and affinity re-chromatography, respectively. The inhibitory effect of the Cu-MT extracts was determined by measuring the tyrosinase activity with CH and CHL as substrates. Tyrosinase activity was determined both spectrophotometrically and polarographically.

4.8.1. Spectrophotometric Determination

The inhibitory effect of the Cu-MT fractions (FII, FIII, FIVc, and FIVc') on tyrosinase biocatalysis is presented in Table 6 and Figure 20. The results (Figure 20) show that the inhibition of tyrosinase activity was obtained using relatively smaller concentrations of the purified Cu-MT fractions FIVc and FIVc' in comparison to that of the partially purified extract FII, with both substrates. The results (Table 6) also show that the I_{50} values obtained for the inhibitory effect of the affinity chromatography fraction (FIVc) and affinity re-chromatography fractions (FIVc') on tyrosinase activity were 2.5 to 9.0 thousands times lower in comparison to that obtained for the heat treatment fraction (FII). In addition, Table 6 shows that an increased inhibitory effect was observed with the more purified Cu-MT fractions as demonstrated by the lower I_{50} and K_i values, obtained with both substrates CH and CHL, in comparison to those obtained for fractions FII and FIII. In addition, the overall findings suggest that the Cu-MT fractions (FII and FIII) showed a higher inhibitory effect on PPO activity with CH as substrate compared to CHL while the purified fractions FIVc and FIVc' showed no significant difference (Table 6) with both substrate. These findings indicate that the inhibitory effect of the Cu-MT extract on tyrosinase activity increased with a concomitant increase in purification of the Cu-MT extract. Furthermore, the overall findings show that the inhibition of PPO activity varied with respect to the substrate present in the reaction environment, i.e. the inhibitory effect was substrate dependent.

Kinetic data on mushroom tyrosinase activity in the presence of three different Cu-MT extracts, including the heat treatment fraction (FII), the affinity chromatography fraction (FIVc), and the affinity re-chromatography fraction (FIVc') is shown in Figure 21 and Table 7. The results (Fig. 21) show that all three Cu-MT fractions showed a mixed

Table 6. Inhibition of tyrosinase activity by copper-metallothionein extracts, obtained from *Aspergillus niger*, determined by spectrophotometer.

Substrate	Fraction	I_{50}[a]	K_i[b]
Catechin			
	Heat treatment (FII)	50.00	4.30
	Ultrafiltration (FIII)	25.00	0.08
	Affinity chromatogram (FIV)	0.02	0.05
	Affinity re-chromatogram (FIVc')	0.02	0.03
Chlorogenic acid			
	Heat treatment (FII)	185.00	2.50
	Ultrafiltration (FIII)	50.00	0.25
	Affinity chromatogram (FIV)	0.03	0.09
	Affinity re-chromatogram (FIVc')	0.02	0.02

[a]Concentration of copper-metallothionein (μg protein/ml) required to inhibit tyrosinase activity by 50%.

[b]Inhibition dissociation constant (μg protein/ml).

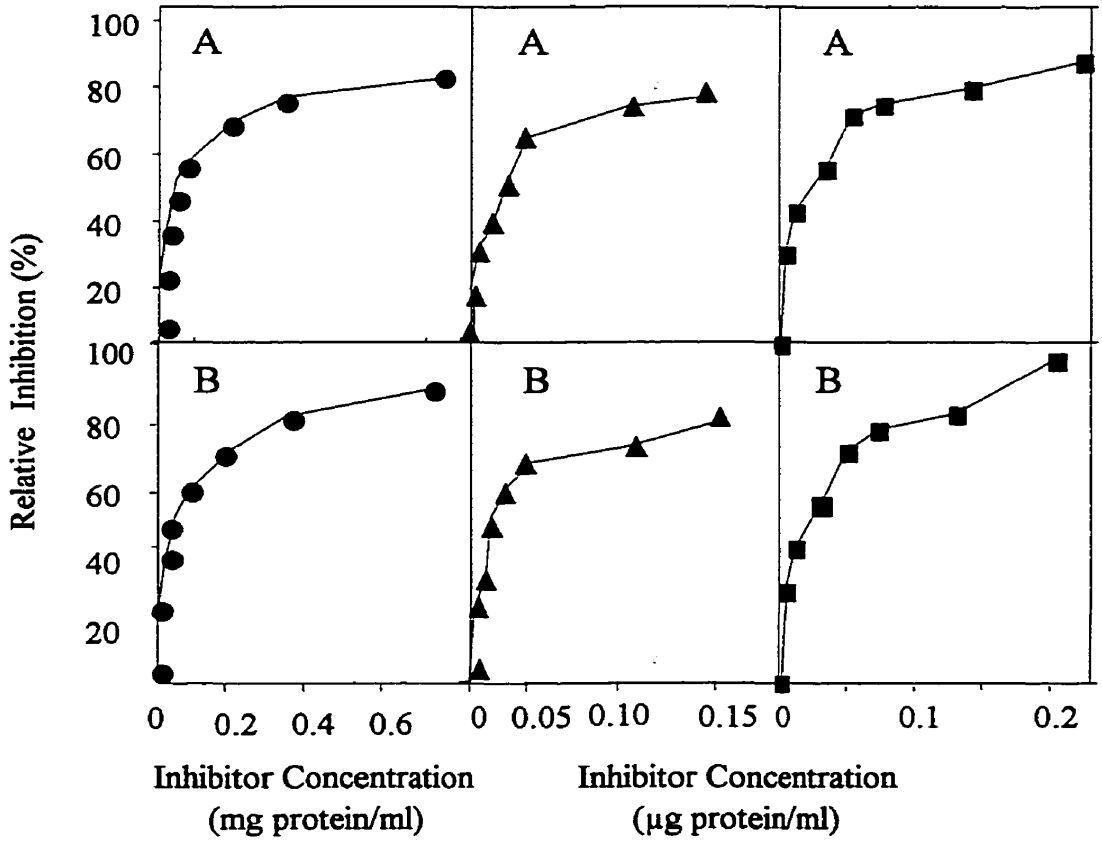


Figure 20. Comparison of the inhibitory effect of the copper-metallothionein fractions FII (●), FIVc (▲) and FIVc' (■) on the mushroom tyrosinase activity, using catechin (A) and chlorogenic acid (B) as substrates, determined by the spectrophotometric method.

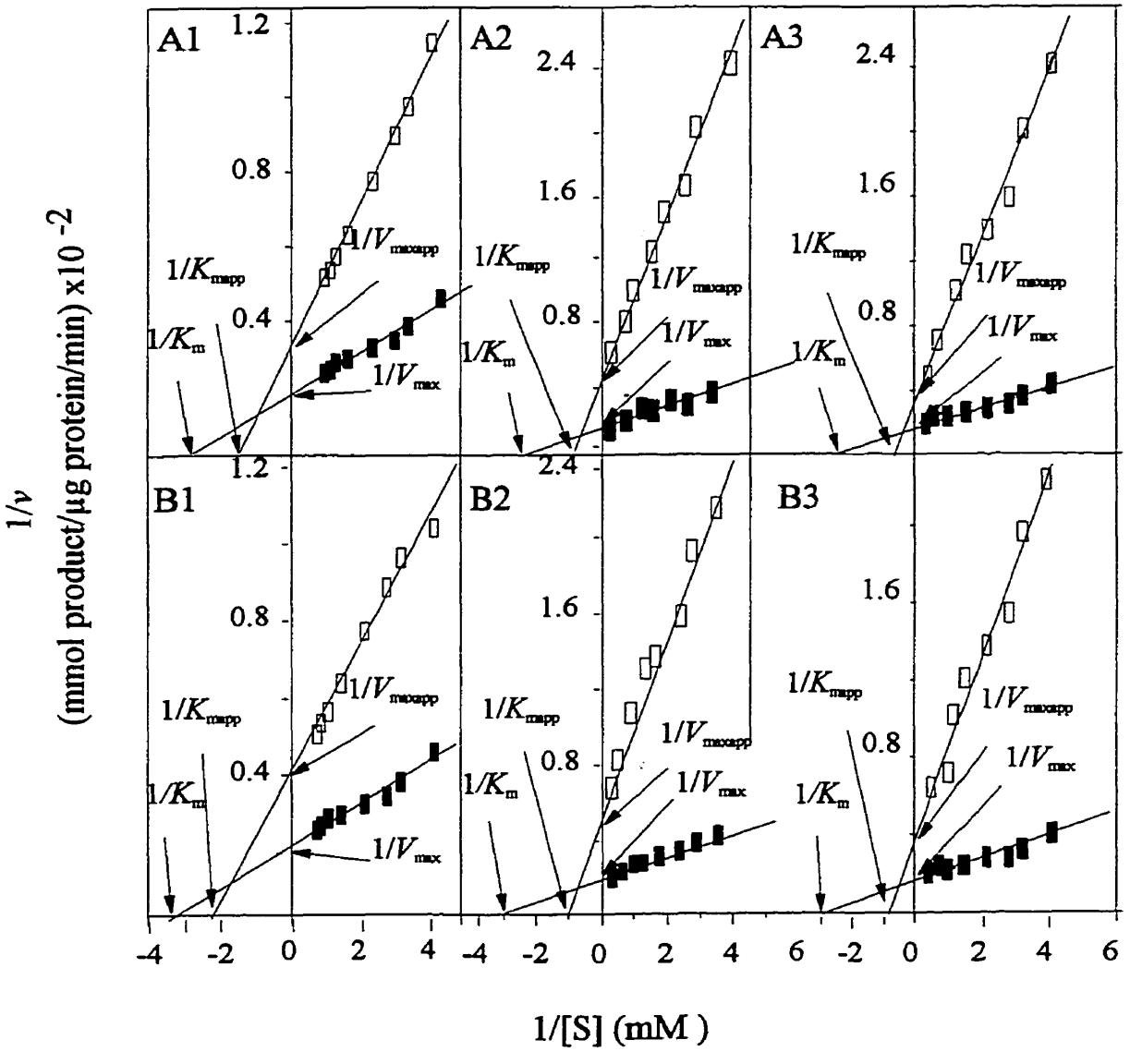


Figure 21. Spectrophotometric measurement of mushroom tyrosinase activity using as substrates catechin (A) and chlorogenic acid (B) with (□) and without (■) the copper-metallothionein fraction FII (1), fraction FIVc (2) and fraction FIVc' (3) as inhibitor.

Table 7: Kinetic parameters of the inhibitory effect of copper-metallothionein extracts on tyrosinase activity, determined by spectrophotometric method.

		Without Cu-MT			With Cu-MT			
Substrate	Fraction	K_m[a]	V_{max}[b]	Enzyme efficiency[c]	K_{mapp}[d]	V_{maxapp}[e]	Enzyme efficiency[c]	Type of inhibition
Catechin								
	FII[f]	0.31	5.12	16.5	0.55	3.33	6.1	mixed
	FIII[f]	0.29	5.00	17.2	1.8	3.62	2.0	mixed
	FIVc[f]	0.30	5.22	17.4	1.1	2.39	2.2	mixed
	FIVc'[f]	0.27	4.98	18.4	1.1	2.53	2.3	mixed
Chlorogenic acid								
	FII[f]	0.33	4.95	15.0	0.48	2.60	5.4	mixed
	FIII[f]	0.35	4.85	13.8	1.93	1.12	0.6	mixed
	FIVc[f]	0.30	4.80	16.0	1.05	2.27	2.2	mixed
	FIVc'[f]	0.31	4.71	15.2	1.05	2.53	2.4	mixed

[a] K_m values for polyphenol oxidase (µg protein /ml).

[b] V_{max} values for polyphenol oxidase in the absence of copper-metallothionein (µmol products/µg protein /ml) x10^{-2}.

[c] Enzyme efficiency V_{max}/K_m or V_{mapp}/K_{maxapp} (x10^{-2}).

[d] K_{mapp} values for polyphenol oxidase in the presence of copper-metallothionein (µg protein/ml).

[e] V_{maxapp} values for polyphenol oxidase in the presence of copper-metallothionein (µmol product /µg protein /ml) x10^{-2}.

[f] Fractions FII, FIII, FIVc and FIVc' were obtained after the crude extract was successively subjected to heat treatment, ultrafiltration, affinity and re-affinity chromatography, respectively.

type inhibitory effect with both CH and CHL as substrates, determined spectrophotometrically. Similar results for the inhibition of tyrosinase activity with both substrates were obtained by Goetghebeur and Kermasha (1996) using an affinity chromatography purified Cu-MT fraction from *A. niger*, determined by the spectrophotometric method. The results also show (Table 7) that the V_{maxapp} values were lower than the V_{max} values for all Cu-MT fractions for both substrates, CH and CHL, determined by spectrophotometric method. Moreover, Table 7 indicates that the effectiveness of the enzyme binding capacities were lower in the presence of Cu-MT fractions than their absence. In addition, the result shows that the enzyme efficiency of the tyrosinase biocatalysis substantially decreased with a concomitant increase in the purity of the Cu-MT fractions with both substrates. These findings suggest that the enzyme was affected by the presence of the Cu-MT which resulted in a decrease in the PPO affinity for the substrates.

4.8.2. Polarographic Determination

Figure 22 and Table 8 shows the polarographic analysis of the inhibitory effect of different Cu-MT fractions (FII, FIVc, and FIVc') on tyrosinase activity. The results (Fig. 22) show that a similar trend for the inhibitory effect of the Cu-MT extracts was observed for the polarographic analyses as that obtained for the spectrophotometric one, i.e. relative lower concentrations of the more purified fractions FIVc and FIVc' inhibited tyrosinase biocatalysis in comparison to that required of the heat-treatment fractions (FII). In addition, Table 8 indicates that the inhibitory effect increased step wise with a concomitant increase in the purity of the Cu-MT fractions as indicated by the I_{50} values which decreased from 800.0 for fraction FII to 0.28 μg protein/ml for fraction FIVc', using the substrate CH. A similar trend for the inhibitory effect of all the Cu-MT fractions on tyrosinase biocatalysis was also observed with the substrate CHL. The results also show that the inhibition efficiency of the affinity and affinity re-chromatography fractions (FIVc and FIVc' respectively) on tyrosinase activity increased by 1.2 to 2.5 thousands times in comparison to that obtained with the heat treatment

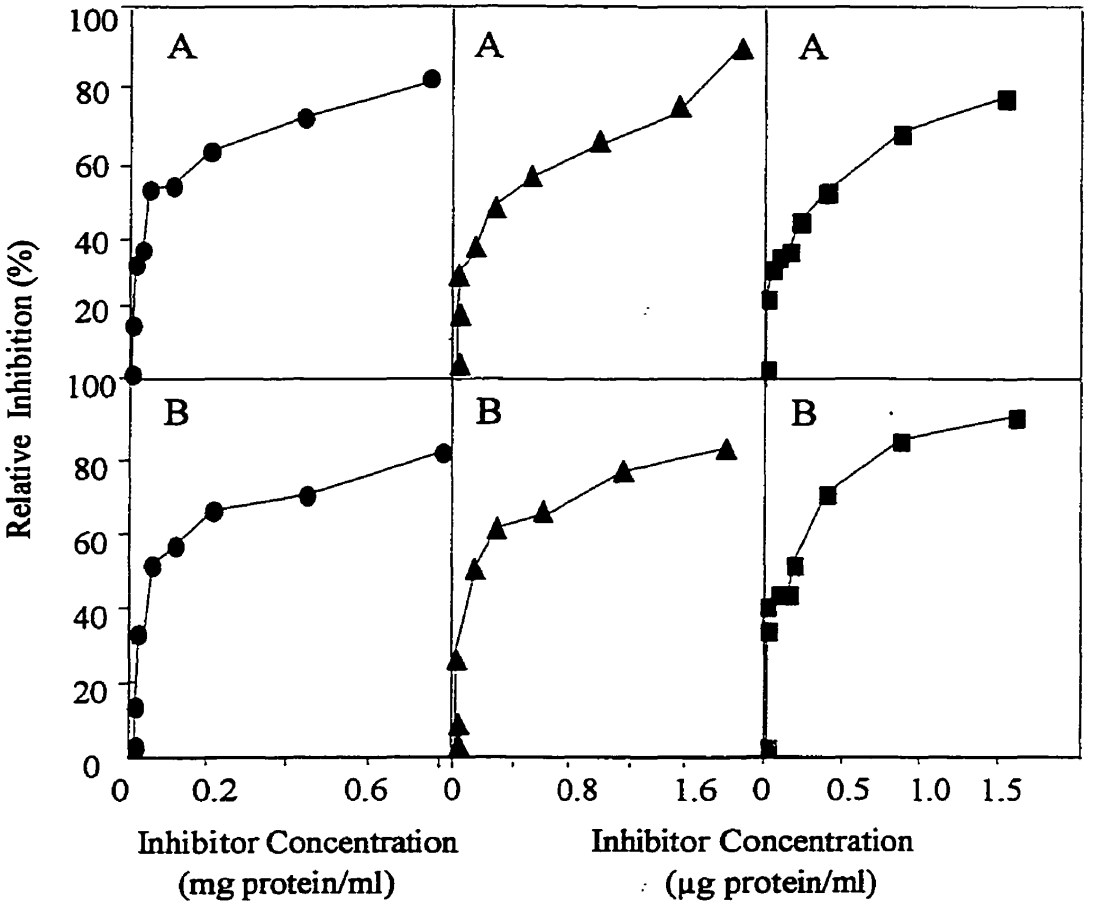


Figure 22. Comparison of the inhibitory effect of copper-metallothionein fractions FII (●), FIVc (▲) and FIVc' (■) on the mushroom tyrosinase activity, using catechin (A) and chlorogenic acid (B) as substrates, determined by the polarographic method.

Table 8. Inhibition of tyrosinase activity by copper–metallothionein extracts, obtained from *Aspergillus niger*, determined by polarographic method.

Substrate	Fraction	I_{50}[a]	K_i[b]
Catechin			
	Heat treatment (FII)	800.00	4.20
	Ultrafiltration (FIII)	55.00	3.20
	Affinity chromatogram (FIV)	0.29	0.07
	Affinity re-chromatogram (FIVc')	0.28	0.03
Chlorogenic acid			
	Heat treatment (FII)	950.00	3.10
	Ultrafiltration (FIII)	139.00	0.70
	Affinity chromatogram (FIV)	0.31	0.07
	Affinity re-chromatogram (FIVc')	0.29	0.05

[a]Concentration of copper-metallothionein (μg protein/ml) required to inhibit tyrosinase activity by 50%.

[b]Inhibition dissociation constant (μg protein/ml).

fraction (FII). In addition, the results also showed that the affinity of tyrosinase for Cu-MT increased with its degree of purification as indicated by the decrease in the K_i values using both substrates. Moreover, the overall results indicate that all the Cu-MT fractions investigated in this study showed a higher inhibitory effect on PPO activity with the spectroscopic method compared to that obtained with the polarographic method.

For the kinetic studies, Figure 23 indicates that all the Cu-MT fractions except FIVc, showed an un-competitive type of inhibition on PPO activity with the substrate CH while with CHL, all the fractions except fraction FII showed a mixed type of inhibition. The results (Table 9) also show that with all the Cu-MT fractions, lower enzyme efficiency was obtained with the substrate CH than with CHL. These overall findings suggest that the affinity of the enzyme for the substrates, CH and CHL, decreased with a concomitant increased in the purity of the Cu-MT fractions.

The overall findings for both spectrophotometric and polarographic analyses show that different types of inhibitory effects were obtained depending on the methods and substrates used for the inhibition assays. The overall results also show that a lower amount of the Cu-MT extracts was needed to inhibit tyrosinase activity in the spectroscopic method than that needed for the polarographic method. In addition, the Cu-MT extracts showed a higher inhibitory effect on tyrosinase activity when CH was used as substrate than CHL.

4.9. Tyrosinase-Biocatalyzed End Products Determination

Figures 24, 25, and 26 show the spectroscopic scanning profile of the enzymatically-catalyzed end products using six different substrates including CF, CT, 4-MCT, L-DOPA, DOPAA and HOPPA in the presence and absence of the purified Cu-MT fraction FIVc'. These results clearly show that in the presence of the Cu-MT, fraction FIVc', the absorption spectra of the end products of each of the six substrates were lower than in the absence of the Cu-MT extract. Table 10 shows that the maximum absorption spectrum of the end-products of the respective substrates shifted towards a lower wavelength in the presence of the inhibitor for all the end-products of substrates except

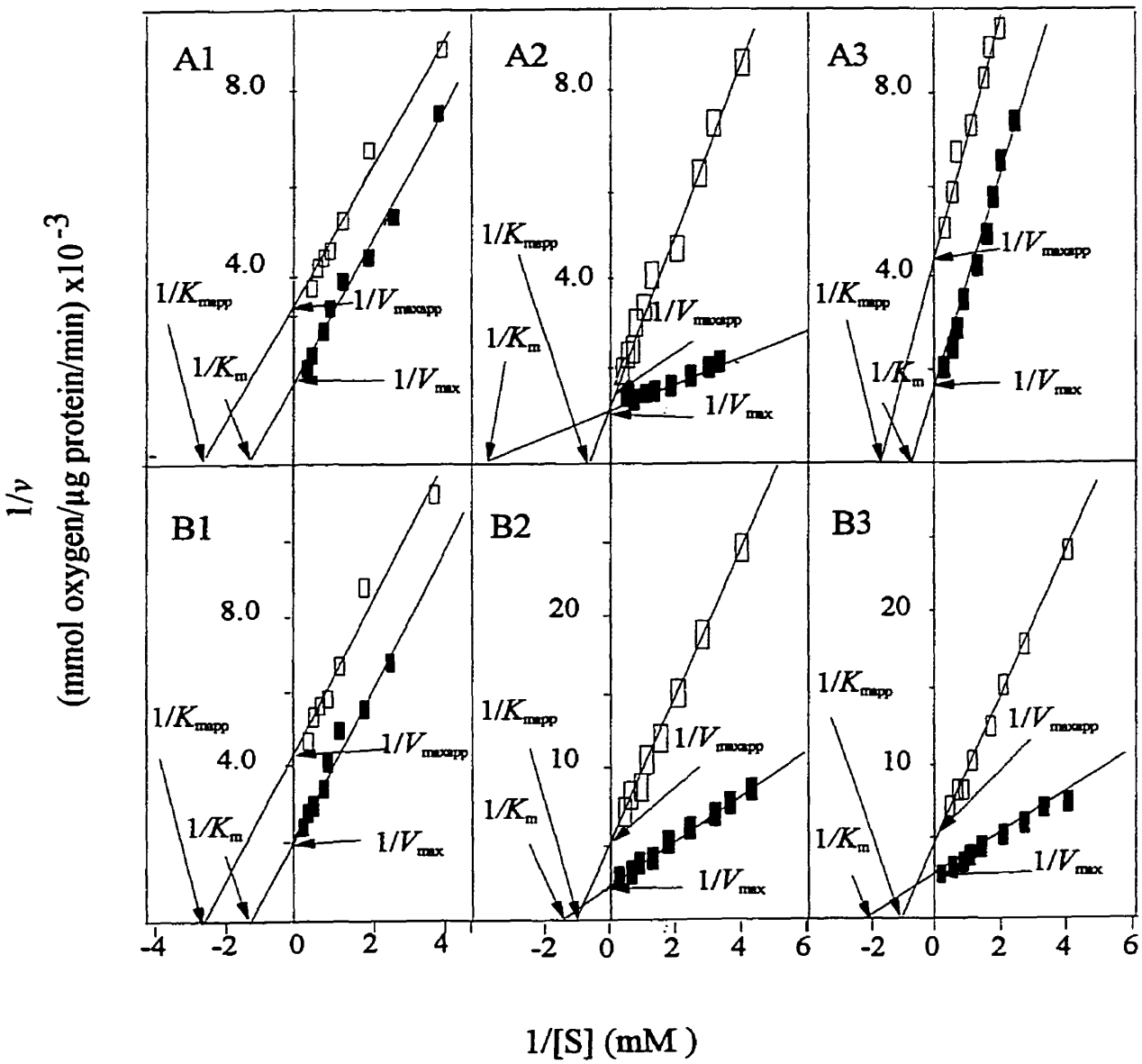


Figure 23. Polarographic measurement of mushroom tyrosinase activity using as substrates catechin (A) and chlorogenic acid (B) with (□) and without (■) the copper-metallothionein fraction FII (1), fraction FIVc (2) and fraction FIVC' (3) as inhibitor.

Table 9. Kinetic parameters of the inhibitory effect of copper-metallothionein extracts on tyrosinase activity, determined by polarographic method.

Substrate	Fraction	Without Cu-MT			With Cu-MT			Type of inhibition
		K_m [a]	V_{max} [b]	Enzyme efficiency [c]	K_{mapp} [d]	V_{maxapp} [e]	Enzyme efficiency [c]	
Catechin								
	FII[f]	0.80	0.63	0.78	0.63	0.26	0.41	uncompetitive
	FIII[f]	0.85	0.61	0.72	0.95	0.21	0.11	uncompetitive
	FIVc[f]	0.79	0.60	0.76	1.80	0.29	0.16	mixed
	FIVc'[f]	0.95	0.65	0.68	0.65	0.20	0.30	uncompetitive
Chlorogenic acid								
	FII[f]	0.71	0.52	0.73	0.35	0.24	0.68	uncompetitive
	FIII[f]	0.69	0.49	0.71	0.95	0.68	0.72	mixed
	FIVc[f]	0.78	0.56	0.72	1.00	0.20	0.20	mixed
	FIVc'[f]	0.68	0.48	0.70	0.91	0.32	0.35	mixed

[a] K_m values for polyphenol oxidase (μg protein /ml).

[b] V_{max} values for polyphenol oxidase in the absence of copper-metallothionein (μmol products/ μg protein /ml) $x10^{-2}$.

[c] Enzyme efficiency V_{max}/K_m or V_{mapp}/K_{maxapp} ($x10^{-2}$).

[d] K_{mapp} values for polyphenol oxidase in the presence of copper-metallothionein (μg protein/ml).

[e] V_{maxapp} values for polyphenol oxidase in the presence of copper-metallothionein (μmol product /μg protein /ml) $x10^{-2}$.

[f] Fractions FII, FIII, FIVc and FIVc' were obtained after the crude extract was successively subjected to heat treatment, ultrafiltration, affinity and re-affinity chromatography, respectively.

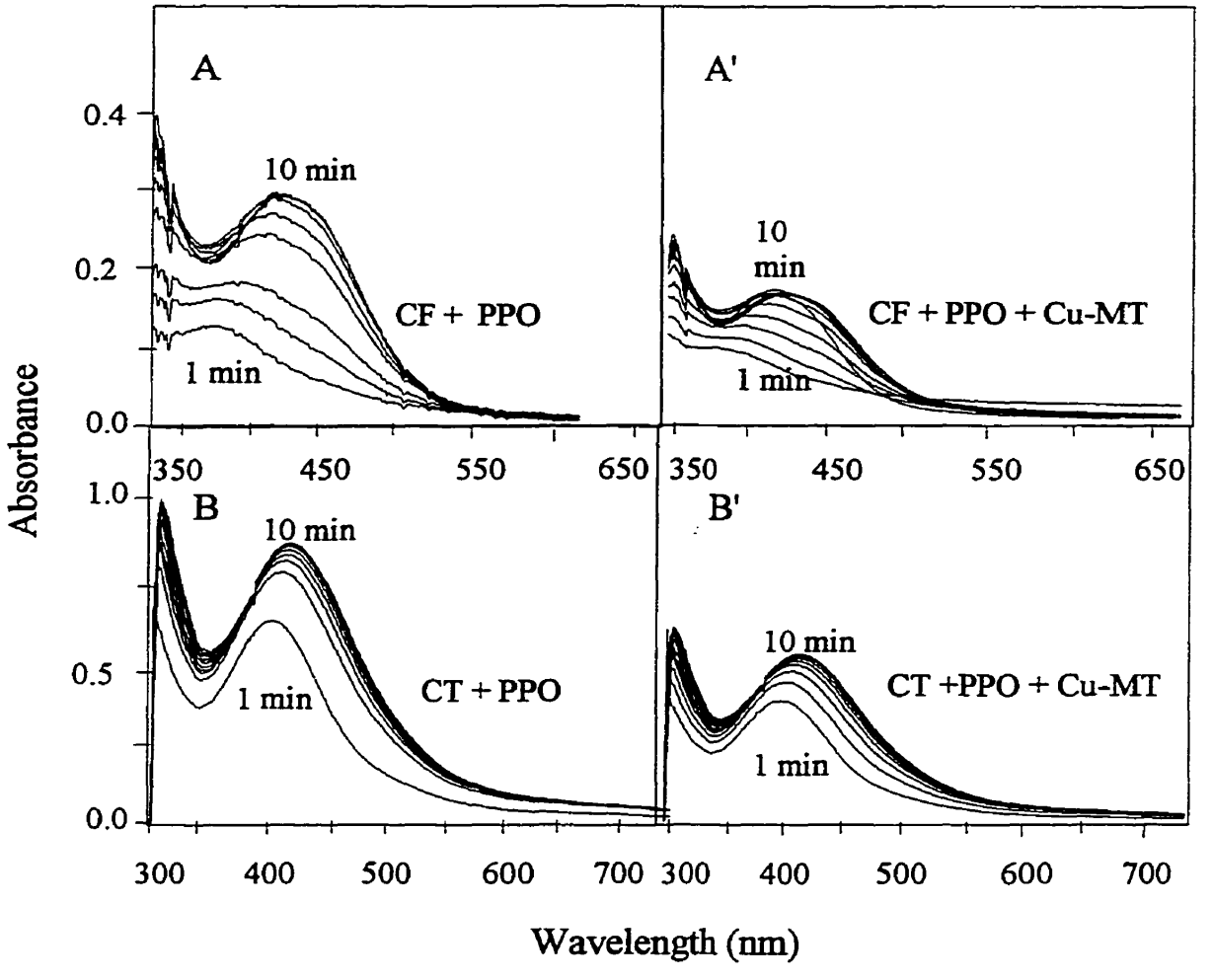


Figure 24. Spectroscopic scanning, at 1 min intervals of the tyrosinase-catalyzed end-products obtained after incubation: caffeic acid and tyrosinase (A), caffeic acid, tyrosinase and copper-metallothionein (A'), catechol and tyrosinase (B), catechol, tyrosinase and copper- metallothionein (B').

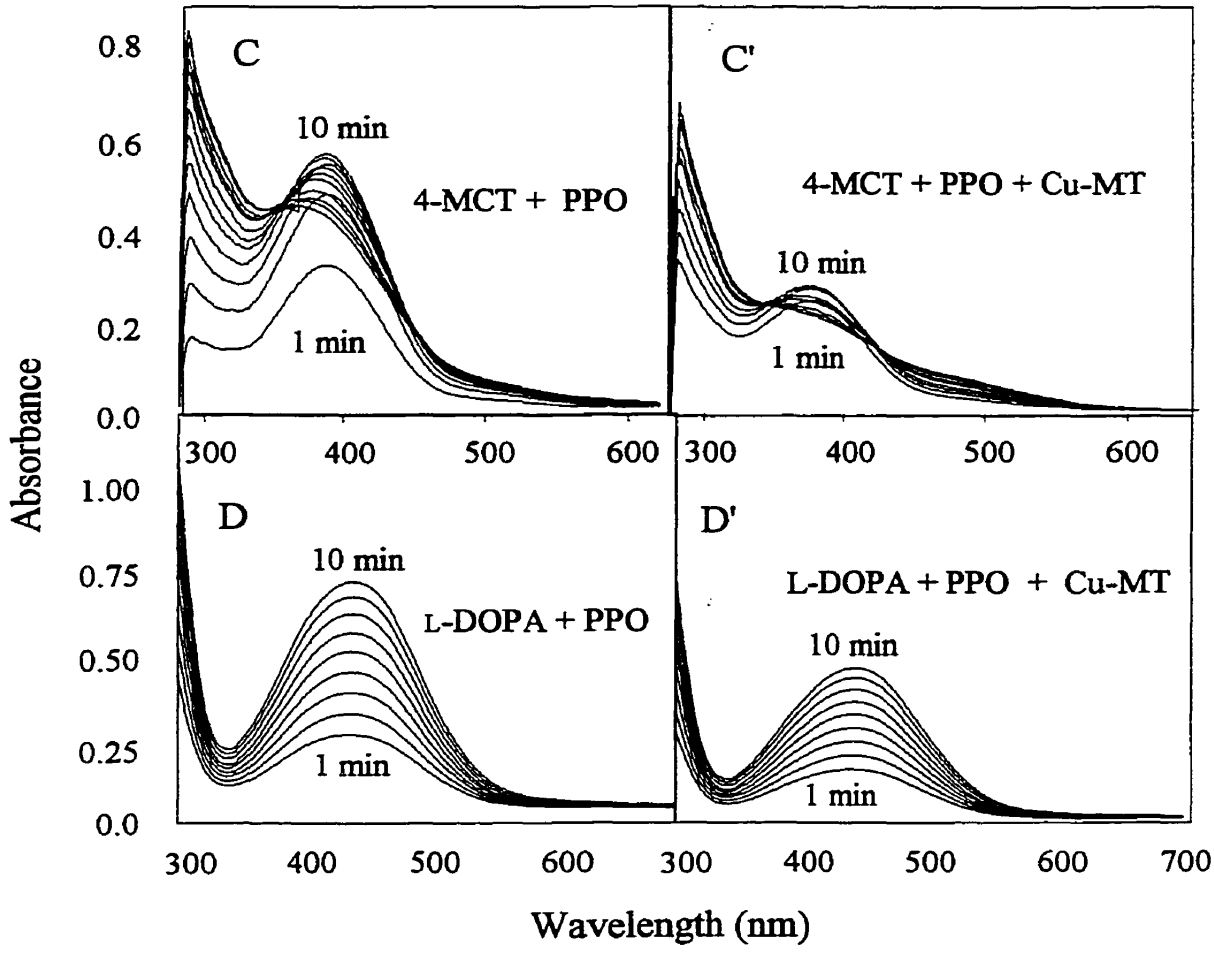


Figure 25. Spectroscopic scanning at 1 min intervals of the tyrosinase-catalyzed end products obtained after incubation: 4-methyl catechol and tyrosinase (C), 4-methyl catechol, tyrosinase and copper-metallothionein (C'), L-3,4-dihydroxyphenyl alanine and tyrosinase (D), L-3,4-dihydroxyphenyl alanine, tyrosinase and copper-metallothionein (D').

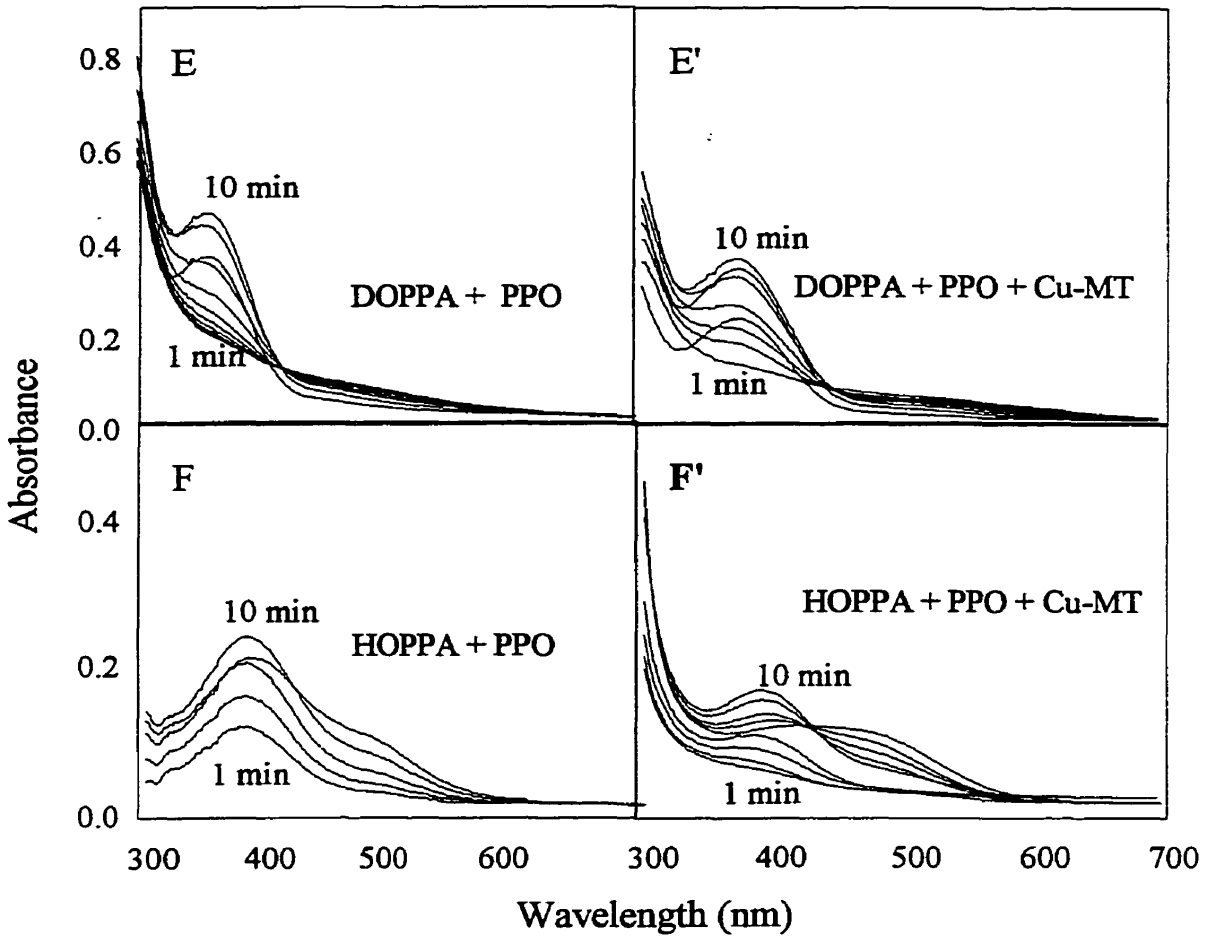


Figure 26. Spectroscopic scanning at 1 min intervals of the tyrosinase-catalyzed end-products obtained after the incubation: 3,4-dihydroxyphenylacetic acid and tyrosinase (E), 3,4-dihydroxyphenylacetic acid, tyrosinase and copper-metallothionein (E'), 3,(*p*-dihydroxyphenyl)-propionic acid and tyrosinase (F), 3, (*p*-dihydroxyphenyl)- propionic acid, tyrosinase and copper- metallothionein (F').

Table 10. The effect of a purified copper-metallothionein (Cu-MT) extract FIVc' on the absorption spectrum of tyrosinase-catalyzed end products, measured by spectrophotometer.

	Without Cu-MT		With Cu-MT	
Substrate	Maximum wavelength	Absorbance (O.D.)	Maximum wavelength	Absorbance (O.D.)
CF[a]	420	0.80	408	0.50
CT[a]	420	0.30	400	0.15
4-MCT[a]	400	0.58	376	0.25
L-DOPA[a]	420	0.70	379	0.35
DOPAA[a]	420	0.45	375	0.37
HOPPA[a]	380	0.22	400	0.15

[a]Caffeic acid (CF); catechol (CT); 4-methyl catechol (4-MCT); L-3,4-dihydroxyphenyl alanine (L-DOPA); 3,4-dihydroxyphenyl acetic acid (DOPAA); 3-(*p*-hydroxyphenyl)-propionic acid (HOPPA).

HOPPA. These findings suggest that since HOPPA is a monophenol, it could have a different mode of interaction with the inhibitor than the diphenolic substrates thereby exhibiting different absorption characteristics. The results also show that in the spectroscopic analysis, tyrosinase biocatalysis was inhibited by the presence of the Cu-MT extract in the reaction mixture as indicated by a lower absorption of the brown-colored-end-products consisting of dark melanin pigments; the occurrence of a lower absorption of the reaction mixture containing the Cu-MT extract suggests an inhibition of tyrosinase activity by Cu-MT as shown by a decrease in the color formation of the enzymatically-catalyzed *o*-quinones, measured spectrophotometrically. The results also show that among the six substrates, the tyrosinase-catalyzed end-products of L-DOPA showed the lowest absorption in the presence of the Cu-MT extract from *A. niger*. Khan (1985) also reported the formation of dopachrome from DOPA, by mushroom PPO activity, at 475 nm.

5. CONCLUSION

This study showed that metal chelating affinity chromatography was an efficient method for the purification of Cu-MT from *A. niger*. In addition, the experimental findings suggested that the excess unbound copper was successfully removed by ultrafiltration procedure. The results indicated that the molecular weight of Cu-MT from *A. niger*, determined by mass spectrometry, was close to that determined by denature polyacrylamide gel electrophoresis. The results also showed that the purified fraction has a copper / protein mass ratio close to those reported from other microbial sources. The data gathered in this study also suggested that the purified Cu-MT from *A. niger* showed certain similarities, including electrophoretic and spectroscopic properties, with those obtained from other eukaryotic cells.

The present study demonstrated that the Cu-MT extract from *A. niger* can be used as an inhibitor for PPO activity. The spectrophotometric assay was more efficient compared to the polarographic assay in terms of limit of detection for the determination of the degree of inhibition of tyrosinase activity. The type and degree of inhibition were dependent upon the substrate and the degree of purity of Cu-MT as well as the method used for the determination of PPO activity. The strong affinity of the induced Cu-MT to bind the copper could explain the inhibitory effect of this protein, acting as a chelating agent, on PPO activity. The literature suggested that another possible mechanism for the inhibitory effect of Cu-MT could be explained by the presence of the cysteine group that could act as a reducing agent and react with *o*-quinones to form less colored, more stable thiol-ester compounds. The results also showed that the maximum absorption spectra of the enzymatically-catalyzed end-products from selected substrates in the presence of Cu-MT showed a lower λ_{max} than that of the original substrates.

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