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Purification and Properties of Phenylpropanoid O-Methyltransferase in *Brassica oleracea*

Emidio DeCarolis

A Thesis

in

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of

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ABSTRACT

Purification and Properties of Phenylpropanoid O-Methyltransferase in *Brassica oleracea*

Emidio DeCarolis

The phenylpropanoid O-methyltransferase from *Brassica oleracea* was purified to apparent homogeneity and the same enzyme was shown to catalyze the O-methylation at the 3-position of caffeic acid and the 5-position of 5-hydroxyferulic acid. Furthermore, two isoforms of the phenylpropanoid O-methyltransferase were separated by ion-exchange chromatography. They catalyzed the *meta* O-methylation of caffeic and 5-hydroxyferulic acids to ferulic and sinapic acids, respectively. Isoforms I and II exhibited different elution patterns from a Mono Q column, distinct apparent pIs on chromatofocusing, different product ratios and different stability on Adenosine-agarose affinity column. However, both isoforms had similar molecular weight (42 Kilodalton) and a pH optimum of 7.5. Substrate interaction kinetics of the more stable isoform I, using 5-hydroxyferulic acid and S-adenosyl-L-methionine, gave converging lines which is consistent with an ordered bi bi mechanism. Product inhibition studies, using isoform I showed competitive inhibition between S-adenosyl-L-methionine and S-adenosyl-L-homocysteine and non-competitive inhibition between the phenylpropanoid substrate and its methylated product. The kinetic patterns are consistent with an ordered bi bi mechanism, where S-adenosyl-L-methionine is the first substrate to bind and S-adenosyl-L-homocysteine is the last product released.

The work described in this thesis has been:

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ABBREVIATIONS

Bis-Tris	:	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
Bisacrylamide	:	N,N'-methylenebisacrylamide
DEAE-Sephacel	:	diethylaminoethyl-Sephacel
DIECA	:	diethylammonium diethyldithiocarbamate
DPM	:	disintegration per minute
EDTA	:	ethylenediaminetetraacetic acid
FA	:	ferulic acid
FPLC	:	fast protein liquid chromatography
5-HFA	:	5-hydroxyferulic acid
HPLC	:	high performance liquid chromatography
KD	:	kilodalton
K_i	:	Dissociation constant of an enzyme-inhibitor complex
K_m	:	Michaelis-Menten constant
OMT	:	O-methyltransferase
pKat	:	pKatal
SA	:	sinapic acid
SAH	:	S-adenosyl-L-homocysteine
SAM	:	S-adenosyl-L-methionine
SDS-PAGE	:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	:	N,N,N',N'-tetramethylethylenediamine
TLC	:	thin layer chromatography
TMV	:	tobacco mosaic virus
Tris	:	tris-(hydroxymethyl)aminomethane

A. INTRODUCTION

Besides the primary metabolic pathways common to all organisms, some biochemical reactions lead to the formation of specific compounds unique to plant species. These compounds include alkaloids, flavonoids, terpenoids, volatile oils, steroids, tannins, quinones, cyanogenic glycosides, glucosinolates and phenylpropanoids [1] and are referred to as secondary metabolites [2]. Several thousands of naturally occurring phenolic compounds have been characterized, but their function remains unclear. Although the majority of known secondary metabolites are of plant origin, many are found in fungi [3], bacteria [4], or marine animals [5].

Among the various substitution patterns of plant secondary metabolites is O-methylation. Enzymatic O-methylation, which is catalyzed by O-methyltransferase (E.C.2.1.1.6), is widely distributed in nature. The physiological significance of O-methylation of plant secondary metabolites is believed to reduce the chemical reactivity of the phenolic hydroxyl group(s), resulting in plant phenolics which are generally less toxic, more lipid soluble and in many instances the methylated compound is a precursor for more complex products [6]. Its role in mammalian tissue involves the extraneuronal inactivation of endogenous catecholamines as well as detoxification of catechol drugs [7]. Phenylpropanoid compounds, which are derived from the aromatic amino acid L-phenylalanine, are among the most common natural constituents of vascular plants. Two of their derivatives, ferulic (3-methoxy-4-hydroxycinnamic) and sinapic

(3,5-dimethoxy-4-hydroxycinnamic) acids are known precursors of lignin (Fig.1). They are formed by the enzymatic O-methylation of caffeic (3,4-dihydroxycinnamic) and 5-hydroxyferulic (3-methoxy-4,5-dihydroxycinnamic) acids, respectively. Phenylpropanoid OMT is known to methylate 5-hydroxyferulic and caffeic acids specifically at the meta position [8] as well as flavonoids and coumarins with o-diphenolic groups [8]. Recent work in this laboratory [9] has demonstrated the stepwise methylation of flavonoid substrates, and the enzymes catalyzing these reactions were shown to be position specific O-methyltransferases. Previous studies with the phenylpropanoid OMT were unable to clearly demonstrate whether the enzyme is position specific due to the lack of protein purification techniques. As a result the question of whether the methylation of caffeic to ferulic and 5-hydroxyferulic to sinapic acids is catalyzed by one or two distinct enzymes has not been thoroughly investigated (Fig. 1).

In the present study,the objective has been divided into two major sections: (A) the purification and characterization of the OMT activity with the aim of establishing whether one or two distinct enzymes are catalyzing the two reactions and (B) the elucidation of the kinetic mechanism and kinetic parameters of the phenylpropanoid OMT. Since no study has dealt with the complete kinetic analysis of the phenylpropanoid OMT it was pertinent to perform substrate interaction kinetics and product inhibition studies.

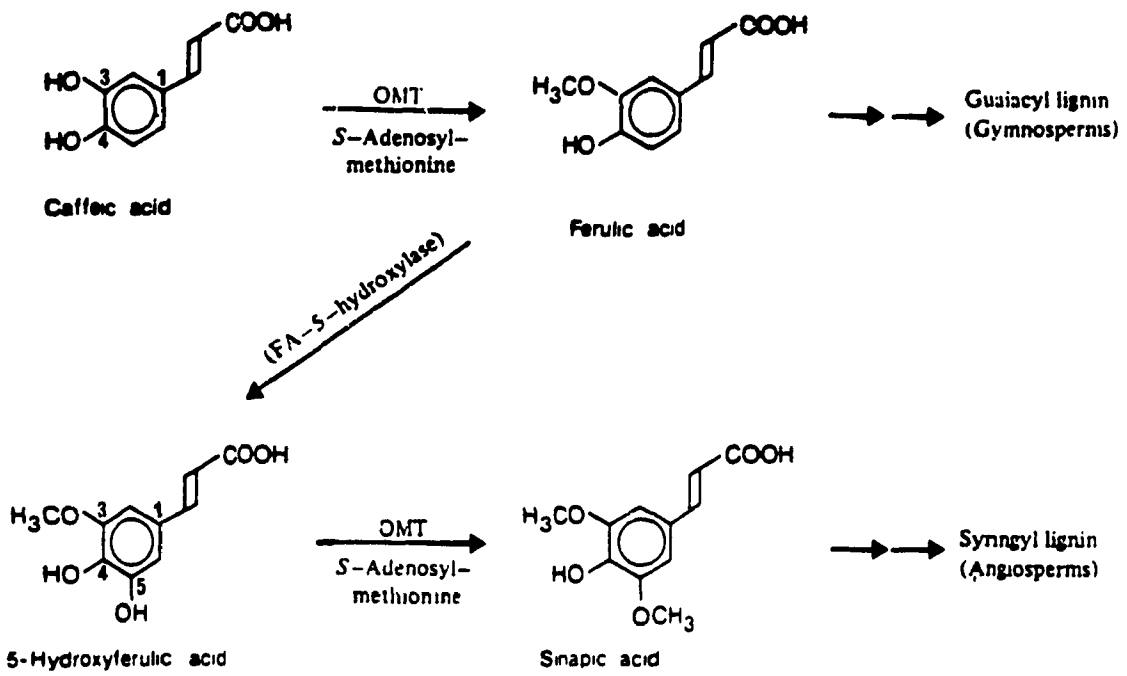


Figure 1. The formation of ferulic and sinapic acids in angiosperms.

B. REVIEW OF LITERATURE

B.1.General Phenylpropanoid Metabolism

It is well documented that the carbon skeleton of phenylpropanoid compounds originates from the aromatic amino acid L-phenylalanine [10]. The latter is deaminated by phenylalanine ammonia-lyase (PAL) to cinnamic acid. Cinnamic acid then undergoes ring substitution in a series of hydroxylation and O-methylation steps yielding various substituted cinnamic acid derivatives (Fig. 2). The hydroxycinnamic acids occur universally in plants, as free and esterified forms (glycosides, amides and cholines) [11]. Substituted hydroxycinnamic acids are important precursors of other naturally occurring phenolic compounds. For example, on reduction, they yield hydroxycinnamoyl alcohols which are the building blocks of lignin, a phenolic polymer which constitutes 20-30% of plant cell walls [12]. Further dehydration of hydroxycinnamoyl alcohols yields phenylpropenes which are constituents of volatile oils of plants and are believed to have hallucinogenic properties [13]. Coumarins are derived from o-hydroxycinnamic acid after subsequent ring closure [14]. Further substitution of o-hydroxycinnamic acid yields substituted coumarins (scopoletin). Some coumarins have found application as anticoagulants and as ointments against skin diseases, to mention a few. Prenylation at position 6 or 8 of 7-hydroxycoumarin gives rise to linear and angular furanocoumarins [14]. The hydroxycinnamic side chain can also

be extended by succesive condensation with malonyl-CoA leading to the formation of ring B of flavonoid compounds [15] and xanthones [13]. Removal of an acetate unit from the side-chain by oxidation of hydroxycinnamic acids yields the corresponding benzoic acid derivatives (Fig.2).

B.2. Significance of O-Methyltransferase in Secondary Metabolism

Following the discovery of S-adenosyl-L-methionine (SAM) as the physiological methyl donor [16] there was a dramatic increase in the number of reports on enzymatic methylations. In mammals, the highest O-methyltransferase activity was observed in the liver [17]. The OMT extracted from this tissue demonstrated a rather broad substrate specificity and generally resulted in the formation of meta-methylated products.

In plants, the incorporation of methyl groups into lignin was first reported by Byerrum in 1954 [18]. The discovery of OMT which methylated caffeic acid at the 3-position in apple cambium [19] and in pampas grass [20] represented a significant finding in the understanding of lignin biosynthesis. This type of OMT was later proven to be directly involved in lignin biosynthesis since an increase in enzyme activity was correlated with increased lignin biosynthesis [8]. In addition, Higuchi and co-workers were able to reveal a significant difference between the OMTs of angiosperms, and gymnosperms. The angiosperm OMT catalyzed the methylation of both caffeic and 5-hydroxyferulic acids to ferulic and sinapic acids, respectively,

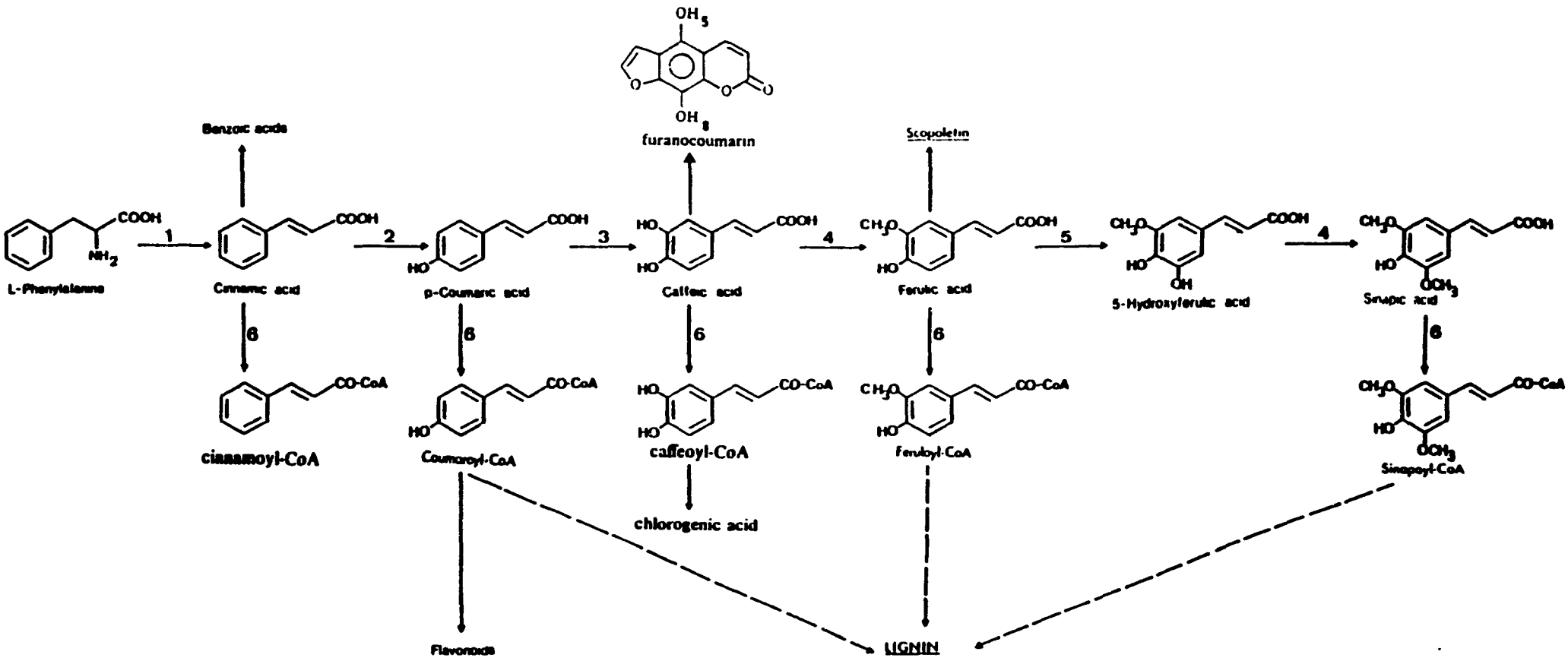


Figure 2. The metabolism of phenylpropanoids;
(1) phenylalanine ammonia-lyase
(2) cinnamic acid 4-hydroxylase
(3) p-coumaric acid 3-hydroxylase
(4) phenylpropanoid O-methyltransferase
(5) ferulic acid 5-hydroxylase
(6) hydroxycinnamate-CoA ligases

Adopted from: Hermann, C., (1986), Ph.D. Thesis, Universite Louis Pasteur, Strasbourg, France.

both of which are lignin precursors. On the other hand, gymnosperm OMT catalyzed only the formation of ferulic acid (Fig. 1).

Several possibilities were proposed to explain the differences existing between angiosperms and gymnosperms: one possibility was that the gymnosperm may lack the 5-hydroxylase of ferulic acid, or that angiosperms may utilize two OMTs; one for the O-methylation of caffeic acid and another for 5-hydroxyferulic acid [21]. Studies conducted on the angiosperm OMT considered it to be a "difunctional" enzyme in view of its ability to methylate both caffeic and 5-hydroxyferulic [22], whereas the gymnosperm OMT was termed "monofunctional" since it showed preference for caffeic acid and did not accept 5-hydroxyferulic acid as substrate [23].

Two forms of an OMT were purified from tobacco cell suspension culture, one exhibited highest activity with 5-hydroxyferulic and caffeic acids whereas the other form methylated predominantly flavonoids in the 7-position [24]. A more complicated picture was reported in virus-infected tobacco leaves, where three O-methyltransferases (OMT I, OMT II and OMT III) were partially purified by anion-exchange chromatography [24]. OMT I exhibited highest activity against 5-hydroxyferulic and caffeic acids, whereas OMT II and III accepted a variety of o-diphenolic substrates [25].

The presence of a phenylpropanoid OMT and a flavonoid OMT has also been demonstrated in soybean cell suspension culture [26] and in tulip anthers [27]. Furthermore, the separation of

the furanocoumarin OMT from the phenylpropanoid OMT was successfully achieved from Ruta graveolens cell suspension culture [28]. It was found that the phenylpropanoid OMT could bind to a ferulic acid ligand immobilized to AH-Sepharose 4B. The furanocoumarin activity peak was found to contain two position-specific OMTs which methylated the 5-hydroxyl and 8-hydroxyl groups (Fig. 2) yielding 8-hydroxybergapten and 5-hydroxyxanthotoxin, respectively [29].

Further evidence of position-specific OMTs was demonstrated in Chrysosplenium americanum. Five distinct flavonol O-methyltransferases were separated from the tissue due to differences in their isoelectric point [30].

B.3. Purification of O-Methyltransferase

O-Methyltransferase is generally assumed to be found in the soluble fraction of the cell [7]. Therefore, there is no special problem with its extraction or solublity. Partial purification of OMT from both animal and plant tissues has been achieved by using classical techniques including ammonium sulfate fractionation, gel-filtration, ion-exchange and affinity chromatography. In plant material, the OMTs investigated have been partially purified but homogeneity of the preparation has seldom been achieved. However, with the use of seven different purification steps utilizing conventional and modern protein purification techniques (FPLC), the three OMTs in virus-infected tobacco leaves were purified to homogeneity in order to raise polyclonal antibodies against them [31].

B.4. The Methyl Donor Group

In 1945 it was demonstrated that guanidoacetic acid could be methylated in rat liver [32]. The methyl donor was reported to be the amino acid, methionine. The effectiveness of methionine as a methyl donor required adenosine triphosphate (ATP) and magnesium ions [16]. It was then shown that the active methionine was actually S-adenosyl-L-methionine (SAM) [33]. The enzyme which catalyzed the formation of SAM was found to be an ATP: L-methionine S-adenosyltransferase [33]. The identification of the latter enzyme in bamboo shoots [8] has also indicated that SAM is the methyl donor with respect to O-methylation reactions in plants.

B.5. Factors Affecting Phenylpropanoid Metabolism

Phenylalanine ammonia-lyase (PAL) catalyzes the deamination of L-phenylalanine which gives trans-cinnamic acid, an important precursor of a variety of metabolic pathways. The end product is usually dependent on such factors as the developmental stage of the producing tissue as well as a number of environmental stimuli [34]. PAL levels are very sensitive to environmental factors (light, bacterial, fungal and viral infection) and hormonal changes, all of which cause rapid increases in PAL levels resulting in the onset of accumulation of specific phenylpropanoid metabolites [35]. For example, PAL levels are increased upon fungal infection which subsequently leads to increases in OMT activity and lignin biosynthesis [36]. Furthermore, infection of hypersensitive tobacco leaves with

tobacco mosaic virus (TMV) resulted in pronounced increases in OMT I, II, and III. The stimulated activities were observed in the cell layers surrounding the necrotic lesions. Increased synthesis of phenylpropanoids in infected plants suggests that lignin biosynthesis at the site of infection acts as a physical barrier against any further invasion [36].

The increases in PAL and OMT activities are generally followed by an almost equally rapid decline [37]. It has been postulated that numerous stimuli are capable of inducing initiation of transcription of the PAL and OMT genes [37]. However, the molecular mechanism underlying the activation and inactivation of gene transcription has not been clearly investigated.

B.6. Enzyme Forms

Proteins are macromolecules, with high molecular weights (ca 10,000 daltons). When the polypeptide chain is formed, it is subjected to many chemical modifications particularly through the reactive amino, carboxyl or hydroxyl groups of several of its amino acids. The presence of disulfide bridges, hydrogen bonding and various other types of interactions between polypeptide chains, all contribute to protein structure which may exist in several distinct alternative and stable forms [38]. All these possibilities raise questions concerning the molecular homogeneity or heterogeneity of any particular protein. Recently, the utilization of techniques that exploit the physical, chemical, immunochemical and kinetic properties of an

enzyme, have successfully revealed that many enzymes exist in multiple form within the same tissue and share catalytic activity [39,40]. These enzymatic activities are known as isozymes or isoforms.

In the general phenylpropanoid metabolism, the formation of hydroxycinnamoyl:CoA esters is mediated by hydroxycinnamate CoA ligase. The occurrence of multiple forms of this enzyme has been demonstrated in Pisum sativum seedlings [41] and populus stems [42]. In addition, phenylalanine ammonia-lyase (PAL) has also been found to exist as multiple forms; three forms were extracted from spinach leaves, two were compartmented in the chloroplasts while the third was located outside the chloroplast [43]. More recently, four forms of PAL were isolated from Phaseolus cell cultures, which were separated according to their different isoelectric points [44]. Multiple forms of catechol O-methyltransferase have been reported in rat liver [45,46]. Multiple forms of plant OMTs have also been reported in tobacco cell suspension [24],TMV infected tobacco leaves [25] and aspen wood [47]. The two phenylpropanoid OMT isoforms extracted from aspen wood were partially purified and it was found that the isoforms differed in enzymatic stability [47].

B.7. Physiological Importance of Phenylpropanoid Compounds in Plants

Presently, there is increasing evidence that phenylpropanoids play an ecological role. Some of these compounds function as allelopathic agents, feeding deterrents and phytoalexins.

For example, ferulic acid in Adenostoma has been shown to function as an allelopathic agent against the growth of Bromus rigidus seedlings [48] and is known to deter feeding of the maize weevil on maize seed [49]. Chlorogenic acid (caffeoylquinic acid) is known for its feeding deterence to the willow-feeding leaf beetle [50]. Furthermore, chlorogenic acid has the potential to be an effective antimicrobial compound against brown rot in apples [51] and is associated with resistance to Phytophora infestans in potato [52]. Essentially, chlorogenic acid production is increased only upon microbial infection.

Phenylpropanoid derived compounds have also been associated with physiological roles. For example, anthocyanins have long been recognized as being important in nature for the attraction of pollinators. Other flavonoids have also been implicated in plant-fungal interactions [53]. Recent studies with flavonoids have associated them with inducing the expression of nodulation genes in leguminous plants [54] and as regulators in the transport of plant growth substances. [55].

B.8. Enzyme Kinetics

Most studies on the kinetic mechanism of the catechol O-methyltransferase have been reported from animal systems [56,57]. The kinetic mechanism commonly reported was the random addition of substrates and release of products. However, the N-methyltransferase from rabbit adrenal, which was resolved into five isoforms having one common kinetic property, namely the

competition between SAM and SAH [58], suggests that SAM must bind prior to the substrate, thus resulting in an ordered bi bi mechanism. Recently, an OMT responsible for the terminal step of antibiotic biosynthesis in Streptomyces fradiae also exhibited an ordered bi bi mechanism [59].

In plants, initial kinetic studies on the OMT from various tissues showed that SAH was a potent inhibitor of SAM at physiological concentrations [60]. In addition, recent reports have demonstrated the complete kinetic analysis involving substrate interaction and product inhibition of a number of position-specific OMTs in flavonoid biosynthesis [61,62]. In contrast, the complete kinetic analysis of the phenylpropanoid OMT has not yet been reported. Preliminary studies using affinity chromatography indicated that ferulic acid ligand immobilized to a AH-Sepharose support could bind the OMT only if SAM was present in the buffer [28]. Based on the latter finding it was concluded that the OMT had to bind SAM first before it could bind the phenolic substrate.

B 9. Pharmacological Properties of Some Phenylpropanoids

Caffeic acid as well as a number of structurally related compounds have been proposed to be the active compounds in plant extracts which demonstrate pharmacological activity. For example, caffeic acid displayed antithyrotropic activity in tests using human thyroid membrane preparations [63]. In addition ferulic and caffeic acids have been shown to inhibit the mutagenicity and cytotoxicity of the known benzo-ring diol

epoxide of polycyclic aromatic hydrocarbons [64]. Recently, compounds containing the caffeic acid moiety have shown antiviral activity by reacting with the viral coat proteins and, as a result, hindering growth and spread of viral infection [65]. Another group of phenylpropanoid conjugates which are characterized by having a caffeoyl and hydroxyphenylethyl moieties, both of which are linked to beta-glucose by ester and glucosidic linkages, respectively, have been reported to exhibit analgesic, hypotensive and euphoric effects [66].

C. MATERIALS AND METHODS

C.1. Plant Material

Seeds of Brassica oleracea L. var. Danish Baldhead (cabbage) were obtained from a commercial source (W.H. Perron, Chomedey, Quebec) and were germinated in potting soil under greenhouse conditions. The plant growth was maintained at a temperature of 15°C +/- 2°C with a good water supply. The first 2-3 fully expanded leaves of six weeks old plants were used for enzyme extraction.

C.2. Chemicals

S-Adenosyl-L-[$^{14}CH_3$]-methionine (47 mCi/mmole) was purchased from ICN Radiochemicals, (Irvine CA). Unlabelled S-adenosyl-L-methionine (SAM) was obtained from Boehringer-Mannheim, (FRG). 5-Hydroxyferulic acid was synthesized as described in section C.3. Caffeic, ferulic, sinapic and 3,4,5-trihydroxycinnamic acids were purchased from Fluka (Ronkonkoma, NY) and their purity was confirmed by TLC and HPLC analysis. Sephadex G-100, DEAE-Sephacel, Polybuffer 74, Mono Q HR 5/5, Mono P, 5/20, Superose 12 HR 15/30 and the Fast Protein Liquid Chromatography (FPLC) system were from Pharmacia (Uppsala, Sweden). Adenosine-agarose affinity support was from P-L Biochemicals (Milwaukee, WI). Dowex 1X2, protein reagent, N,N,N',N'-tetramethylethyl-ethylenediamine (TEMED), ammonium persulfate and the Mini-Protean II Slab Gel Electrophoresis were all from Bio-Rad (Richmond, CA). Bis-Acrylamide and acrylamide (99.9%) were

purchased from Biotech (Fair Lawn, NJ). All other chemicals used were of analytical grade.

C.3. Synthesis of 5-hydroxyferulic acid

3-methoxy-4,5-dihydroxycinnamic acid (5-HFA) was prepared according to Vorsatz [67]. Briefly, 3-methoxy-4,5-dihydroxybenzaldehyde was dissolved in anhydrous pyridine to which was added malonic acid and aniline. The condensation reaction was allowed to take place in a vessel which was placed in a water bath at 55°C for approximately 7 hours. Dilution of the reaction with water and acidification at 4°C yielded crystalline 5-hydroxyferulic acid. The purity of the product was identified as 5-hydroxyferulic acid by comparing its R_f value with that of authentic compound, by similar retention time with standard compound using HPLC and by ^{1}H-NMR analysis (Fig.3 and Table 1).

C.4. Extraction And Detection of Phenylpropanoids in Young Cabbage Leaves

Young cabbage leaves were frozen in liquid nitrogen and ground to a fine powder. Phenylpropanoids, being relatively polar, were extracted with 85% aqueous methanol. The alcoholic extract was filtered through nylon mesh and concentrated to an aqueous residue by flash evaporation (30°C) under reduced pressure. Initially, the aqueous extract was hydrolyzed with 1M NaOH in a N_2 atmosphere for 2 hours at room temperature. The alkaline extract was acidified with 2N HCl and heated for 30 min at ca. 95°C [68]. The acid hydrolyzate was then extracted with

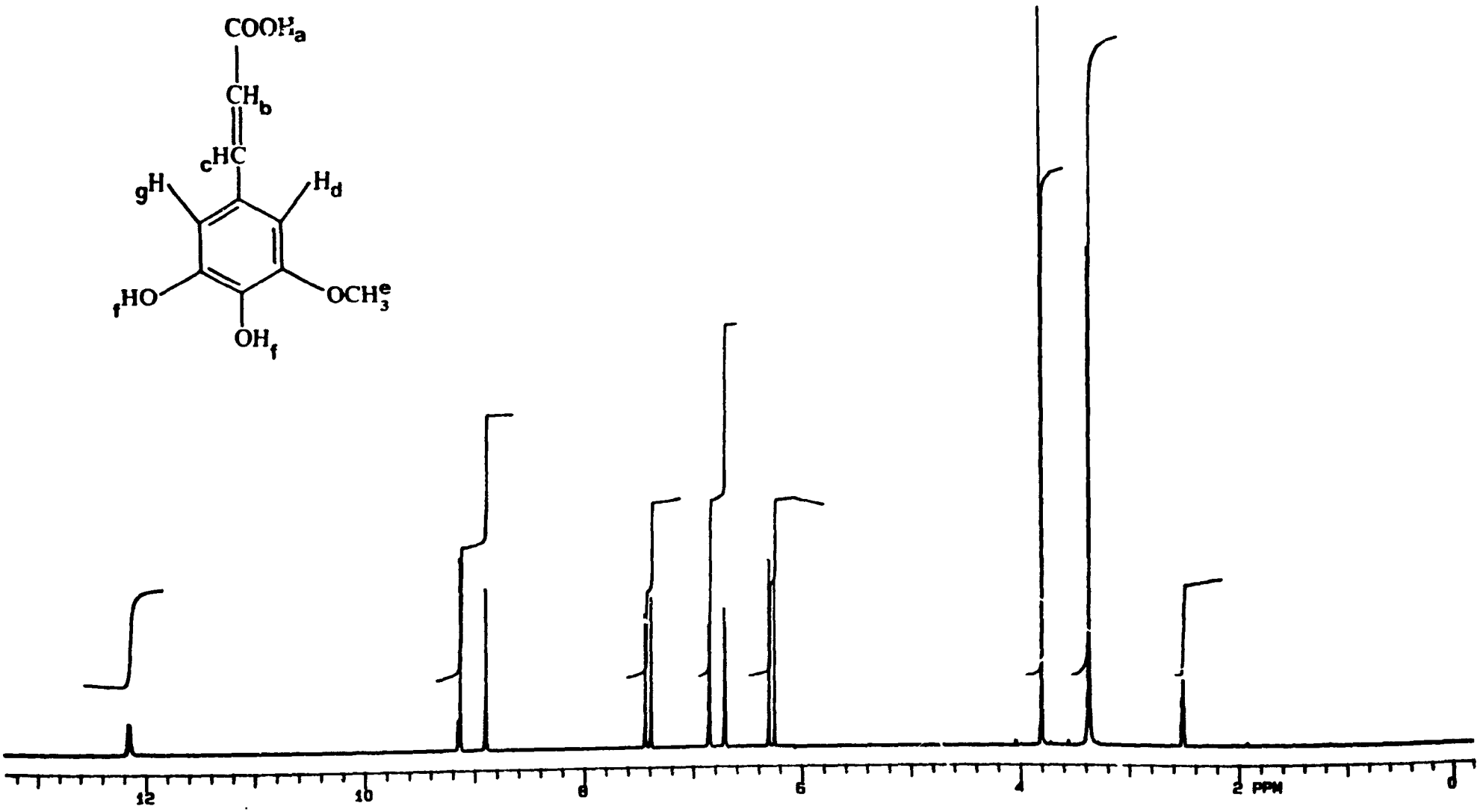
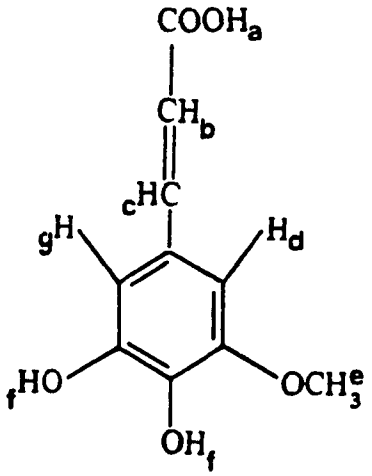


Figure 3. ^{1}H-NMR spectrum of 5-hydroxyferulic acid.

Table 1. ^{1}H-NMR (400 MHz) chemical shifts of 5-hydroxyferulic acid in deuterated dimethylsulfoxide[a].

Hydrogen	Chemical Shift (ppm)
H_a	12.14
H_b	6.26, J=15.9 Hz
H_c	7.40, J=15.9 Hz
H_d & H_g	6.83, J=1.8 Hz
H_e	3.79
H_f	8.89, 9.11
Solvent	2.49

[a] All chemical shifts are relative to dimethylsulfoxide.



diethylether using a continuous liquid-liquid extractor for 4 hours. The ether extract which contained the phenolic aglycones was flash evaporated and the residue was taken up in methanol. The phenolic constituents were identified by TLC using benzene-acetic acid-water (2:3:1 v/v/v) as the solvent system. The developed chromatograms were visualized under UV-light (366 nm). Free phenylpropanoids were identified by cochromatography with authentic samples. Caffeic, ferulic and sinapic acids were found to be the major phenolics of the hydrolyzed extract.

C.5. Buffer Systems

The following buffers were used: A, 200mM Tris-HCl, pH 7.6 containing 5mM ethylenediamine tetraacetic acid (EDTA), 10mM diethylammonium diethyldithiocarbamate (DIECA) and 14mM 2-mercaptoethanol; B, 50mM Tris-HCl, pH 7.6 containing 14mM 2-mercaptoethanol; C, Polybuffer 74-iminodiacetic acid (1:10 v/v), pH 4.0 containing 14mM 2-mercaptoethanol. All buffers used were degassed and filtered through a 0.45 μm filter before use.

C.6. Enzyme Extraction

Unless stated otherwise, all extraction and purification procedures were carried out at 4^{o}C. In general, ca. 100g of young deveined leaves were homogenized in a Waring blender containing Polyclar AT (10% w/w) and buffer A (1:4 w/v). The slurry was filtered through a double layer of nylon mesh and centrifuged at 15,000g for 15 min. The supernatant was collected and gently stirred for 15 min with Dowex 1X2 (10% w/v)

which had previously been equilibrated in buffer A, then filtered through glass wool. The filtrate was fractionated with solid ammonium sulfate and the protein which precipitated between 35 and 80% salt saturation was collected by centrifugation at 15,000g for 15 min.

C.7. Enzyme Purification

C.7.1. Chromatography on Sephadex G-100

The protein pellet was resuspended in the minimum volume of buffer B and then chromatographed on a Sephadex G-100 column (5x55cm) which had previously been equilibrated in the same buffer. The column was developed for 12 hours at a flow rate of 2.5 mL/min. Fractions of 5 mL were collected and assayed for OMT activity. Enzyme assays were conducted against 5-hydroxyferulic and caffeic acids. The protein fractions with OMT activity were pooled and concentrated by precipitation with 80% ammonium sulfate. The resulting pellet was resuspended in buffer B and desalted on a Sephadex G-25 column. Alternatively, the Sephadex G-100 fractions with OMT activity were concentrated by Ultrafiltration (Amicon Corp., Mass.) using a membrane filter type PM 30.

C.7.2. Chromatography on DEAE-Sephacel

The protein fractions exhibiting OMT activity were recovered from the gel filtration step and were subjected to anion-exchange chromatography on a DEAE-Sephacel column (2.5x8cm) which had previously been equilibrated in buffer B. The column

was then washed with the same buffer until the absorbance at 280 nm returned to base line. The bound proteins were with 500 mL of a linear (0-500 mM) gradient of KCl in buffer B. Fractions of 3 mL were collected and assayed for OMT activity using both substrates.

C.7.3. Chromatography on Mono Q

The active fractions from the DEAE-Sephacel column were concentrated by Ultrafiltration and desalted on a Sephadex G-25 column which had previously been equilibrated with buffer B. The desalted enzyme protein was subjected to anion-exhange chromatography on a Mono Q column which had previously been equilibrated with buffer B. After loading the protein onto the column, buffer B was passed through until all unbound protein was eliminated. The bound protein was eluted using a 50 mL linear (0-300 mM) gradient of KCl in buffer B, at a flow rate of 0.5 mL/min. One-mL fractions were collected and assayed for OMT activity, using the two substrates.

C.7.4. Affinity Chromatography

The active fractions which eluted from the Mono Q column were pooled and applied to an Adenosine-agarose affinity column (1x7cm) which had previously been equilibrated with buffer B. The column was washed with 15 mL of the same buffer, at a flow rate of 0.5 mL/min, followed by another 15 mL of 500 mM KCl in buffer B, so as to remove any contaminating proteins other than the OMT which may be bound to the support. Once all of the

unbound protein passed through, the bound proteins were eluted using a 50 mL linear (0-0.5 mM) gradient of SAM in buffer B. One-mL fractions were collected and assayed for OMT activity using both substrates.

C.7.5. Chromatofocusing on Mono P

The OMT activity which eluted from DEAE-Sephacel or Mono Q was concentrated by Ultrafiltration, then desalted on Sephadex G-25 prior to being applied to a chromatofocusing, Mono P column. The latter was washed with 3 column volumes of buffer B and the bound protein was eluted at a flow rate of 0.3 mL/min with 60 mL of buffer C which generated a linear gradient of pH 7-4. One-mL fractions were collected in 0.5 mL of 0.2 M Tris-HCl, pH 8.0 and assayed for OMT activity against the two substrates.

The active OMT which eluted from the Mono P column contains the ampholyte-type buffer which is responsible for the pH gradient. Since this buffer interferes with the enzyme activity, it was removed by subjecting the ampholyte containing OMT fractions to chromatography on Mono Q, as described in C.7.3.

C.8 O-Methyltransferase Assay

The assay for OMT activity consisted of the phenolic substrate (100 μM), S-adenosyl-L-methionine (100 μM containing 0.05 μCi), and up to 50 μg of the enzyme protein in a final volume of 100 μL. The enzyme reaction mixture was started by

the addition of protein and the mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 10 μL of 6N HCl. The O-methylated products formed were separated from unreacted SAM by extraction in 250 μL of ethylacetate and vortexed for 1 min. The organic phase containing the methylated product was separated from the aqueous layer by a 2 min centrifugation using the Eppendorf Centrifuge model 3200. An aliquot of 100 μL of the organic phase was transferred to 3.5 mL of a toluene based scintillation liquid and counted for radioactivity, using an LKB 1217 Rackbeta liquid scintillation counter, after correction for quench.

C.9. Identification of Reaction Products

The remaining organic layer was used to identify the reaction product. Several assays demonstrating high enzymatic activity were pooled in order to obtain enough radioactivity for autoradiography. The organic layer was evaporated to dryness and dissolved in 20 μL of absolute methanol. The latter was cochromatographed with authentic samples on TLC plates (cellulose:silica, 1:1 w/w) using benzene-acetic acid-water (2:3:1 v/v/v) as the solvent system. The developed chromatograms were visualized under UV-light (366nm) and then autoradiographed on X-ray film.

C.10. Gel Electrophoresis

C.10. 1. Native Gel Electrophoresis

The Bio-Rad Mini-Protean II electrophoresis system was used

with 7.5% polyacrylamide slab gels. The gels were loaded with the enzyme protein from different steps of purification, and the protein was allowed to migrate through the gel at 4°C. A scalpel was used to slice the gel horizontally into 2 mm-wide strips. Each strip was transferred to an Eppendorf tube containing 750 μL of buffer B and stored at 4°C overnight. This incubation period allowed the protein to diffuse out of the polyacrylamide gel. The strips containing OMT activity were identified after the supernatant of each Eppendorf tube was assayed for OMT activity using both substrates [69].

C.10.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE slab gels were prepared using the Mini-Protean II electrophoresis system. A 4% stacking gel and a 12% separating gel were used to resolve the proteins from different stages of purification under denaturing conditions. The protein markers (Sigma) were also resolved according to their molecular weight and consisted of bovine serum albumin (66 KD), ovalbumin (45 KD), glyceraldehyde-3-phosphate dehydrogenase (36 KD), carbonic anhydrase (29 KD), trypsinogen (24 KD), trypsin inhibitor (20.1 KD) and lactalbumin (14.2 KD). Based on the migration of the OMT with respect to the migration of the standard proteins it is possible to estimate the molecular weight of the OMT [70]. The gels were stained with Coomassie blue. Alternatively, silver nitrate stain was used for greater sensitivity.

C.11. Molecular Weight Determination Based on Gel Filtration

The molecular weight of the partially purified OMT was determined by gel filtration [71] on a calibrated Superose 12 HR 16/50 column using the FPLC system. The column had previously been equilibrated with buffer B. The column was previously calibrated with the following standard proteins: bovine serum albumin (67 KD), ovalbumin (43 KD), chymotrypsinogen A (25 KD), and ribonuclease A (13.7 KD). The void volume of the column was determined by the elution of a sample of blue dextran (2,000 KD).

$$Kav = \frac{V_e - V_o}{V_t - V_o} \qquad (1)$$

K_{av} = is a ratio comparing elution volume parameters of the protein of interest with the values obtained for several known protein standards, V_t = total bed volume, V_o = column void volume and V_e = elution volume for protein. The K_{av} values were calculated for the standard proteins and a plot of K_{av} versus Log (MW) was plotted. The molecular weight of the OMT was extrapolated from the plot.

C.12. Protein Estimation

The protein concentration was estimated according to the method of Bradford [72] using the Bio-Rad reagent and bovine serum albumin as standard protein.

C.13. Analysis of Kinetic Data

It was established that the reaction velocity was linear with respect to time for at least 60 min at all substrate concentrations. Substrate interaction kinetics was fitted to equation (2) [73] which represents a bi bi mechanism.

$$V = \frac{V_{max}[A][B]}{K_{ia}K_b + [A]K_b + [B]K_a + [A][B]} \qquad (2)$$

A and B are the varied substrates, K_a and K_b are the respective Michaelis-Menten constants, V_{max} is the maximum velocity and $K_{ia}K_b$ is an interaction term. All data from inhibition studies were fitted to the equations for competitive (3) and non-competitive (4) inhibition.

$$V = \frac{V_{max}}{1 + K_a/[A] + K_a[I]/K_I[A]} \qquad (3)$$

$$V = \frac{V_{max}}{1 + K_a/[A] + K_a[I]/K_{ia} + [I]/K_I} \qquad (4)$$

In equations (3) and (4) A represents the variable substrate, I the product (inhibitor), K_a and V_{max} are the Michaelis constants and maximum velocity, respectively. The K_I is the inhibitor constant [73]. The data are presented as double-reciprocal plots which were fitted by linear least squares analysis and to the appropriate equation. The kinetic constants K_a, K_b, K_{ia},

K_{ip} and K_{iq} were calculated from intercept and slope replots of the generated data [73].

D. RESULTS

D.1. Purification and Characterization of the Phenylpropanoid O-Methyltransferase

D.1.1. Optimization of O-Methyltransferase Assay

Preliminary studies on optimizing assay conditions were carried out on a partially purified enzyme preparation. The enzyme assay was based on product formation with time at different concentrations of enzyme. Linearity was observed up to 50 μg/assay (in 50 mM Tris-HCl pH 7.5) and with time up to 120 min. All purification steps were assayed with 5-hydroxyferulic and caffeic acids with the aim of separating the two activity peaks.

D.1.2. Effect of 2-Mercaptoethanol

2-Mercaptoethanol was added to the extraction buffer, as well as the buffers used in purification, so as to retard oxidation of the native -SH groups of the partially purified OMT. The optimal OMT activity was observed at a concentration of 14 mM 2-mercaptoethanol (Table 2). A higher concentration did not result in any significant increase in enzyme activity.

D.1.3. Enzymatic Synthesis of Ferulic and Sinapic Acids

The enzymatic O-methylation of caffeic and 5-HFA acids was demonstrated using a partially purified OMT extract. Figure 4 is a photograph of the autoradiographed reaction products. The

Table 2: Effect of 2-Mercaptoethanol on OMT Activity[a,b].

Substrate[c]	Concentration of 2-Me (mM)	Relative Activity (%)
5-Hydroxyferulic acid	0	1
Caffeic acid	0	2
5-Hydroxyferulic acid	1	49
Caffeic acid	1	52
5-Hydroxyferulic acid	5	52
Caffeic acid	5	57
5-Hydroxyferulic acid	14	100[d]
Caffeic acid	14	100[e]
5-Hydroxyferulic acid	20	96
Caffeic acid	20	94

[a] A partially purified OMT (Sephadex G-100) was used.
[b] The standard enzyme assay was used as described in C.8.
[c] Both substrates were assayed at 100 μM final concentration.
[d] Represents a specific activity of approximately 4.4 pKat/mg.
[e] Represents a specific activity of approximately 1.5 pKat/mg.

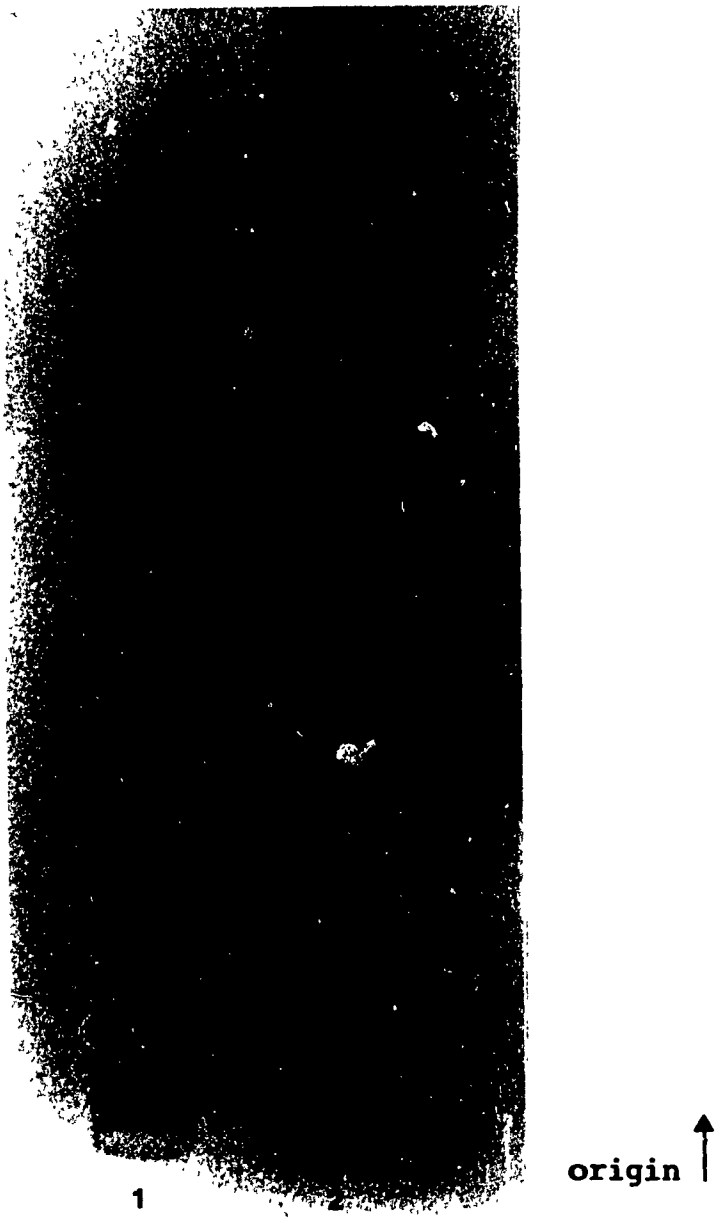


Figure 4. Photograph of an autoradiograph of the chromatographed reaction products using a partially purified OMT preparation with caffeic (1) and 5-hydroxyferulic (2) acids as the substrates. The assay mixture contained 100 μM phenolic substrate, 100 μM SAM (containing 110,000 DPM) and up to 50 μg protein. The mixture was incubated for 30 min at 30°C. The reaction products were identified as ferulic acid (A) and sinapic acid (B) by comparing their R_f values with those of authentic ferulic acid (R_f=0.69) and sinapic acid (R_f=0.71) and co-chromatographed on cellulose:silica plates (50:50) in benzene:acetic acid:water (2:3:1, v/v/v).

autoradiograph demonstrates that ferulic and sinapic acids are the major products formed from caffeic and 5-HFA, respectively. A quantitative value for the amount of product formed could not be obtained however based on the product ratio (sinapic acid;ferulic acid), 5-HFA showed approximately 3 times greater activity (table 3) than caffeic acid, at this stage of purification suggesting the 5-HFA to be the preferred substrate for the OMT.

D.1.4. Enzymatic Synthesis of 5-Hydroxyferulic and Sinapic Acids

Since trihydroxycinnamic acid (THCA) acts as a substrate for the OMT, it was considered important to follow the sequence of reaction products and the intermediate products formed (Fig. 5). Figure 6 shows trace amounts of 5-HFA were formed after a 10 min incubation period, whereas sinapic acid was formed after 15 min incubation. After 60 min of incubation, it appears that the rate of sinapic acid formation (based on the intensity of the reaction product formed) was greater than that of 5-HFA, suggesting that 5-HFA is an intermediate product which is quickly utilized in the synthesis of sinapic acid. Furthermore, this experiment suggested that the partially purified OMT extract used might contain more than one enzyme involved in the O-methylation of the 3-and-5 hydroxyl groups of THCA.

D.1.5. Purification of the Phenylpropanoid O-Methyltransferase

Many plant tissues contain varying concentrations of plant

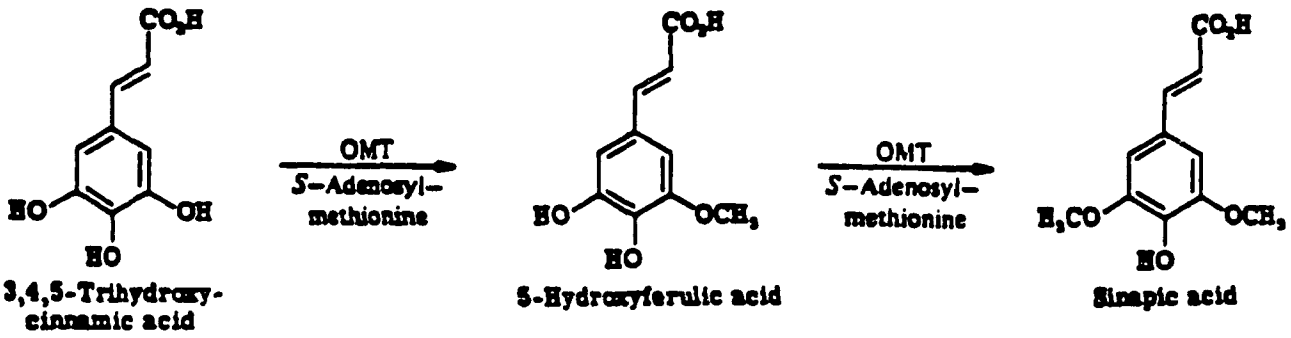


Figure 5. O-Methylated products of 3,4,5-Trihydroxycinnamic acid.

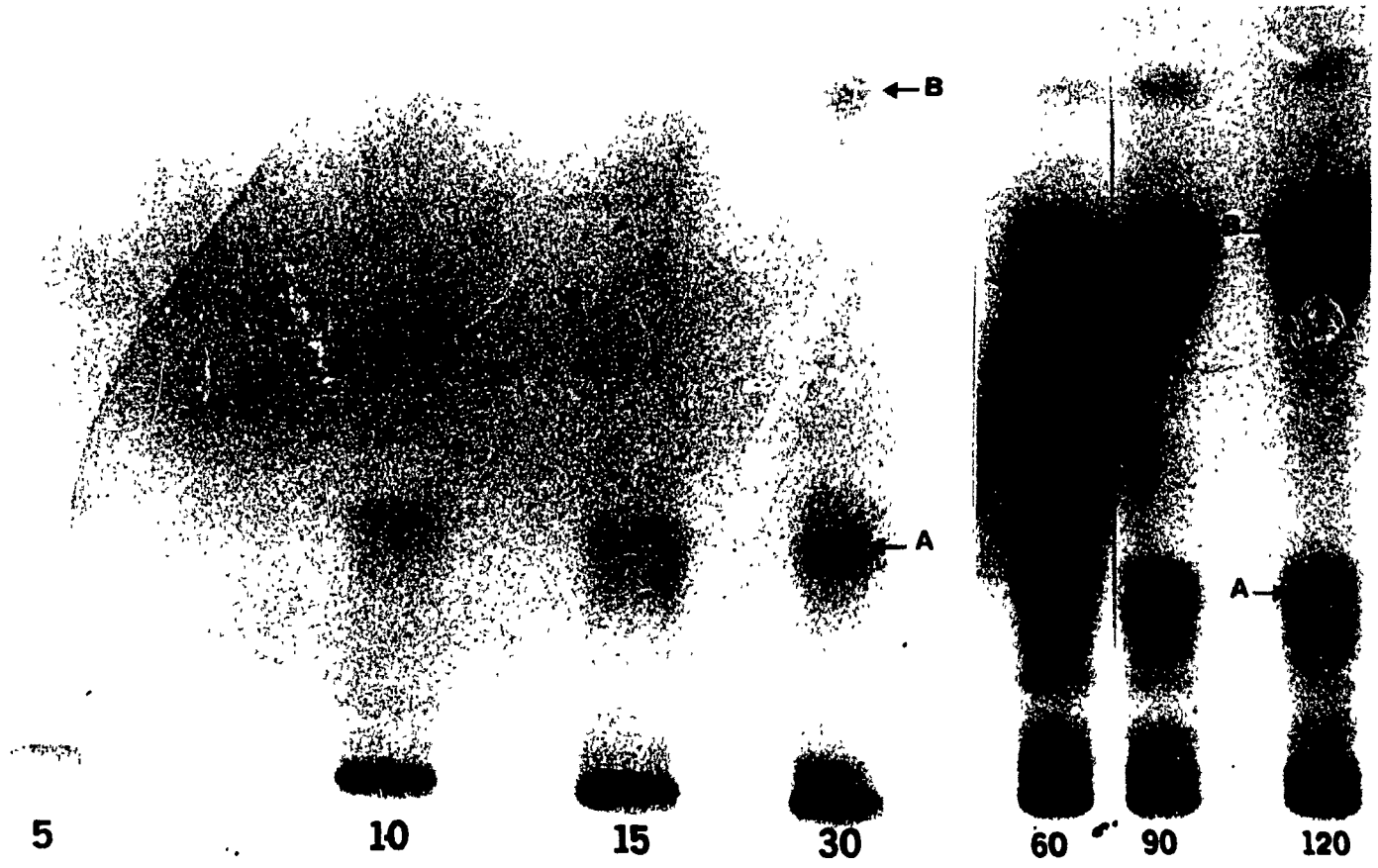


Figure 6. Photograph of an autoradiograph of the chromatographed reaction products over a time course (min) using a partially purified OMT preparation. The assay mixture contained 100 μM 3,4,5-trihydroxycinnamic acid ,100 μM SAM (containing 110,000 DPM) and up to 50 μg protein. The assay mixture was incubated for 30 min at 30°C. The major reaction products were identified as 5-HFA (A) and SA (B) by comparing their R_f values with those of authentic 5-HFA (R_f=0.23) and SA (R_f=0.71) and co-chromatographed on cellulose:silica plates (50:50) in benzene:acetic acid:water (2:3:1, v/v/v).

pigments and polyphenols, all of which are known to inactivate proteins. Previous studies in this laboratory have shown that diethylammonium diethyldithiocarbamate (DIECA), Polyclar AT and Dowex 1X2, are required in the homogenate so as to prevent "browning" which is caused by the presence of phenol-bound proteins. These reagents are also responsible for maintaining optimal OMT activity and stability during extraction of the OMT [74].

D.1.6. Purification Procedure

Initially, the purification of the suspended protein pellet exhibiting OMT activity made use of Sephadex G-25 as the initial gel filtration step. However, the enzyme activity was confined to the major protein peak which also included plant pigments and other contaminating proteins. Separation of the pigment peak from the OMT activity peak was partially achieved by replacing Sephadex G-25 with Sephadex G-100 which has greater discriminating ability due to its higher molecular weight cut-off point (Fig. 7).

This was followed by chromatography on DEAE-Sephacel which eliminated most of the extraneous proteins and all of the contaminating pigments (Fig. 8). All of the OMT activity was confined to a discrete peak which eluted at approximately 290 mM KCl.

Chromatography of the partially purified preparation (DEAE-Sephacel) on Mono Q resulted in the separation of two distinct O-methylating activities which eluted at 120 mM KCl (isoform I)

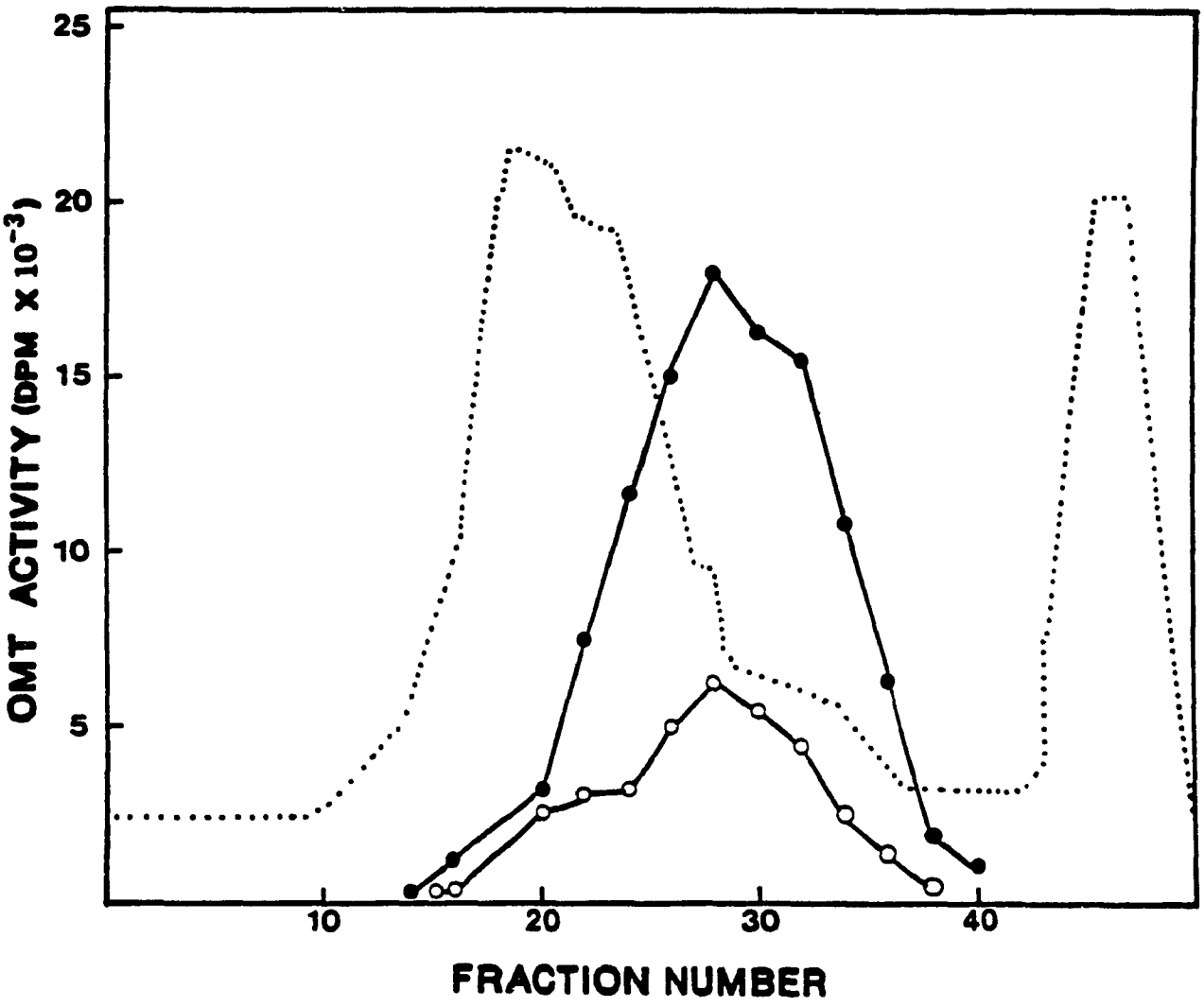


Figure 7. Elution profile of the OMT activities after gel filtration on Sephadex G-100.

Fractions were collected and assayed for OMT activity against 5-hydroxyferulic acid (—●—) and caffeic acid (—○—) as substrates. The absorbance (.....) was monitored at 280 nm. Refer to Table 3 for the amount of protein applied and recovered from the G-100 column.

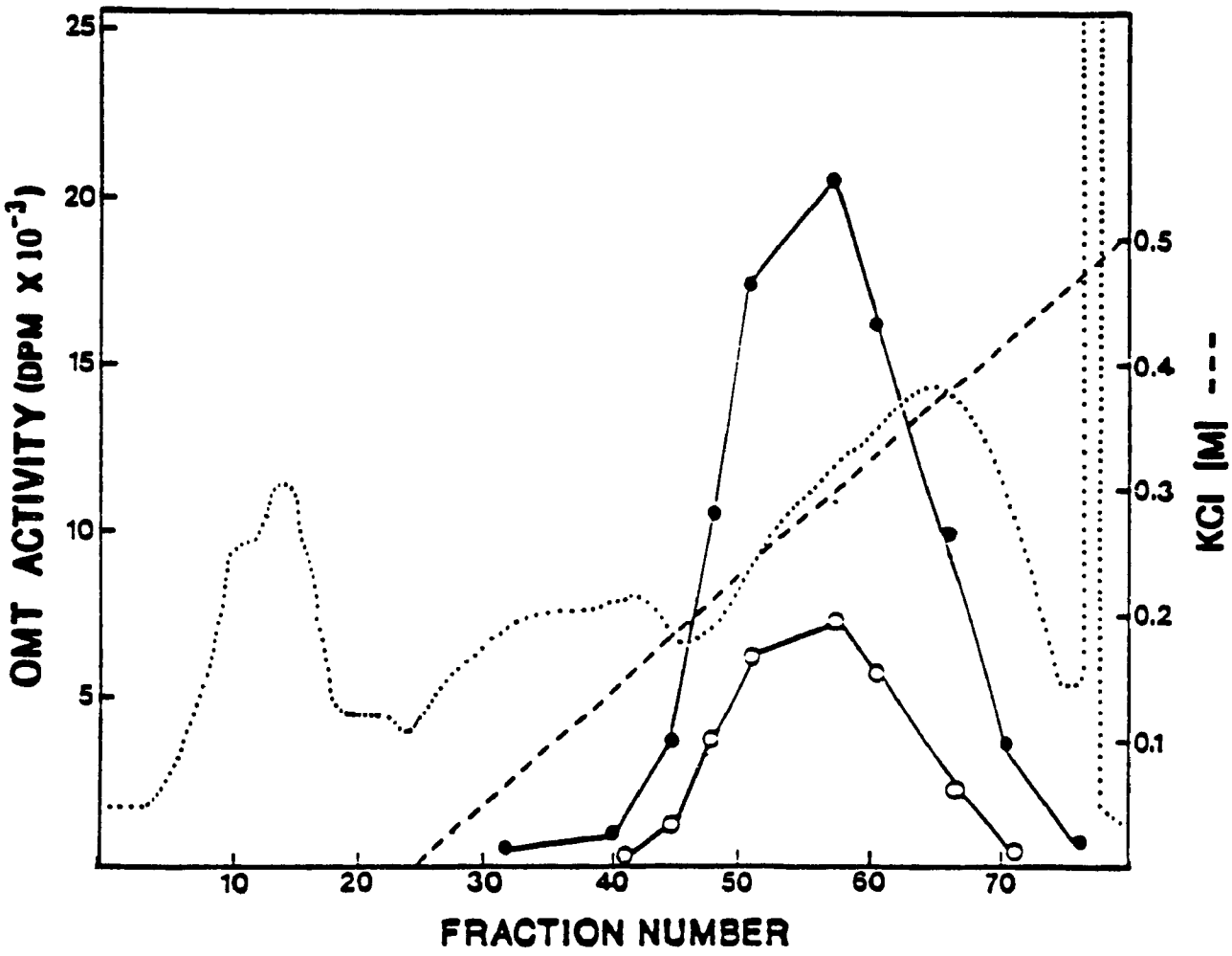


Figure 8. Elution profile of the OMT activities after ion-exchange chromatography on DEAE-Sephacel.

The bound proteins were eluted using a linear salt gradient of 0-0.5 M KCl (- - - -). OMT activity was assayed using 5-hydroxyferulic acid (—●—) and caffeic acid (—○—) as substrates. The absorbance (·····) was monitored at 280 nm. Refer to Table 3 for the amount of protein applied and recovered from the DEAE-Sephacel column.

and 150 mM KCl (isoform II), (Figure 9). However the resolution of the two isoforms does not suggest the separation of two distinct enzyme activities since both proteins catalyzed the O-methylation of caffeic acid to a lesser extent than 5-HFA. However, isoform II differed from isoform I in its affinity for 5-HFA (see D.2.2.).

Adenosine, in addition to being one of the precursors in S-adenosyl-L-homocysteine synthesis is also an inhibitor of the phenylpropanoid OMT (see D.2.11.), suggesting a specific interaction between the nucleotide and the OMT. It therefore appeared reasonable to use an adenosine matrix as a possible affinity ligand for the purification of the OMT. Further purification of each isoform was achieved by affinity chromatography on Adenosine-agarose column, and the OMT activity was eluted with 0.3 mM SAM in the same buffer (Fig. 10). While both isoforms appeared to be homogeneous at this stage of purification (Fig. 17), isoform I exhibited a significant increase in specific activity whereas, Isoform II was found to be unstable.

Table 3 is representative of a typical purification procedure for the phenylpropanoid OMT activities from cabbage tissue. Although the amount of total protein varied in different extractions, the product ratio, purification-fold and yield or recovery remained consistent among the several purification procedures conducted. The increase in specific activities was taken as a measure of the purification-fold brought about by each step. The protein fractions containing

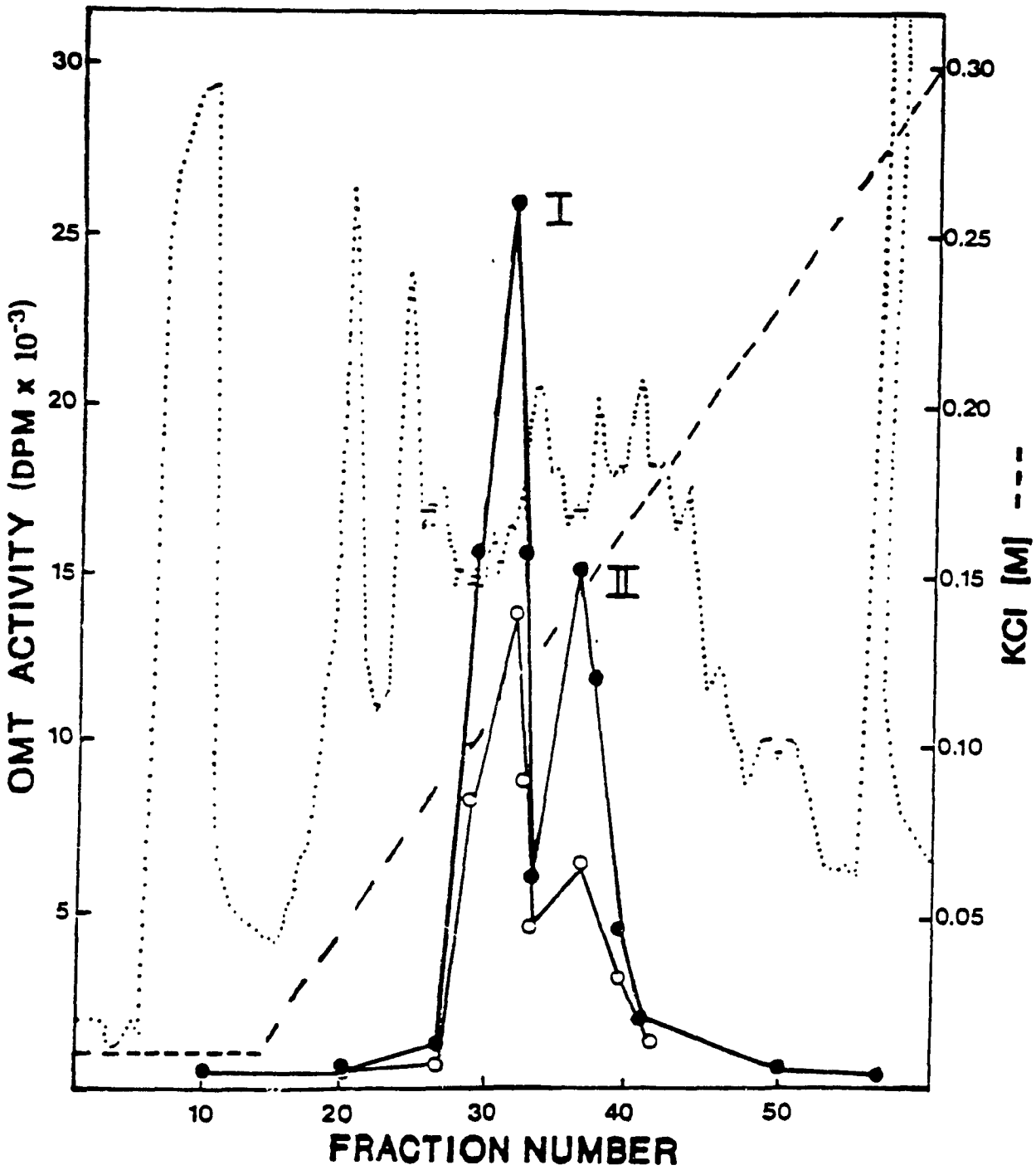


Figure 9. Elution profile of the OMT activities after ion-exchange chromatography on Mono Q HR 5/5 column.

The bound proteins were eluted using a linear salt gradient of 0-0.3 M KCl (- - - -). OMT activity was assayed using 5-hydroxyferulic acid (—●—) and caffeic acid (—○—) as substrates. The absorbance (·····) was monitored at 280 nm. Refer to Table 3 for the amount of protein applied and recovered from the Mono Q column.

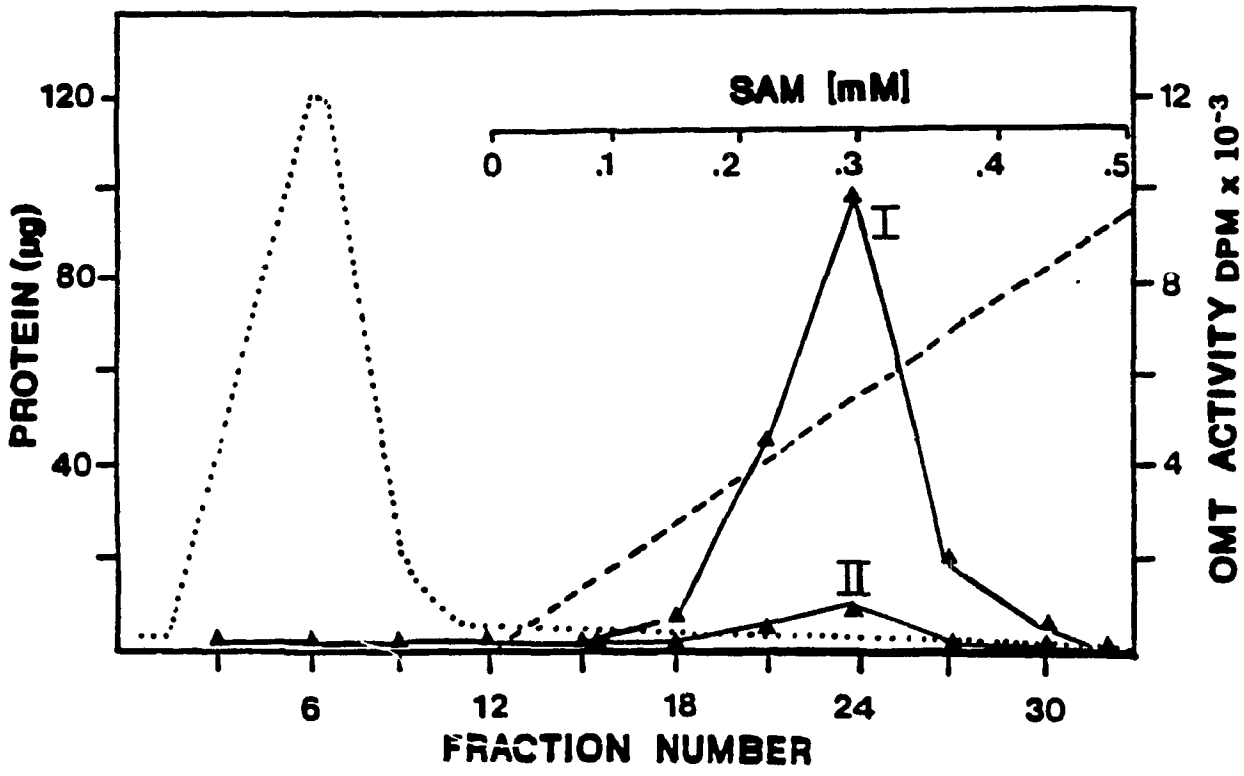


Figure 10. Elution profile of the OMT activities after Adenosine-agarose affinity chromatography.

The bound proteins were eluted using a linear 0-0.5 mM S-adenosyl-L-methionine gradient (- - - -). OMT activities were assayed using 5-hydroxyferulic acid (—▲—). The absorbance (·····) was monitored at 280 nm. Refer to Table 3 for the amount of protein applied to the Adenosine-agarose column. The total protein recovered is less than 25 µg for each isoform.

Table 3. Purification Of The Phenylpropanoid-OMT from *Brassica oleracea*[a]

Step	Total Protein (mg)	Specific Activity (pKat/mg) SA	FA	Total Activity (pKat) SA	FA	Ratio[b] (SA/FA)	Purification -Fold	Yield (%)
Dowex	600	1.20	0.40	720	240	3.0	-	100
Sephadex G-100	140	4.40	1.50	616	210	2.9	4	86
DEAE-Sephacel	9.4	12.5	4.20	118	39.5	3.0	10	17
Mono Q								
I	2.5	19.0	10.8	48.0	27.0	1.8	16	9
II	2.1	16.0	6.10	34.0	13.0	2.8	13	5
Adenosine-Agarose								
I	< 0.025[c]	>6.8x10^3	>3.9x10^3	<171	<98.0	1.8[d]	>5660	<24
II	< 0.025	No Activity		-	-	-	-	-

[a] The O-methyltransferase activities were assayed as described in the Methods section using 5-hydroxyferulic and caffeic acids. The yield (%) and purification-fold are based on the enzyme activity with 5-hydroxyferulic acid.

[b] SA, sinapic acid; FA, ferulic acid.

[c] The minimum detectable value using the Bio-Rad method.

[d] Based on DPMs.

OMT activity were partially purified after Sephadex G-100, DEAE-Sephacel and Mono Q which resulted in the specific activity being 16-fold (isoform I) greater than the crude extract. However, further purification of the OMT activities was achieved after adenosine-agarose affinity chromatography. The specific activity increased 360-fold over that of the Mono Q and resulted in an overall purification-fold greater than 5660, for isoform I when compared with the crude protein extract. The recovery of the enzymatic activity for isoform I from the latter column was less than 24%. Despite the instability of isoform II it was still detectable by its very low DPMs recorded. As a result of the enzymatic instability the specific activity nor product ratio could be determined for isoform II.

D.1.7. Electrophoretic Analysis

Enzyme purification was monitored by SDS-PAGE. With the exception of adenosine-agarose, all other steps of purification are illustrated (Fig. 11). Despite the increase in enzymatic activity (see Table 3) the protein profiles of each step show that purification to homogeneity of the OMT could not be achieved with these steps alone.

D.2. Characterization of the OMT Isoforms

D.2.1. Isoelectric Point

The DEAE-Sephacel fractions containing OMT activity were subjected to chromatofocusing, on a Mono P column. Isoforms I and II were resolved at pH 4.9 and 4.7, respectively (Fig.12).

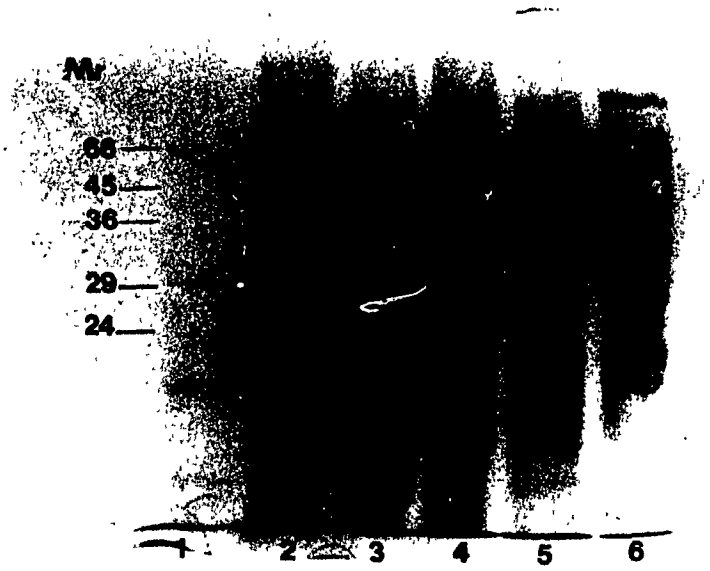


Figure 11. Photograph of the protein profile of the different steps of purification submitted to denaturing conditions on 12% acrylamide gels using Mini-Protean II and stained with Commassie blue. Approximately 30 μg of protein was applied to each lane.

Lane 1. Standard protein markers: bovine serum albumin (66 KD), ovalbumin (45 KD), glyceraldehyde-3-phosphate dehydrogenase (36 KD), carbonic anhydrase (29 KD) and trypsinogen (24 KD).

Lane 2. Dowex 1X2

Lane 3. Sephadex G-100

Lane 4. DEAE-Sephacel

Lane 5. Isoform I using Mono Q

Lane 6. Isoform II using Mono Q

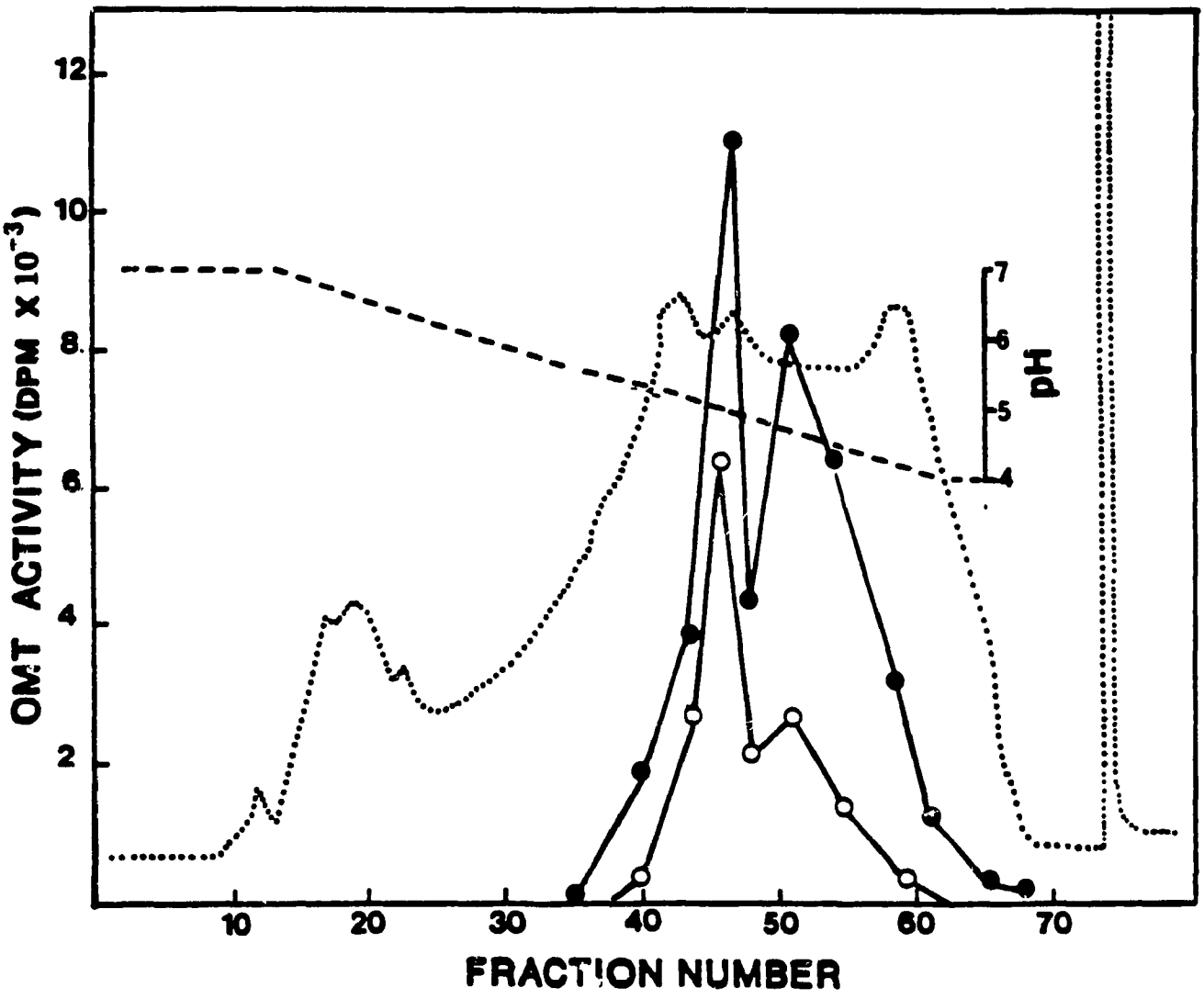


Figure 12. Elution profile of the OMT activities after chromatofocusing on Mono P 5/20 FR column.

The bound proteins were eluted with buffer C by a gradient (----) between pH 7.0 and pH 4.0. OMT activity was assayed with 5-hydroxyferulic acid (—●—) and caffeic acid (—○—). The absorbance (·····) was monitored at 280 nm. The amount of protein applied to the Mono P column was estimated at 2.5 mg and approximately 0.7 mg of total protein was recovered.

D.2.2. Product Ratio

The ratio of sinapic acid to ferulic acid activities (SA:FA) is very useful as an indicator for the ability to form syringyl lignin. The ratio of (SA:FA) formed remained constant (ca. 3) during the early steps (Dowex 1X2, Sephadex G-100 and DEAE-Sephacel) of enzyme purification (Table 3). Further purification of the enzyme on Mono Q resulted in a decline of the (SA:FA) ratio for isoform I, in contrast with that of isoform II. Similar behavior was observed on chromatofocusing of both isoforms (Table 4). Furthermore, the homogeneous isoform I experienced no change in product ratio (Table 3), while due to the instability of isoform II a product ratio could not be found. The fact that the product ratio of isoform I differed from isoform II and their differences in stability can be taken as an indication of the existence of two distinct forms of the enzyme.

D.2.3. Substrate Specificity

The activity of both isoforms (Mono Q fractions) was found to be quite similar against a variety of substrates when assayed at 100 μM concentration. The preference of the isoforms was found to be in the following descending order: 5-hydroxyferulic acid > caffeic acid > quercetin > trihydroxycinnamic acid > cinnamic acid > esculetin. Such compounds as chlorogenic, m-coumaric, 2,5- and 3,4-dihydroxybenzoic acids as well as hydroxyanthraquinones did not serve as methyl acceptors (Table 5).

Table 4: Product Ratio (sinapic acid/ferulic acid) of the Two Isoforms[a,b].

	Mono Q	Mono P
Isoform I	1.8	1.7
Isoform II	2.8	2.5

a The standard enzyme assay was used as described in C.8.
b The partially purified isoforms were used after Mono Q.

Table 5. Substrate Specificity of Isoform I And Isoform II[a]

Substrate	Relative Activity (%)	
	Isoform I	Isoform II
5-Hydroxyferulic acid	100	100
Caffeic acid	74.0	45.0
Trihydroxycinnamic acid	15.0	14.0
Chlorogenic acid	13.0	13.0
m-Coumaric acid	14.0	10.0
p-Coumaric acid	8.0	5.8
Gallic acid	4.0	4.0
2,3-dihydrobenzoic acid	5.0	4.0
Esculetin	19.0	17.5
Scopoletin	4.0	4.2
Daphnetin	8.5	5.3
Quercetin	68.0	55.0
Myricetin	60.0	53.0
Luteolin	50.0	41.0
Kampferol	3.3	3.7
Tamarixetin	4.5	4.0
1,2-dihydroxyanthraquinone	4.1	4.4
1,2,4-Trihydroxyanthraquinone	3.4	4.0

a The partially purified isoforms were used after Mono Q.

D.2.4. pH Optimum

The O-methylation reaction was measured against 5-hydroxyferulic acid (5-HFA) in the presence of Bis-Tris (pH 5.5-8.0), imidazole (pH 6.5-8.0), phosphate (pH 6.5-8.0), Tris-HCl (pH 7.0-9.0) and glycine-NaOH (pH 8.0-10.0). Figure 13 shows OMT activity in various buffers over a pH range of 5.5-10.0. The optimal enzymatic activity was found between pH 7.0-7.5 in Tris-HCl.

D.2.5. Enzyme Stability

The partially purified isoforms I and II (Mono Q fractions) maintained 90% of their activity over a period of 72 hours at 0-4°C. Furthermore, the addition of 10% glycerol (v/v) and 10mM dithioerythritol maintained the enzyme activity for one month when stored at -20°C. On the other hand, upon elution from Adenosine-agarose, isoform I maintained its activity for 24 hr whereas isoform II lost more than 85% of its activity when both were stored at 0-4°C in 10% glycerol and 10 mM dithioerythritol.

D.2.6. Thermostability

The stability of the partially purified isoforms I and II was also tested at 45°C. Figure 14 illustrates the OMT activity of each isoform at different incubation periods. The results indicate that more than 50% of the OMT activity of both isoforms was lost within the first 5 min and approximately 75% of the activity of both isoforms was lost after 10 min. After 20 min the two isoforms were completely inactived. These results

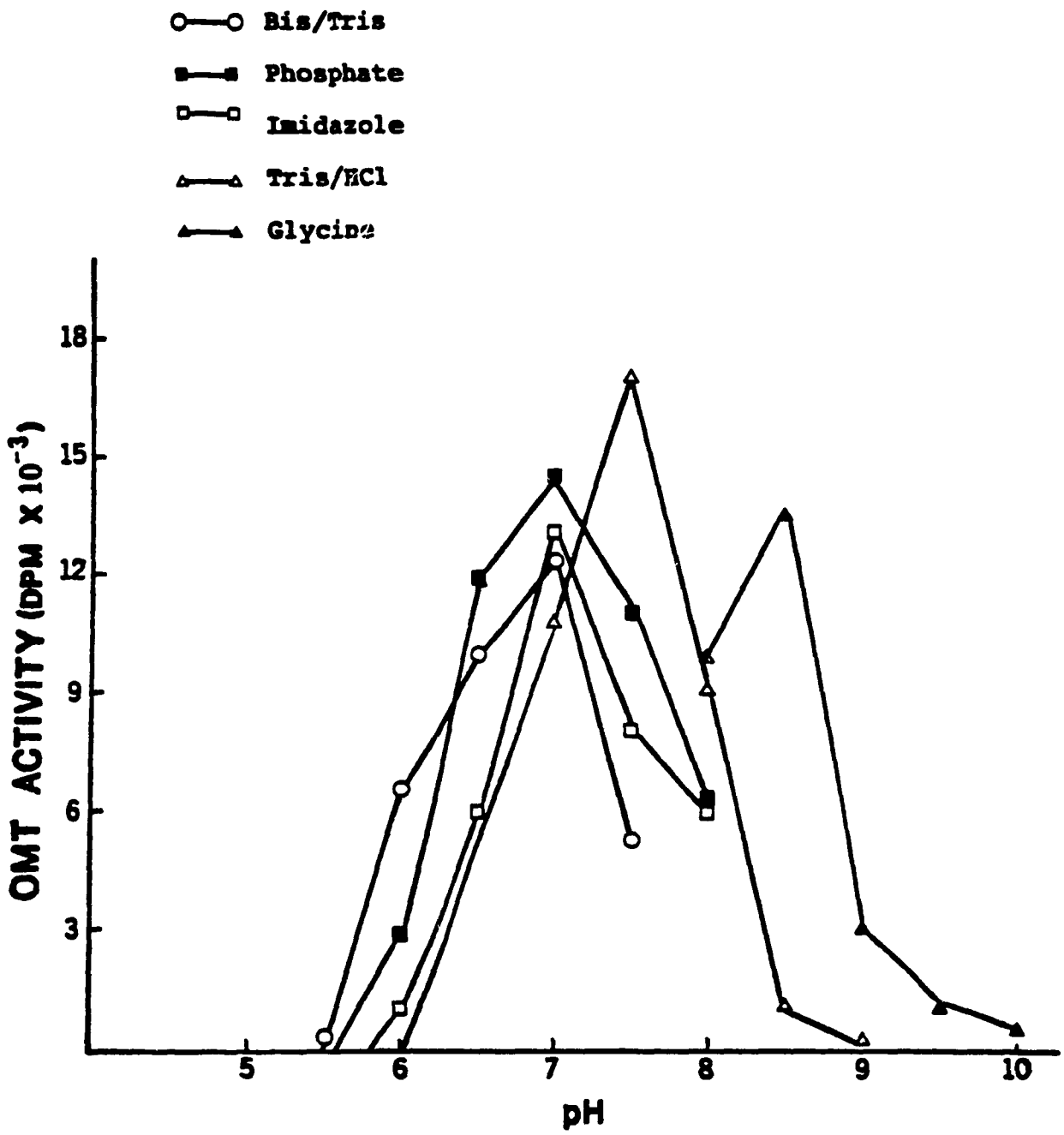


Figure 13. pH optimum of the partially purified phenylpropanoid O-methyltransferase. DEAE-Sephacel fractions exhibiting OMT activity were used. The assay mixture contained 100 μM of 5-hydroxyferulic acid, 100 μM SAM (containing 0.05 μM) and up to 50 μg of protein. The assay mixture was incubated for 30 min at 30°C.

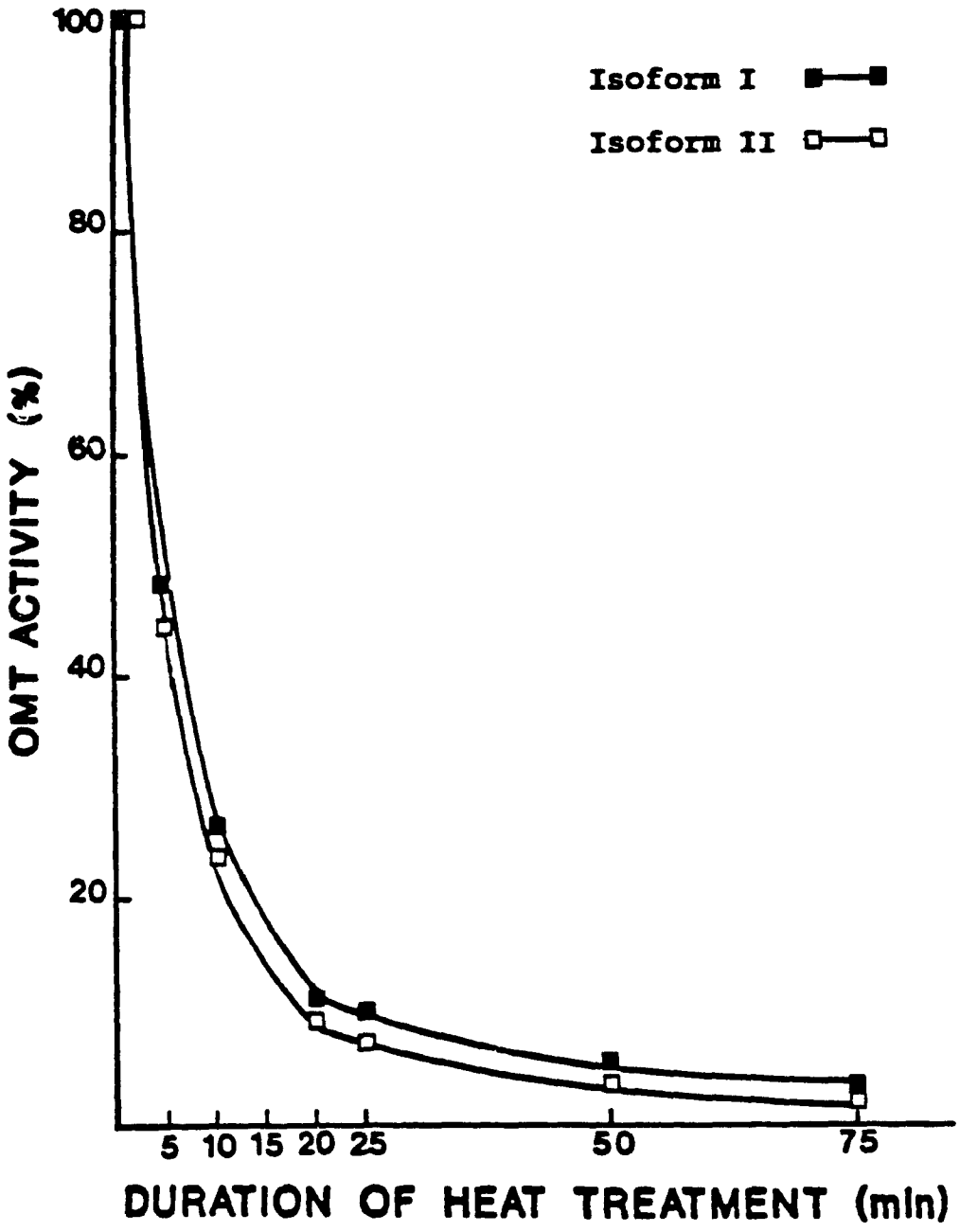


Figure 14. Thermal stability of the partially purified Isoforms I and II. After heating at 45°C for the indicated time periods, OMT activity of each isoform was assayed using 100 μM 5-hydroxyferulic acid 100 μM SAM (containing 0.05 μC_i) and up to 50 μg protein for 30 min at 30°C.

suggest that both isoforms have the same sensitivity towards inactivation by heat.

D.2.7. Molecular Weight

The molecular weight of each partially purified isoform was determined by gel filtration on a calibrated Superose 12 HR 16/50 using the FPLC system (Fig. 15). Both isoforms had the same elution volume (ca 51.7 mL) which corresponded to a molecular weight of 56,000 daltons +/- 3,000 daltons (Fig. 16). In addition, the molecular weight of isoforms I and II was estimated by SDS-PAGE. The homogeneous isoforms I and II which eluted from the Adenosine-agarose column were applied to an SDS-PAGE. Isoforms I and II migrated as single bands on the gel when stained with silver nitrate (Fig. 17). The Rf values of both isoforms were identical and corresponded to a molecular weight of 42,000 daltons +/- 2,000 daltons when compared with standard proteins (Fig. 18). See discussion concerning the difference in molecular weight of the native and denatured enzyme.

D.2.8. Electrophoretic Analysis Using Native Gels

Both isoforms I and II (Mono Q fractions) were pooled, desalted and concentrated to ca. 500 μL using Centricon tubes. The combined enzyme activity was applied to non-denaturing gels (section C.10.1.). OMT activity against 5-HFA and caffeic acids was localized in the same region of the gel suggesting that isoforms I and II behaved similarily in the native form.

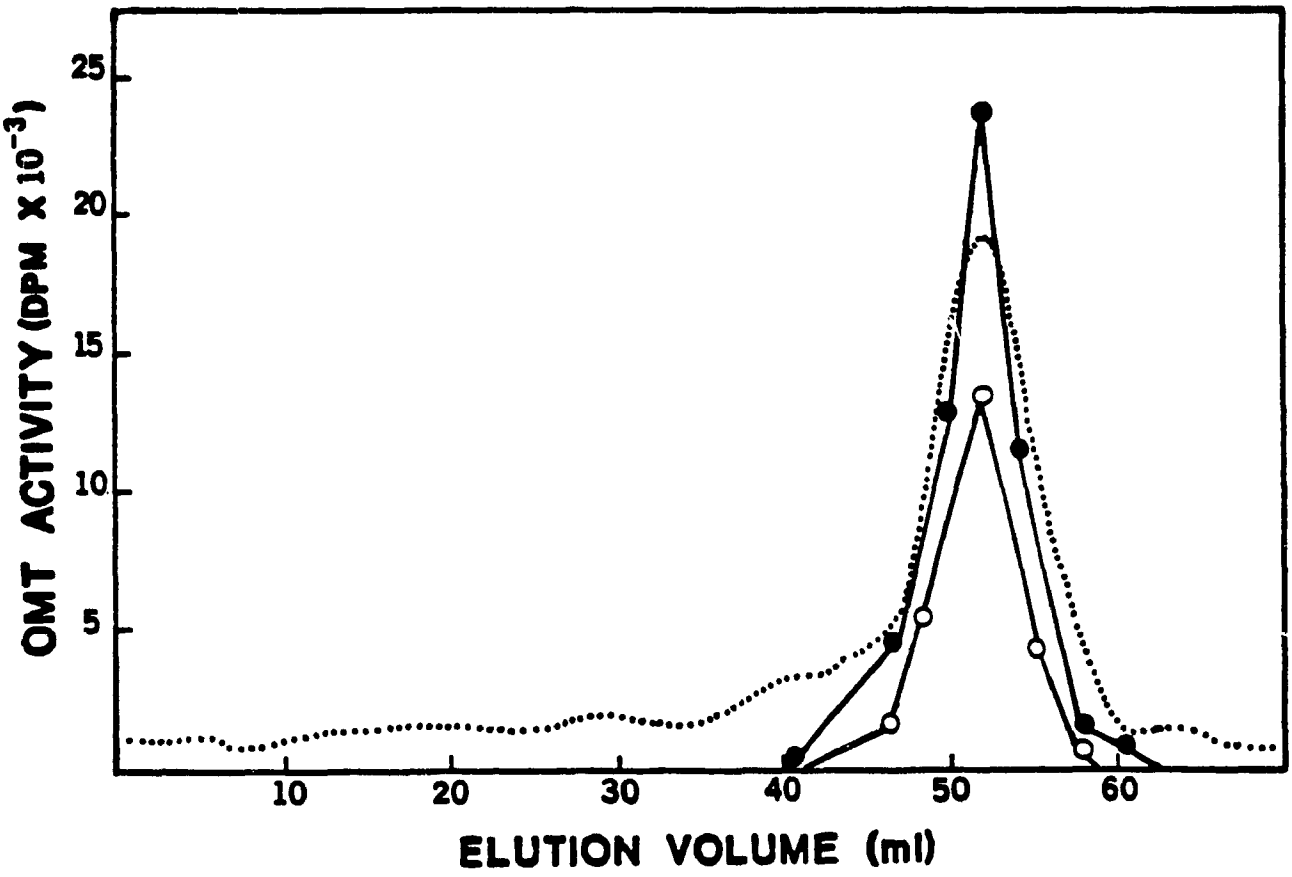


Figure 15. Elution profile of Isoform I or II using Superose HR 16/50 Column.

Each OMT isoform was applied to a calibrated Superose HR 16/50 column using the FPLC system. OMT activity was assayed using 5-hydroxyferulic acid (—●—) and caffeic acid (—○—). The elution volume was used to estimate the native moloecular weight of the OMT isoforms. The absorbance (·····) was monitored at 280 nm. Approximately 2.5 mg of each isoform in buffer B was applied to the Superose 12 column.

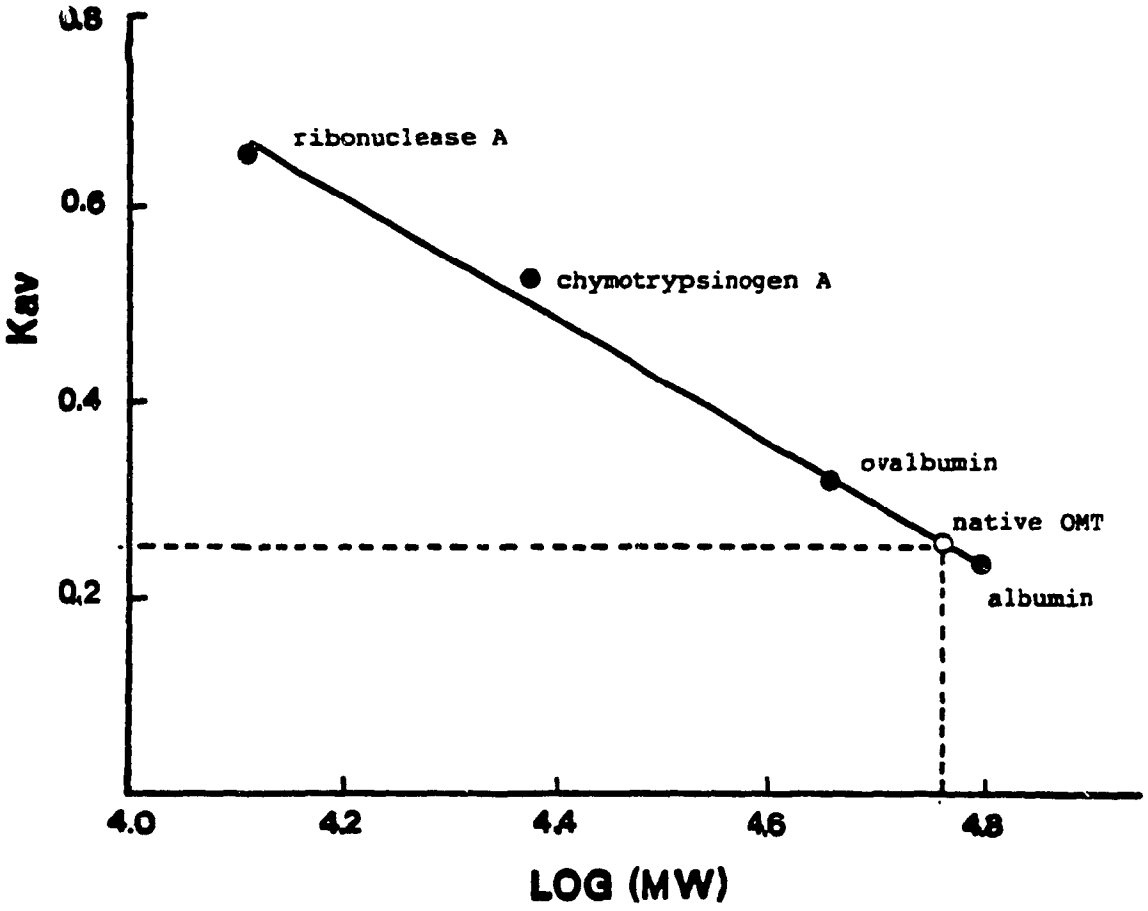


Figure 16. Determination of molecular weight of the native phenylpropanoid OMT isoforms.

Using a calibrated Superose 12 HR 16/50 column on the FPLC system. (--○--) K_{av} of the native OMT and (—●—) K_{av} of protein standards.

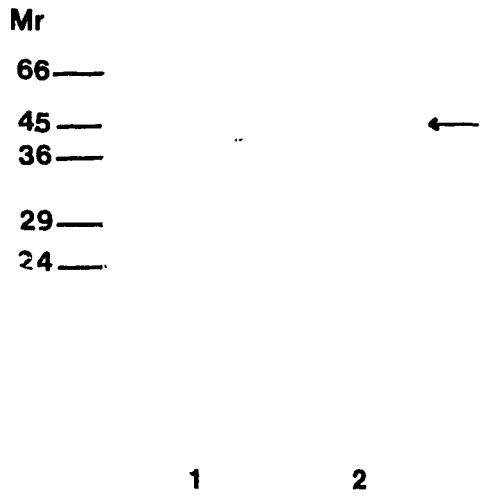


Figure 17. Photograph of the protein profile under denaturing conditions of the OMT isoforms which eluted from Adenosine-agarose affinity chromatography. Acrylamide (12%) gels were used on the Mini-Protean II. The amount of protein applied was ca. 200 ng bovine serum albumin equivalent as determined from serial dilution of a standard protein and stained with silver nitrate.

Mr — Standard protein markers: bovine serum albumin (66 KD), ovalbumin (45 KD), glyceraldehyde-3-phosphate dehydrogenase (36 KD), carbonic anhydrase (29 KD), trypsinogen (24 KD).

Lane 1 — Isoform I

Lane 2 — Isoform II

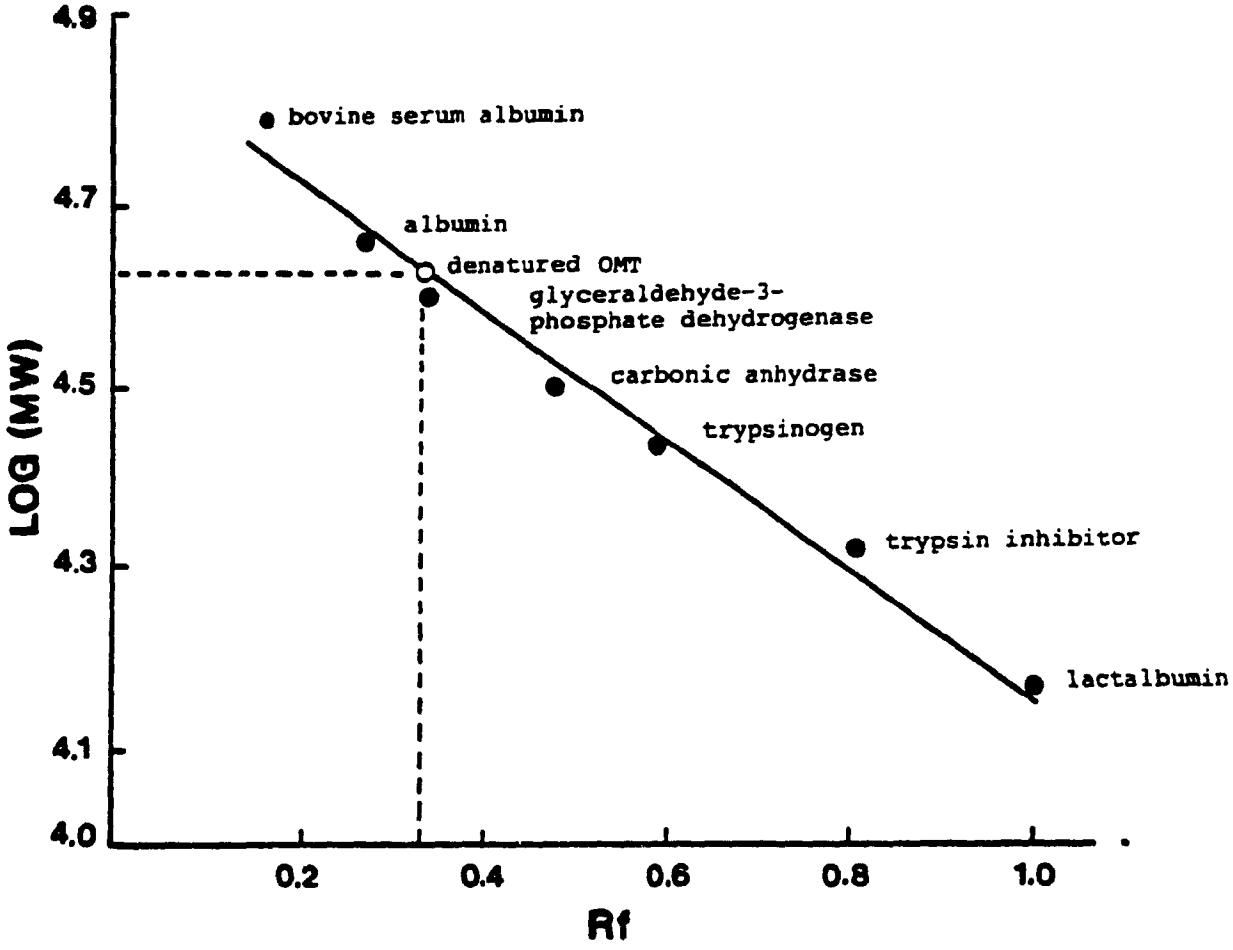


Figure 18. Determination of molecular weight of homogeneous phenylpropanoid OMT isoforms.

Comparison of the electrophoretic mobilities of the OMT isoforms with the standard protein markers. (⊢-O-⊣) R_f value of denatured OMT and (—●—) R_f value of denatured protein standard.

D.2.9. Effect of Metal Ions

The effect of metal ions on the enzyme activity of isoforms I and II is shown in Table 6. The addition of Mg^{+2} (1 and 10 mM) had no effect on O-methylation reaction. Other divalent metal ions such as Mn^{+2} and Cu^{+2} also showed no effect, with the exception of Zn^{+2} which appeared to significantly inhibit the enzyme reaction. Addition of EDTA did not inhibit the enzyme activity, suggesting that metal ions are not required for enzymatic activity.

D.2.10. Effect of Sulfhydryl Reagents

As was previously mentioned (section D.1.2.) 2-mercaptoethanol or dithioerythritol is essential for optimal enzymatic activity. Therefore, it was considered important to investigate the effect of sulfhydryl-binding reagents on the OMT activity of isoforms I and II. The enzyme activity of either of the isoforms was completely inhibited by the addition of 1 mM iodoacetate (Table 7) suggesting the presence of at least one sulhydryl group at the active site of the OMT. This inhibition was not restored after the addition of 2-mercaptoethanol. On the other hand, neither N-ethylmaleimide nor iodoacetamide demonstrated any significant inhibition when tested.

D.2.11. Effect of Inhibitors

The reaction products ferulic and sinapic acids proved to be poor inhibitors of their respective O-methylation reaction (section E.1.2.). On the other hand, the second product of the

Table 6: Effect of Divalent Cations on the Activities of Isoform I and Isoform II[a].

Addition	Concentration (mM)	Isoform I (%)	Isoform II (%)
No addition	-	100	100
Mg^{+2}	1	110	113
Mg^{+2}	10	98	102
Mn^{+2}	1	106	105
Mn^{+2}	10	61	89
Ca^{+2}	1	119	106
Ca^{+2}	10	72	75
Cu^{+2}	1	79	79
Co^{+2}	1	79	81
Zn^{+2}	1	10	19
EDTA	1	91	98
EDTA	10	81	89

[a] The partially purified isoforms were used after Mono Q.

Table 7: Effect of Sulfhydryl-Binding Reagents on the Activities of Isoform I and Isoform II[a].

-SH Reagent	Concentration (mM)	Isoform I (%)	Isoform II (%)
Control	-	100	100
Iodoacetate	1	2.5	4.0
Iodoacetate + DTT[b]	1,10	2.3	2.5
Iodoacetamide	1	102	110
N-Ethylmaleimide	1	108	111

[a] The partially purified isoforms were used after Mono Q.
[b] Dithioerythritol.

reaction, S-adenosyl-L-homocysteine (SAH), was a potent inhibitor of the reaction (K_i 15 μM). Adenosine, a precursor of SAH synthesis, also inhibited the O-methylation reactions but to a lesser extent than SAH as witnessed by its K_i (5.5 mM).

D.2.12 Presence of OMT Isoforms and Sinapine Content in Relation to Developmental Stages

The major constituent in seeds of the Brassicacea family is sinapine, a choline ester of sinapic acid [75]. However, as the tissue goes from a developing embryo to a developing seedling, sinapine is rapidly eliminated by hydrolysis. Germinating seeds (ca. 4 days) were extracted simultaneously for detection of sinapine and for isoforms I and II. The same test was also performed on developing seedlings, (ca. 12 days). Isoforms I and II were detected in both germinating and developed seedlings. While the presence of sinapine was clearly demonstrated in germinating seedlings, it could not be detected in developing seedlings.

D.3. Separation And Partial Characterization of the Flavonoid O-Methyltransferase

The general structure of flavonoid compounds consists of two benzene rings (namely, A and B) linked to a three-carbon unit formed into a pyrone ring (Fig. 19). The substitution patterns of the B ring generally resemble those of common cinnamic acids whereby the C-4' usually possesses a hydroxyl group and is rarely methylated, while the C-3' and C-5' may bear either a

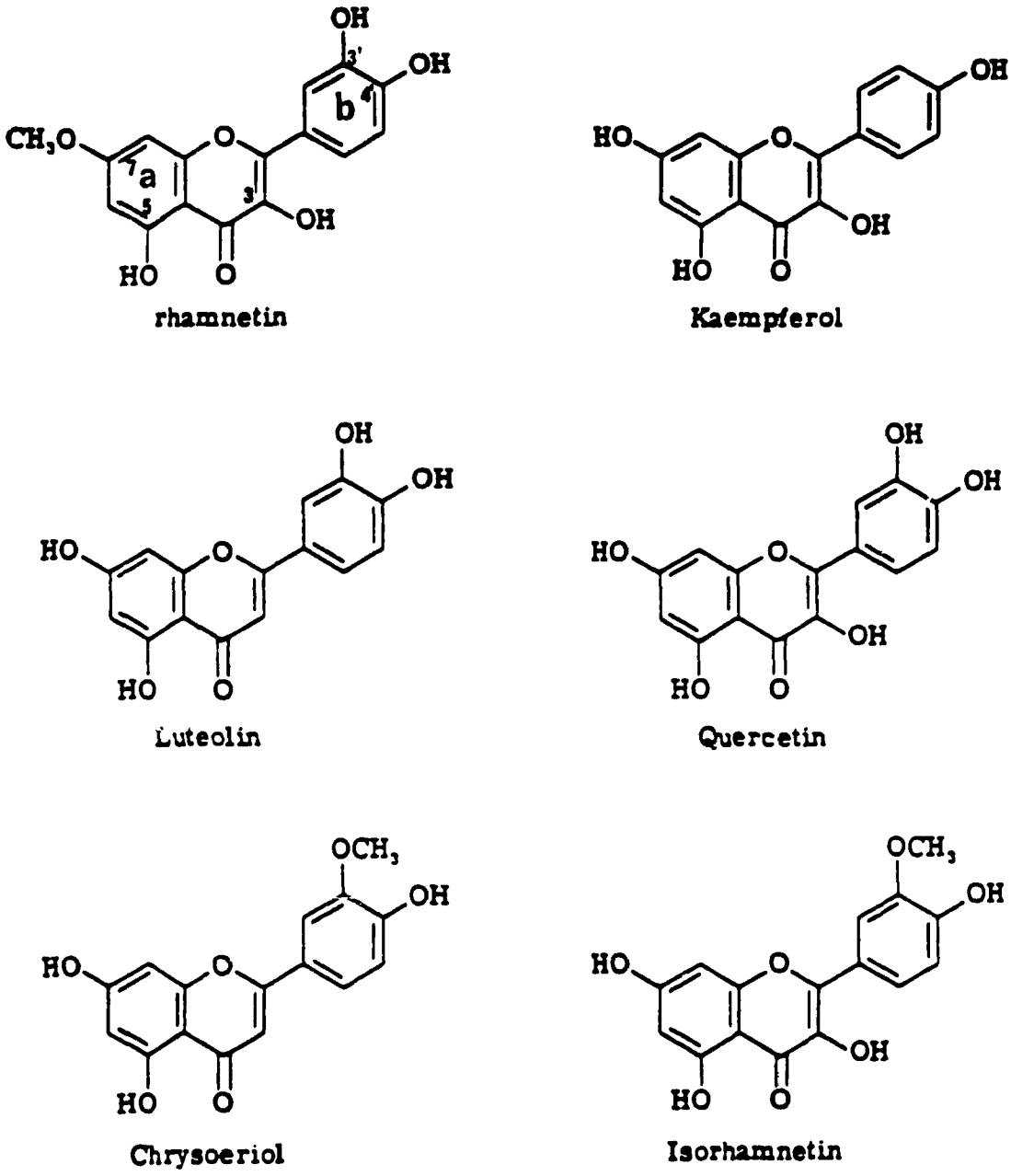


Figure 19. Structures of several flavonoid compounds.

hydroxyl or methoxyl groups. Flavonoid OMTs have been purified from soybean cell suspension culture [26] and Tulipa anthers [27]. While purifying the phenylpropanoid OMT, we also proceeded to the purification of the flavonoid OMT. The active fractions exhibiting flavonoid OMT activity which eluted from Sephadex G-100 were applied to Mono Q column (Fig. 10) and subsequently to Superose 12 column. Our purification procedure resulted in a 100-fold increase in enzyme activity as compared to the crude extract (Table 8) and enabled its partial characterization.

D.3.1. Substrate Specificity

The flavonoid OMT exhibited a pronounced preference for flavonoid substrates as compared with phenylpropanoids (Table 9). Furthermore, it appears that O-methylation of the flavonoid substrates is occurring on the B ring carrying O-dihydroxyl groups (Fig. 19). Preliminary identification of the reaction product of luteolin, by cochromatography with a reference sample of chrysoriol, suggests that O-methylation is occurring at the 3'-hydroxyl of ring B. The apparent K_m for luteolin was found to be 20 μM as determined from a double-reciprocal plot.

D.3.2. pH Optimum

The pH optimum for the O-methylation of luteolin was studied in several buffers ranging from pH 5.5 to pH 10 (Fig. 20). Optimum OMT activity was found to be at pH 8.0 in 0.1 M phosphate buffer.

Table 8: Purification of the Flavonoid OMT in Brassica oleracea[a]

Step	Total Protein (mg)	Specific Activity (pKat/mg)	Yield (%)	Purification (Fold)
Dowex	130	0.50	100	-
Sephadex-G-100	22.5	2.1	73	4
Mono Q	3.0	14.2	66	28
Superose-12	0.35	52.0	28	104

[a] Using the standard enzyme assay as described in C.7. and luteolin as substrate at 50 μM final concentration.

Table 9: Substrate Specificity of the Flavonoid OMT[a,b].

Substrate	Relative Activity (%)
Luteolin	100
Quercetin	53
Isorhamnetin	8
Rhamnetin	39
Kaempferol	9
Tamarixetin	8
Genistein	9
5-Hydroxyferulic acid	20
Caffeic acid	28

[a] The partially purified flavonoid OMT was used after chromatography on Mono Q column.
[b] The standard enzyme assay was used as described in C.8.

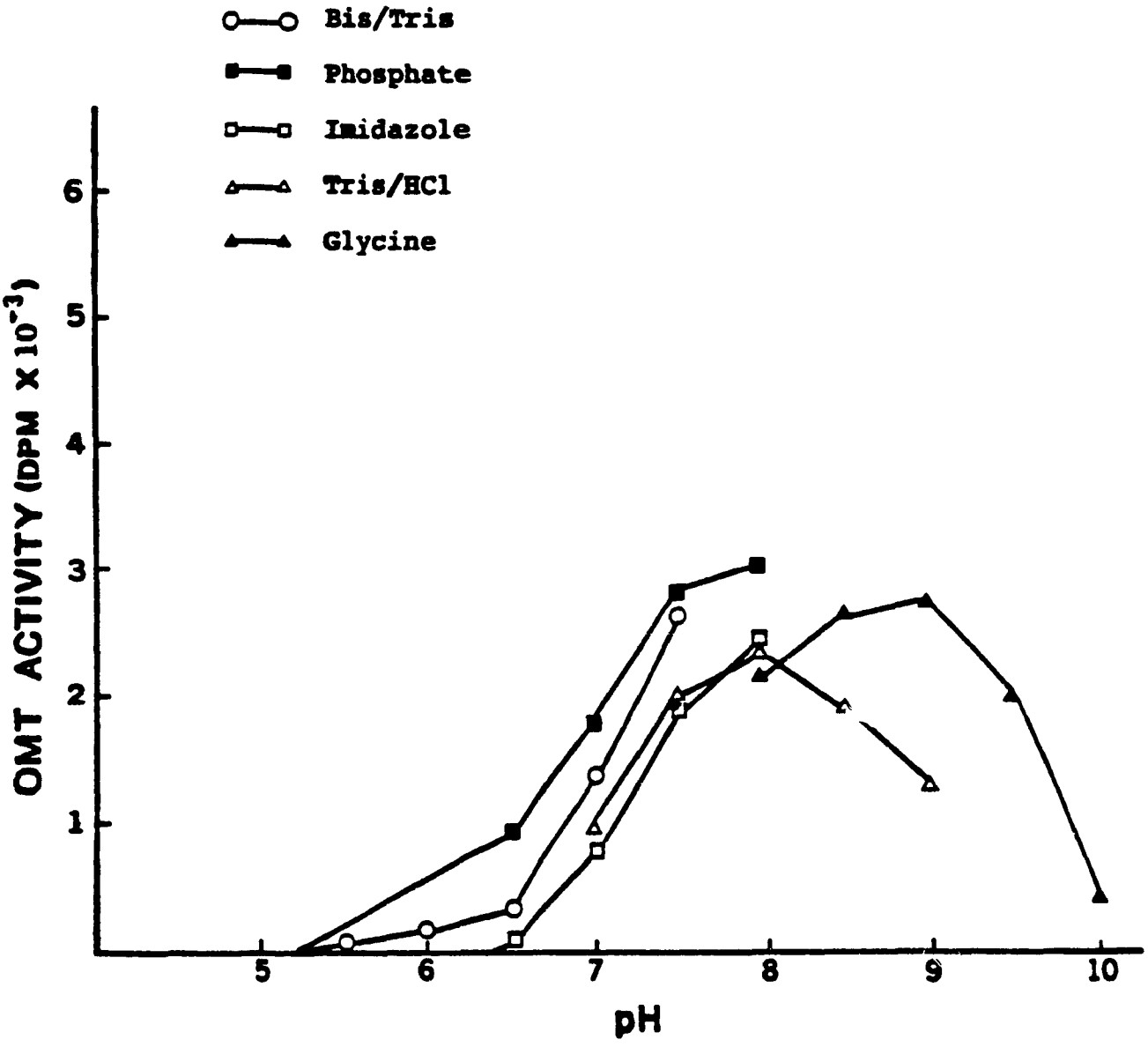


Figure 20. pH Optimum of the partially purified flavonoid OMT. The assay mixture was incubated for 30 min at 30°C and contained 50 μM luteolin, 100 μM SAM (containing 0.05 μC_i) and up to 50 μg protein.

D.3.3. Effect of Metal Ions And Sulfhydryl Reagents on Flavonoid OMT Activity

The effect of metal ions and sulfhydryl reagents on the flavonoid OMT activity are shown in Table 10. The flavonoid OMT activity was enhanced with the addition of Mg^{+2} ions. Furthermore, the enzyme was strongly inhibited by Ca^{+2} ions and partially inhibited by Mn^{+2}, Cu^{+2} and Co^{+2} ions. EDTA proved to completely inhibit the flavonoid OMT, whereas iodoacetate had no significant effect.

D.3.4. Stability

Unlike the phenylpropanoid OMT isoforms which were stable for one month, the flavonoid OMT retained enzyme activity for only 24 hr when stored in 10% glycerol, 10 mM dithioerythritol at 0-4^{o}C.

Table 10: Effect of Divalent Cations and Sulfhydryl-Reagents on the Flavonoid OMT[a] Activity.

Addition	Concentration (mM)	Relative Activity (%)
Control	-	100
Mg^{+2}	1	125
Mg^{+2}	10	121
Mn^{+2}	1	56
Mn^{+2}	10	53
Ca^{+2}	1	20
Ca^{+2}	10	12
Cu^{+2}	1	49
Co^{+2}	1	41
Zn^{+2}	1	10
EDTA	1	6
EDTA	10	6
Iodoacetate	1	87
N-Ethylmalimide	1	93

[a] The partially purified flavonoid OMT was used after chromatography on Mono Q column.

E.1. Kinetic Analysis of The Phenylpropanoid O-Methyltransferase

Steady state kinetic studies for the OMT catalyzed conversion of 5-hydroxyferulic acid (5-HFA) to sinapic acid were performed with the stable and partially purified isoform I.

E.1.1. Substrate Interaction Kinetics

In the absence of inhibitor the activity data were analyzed by double-reciprocal plots for an intersecting or parallel pattern with 5-HFA as the variable substrate and several fixed concentrations of SAM (Fig. 21). The converging lines obtained from the graphical analysis suggest that the mechanism to best fit the data is a sequential binding mechanism [73]. The experimental data were therefore represented by equation (2), the rate equation for a sequential bireactant mechanism (section C.7.13) [73]. The plot represents N=3 and the actual data is the mean value (SD 9%).

E.1.2. Product Inhibition Studies

The order of substrate binding and product release was obtained from product inhibition studies. The different plots represent N=3 and the datum is the mean value (SD 10%). The results from Figure 22a indicate that S-adenosyl-L-homocysteine (SAH) is a competitive inhibitor with respect to S-adenosyl-L-methionine (SAM), furthermore, SAH was found to be non-competitive with respect to 5-HFA (Fig.22b). Sinapic acid was also found to be a non-competitive inhibitor with respect to SAM

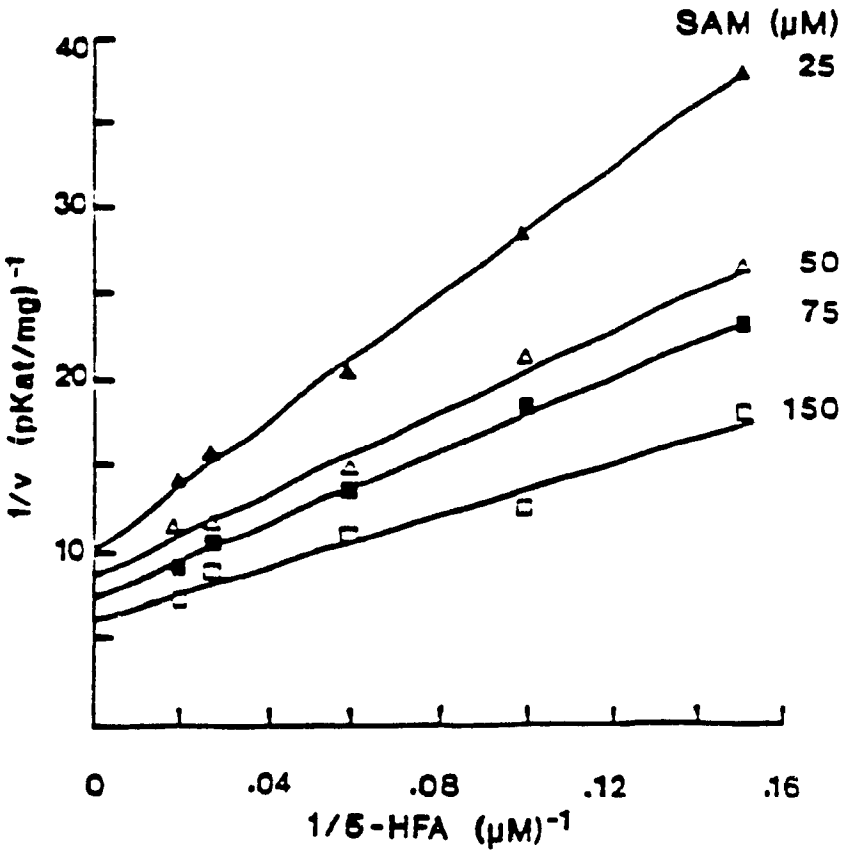
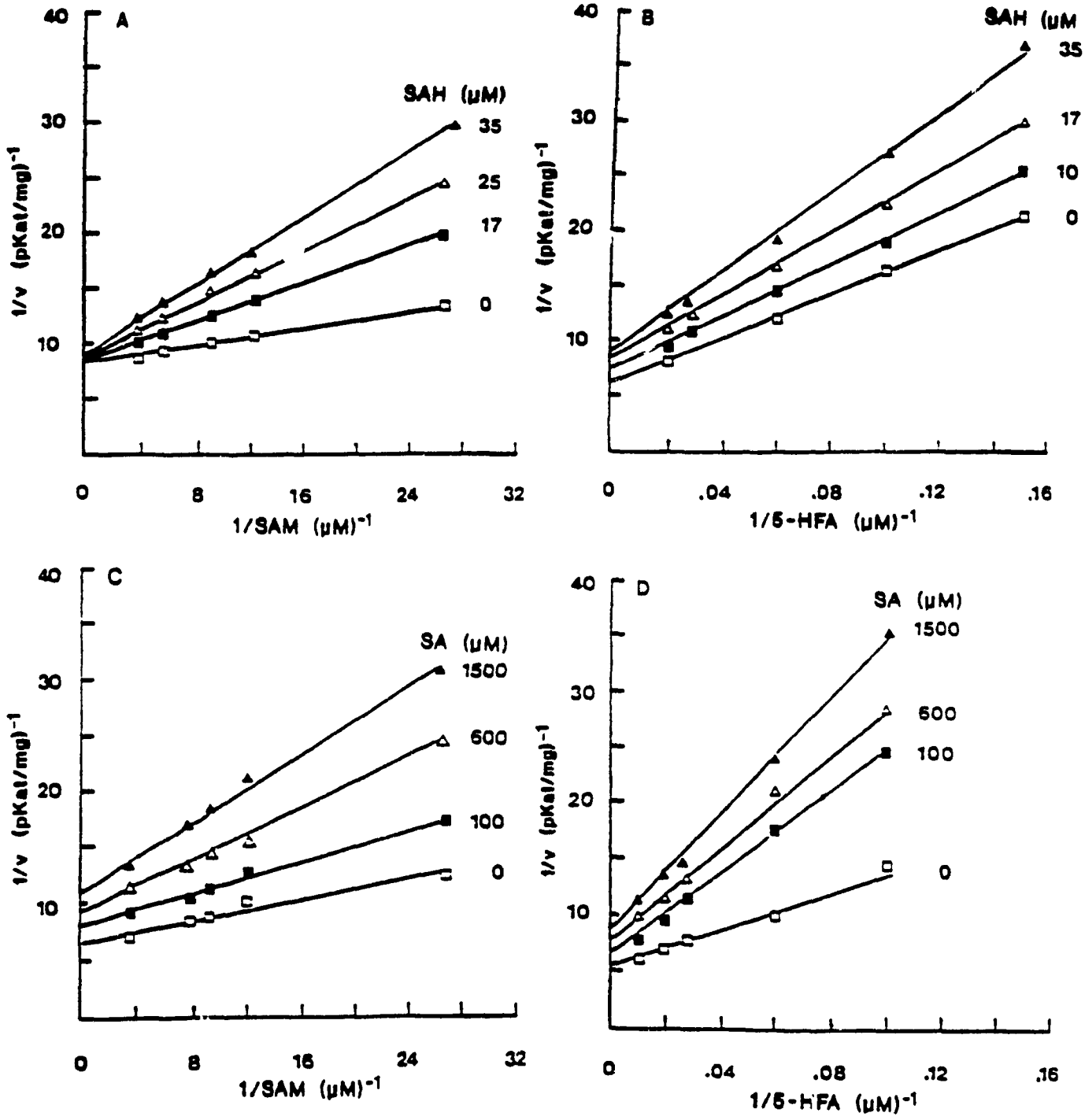


Figure 21. Double-reciprocal of initial velocity (v) versus 5-hydroxyferulic acid (5-HFA) concentration for the O-methylation of 5-HFA. S-adenosyl-L-methionine concentrations were fixed with 5-hydroxyferulic acid as the variable substrate. Varying concentrations of S-adenosyl-L-methionine were used containing 0.05 μC_i. The assay mixture was incubated for 30 min at 30°C.

Figure 22. Product inhibition studies of the phenylpropanoid OMT.

(A) Inhibition by S-adenosyl-L-homocysteine (SAH) with S-adenosyl-L-methionine (SAM) as the variable substrate at fixed 5-hydroxyferulic acid concentration (35 μM). Varying concentrations of SAM were used containing 0.05 μC_i.
(B) Inhibition by SAH with 5-hydroxyferulic acid as the variable substrate at fixed SAM concentrations (0.05 μC_i containing 100 μM SAM).
(C) Inhibition by sinapic acid with SAM as the variable substrate at fixed 5-hydroxyferulic acid concentration (35 μM). Varying concentrations of SAM were used containing 0.05 μC_i.
(D) Inhibition by sinapic acid with 5-hydroxyferulic acid as the variable substrate at a fixed SAM concentration (100 μM containing 0.05 μC_i). All assay mixtures were incubated for 30 min at 30°C.


A
SAH (μM)
35
25
17
0
1/v (pKat/mg)⁻¹
1/SAM (μM)⁻¹
B
SAH (μM
35
17
10
0
1/5-HFA (μM)⁻¹
C
SA (μM)
1500
600
100
0
1/SAM (μM)⁻¹
D
SA (μM)
1500
600
100
0
1/5-HFA (μM)⁻¹

(Fig. 22c) and 5-HFA (Fig. 22d). These kinetic patterns are consistent with an ordered bi bi mechanism where SAM binds first, followed by 5-HFA with the subsequent release of sinapic acid and SAH (Fig. 23).

The kinetic constants K_a, K_b, K_{ia}, K_{ip}, K_{iq} and V_{max} were determined by replotting slopes and intercepts versus substrate or inhibitor concentrations. The K_m (K_a and K_b), the dissociation constants (K_{ia}, K_{ip} and K_{iq}) as well as V_{max} are listed in Table 11.

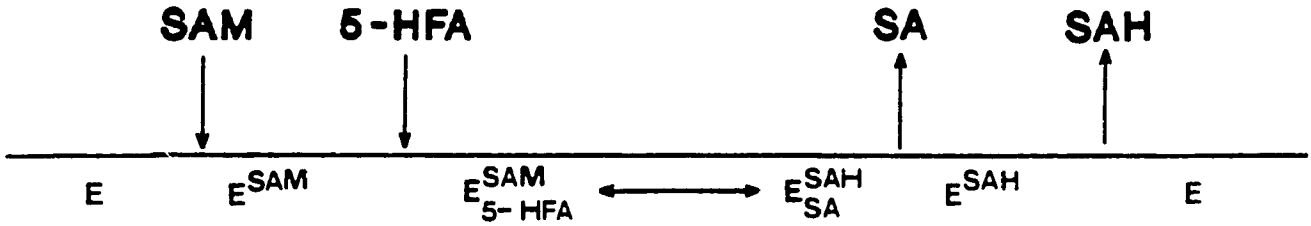


Figure 23. The proposed kinetic mechanism of the phenylpropanoid O-Methyltransferase; both SAM followed by 5-HFA must bind prior to release of SA and SAH from the enzyme (E).

SAM : S-adenosyl-L-methionine
5-HFA: 5-Hydroxyferulic acid (phenylpropanoid substrate)
SA : Sinapic acid (O-methylated phenylpropanoid)
SAH : S-adenosyl-L-homocysteine

Table 11: Kinetic Parameters of the Phenylpropanoid-OMT[a]

Kinetic Parameter	
K_a	55 μM
K_b	20 μM
K_{ia}	100 μM
K_{iq}	15 μM
K_{ip}	2,000 μM
V_{max}	14 pKat/mg

[a] Isoform I was used in determining the kinetic parameters from chromatography on Mono Q column.

a) S-adenosyl-L-methionine
b) 5-hydroxyferulic acid
p) sinapic acid
q) S-adenosyl-L-homocysteine

F. DISCUSSION

Angiosperm phenylpropanoid OMT has been considered "difunctional" O-methyltransferases in view of its capacity to methylate caffeic and 5-hydroxyferulic acids [76]. This concept has been maintained in most previous work despite the low purification-folds achieved, 100-fold in bamboo [22], 75-fold in spinach beet [60] and 11-fold in mistletoe [77].

The experiments described here have demonstrated the presence of one phenylpropanoid OMT in cabbage leaves which mediated the transfer of the methyl of S-adenosyl-L-methionine to the hydroxyl groups at position 3 of caffeic acid and at position 5 of 5-hydroxyferulic acid. The cabbage OMT was purified by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, DEAE-Sephacel, Mono Q and Adenosine-agarose. This resulted in a purification-fold greater than 5660 and yielded a homogeneous OMT. Furthermore, two OMT activities were separated by anion-exchange chromatography on Mono Q column; both of which were active against 5-hydroxyferulic and caffeic acids (Fig. 10). Since an isoform is defined as multiple molecular forms of an enzyme derived from the same tissue and catalyzing the same activity [40], we felt it appropriate to identify the two forms as isoform I and isoform II.

Despite having identical molecular weights, both isoforms exhibited distinct properties: (a) both isoforms eluted at specific salt (KCl) concentrations from the Mono Q column (Fig. 10), (b) different apparent pI were observed on

chromatofocusing, (c) the differences in product ratios observed during enzyme purification indicates the existence of two distinct forms of the phenylpropanoid OMT, (d) whereas isoform I exhibited a significant increase in specific activity (Table 3) isoform II lost its catalytic activity possibly due to instability. These differences confirm the presence of two distinct forms of the OMT. These isoforms have been consistently reproduced even after excluding ammonium sulfate precipitation, thus reducing the possibility of being an artifact resulting from protein aggregation.

To our knowledge, this is the first reported instance where the phenylpropanoid OMT has been purified to homogeneity with the aim of studying its specificity for phenylpropanoids. Furthermore, our purification protocol enabled the resolution of two isoforms of the phenylpropanoid OMT.

Multiple forms of the phenylpropanoid OMT [24,25,47] have been previously observed in several tissues. Kuroda et al. [47] were able to separate two isoforms in aspen wood by applying anion-exchange chromatography to a partially purified OMT extract. However, due to enzymatic instability they were able to purify and characterize only one of the enzyme forms. The polymorphism observed by the phenylpropanoid OMT in these studies suggests it is not an artifact, but reflects some physiological state <u>in vivo</u>. In view of the recent studies with isoforms of pectate lyase demonstrating that disruption of the gene coding for the active enzyme form resulted in the loss of activity of both isoforms, [78] it could be postulated that one

gene is responsible for the coding of the OMT, and different enzyme forms are the result of physical changes to the protein.

The substrate specificity of the phenylpropanoid OMT in cabbage demonstrated the highest OMT activity with 5-hydroxyferulic acid followed by caffeic acid (Table 5) while para and meta-coumaric acids and derivatives of benzoic acid did not serve as good methyl acceptors. Flavonoids like quercetin proved to be good methyl acceptors. However, this does not imply that the OMT preparation is contaminated with a flavonoid OMT. It suggests that the phenylpropanoid OMT exhibits a fairly wide range of substrate specificity. The results obtained from substrate specificity studies indicate that the substrates must satisfy certain structural requirements for maximal activity of the phenylpropanoid OMT. These are: (a) o-dihydroxy phenolics as in the case of 5-hydroxyferulic and caffeic acids as well as the B ring of quercetin, (b) a 3-carbon side chain attached to the phenolic ring, since neither gallic acid nor benzoic acid derivatives were methylated to any significant extent, (c) a free carboxyl group at the end of the side chain, since esterification (chlorogenic acid) or lactonization as in coumarins greatly reduced the activity.

The efficiency with which the phenylpropanoid OMT catalyzed the O-methylation of 5-hydroxyferulic acid and caffeic acids, suggests that this enzyme is involved in the sequence of reactions leading to lignin formation.

Phenylpropanoid OMTs might be characterized as follows: the pH optimum ranges from 6.5 to 8.0 as observed in tobacco cell

suspension [24], in tobacco leaves infected with TMV [25], aspen wood [47] and spinach beet leaves [60]. The lignin type OMT found in tobacco, aspen wood and spinach leaves generally do not require Mg^{+2} ions [24,25,47,60] with the exception of bamboo OMT [21]. Sulfhydryl group reagents generally inhibit plant OMTs to various extents as observed in cell cultures of Ruta graveolens [29], aspen wood [47] and spinach beet leaves [60]. The effect of EDTA on the OMTs is not always the same; some showed no effect as in the soybean cell suspension cultures [26] but others were moderately activated as in pine seedlings [23]. The cabbage OMT activities described here appear to fulfill the general requirement of a phenylpropanoid-specific OMT.

The product ratio of sinapic acid to ferulic acid was decreased during purification (Table 4) suggesting the elimination of contaminating 5-hydroxyferulic acid activity contained in the crude and partially purified extract. However, the product ratio observed in cabbage is in accordance with other angiosperms such as; bamboo [21], aspen wood [47] and mistletoe [77] whereby the sinapic acid to ferulic acid ratio is greater than one indicating a preferential formation of syringyl lignin in these plants.

The molecular weight of isoforms I and II was estimated by molecular sieving (Figs. 15 and 16) and SDS-gels (Figs. 17 and 18. Under denaturing conditions the isoforms had identical values, 42 Kdaltons. However the molecular weight value of the native isoforms was estimated to be 56 Kdaltons. The 1.3-fold difference observed in molecular weights between the native and

denatured forms is difficult to explain but it may be the result of protein aggregation of the native OMT. Hermann et al. [31] also observed a 1.5-fold difference between the native form and the denatured form of the phenylpropanoid OMT in TMV infected tobacco leaves. The latter authors explained that the difference may be a result of monomers or dimers which make up the native OMT. Our results suggest that the single band observed on SDS-PAGE can either be a monomer or a homodimer. If the OMT were a homodimer it would yield a native molecular weight much higher than the experimentally determined one, thus our results suggest the OMT to be a monomer.

Except for the caffeic acid OMT of Ruta graveolens [28], there has been no thorough investigation of the kinetic mechanism or kinetic parameters of the phenylpropanoid OMT. The kinetic studies of cabbage OMT were performed on isoform I using 5-hydroxyferulic acid as the substrate. Substrate interaction kinetics gave converging lines (Fig. 21) suggesting a sequential mechanism. However, a sequential mechanism can either be random or ordered binding. Based on Cleland's rules [79], an ordered binding mechanism would consist of one competitive and three noncompetitive product inhibition patterns while a random mechanism consists of four competitive product inhibition patterns. Furthermore, the order of substrate binding and product release was determined also from product inhibition patterns (Fig. 22, A-D). Cleland's rules state that a change in the slopes is indicative of competitive inhibition (Fig. 22 A); while a change in slope and y-intercept is noncompetitive

inhibition (Fig. 22, B,C,D) [80]. The product inhibition patterns obtained for the phenylpropanoid OMT suggest an ordered binding mechanism. Since S-adenosyl-L-homocysteine (SAH) was a competitive inhibitor of S-adenosyl-L-methionine (SAM) (Fig. 22 A) this suggests that SAM is the first substrate to bind and SAH is the last product released. Thus, the phenylpropanoid substrate binds second while the methylated product is released prior to SAH release (Fig. 23). It is interesting to note that phenylpropanoid OMT mechanism elucidated here is in accordance with the OMT mechanism deduced from affinity chromatography by Sharma and Brown [28]. In addition, the kinetic mechanism of cabbage OMT is similar to that of flavonoid OMTs [9,62,81], while the catechol O-methyltransferase of rat liver exhibits a random bi bi mechanism [56] which is unlikely for the cabbage OMT based on the product inhibition patterns.

The K_m of 5-hydroxyferulic acid for the phenylpropanoid OMT was found to be 20 μM. Other K_m values for 5-hydroxyferulic acid with OMTs in various tissue are; 10 μM in bamboo [22], 310 μM in aspen wood [47] and 65 μM in TMV infected tobacco leaves [82]. The inhibition constant for SAH (K_{iq}) was low suggesting that the O-methylation reaction is strongly inhibited at low concentrations of SAH. However, the inhibition by sinapic acid (K_{ip}) was found to be unusually high when compared with other K_{ip} constants of O-methyltransferases; 37 μM for macrocin [59], 15 μM for 3,7-dimethylquercetin [62] and 30 μM for 5-O-methylgenistein [81]. This indicates that the cabbage OMT continues to synthesize sinapic acid, despite its high level in

the plant cell. Sinapic acid has been shown to be an efficent substrate for other enzymes involved in lignin biosynthesis, namely para coumarate:CoA ligase [83], cinnamyl CoA reductase [84] and cinnamyl alcohol dehydrogenase [85].

The phenylpropanoid and flavonoid OMTs were separated due to differences in their chromatographic properties. In addition, the flavonoid OMT (FOMT) differed from the phenylpropanoid OMT (POMT) by being completely inhibited by EDTA, having a pH optimum at 8.0 in phosphate buffer. Furthermore, whereas iodoacetate was a potent inhibitor of the phenylpropanoid OMT, it had no effect on the flavonoid OMT. Our results are in accordance with the results obtained with soybean cell suspension culture [26] whereby two distinct O-methyltransferases are present in cabbage leaves. The phenylpropanoid activities are specific for the O-methylation of substituted cinnamic acids and are involved in lignin biosynthesis whereas the flavonoid OMT methylates luteolin and quercetin (Fig. 24). The presence of the two specific O-methyltransferases points to the possibility that methylation in cabbage leaves is occuring at both the C-9 and C-15 stages.

F.1. Possible role of the phenylpropanoid O-methyltransferase activities.

Members of the Brassicaceae are known to accumulate sinapine, the choline ester of sinapic acid, during the main growth phase of the developing embryo. Furthermore, sinapine synthesis is dependent on the availability of sinapic acid [86]. The existence of two forms of the phenylpropanoid O-

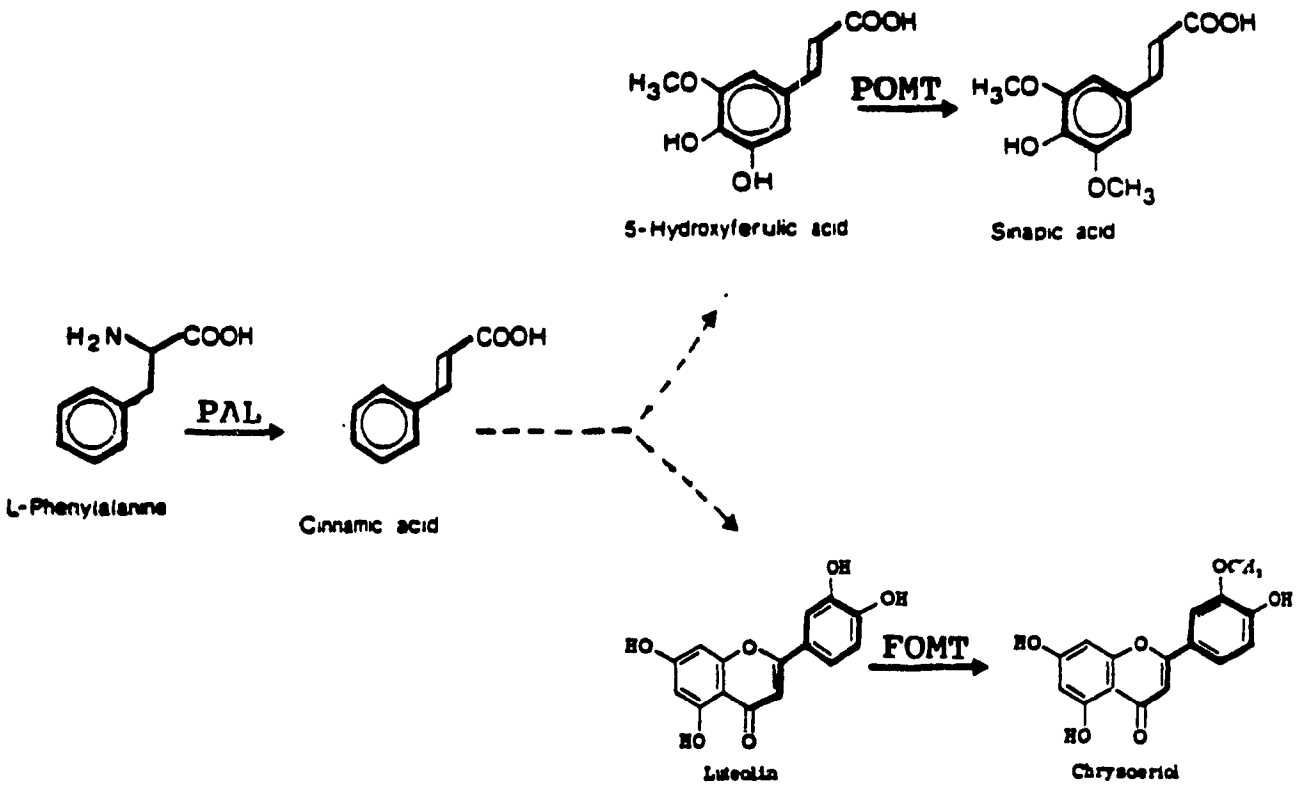


Figure 24. The metabolic relationship of the phenylpropanoid (C-9) and flavonoid (C-15) OMTs in cabbage leaves.

methyltransferase both of which catalyze the O-methylation of 5-hydroxyferulic acid although to different extents (Table 3) suggests the presence of two spatial cytoplasmic compartments. One of these compartments may be specialized in the biosynthesis of sinapine, whereas the other may catalyze the O-methylation of both caffeic and 5-hydroxyferulic acids leading to the production of lignin precursors (ferulic and sinapic acids). Preliminary studies have shown that isoforms I and II are present at all stages of plant growth, however, the detection of sinapine was only found in germinating seedlings (ca. 4 days old). These results suggest that sinapine and lignin synthesis are under developmental control. Furthermore, it appears that both isoforms are involved in lignin or sinapine biosynthesis (Fig. 25) although the possibility that each isoform is specific for one of the two pathways cannot be ruled out.

At present, there is no evidence linking the isoforms with a specific pathway but identification of each isoform within the cell may be helpful in resolving this problem. Evidence for the compartmentation of the isoforms should be derived from immunocytochemical studies. Antibodies raised against the homogeneous isoforms can be used for *in vivo* localization of the enzyme forms, as has been demonstrated with the isoforms of anthranilate synthase [87]. This may possibly explain the physiological role of the isoforms in *Brassica oleracea*.

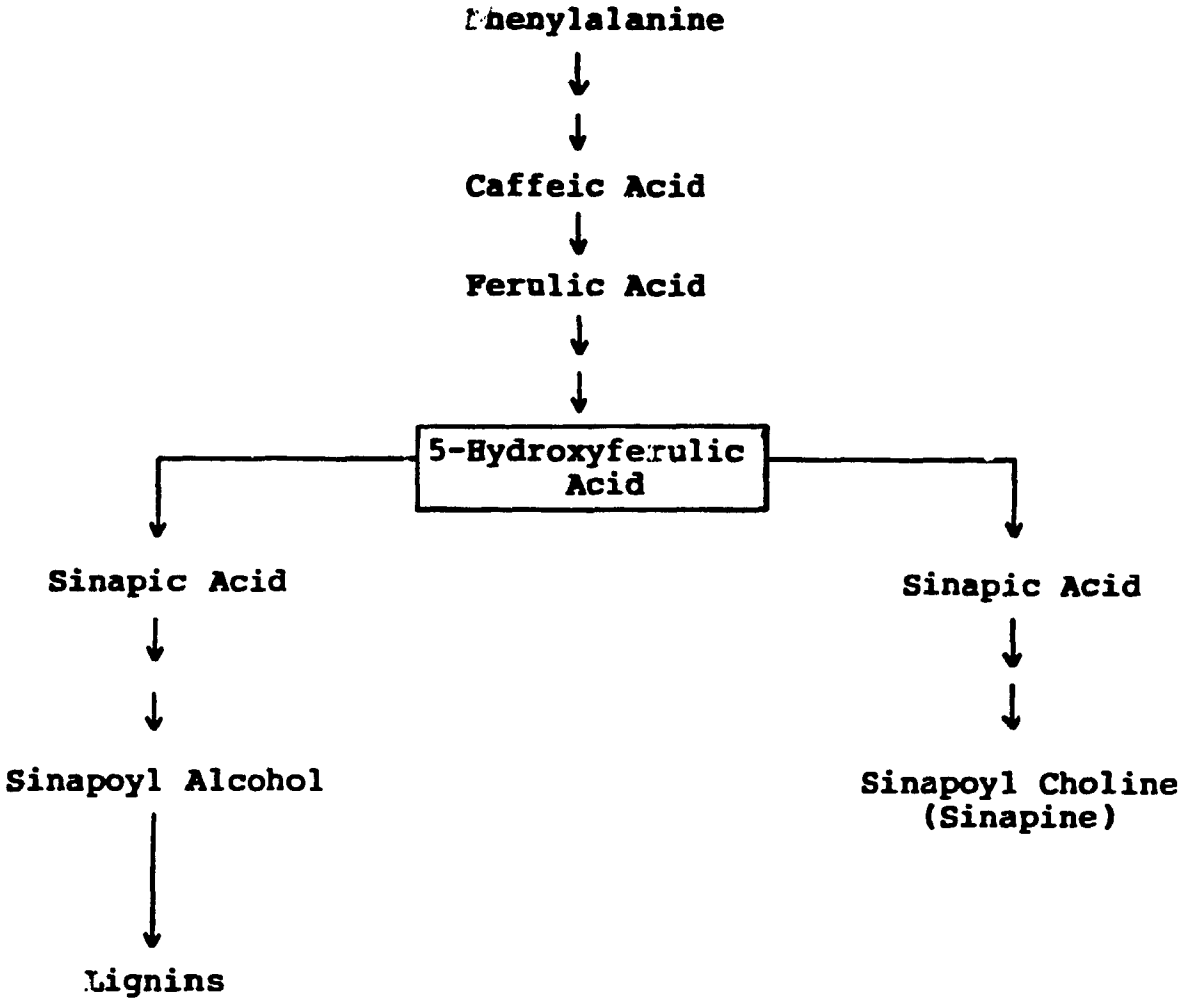


Figure 25. Possible biosynthetic pathways of sinapic acid.

G. CONCLUSION

Relatively few enzymes have been purified to homogeneity although its potential has been recognized for some time. The work completed in this thesis has produced a relatively complete picture with respect to the purification and kinetic properties of the phenylpropanoid OMT in Brassica oleracea. Homogeneous enzyme was achieved through a combination of conventional and modern protein purification techniques. Furtnermore, the purification procedure enabled the separation of different forms of the enzyme suggesting a type of heterogeneity existing for the phenylpropanoid OMT. The ordered bi bi mechanism for the OMT was rigorously established and kinetic parameters were determined. The information obtained on the difunctional OMT's preference for sinapic acid formation and its metabolite pool dynamics has been integrated with the aim of developing a model for lignin and possibly sinapine biosynthesis in Brassica oleracea.

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