Inhibitory effect of coumarin derivatives on apple (cv. Idared) polyphenol oxidase

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Summary

Inhibitory effect of 32 coumarin derivatives (20 Schiff bases, 5 thiosemicarbazides, 5 thiazolidinones, and their precursors, 7-hydroxy-4-methylcoumarin and 4-methylcoumarin-7-yl hydrazine carboxylate) on partially purified apple polyphenol oxidase was investigated. Thirteen coumarin derivatives inhibited polyphenol oxidase (5 Schiff bases, 5 thiosemicarbazides, 1 thiazolidinone, 4-methyl-7-hydroxycoumarin and 4-methylcoumarin-7-yl hydrazine carboxylate), while 19 derivatives showed no effect on enzyme activity. The most effective inhibitors were thiosemicarbazides, with 4-methyl-1-(2-(4-methyl-2-oxo-*2H*-chromen-7-yloxy)acetyl) thiosemicarbazide (compound C23) being the most prominent inhibitor (IC_{50} = 10.45 μM). The importance of thiosemicarbazide moiety as crucial structure element for strong apple PPO inhibition was confirmed by its cyclisation to thiazolidinone bearing the same substituents as corresponding thiosemicarbazide. Capture of the sulphur atom of thiosemicarbazide group within tiazolidinone ring caused significant loss of inhibitory effect against apple PPO.

Keywords: apple polyphenol oxidase, coumarin derivatives, inhibition

Introduction

Polyphenol oxidases or tyrosinases (PPOs) are oxidoreductases widely distributed among plants, animals, fungi and bacteria. These enzymes with a binuclear copper centre are able to insert oxygen in an *ortho*-position of an existing hydroxyl group in aromatic ring, followed by oxidation of diphenol to the corresponding quinone (Mayer, 2006; Strelec et al., 2014; Molnar et al., 2016). Besides their positive developmental and defensive physiological role in various organisms, such as sclerotization and pigmentation of insect cuticles, wound healing, or defence against herbivores and pathogens in plants, PPOs has negative impact in food industry. Due to the oxidation of phenolic compounds to the corresponding quinones, PPOs are responsible for enzymatic browning of fruits and vegetables during ripening, handling, storage and processing (Kim et al., 2005; Mayer, 2006; Queiroz et al., 2008; Molnar et al., 2010; Liu et al., 2012). In addition to the development of undesirable colour and flavour, the quinones produced in the browning reaction may irreversibly react with the amino and sulfhydryl groups of proteins, leading to decreased protein digestibility and essential amino acid (lysine and cysteine) bioavailability. This affects nutritional quality, reduces the consumer's acceptability and subsequently causes significant economic loss, both to food producers and to food processing industry (Kim et al., 2005; Queiroz et al., 2008). Therefore, the need for search of effective PPO inhibitors in the food field still exists.

Coumarins are plant derived natural product extensively used as a biologically active compounds due to their diverse structural features and versatile biological properties, such as anti-inflammatory, antioxidant, vaso-relaxant, cytotoxic, anti-HIV, anti-tubercular, antifungal, antimicrobial, as well as PPO inhibiting activity (Chang, 2009; Šarkanj et al., 2013; Asif, 2014; Molnar et al., 2014; Čačić et al., 2014).

Natural coumarin derivatives reported to inhibit PPO are: a) alosein a hydroxycoumarin glucoside isolated from *Aloe vera* (Jones et al., 2002), b) 9-hydroxy-4-methoxypsoralen isolated from the roots of *Angelica dahurica* (Piao et al., 2004), c) 8'-epicleomiscosin A and cleomiscosin A isolated from the aerial parts of *Rhododendron collettianum* (Ahmad et al., 2004), d) umbelliferone (Sollai et al., 2008), and e) 3-hydroxycoumarin found in some plants (Asthana et al., 2015).

Synthetic coumarin derivatives found to inhibit PPO activity are coumarin-resveratrol hybrids (Fais et al., 2009), halogenated phenylcoumarins (Matos et al., 2011), coumarin derivatives bearing various moieties on 3-C atom of coumarin ring (Liu et al., 2012; Matos et al., 2015), as well as 7-C atom substituted umbelliferone analogues (Ashraf et al., 2015).

Since, several coumarin derivatives bearing thiosemicarbazide or thiazolidinone moieties on 7-C atom of coumarin ring were found to be very a good anti-browning agent of fresh apple (cv. Idared) slices, and their anti-browning activity was attributed to

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PPO inhibition (Molnar et al., 2010), we investigated inhibitory activity of 32 novel coumarin derivatives substituted at position-7 of coumarin ring on partially purified apple (cv. Idared) polyphenol oxidase.

Materials and methods

Chemicals

L-3,4-dihydroxyphenylalanine (L-DOPA), (+)-catechin, (-)-epicatechin, caffeic acid, kojic acid, chlorogenic acid, 4-methylcatechol and polyvinylpyrrolidone (PVP) were purchased from Sigma-Aldrich (USA), L-tyrosine, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic dihydrate from Merck (Germany), Triton X-100 from Fluka (Germany), Bradford reagent from Bio-Rad (Germany), while ammonium sulphate (AmS), acetone and dimethyl sulfoxide (DMSO) from Kemika (Croatia).
Coumarin derivatives were synthesized and characterized as described previously (Čačić et al., 2010; Šarkanj et al., 2013; Molnar et al., 2014).
Standard stock solutions (5 mM) of coumarin derivatives were prepared with DMSO prior to inhibition studies.

Partial purification of apple polyphenol oxidase

Apples (*Malus domestica cv. Idared*) of commercial maturity marked as Croatian product, were obtained from the local market "Konzum" in Osijek, Croatia, and used for polyphenol oxidase purification. Apple slices (100 g, after peeling and core removal) were homogenised with 100 mL of 0.1 M phosphate buffer, pH 6.8, containing 10 mM ascorbic acid, 0.1% polyvinylpyrrolidone (PVP) and 0.1% Triton X-100 in a Waring blender (Waring Products, USA) at 5000 rpm for 3 min. The homogenate was filtered through cheesecloth, and filtrate clarified by centrifugation (15 000 g, 20 min, 4 °C). Obtained supernatant was treated with solid ammonium sulphate (AmS) from 30% to 80% saturation. Precipitate collected by centrifugation (15 000 g, 20 min, 4 °C) was dissolved in small amount of 0.1 M phosphate buffer, pH 6.8, and excess of AmS in solution was removed by desalting procedure using PD-10 columns (Amersham Biosciences, Sweden). Enzyme in desalted protein solution was precipitated by addition of 3 volumes of ice cold acetone (-20 °C), and brownish precipitate collected by centrifugation (15 000 g, 20 min, 4 °C) containing apple PPO was stored at -20 °C, and used for preparation of enzyme solution prior to analysis. All extraction and purification procedures were carried out at 4 °C, until otherwise stated.

Polyphenol oxidase activity assay

PPO activity was determined by measuring the initial rate of dopachrome formation indicated by an increase in absorbance at 475 nm (Strelec et al., 2014). The blank contained 1.0 mL of substrate (10 mM L-DOPA in 0.1 M phosphate buffer, pH 6.8), and the sample contained 0.5 mL of substrate (20 mM L-DOPA in 0.1 M phosphate buffer, pH 6.8), 0.45 mL of 0.1 M phosphate buffer, pH 6.8, and 0.05 mL of enzyme solution (0.24 mg/mL). The reaction was carried out at 25 °C and increase in absorbance at 475 nm was measured using a double-beam spectrophotometer Specord 200 (AnalytikJena, Germany). Change in absorbance was recorded every 10 s for 100 s and enzyme activity was measured from the linear portion of the curve. One unit of PPO activity was defined as the change in absorbance of 0.001 per min per mL of enzyme. Activity measurements were carried out in triplicate.

Polyphenol oxidase substrate specificity determination

Substrate specificity of partially purified apple PPO was determined using 7 different substrates: L-DOPA, (+)-catechin, (-)-epicatechin, caffeic acid, chlorogenic acid, 4-methylcatechol and L-tyrosine. Substrate solutions (10 mM) were prepared in 0.1 M phosphate buffer, pH 6.8, and PPO activity at 2.5 mM final concentration of substrate in reaction mixture was assayed as described previously, except for L-tyrosine as substrate.
Substrate specificity of apple PPO toward L-tyrosine, was determined according to Duckworth and Coleman (1970), with minor modification. Reaction mixture contained 500 µL of 5 mM L-tyrosine dissolved in 0.1 M phosphate buffer, pH 6.8, 400 µL of 0.1 M phosphate buffer, pH 6.8, and 100 µL of enzyme solution (0.24 mg/mL). Change in absorbance at 475 nm was recorded every 30 s for 15 min and enzyme activity was measured from the linear portion of the curve

Protein content determination

Protein concentration was determined by the Bradford (1976) method with bovine serum albumin as standard.

Inhibition studies

Polyphenol oxidase activity was measured in the presence of 32 coumarin derivatives. Inhibitors dissolved in DMSO at 5 mM concentration, were

added (0.05 mL) to the reaction mixture containing 0.5 mL of substrate (20 mM L-DOPA in 0.1 M phosphate buffer, pH 6.8), 0.4 mL of 0.1 M phosphate buffer, pH 6.8, and 0.05 mL of enzyme solution (0.24 mg/mL), and PPO activity was assayed as described previously. Final concentration of inhibitors in the reaction mixture was 0.25 mM, and of DMSO 5%. Controls without inhibitor containing 5% DMSO were routinely carried out.

For coumarin derivatives showing significant inhibition of PPO activity at 0.25 mM concentration (greater than 30%), IC_{50} value (a concentration giving 50% inhibition of PPO activity) was determined by interpolation of the dose-response curves.

The percent of inhibition of PPO reaction was calculated as following:

$$\text{Inhibition rate (\%)} = [1 - [(S - B)/(C - B)]] \times 100$$

where S, B, are the absorbance's for sample and blank, and C absorbance for control without inhibitor, but containing appropriate volume of DMSO dependent on the final concentration of inhibitor (0.025, 0.05, 0.1, 0.25 and 0.5 mM) in the reaction mixture. All measurements were performed in triplicate for each concentration and averaged before further calculation.

Results and discussion

Synthesis of coumarin derivatives

Modification of 7-hydroxy-4-methylcoumarin was performed via substitution of hydroxyl group in position C-7 (Fig. 1). Schiff bases ((*E*)-*N*-2-aryliden-2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetohydrazides) were synthesized from hydrazide A2 employing various aldehydes, thus yielding Schiff bases with various substituents (Čačić et al., 2010). Thiosemicarbazides (4-substituted-1-(2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)acetyl)thio-semicarbazides) were synthesized from the same hydrazide (A2) by reaction with different isothiocyanates and subsequently cyclised to corresponding thiazolidinones ((*Z*)-2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)-*N*-(4-oxo-2-substituted thiazolidin-3-yl)acetamides) (Šarkanj et al., 2013).

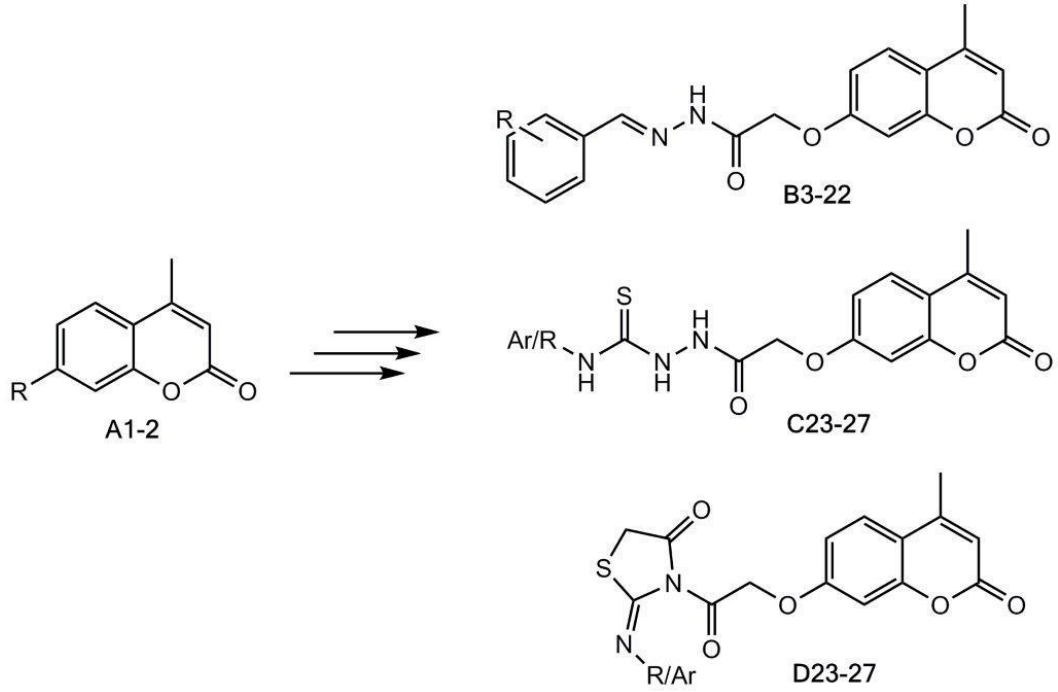

Fig. 1. Synthetic route for tested coumarin derivatives

(A1/R = -OH; A2/R = $-OCH_2CONHNH_2$; B3/R = 3-OH; B4/R = 4-OH; B5/R = $2\text{-}OCH_3$; B6/R = $4\text{-}OCH_3$; B7/R = $2,3\text{-}(OH)_2$; B8/R = $2,4\text{-}(OH)_2$; B9/R = $2,5\text{-}(OH)_2$; B10/R = $3,4\text{-}(OH)_2$; B11/R = $3,5\text{-}(OH)_2$; B12/R = $3\text{-}OCH_3$-4-OH; B13/R = 3-phenoxy; B14/R = $3,4,5\text{-}(OCH_3)_3$; B15/R = 2-Br; B16/R = 3-Br; B17/R = 4-Br; B18/R = 2-F; B19/R = 3-F; B20/R = 4-F; B21/R = styryl; B22/R = $4\text{-}N(CH_3)_2$; C/D23/R = CH_3; C/D24/R = CH_2CH_3; C/D25/R = phenyl; C/D26/R = 4-methylphenyl; C/D27/R = 4-methoxyphenyl)

Partial purification of apple polyphenol oxidase

Polyphenol oxidase was partially purified from apple (*Malus domestica* cv. Idared) by ammonium sulphate and acetone precipitation. The result of PPO purification are summarised in Table 1. Specific activity of crude extract was 642.82 U/mg. Ammonium sulphate precipitation (30% to 80%) resulted in 3.45-fold increase in specific activity to an average of 2217.37 U/mg with recovery of 83.34% of the crude extract. Further increase in PPO specific activity was obtained by acetone precipitation. Partially purified apple PPO of acetone precipitate showing specific activity of 6234.45 U/mg was purified 9.70-fold, with recovery of 61.61% of the crude extract.

Similar increase in PPO activity (3-fold), with 85.7% recovery, after ammonium sulphate purification (from 30% to 85%) was reported for PPO isolated from apple cv. Bramley seedlings (Ni Eidhin et al., 2006). Acetone precipitation of peach PPO from crude extracts was found to cause 2.66-fold increase in enzyme activity (Wong et al., 1971). This is similar to the increase detected in our study, where additional 2.81-fold increase in PPO activity was obtained after acetone precipitation.

Substrate specificity of partially purified apple (cv. Idared) polyphenol oxidase

Partially purified apple PPO was able to oxidise a wide variety *o*-diphenols (Table 2), but was found completely devoid of monophenolase activity toward L-tyrosine as substrate. Substrate specificity of apple PPO toward o-diphenols (catechin>epicatechin> 4-methylcatehol> chlorogenic acid>caffeic acid> L-DOPA) was found similar to the PPO specificity reported by Ni Eidhin et al. (2006) and Zucca et al. (2013). Moreover, the same authors reported the absence of PPO activity toward L-tyrosine as substrate, what additionally confirms results obtained in the present study. The lack of apple PPO activity toward L-tyrosine as substrate were also reported by Rocha et al. (1998) and Rocha and Morais (2001). The most probable reason for the observed lack of apple PPO activity toward L-tyrosine as substrate is loss of enzyme activity during the purification procedure (Nicholas, 1994).

Inhibitory effect of coumarin derivatives on apple polyphenol oxidase

All synthesized compounds were tested on inhibitory activity against partially purified apple PPO, at 0.25 mM concentration in the reaction mixture with 10 mM L-DOPA as substrate. Results are summarised in Table 3. Thirteen coumarin derivatives inhibited polyphenol oxidase, while 19 derivatives showed no effect on enzyme activity. The most effective inhibitors were thiosemicarbazides (C23-27) and thiazolidinone (D27), while lower inhibiting activity (< 20%) showed 5 Schiff bases (B8, B15, B16, B19, and B22), and the lowest activity was detected with 7-hydroxy-4-methyl-coumarin (A1) and 4-methyl-coumarin-7-yl hydrazine carboxylate (A2).

Table 1. Partial purification of apple polyphenol oxidase*

Purification step	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude	67200.05	104.54	642.82	100.00	1.00
Ammonium sulphate precipitation	56006.77	25.26	2217.21	83.34	3.45
Acetone precipitation	41400.05	6.64	6234.45	61.61	9.70

*PPO activity was assayed using 10 mM L-DOPA in 0.1 M phosphate buffer, pH 6.8. Values are typical recovery results.

Table 2. Substrate specificity of partially purified apple (cv. Idared) polyphenol oxidase

Substrate	λ_{max} (nm)	PPO activity (U/mL)
4-Methylcatechol	420	3387
Chlorogenic acid	420	1687
(+)-Catechin	420	6392
(-)-Epicatechin	420	5673
Caffeic acid	420	1260
L-DOPA	475	160
Tyrosine	475	n.d.*

*n.d. = not detectable.

Table 3. Inhibitory effect of coumarin Schiff bases, thiosemicarbazides and thiazolidinones on apple polyphenol oxidase activity

Compounds	PPO inhibition (%)*
A1	4.52
A2	4.29
B3-B7, B9-B14, B17-B18, B20-B21	**NI
B8	19.25
B15	12.47
B16	19.23
B19	14.91
B22	12.42
C23	95.12
C24	59.56
C25	37.87
C26	35.02
C27	63.00
D23-D26	NI
D27	45.92

*Percent of inhibition of PPO reaction at the 0.25 mM concentration of selected coumarin derivatives. Results present mean of three independent activity determinations;** Not inhibiting at 0.25 mM concentration.

7-hydroxy-4-methylcoumarin (A1) slightly inhibited PPO (~4%) at 0.25 mM concentration. Addition of hydrazyde group to 7-OH group of 7-hydroxy-4-methyl-coumarin (4-methylcoumarin-7-yl hydrazine carboxylate; compound A2) showed no effect to the increase of inhibitory activity. 7-hydroxy-4-methyl-coumarin and its hydrazide were only precursors in synthesis of Schiff bases and thiosemicarbazides, and their activity was determined in order to confirm that, not only substitution, but type of substituents as well has a significant influence on PPO inhibition.

Since research of Tišma et al. (2014) showed the potency of 2 coumarin Schiff bases dihydroxylated at benzene ring to inhibit laccase activity with ABTS as substrate, a series of Schiff bases with various substituents on benzene ring (hydroxy, metoxy, fenoxy, bromine, fluorine, styryl and dimethyl-amino) in the present study were examined on inhibition efficiency against apple PPO. However, Schiff bases were not found as promising PPO inhibitors. From the 20 Schiff bases investigated, only five of them (B8, B15, B16, B19, and B22) exhibited slight inhibitory activity against PPO (Table 3). Compound B8 bearing hydroxyl groups in 2- and 4-position, and compound B16 containing bromine in 3-position of benzene ring exhibited the highest inhibitory activity (~20%). When bromine was replaced with fluorine in position-3 of benzene ring (compound B19) inhibitory activity was lower (~15%). The lower inhibitory activity (~12%) was found for compounds B15 and B22. Compound B15 contained bromine in 2-position, while compound B22 dimethyl amino group in 4-position of benzene ring. Coumarin Schiff bases dihydroxylated at 2,3- (compound B7), 2,5- (compound B9), and 3,4-position of benzene ring (compound B10) examined in the present study were previously reported to inhibit laccase activity with ABTS as substrate 20.8, 79.3 and 64.3%, respectively. However, when inhibitory effect of these compounds was tested with L-DOPA as laccase substrates, there was no inhibition (Tišma et al., 2014). Thus, it seems quite possible that observed poor inhibition of apple PPO by coumarin Schiff bases simply reflect inability of these compounds to significantly inhibit diphenolase activity.

Coumarin thiosemicarbazides (C23-27) were found as the most prominent inhibitors of apple PPO (Table 3). At 0.25 mM concentration in the reaction mixture, compounds C23-27 inhibited PPO activity 95.12, 59.56, 37.87, 35.02 and 63%, respectively. Inhibitory activity of thiosemicarbazides was found to be dependent on the substituent attached at 4-position of thiosemicarbazide chain. Methyl (C23) and ethyl (C24) group attached at 4-position of thiosemicarbazide chain showed greater inhibitory activity than phenyl (C25) or 4-methylphenyl (C26) substituents, with compound C23 being the most prominent inhibitor. Attachment of 4-methoxyphenyl at 4-position of thiosemicarbazide chain (C27) caused increase in inhibitory activity compared to aryl substituents (phenyl (C25) or 4-methylphenyl (C26)), being almost equal to the inhibitory activity detected with compound C24. In addition, inhibitory effect was found to be dependent on the length of alkyl substituent. Increase in the length of alkyl substituent (methyl to ethyl; C23 and C24, respectively) caused significant decrease in inhibitory activity. Similar dependence was reported by Liu et al. (2008, 2009) who found gradual decrease in inhibitory activity of mushroom tyrosinase with the increase of the length of alkyl chain of

alkylidenethiosemicarbazides, and enhancement in inhibitory activity of 1-(1-phenylethylidene)thiosemicarbazides when methoxy group was attached at 4-position on benzene ring. Enhancement in inhibitory activity of phenylmethylenethiosemicarbazones against mushroom tyrosinase, due to addition of methoxy group at 4-position of benzene ring was reported by Yi et al. (2009). If inhibitory activities of alkylidenethiosemicarbazides bearing methyl- or ethyl-moieties are compared with those of 1-(1-arylethylidene) thiosemicarbazides bearing phenyl- or methyl-phenyl-moieties (Liu et al., 2008; 2009), it can be observed that methyl and ethyl groups shows greater inhibitory activity than phenyl or 4-methylphenyl substituents. This confirms results of the present investigation where methyl and ethyl group attached at 4-position of thiosemicarbazide chain, showed greater inhibitory activity than phenyl or 4-methylphenyl substituent.

Due to the great inhibitory activity against apple PPO, IC_{50} value of coumarin thiosemicarbazides were determined (Table 4). All coumarin thiosemicarbazides (C23-27) showed sub-millimolar values of IC_{50}, with compound C23 as the most potent inhibitor causing 50% inhibition of polyphenol oxidase activity at 10.45 μM concentration. Observed inhibiting concentration was about 10-fold greater than those of kojic acid which was used as positive control (IC_{50} = 103.63 ± 5.52 μM).

Literature survey for IC_{50} values of various coumarin derivatives inhibiting PPO (Table 5) showed that compound C23 examined in the present study belongs to the group of potent coumarin derivatives inhibiting PPO. This includes 5,7-dihydroxy-3-(3-thiophenyl) coumarin with IC_{50} value of 0.19 μM (Matos et al., 2015), (Z)-3-((7-hydroxy-2-oxo-*2H*-chromen-4-yl) methyl)-5-(4-hydroxybenzylidene)thiazolidine-2,4-dione with IC_{50} value of 0.25 μM (Molnar et al., 2016), 8'-epicleomiscosin A with IC_{50} value of 1.33 μM (Ahmad et al., 2004), (Z)-3-((7-hydroxy-2-oxo-*2H*-chromen-4-yl)methyl)-5-(3-hydroxybenzylidene)thiazolidine-2,4-dione with IC_{50} value of 1.63 μM (Molnar et al., 2016), 2-(1-(coumarin-3-yl)ethylidene)hydrazine-carbothioamide causing 50% of inhibition of mushroom tyrosinase at 3.44 μM concentration (Liu et al., 2012) and 2-oxo-2-[(2-oxo-2H-chromen-7-yl)oxy] ethyl-2,4-dihydroxybenzoate with IC_{50} of 8.96 μM (Ashraf et al., 2015).

Table 4. Concentration of selected coumarin derivatives causing 50% inhibition of polyphenol oxidase activity (IC_{50})

Compound	IC_{50} (μM)*
C23	10.45 ± 1.22
C24	147.27 ± 20.03
C25	523.64 ± 68.87
C26	591.58 ± 72.09
C27	143.91 ± 11.33
D27	364.21 ± 74.54
Kojic acid	103.63 ± 5.52

*Values were determined from logarithmic concentration–inhibition curves and are given as means ± SD of three experiments.

Table 5. Literature overview of natural and synthetic coumarin derivatives causing 50% inhibition of polyphenol oxidase (tyrosinase) activity with L-DOPA as substrate (IC_{50})

Compound	IC_{50} (μM)	References
5,7-dihydroxy-3-(3-thiophenyl)coumarin	0.19	Matos et al., 2015
(Z)-3-((7-hydroxy-2-oxo-*2H*-chromen-4-yl)methyl)-5-(4-hydroxybenzylidene)thiazolidine-2,4-dione	0.25	Molnar et al., 2016
8'-epicleomiscosin A	1.33	Ahmad et al., 2004
(Z)-3-((7-hydroxy-2-oxo-*2H*-chromen-4-yl)methyl)-5-(3-hydroxybenzylidene)thiazolidine-2,4-dione	1.63	Molnar et al., 2016
2-(1-(coumarin-3-yl)ethylidene)hydrazine carbothio-amide	3.44	Liu et al., 2012
2-oxo-2-[(2-oxo-*2H*-chromen-7-yl)oxy]ethyl-2,4-dihydroxybenzoate	8.96	Ashraf et al., 2015
4-methyl-1-(2-(4-methyl-2-oxo-*2H*-chromen-7-yloxy)acetyl)thiosemicarbazide (compound C23)	10.45	present study
Cleomiscosin A	18.69	Ahmad et al., 2004
6-Bromo-8-hydroxy-3-(4'-hydroxyphenyl)coumarin	215.00	Matos et al., 2011
3-(3',4',5'-trihydroxyphenyl)-6,8-dihydroxycoumarin	270.00	Fais et al., 2009
Umbelliferone	420.00	Masamoto et al., 2003
Coumarin	8300.00	Masamoto et al., 2003

However, in the comparison with the lowest IC_{50} values reported for various alkyl- and aryl-thiosemicarbazides inhibiting mushroom tyrosinase (IC_{50} = 0.086 μM) (Liu et al., 2008; 2009), or phenylmethylenethiosemicarbazones (IC_{50} = 0.18 μM) (Yi et al., 2009), compound C23 shows only moderate potency of PPO inhibition.

Obtained data clearly implies the importance of thiosemicarbazide moiety as crucial structure element for strong inhibition of PPO. Moreover, due to the similarity of data in the present study with those one reported by Liu et al. (2008; 2009; 2012) it can be supposed that tested thiosemicarbazides act as irreversible inhibitors of PPO. Namely, the sulphur atom of thiosemicarbazide group could chelate with the dicopper nucleus in the active site of PPO, leading to the loss of enzyme activity (Liu et al., 2012).

Additional support to the importance of thiosemicarbazide moiety as crucial structure element for strong PPO inhibition, came in the present study when thiosemicarbazide group of coumarin derivatives (C23-27) was cyclised and coumarin thiazolidinones (D23 - 27) bearing the same substituents as their corresponding thiosemi-carbazides were obtained (Fig. 1, Table 3). Capture of the sulphur atom of thiosemicarbazide group within tiazolidinone ring caused complete loss of inhibitory effect observed for corresponding thiosemicarbazides bearing methyl (C23 → D23), ethyl (C24 → D24), phenyl (C25 → D25) or 4-methylphenyl (C26 → D26) substituents. However, when 4-methoxyphenyl group as substituent was present at thiazolidinone ring (compound D27), significant inhibition of apple PPO (45.92%), but lower than those one of corresponding thiosemicarbazide (C27; 63%), could be observed. This indicates that methoxyphenyl group attached at thiazolidinone ring or thiosemicarbazide obviously has different inhibiting mechanism, then corresponding thiosemicarbazides bearing methyl, ethyl, phenyl or 4-methylphenyl substituents.

Due to the significant inhibitory activity against apple PPO (45.92%), IC_{50} value of compound D27 was determined (Table 4). The IC_{50} value of compound D27 was 364 μM, which was better than those one detected for umbelliferone (420 μM; Table 5), but in comparison to methoxyphenyl coumarin thiosemicarbazide substituted at 4-position of thiosemicarbazide chain (thiosemicarbazide analogue), inhibitory activity was 2.5-fold decreased. This data, additionally confirms the importance of thiosemicarbazide moiety as crucial structure element for strong PPO inhibition.

Conclusions

The present study investigated inhibitory effect of 7-hydroxy-4-methyl substituted coumarins (Schiff bases, thiosemicarbazides, and thiazolidinones) against partially purified apple polyphenol oxidase. Coumarin thiosemicarbazides were found to be a potent inhibitors of apple PPO, with compound C23 (4-methyl-1-(2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetyl)thiosemicarbazide) being the most prominent one (IC_{50} = 10.45 μM). Inhibitory activity of thiosemicarbazides against apple PPO was found to be dependent on the substituent (alkyl, aryl) attached at 4-position of thiosemicarbazide chain, as well as on the length of alkyl substituent. Alkyl (methyl and ethyl) groups attached at 4-position of thiosemicarbazide showed greater inhibitory activity than aryl- (phenyl or 4-methylphenyl) substituents. Increase in the length of alkyl substituent (methyl to ethyl) caused significant decrease in inhibitory activity. Cyclisation of thiosemicarbazide group to the thiazolidinone ring bearing the same substituents as its corresponding thiosemicarbazide caused significant decrease in apple PPO inhibitory activity. These results imply the importance of thiosemicarbazide moiety as crucial structure element for strong PPO inhibition, and suggest that further development of coumarin compounds bearing thiosemicarbazide moiety may be of interest.

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