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Extraction and Quantitation of Coumarin from Cinnamon and its Effect on Enzymatic Browning in Fresh Apple Juice: A Bioinformatics Approach to Illuminate its Antibrowning Activity

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Supporting Information

ABSTRACT: Enzymatic browning by polyphenoloxidase (PPO) affects food quality and taste in fruits and vegetables. Thus, the study was designed to reduce browning in apple juice by coumarin. The ethanolic extract of cinnamon was prepared and its coumarin content was quantitated by HPLC, using authentic coumarin (AC) as standard. The effect of cinnamon extract (CE) and AC on enzymatic browning, its time dependent effects, and the specific activity of PPO and peroxidase (POD) were studied in apple juice. The docking of coumarin with PPO and POD was also performed to elucidate its antibrowning mechanism. The CE (73%) and AC (82%) showed better reduction in browning, maintained its antibrowning effect at all time points, and significantly ($p < 0.05$) reduced the specific activity of PPO and POD when compared with controls. Coumarin showed strong interaction with binding pockets of PPO and POD, suggesting its potential use as inhibitor to enzyme mediated browning in apple juice.

KEYWORDS: *apple juice, browning, polyphenoloxidase, peroxidase, coumarin, docking*

INTRODUCTION

Browning is an enzymatic process which occurs in fruits and vegetables by the enzyme polyphenoloxidase (PPO, EC 1.10.3.1). PPO is a widespread copper-enzyme, containing two copper ions. It has been observed that the level of phenolics and their oxidizing enzyme PPO are key players in the browning process of various fruits and vegetables.[1−3] Apple is one of the most frequently consumed fruit all over the world.[4] Apple and its juice are highly susceptible to enzymatic browning reaction because apple PPO rapidly oxidizes *o*-diphenols to *o*-quinones and a subsequent nonenzymatic condensation with amino acids, proteins, or other compounds to form insoluble browning pigments of melanin.[5,6] This PPO-catalyzed browning gives not only a negative effect on appearance but also impairs the sensory properties including odor and texture.[7,8] This suggests that enzymatic browning decreases both acceptability and nutritional quality of the fresh juice.

The juice content of apple fruit depends on water present in the fruit.[9] The apple cultivars varied significantly in juice content, and the maximum juice content (58.54%) was recorded in red delicious. In addition, red delicious apples have been used in many other browning inhibition studies due to their extreme sensitivity.[10] Thus, the use of antibrowning agents is required for industrialization of red delicious apple based fruit products.

The molecular target for antibrowning process is the inhibition or minimization of key enzyme PPO, substrates, or the end products of browning reaction.[11−14] It has been reported that sulfite-containing additives are commercially used to inhibit PPO activity in vegetables and fruits.[15] Even though bisulfites act as effective antibrowning agents, they are harmful to human health, especially in asthmatic patients.[16] The most common response to sulphites not only cause asthma but can include flushing, urticaria, angioedema, tearing, runny nose, abdominal pain, seizures, and anaphylaxis. Hence, these compounds have been restricted by the Food and Drug Administration (FDA) due to the possibility of their associated potential hazards.[17] Therefore, a potent naturally derived PPO inhibitor(s) is essential to overcome PPO mediated browning of fruit juice. Also, there is an increasing demand of consumers for substituting authentic compounds with natural substances as food ingredients.[12]

Natural antioxidants including tocopherols, carotenoids, flavonoids, isoflavonoids, cinnamic acids, and coumarins, etc., received more and more attention in recent years, owing to their high radical-scavenging activity and various pharmacological functions.[18,19] It has been observed that ascorbic acid reduces enzymatic browning.[20] Thus, we assume that naturally derived phenolics like coumarin would inhibit PPO mediated enzymatic browning in fresh apple juice.

Coumarin (1,2-benzopyrone) (Figure 1) is a phenolic substance present in a wide variety of plants. It consists of an aromatic ring fused to a condensed lactone ring. Since 1954, coumarin has been regarded as a toxic substance by the FDA. Coumarin is known to develop tumor, hepatotoxicity, and necrosis in rodents.[21] However, it has been noted that the rat model is not appropriate to compare with humans for coumarin metabolism.[22] In vitro studies in human and monkey liver slices

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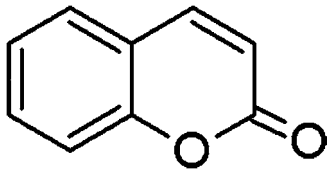

Figure 1. Structure of coumarin.

and/or hepatocytes showed resistance against coumarin toxicity, which correlates with coumarin 7-hydroxylation and suggests detoxification of coumarin. Various tests have shown that coumarin and its metabolites are nonmutagenic, exert antitumor activity over human tumor cell lines, and do not produce hepatotoxicity even in individual with deficient 7-hydroxylase activity,[21,23,24] revealing the nontoxic effect of coumarin to humans.

It has been mentioned that coumarin is an "active principle" by the Council of Europe, and the maximum permitted concentrations in foodstuffs are given in Annex II of European Directive (88/388/EEC). The general limit for coumarin in food and nonalcoholic beverages is 2 mg/kg; however, in alcoholic beverages and certain caramel confectionery, the permitted limit is 10 mg/kg. The main potential sources of coumarin in the diet are the coumarin content of cinnamon. Over a long period of time, less than 5% of solid food would be flavored with cinnamon or other ingredients capable of imparting the 2 mg/kg maximum concentration of coumarin. In the past few years, coumarin has received much attention for their diverse bioactivities,[25,26] which include antibacterial, antiviral, antithrombotic, vasodialatory, antioxidant, and inhibitor for lipoxygenase and polyoxygenase.[27,28] Coumarin is also used as a flavoring agent in food and as a fixative fixes the natural taste, flavor, and aroma. Masamoto et al.[29] have found the structur−activity relationship of 18 coumarin derivatives for their inhibitory activity on mushroom PPO. However, no reports have been found on the inhibitory effect of coumarin on apple PPO and POD activities so far.

The present study is aimed to explore the effect of coumarin on enzymatic browning in fresh apple juice. We extracted coumarin from cinnamon and quantitated by high performance liquid chromatography (HPLC) using pure authentic coumarin as standard. The effect of cinnamon extract and authentic coumarin on enzymatic browning in fresh apple juice, its time dependent effects, and the specific activity of PPO and POD were studied. The molecular docking of coumarin with PPO and POD was performed to illustrate its mechanism of antibrowning activity.

MATERIALS AND METHODS

Fruits and Chemicals. Red delicious apples at commercial maturity stage and cinnamon were purchased from the local market and stored at 4 °C until processed. Authentic coumarin (purity 99%) was obtained from the Sisco Research Laboratories (Mumbai, India). All other chemicals and solvents were of analytical grade and were purchased from Himedia Laboratories (Mumbai, India).

Coumarin Extraction. Coumarin extraction from cinnamon was performed based on the standard extraction procedure by Celeghini et al.[30] with slight modification. Powdered cinnamon (2.5 g) was mixed with ethanol−water (1:1; v/v, 25 mL) and subjected to vigorous shaking and left at 37 °C for 10 min. The material was filtered using Whattman filter paper no.1.

HPLC Analysis. The obtained filtrate was analyzed directly by HPLC-UV. A modular Shimadzu LC-10 system comprised of a LC-10AD pump, a CTO-10A column oven, a SPD-10A UV detector, a CBM-10A interface, and a LC-10 Workstation was utilized. A LC-18 column (250 mm × 4 mm i.d. × 5 mm) from Supelco (Bellefonte, USA) was employed at 30 °C. Separations were done in the isocratic mode, using acetonitrile:water (40:60; v/v) at a flow rate of 1 mL min^{-1}, with an injection volume ("loop") of 20 μL; UV detection was at 274 nm using pure coumarin as standard.

Apple Juice Preparation. Apples were washed with chlorinated water (200 mg L^{-1}) and cut into four pieces, and the seeds were carefully removed. Then 50 g of apple flesh was blended with 100 mL of distilled water. The homogenate was centrifuged for 30 min at 1000*g* at 4 °C. The supernatant was recovered, filtered through Whatman no. 1 paper, and stored at 4 °C (airtight vials) until the completion of experiment.

Biochemical Analysis. *Measurement of Browning.* The degree of browning was determined by the method of Coseteng and Lee.[5] Briefly, 10 mL of supernatant was mixed with different volume (0.5, 1, 2, and 3 mL) of CE and AC (0.012 mg per 3 mL) and ethanol−water (1:1; v/v, 3 mL) and incubated at room temperature for 1 h. The absorbance was measured spectrophotometrically at 420 nm. To evaluate time dependent antibrowning effect of cinnamon extract, an effective inhibitory concentration of cinnamon extract and authentic coumarin were selected and added at the beginning of apple juice preparation. The absorbance was measured at different time (0, 12, 24, 36, and 48 h) intervals using controls, i.e., apple juice (A) and apple juice with ethanol (A + E).

Enzyme Extraction. Some modifications were introduced to the extraction procedure described by Galeazzi and Sgarbieri[31] and Sanches-Ferrer et al.[32] First, 5 g of apple flesh was treated with the optimized concentration of cinnamon extract and authentic coumarin and homogenized in 10 mL of 0.05 M sodium phosphate buffer (pH 6.5) containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.5% PVPP, and 6% Triton X-114 and homogenized in an ice bath using a homogenizer. Then the homogenate was filtered through cheese cloth, and the filtrate was centrifuged at 16000*g* for 30 min at 4 °C. The supernatant was used as the crude enzyme extract, and its protein content was measured and stored at −20 °C until used.

Protein Determination. Protein content in crude enzyme solution was quantified by Lowry et al.[33] with bovine serum albumin as standard. The absorbances obtained were evaluated by graphic interpolation on a calibration curve at 650 nm.

Enzyme Assay. PPO enzymatic activity was measured using catechol as the exogenous substrate according to Kumar et al.[34] with some modifications. The reaction medium contained 200 μL of enzyme extract and 800 μL of 50 mM catechol in 50 mM phosphate buffer (pH 6.5) and was incubated at 37 °C for 120 min. The blank, control, and sample contained 1 mL of substrate solution. PPO activity was measured by increased absorbance at 420 nm using a spectrophotometer (Beckman DU 650, USA). One unit of PPO activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 OD min^{-1}.

The POD activity of the crude enzyme was also determined spectrophotometrically using the modified method of Aydin and Kadioglu.[35] The assay mixture contained 20 mM guaiacol, an equal volume of 40 mM hydrogen peroxide, and 50 mM sodium phosphate buffer (pH 5). Changes in the absorbance at 475 nm were monitored for 3 min using a spectrophotometer after the crude enzyme solution was added. One unit was defined as the amount of enzyme that caused a change in absorbance of 0.001 OD min^{-1}, and the enzyme activity was expressed as units of enzyme mg^{-1} protein.

Molecular Docking Studies. *Preparation of Ligand Structures.* The small-molecule topology generator Dundee PRODRG 2 server[36] was used for ligand optimization, a tool for high-throughput crystallography of protein−ligand complexes which collected input from existing coordinates or various two-dimensional formats and automatically generated coordinates and molecular topologies suitable for X-ray refinement of protein−ligand complexes.

Three-Dimensional Structure Modeling. BLAST analysis was performed for apple PPO (accession no. P43309.1) and POD (accession no. ABP87792) sequence. Four structurally determined

polyphenoloxidases such as grape (2P3X; 64% identity), sweet potatoes (1BT1; 59% identity), *Bacillus megaterium* (4HD4; 27% identity), and sea snail (1LNL; 25% identity) and five structurally determined peroxidases, including cytosolic ascorbate peroxidase of pea (1APX; 80% identity), cytosolic ascorbate peroxidase of soybean (2GHC; 79% identity), chloroplastic ascorbate peroxidase of tobacco (1IYN; 42% identity), cytochrome C peroxidase of yeast (2EUT; 34% identity), and peroxidase of *Arabidopsis* (1PA2; 32% identity) were found with sequence similarity to that of apple PPO and POD, respectively. The sequences of grape PPO (2P3X) and pea POD (1APX), which showed significant structural similarity, were used as structural template in the homology modeling of apple PPO and POD.

Binding Site Prediction. The probable binding sites of preferred targets grape PPO (2P3X) and pea POD (1APX) receptors were searched using Q-site Finder to predict the ligand-binding site. Q-site Finder includes a graphical user interface, flexible interactive visualization, as well as on-the-fly computation for user uploaded structures. It is important to keep the predicted ligand-binding site as small as possible without compromising accuracy for a range of applications such as molecular docking, de novo drug design, and structural identification and comparison of functional sites.[37] Q-site Finder works by binding hydrophobic probes to the protein and finding clusters of probes with the most favorable binding energy. These consist of active sites on protein surfaces and voids covered in the interior of proteins. The individual probe sites relate most closely to the favored high-affinity binding sites on the protein surface. These favorable binding sites relate to locations where a putative ligand could bind and optimize its Van der Waals interaction energy.

Protein–Ligand Interaction Using Autodock. The docking studies were conceded by Autodock tools[38] (ADT) version v.1.5.4 and Autodock v.4.2 programs (Molecular Graphics Laboratory, The Scripps Research Institute, CA). The searching grid extended above the preferred target proteins; polar hydrogen was added to the ligand moieties. Kollman charges were assigned, and atomic solvation parameters were added. Polar hydrogen charges of the Gasteiger-type were assigned, and the nonpolar hydrogen was merged with the carbons and the internal degrees of freedom and torsions were set. Coumarin was docked to target protein complex (2P3X and 1APX) with the molecule considered as a rigid body and the ligand being flexible. The search was extended over the whole receptor protein used as blind docking. Affinity maps for all the atom types present, as well as an electrostatic map, were computed with a grid spacing of 0.375 Å. The search was carried out with the Lamarckian genetic algorithm; populations of 150 individuals with a mutation rate of 0.02 were evolved for 10 generations. Evaluation of the results was done by sorting the different complexes with respect to the predicted binding energy. A cluster analysis based on root-mean-square deviation values, with reference to the starting geometry, was subsequently performed and the lowest energy conformation of the more populated cluster was considered as the most trustable solution.

Statistical Analysis. Values are expressed as means ± SD. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Quantitative Analysis of Coumarin. The presence of coumarin in the cinnamon extract was determined by HPLC analysis. Coumarin was identified by matching the retention time of compounds in the cinnamon extract to the pure standard coumarin. Coumarin content of cinnamon extract showed a similar trend of peak as that of standard coumarin. The retention time for coumarin content of cinnamon and standard coumarin was 10.080 and 9.940 min, respectively. This data indicated that existence of coumarin in the ethanolic extract of cinnamon. The quantitated amount of coumarin in the extract was 0.415%.

Antibrowning Effect of Coumarin. Concentration and time dependent effects of cinnamon extract on PPO mediated browning are shown in parts a and b of Figure 2, respectively.

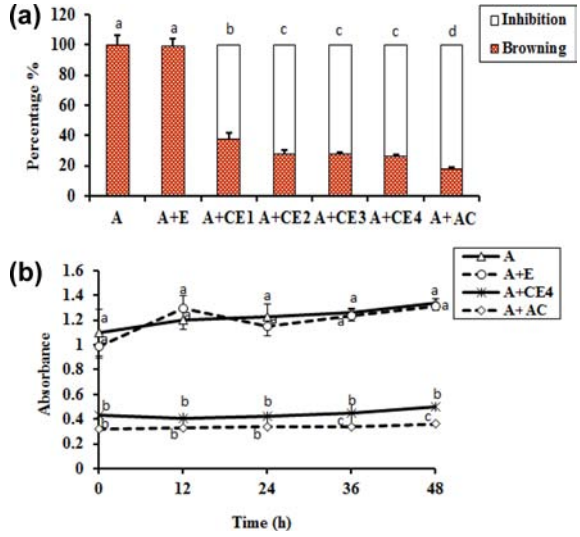

Figure 2. (a) Antibrowning effect of authentic coumarin and cinnamon extract at various concentrations after a period of 1 h incubation. Values are means ± SD ($n = 6$ in each group). c ($p < 0.05$) as compared to a and b. d ($p < 0.05$) as compared to a, b, and c. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A, apple juice; CE, cinnamon extract (CE1−2 μg, CE2−4 μg, CE3−8 μg, CE4−12 μg); AC, authentic coumarin; E, ethanol. (b) Antibrowning effect of authentic coumarin and cinnamon extract on apple juice with time. Values are means ± SD ($n = 6$ in each group). b ($p < 0.05$) as compared to a. c ($p < 0.05$) as compared to a and b. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A, apple juice; CE, cinnamon extract; AC, authentic coumarin; E, ethanol.

On the basis of the coumarin content in CE (by HPLC analysis), different volumes of CE (0.5, 1, 2, and 3 mL) at the concentrations of 2, 4, 8, and 12 μg of coumarin were used to identify effective inhibitory concentration. We incubated supernatant of apple juice with different concentrations (2, 4, 8, and 12 μg) of coumarin and compared with pure standard coumarin (12 μg). Interestingly, we observed significantly reduced absorbance (browning) of apple juice with all concentration of coumarin in cinnamon extract (Figure 2a). However, apple juice with 12 μg of coumarin showed a better reduction ($p < 0.05$) in browning when compared with control. The antibrowning effect of authentic coumarin was much better when compared with control.

An optimized concentration of coumarin (12 μg) was taken for the assessment of its antibrowning effect at different time periods (Figure 2b). Apple juice with optimized volume of cinnamon extract and authentic coumarin displayed significantly reduced browning intensity at all time points (0, 12, 24, 36, and 48 h) when compared with control, i.e., apple juice without coumarin and with ethanol/water. The inhibition of browning in apple has been reported by several researchers.[39,40] Reducing agents, antioxidants, and enzymatic inhibitors prevent browning by chemically reducing the *o*-quinones to colorless diphenols. The natural phenolics coumarin inhibits activity of lipoxygenase, process of lipid peroxidation, and scavenges superoxide and hydroxyl radicals and chelate metal ions,[27]

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suggesting the antioxidant property of coumarin might reduce the extent of browning in apple juice.

Effect of Coumarin on PPO and POD Activity. PPO and POD are the enzymes involved in the browning process. The specific activities of these enzymes are presented in Figure 3a,b.

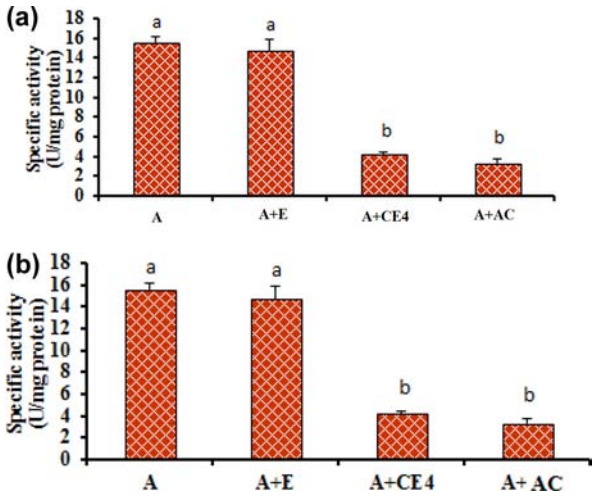

Figure 3. (a) Effect of coumarin on polyphenoloxidase activity in apple juice. Values are expressed as means ± SD (n = 6 in each group). b (p < 0.05) as compared to a. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A, apple juice; CE, cinnamon extract; AC, authentic coumarin; E, ethanol. (b) Effect of coumarin on peroxidase activity in apple juice. Values are expressed as means ± SD (n = 6 in each group). b (p < 0.05) as compared to a. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A, apple juice; CE, cinnamon extract; AC, authentic coumarin; E, ethanol.

The enzymes were assayed using substrate with and without coumarin. Interestingly, cinnamon extract treated samples showed significantly low activities of PPO (73%) and POD (59%) when compared with control, whereas the authentic coumarin inhibited PPO (77.5%) and POD (63%) much better (p < 0.05) than coumarin extract of cinnamon. This suggests that pure coumarin inhibits the enzymes of browning process.

The elevated POD level in the fresh apple juice infers the contribution of peroxidase to enzymatic browning in apple juice.[1−3] Browning occurs almost instantly when the cell structure is destroyed, and the enzyme and substrate are mixed. In our study, coumarin treated apple juice displayed 59% of inhibition when compared with control, suggesting the inhibitory action of coumarin on browning reaction catalyzed by POD.

PPO activity in coumarin treated apple juice was significantly (p < 0.05; 73%) reduced when compared to control, revealing that coumarin could serve as a potential inhibitor of PPO and thereby prevent enzymatic browning. The PPO inhibitory mechanisms of some proteins (peptides or amino acids) and compounds structurally similar to phenolic substrates have proposed that proteins can exert an inhibitory effect on PPO activity by chelating the essential copper at the active site of PPO and the substrate−similar compounds can work through competitive inhibition.[41] Currently, coumarin derivative, umbelliferone, a monophenolase precursor of esculetin, behaved as a competitive inhibitor for the monophenolase activity of mushroom PPO.[42] Apart from this, esculetin also showed in vitro inhibitory effect on melanogenesis in cultured B16 melanoma cells due to the competitive inhibition of 3,4-dihydroxyphenylalanine (DOPA),[29,43] whereas the oxidized esculetin acts as a possible PPO substrate rather than an inhibitor, concluding that esculetin is not a true inhibitor of PPO.[44] Lukinac et al.[45] have reported that synthesized coumarin derivatives act as potent tyrosinase inhibitor. These findings reveal an interesting controversy in whether coumarin derivatives act as substrate or inhibitor of PPO. It has been studied that PPO contains copper in its active site, which is essential for enzyme activity. The copper maintains equilibrium between enzyme-Cu^{2+} and enzyme-Cu^{+} during enzymatic browning.[41,46] Therefore, a possibility is that the highly electronegative oxygen atom of coumarin (1,2-benzopyrone) might extensively interact with Cu^{2+} ion of PPO and thereby alter the catalytic activity of PPO in reaction to the complexation property of coumarin.

Molecular Docking Analysis. To confirm and to elucidate the binding efficacy of coumarin on the browning enzymes (PPO and POD), docking studies were performed with the structural analogues of apple PPO and POD. Coumarin was docked at the active site of PPO and POD. The glide energy for PPO and POD was −7.49 and −10.86 kcal mol^{-1}, respectively. The more negative interaction energy indicates the more favorable binding of coumarin with the browning enzymes. The coumarin−PPO complex had one hydrogen bond interaction, which was observed between the ligand and the active site residue Tyr 306 and the distance was 1.9 Å, whereas coumarin displayed three hydrogen bond interaction with POD, two hydrogen bonds with His 169, and one hydrogen bond with Ser 173 in the binding pockets of the enzyme and the distances were observed to be 1.9 and 2.1 Å at the His 169 residue and 2.7 Å at the Ser 173 residue. The docking data clearly illustrated how the ligand (coumarin) binds to the active site of PPO and POD, in addition to the suggested complexation property with the metal ion Cu^{2+}. From this, it is evident that coumarin might be a potent inhibitor of the browning enzymes. Synthetic chemicals based antibrowning agents are hazardous to humans. Thus, the utilization of "cinnamon derived coumarin" will be promised as a potent natural inhibitor for the prevention of browning in red delicious apple juice caused by PPO and POD. The introduction of coumarin in apple drinks or beverages would reduce the browning and thereby extend the shelf life of the product. Ours is the first report demonstrating that the cinnamon derived coumarin bears antibrowning action and would serve as a natural food additive for the industries to preserve apple based fruit juice.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures showing the docking of coumarin with apple PPO (S1a) and POD (S1b), illustrate the molecular interaction of coumarin with the browning enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CE, cinnamon extract; AC, authentic coumarin; PPO, polyphenol oxidase; POD, peroxidase; HPLC, high performance liquid chromatography; BLAST, basic local alignment search tool; ADT, Autodock tools; DOPA, 3,4-dihydroxyphenylalanine

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Due to a production error, this paper published May 24, 2013 with a typographical error in the presentation of protein 2P3X. The corrected version published May 28, 2013.