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Carbonic anhydrase inhibitors. Inhibition of human erythrocyte isozymes I and II with a series of antioxidant phenols

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ABSTRACT

The inhibition of two human cytosolic carbonic anhydrase (hCA, EC 4.2.1.1) isozymes I and II, with a series of phenol derivatives was investigated by using the esterase assay, with 4-nitrophenyl acetate as substrate. 2,6-Dimethylphenol, 2,6-diisopropylphenol (propofol), 2,6-di-*t*-butylphenol, butylated hydroxytoluene, butylated hydroxyanisole, vanillin, guaiacol, di(2,6-dimethylphenol), di(2,6-diisopropylphenol), di(2,6-di-*t*-butylphenol), and acetazolamide showed K_I values in the range of 37.5–274.5 μM for hCA I and of 0.29–113.5 μM against hCA II, respectively. All these phenols were non-competitive inhibitors with 4-nitrophenylacetate as substrate. Some antioxidant phenol derivatives investigated here showed effective hCA II inhibitory effects, in the same range as the clinically used sulfonamide acetazolamide, and might be used as leads for generating enzyme inhibitors possibly targeting other CA isoforms which have not been yet assayed for their interactions with such agents.

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1. Introduction

Carbonic anhydrase (EC 4.2.1.1., CA) is a pH regulatory/metabolic enzyme in all life kingdoms, being found in organisms all over the phylogenetic tree.[1] It catalyzes the hydration of carbon dioxide to bicarbonate and the corresponding dehydration of bicarbonate in acidic medium with regeneration of CO_2.[1,2] At least 16 CA isozymes were described up to now in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX.[2,3] The first one is found primarily in red blood cells but also in many other secretory tissues of the gastrointestinal tract, kidneys, lungs, eye, CNS, etc.,[1,2] whereas the second one is a tumor-associated isoform.[1,3–5] Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion.[1–5] Many such CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis.[1,4]

Our groups recently investigated the interaction of all 16 mammalian CA isozymes with several types of phenols, such as the simple phenol and several of its substituted derivatives, for example, clioquinol, diphenols, paracetamol, salicyclates and some of their derivatives.[6] Here we extend these earlier investigations to a novel series of phenols, some of which are widely used as antioxidant food additives or as drugs. Propofol for example, a compound investigated in this study, is a phenol widely employed as drug. Propofol (2,6-diisopropylphenol) is structurally similar to many food additives, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol. In addition, propofol has been shown to attenuate experimental reperfusion injury in the cerebral cortex.[2c] The most commonly used antioxidants at the present time are BHA, BHT, propyl gallate and *tert*-butylhydroquinone.[7b–9] Vanillin is another phenol derivative which is frequently used in food and drug industry due to its pleasant aroma. However, vanillin is used not only as a flavouring ingredient but also to mask undesirable off-flavours developed during storage of products susceptible to oxidative degradation.[9] It was previously demonstrated that such phenol derivatives possess strong antioxidant properties.[7b–9]

In the present study we have purified human CA I and II (hCA I and hCA II) from erythrocytes and examined the in vitro inhibition effects of some antioxidant phenols mentioned above on these enzymes, using the esterase activity of hCA I and II, with 4-nitrophenyl acetate as substrate.

2. Results and discussion

2.1. Chemistry

The rationale of investigating phenols as CA inhibitors (CAIs) is due to the fact that the simple phenol (PhOH) has been shown to

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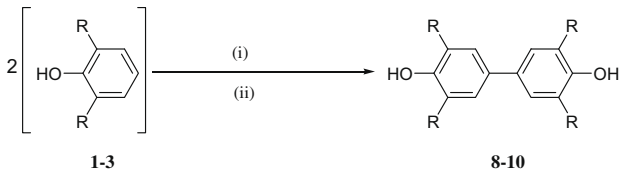
be the only competitive inhibitor with CO_2 as substrate for the main isoform of CA, that is, human CA II (hCA II).[10] In a very elegant study, Christianson's group reported the X-ray crystal structure for the adduct of hCA II with phenol,[10] showing this compound to bind to CA by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the enzyme active site, through a hydrogen bond as well as through a second hydrogen bond to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes.[1–4] The phenyl moiety of phenol was found to lay in the hydrophobic part of the hCA II active site, where CO_2, the physiologic substrate of the CAs, binds in the pre-catalytic complex, explaining thus the behaviour of phenol as a unique CO_2 competitive inhibitor. Only recently, our groups investigated the interactions of phenol and some of its substituted derivatives (as well as bicyclic phenols) with all mammalian isozymes, CA I–XV,[6] evidencing some low micromolar/submicromolar inhibitors as well as the possibility to design isozyme-selective CAIs. Indeed, the inhibition profile of various isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols.[6] It appeared thus of interest to extend the previous studies,[6] including in this investigation phenols with clinical and antioxidant applications as food additives, such as compounds **1–3**.[11] Other structurally related derivatives such as propofol **2,** guaiacol **6** and vanillin **7**, as well as dimeric phenol derivatives **8**, **9**, **10** were also included in our study. Compounds **8–10** were synthesized by the literature procedure (Scheme 1).[7a]



1-3 8-10

Scheme 1. Preparation of biphenyl diphenols **8**–**10** by oxidation of phenols **1**–**3**. Regents and conditions: (i) CuCl(OH)·TMEDA/CH_2Cl_2; (ii) $Na_2S_2O_4$.

Oxidation of the phenol derivatives **1–3** with catalytic CuCl(OH). TMEDA (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) in the presence of $Na_2S_2O_4$ gave the biphenyl diphenols **8–10** in high yields (see Experimental for details).

2.2. CA purification, assay and inhibition with phenols 1–10

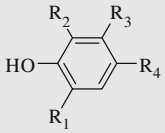
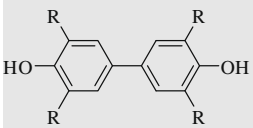
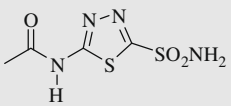
The purification of the two CA isozymes used here was performed with a simple one step method by a Sepharose-6B-aniline-sulfanilamide affinity column chromatoghrapy.[12] hCA I was purified, 105.5-fold with a specific activity of 975.92 EU mg^{-1} and overall yield of 51.7% and hCA II was purified, 646.5-fold with a specific activity of 5980 EU mg^{-1} and overall yield of 55% (Table 1).[13–15] Inhibitory effects of antioxidant phenolic compounds on enzyme activities were tested under in vitro conditions; K_i values were calculated from Lineweaver–Burk graphs and are given in Table 2.[16,17]

We report here the first study on the inhibitory effects of antioxidant phenols of type **1–10** on the esterase activity of hCA I and II. The sulfonamide CAI acetazolamide **11**[1] has been used as a neg-

Table 1
Summary of purification procedure for human carbonic anhydrase izoenzymes (hCA I and hCA II) by a Sepharose-6B-aniline-sulfanilamide affinity column chromatography

Purification steps		Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate		181	45	19.56	880.2	8145	9.25	100	1
Sepharose-6B-aniline-sulfanilamide affinity column chromatography	hCA I	527	8	0.54	4.32	4216	975.92	51.7	105.5
	hCA II	897	5	0.15	0.75	4485	5980	55	646.5

Table 2
hCA I and II inhibition data with phenols **1**-**10** and acetazolamide **11**, by an esterase assay with 4-nitrophenylacetate as substrate[14]

Structure	Cmpnd.	R	R_1	R_2	R_3	R_4	K_I (μM)[a]	
							hCA I	hCA II
	**1**	–	CH_3	CH_3	H	H	198.3	113.5
	2	–	$CH(CH_3)_2$	$CH(CH_3)_2$	H	H	98.9	19.5
	3	–	$C(CH_3)_3$	$C(CH_3)_3$	H	H	274.5	0.51
	4	–	$C(CH_3)_3$	$C(CH_3)_3$	H	CH_3	245.2	0.63
	5	–	$C(CH_3)_3$	$C(CH_3)_3$	H	OCH_3	63.7	0.42
	6	–	H	OCH_3	H	H	60.2	2.94
	7	–	H	OCH_3	COH	H	55.6	1.25
	**8**	CH_3	–	–	–	–	92.8	4.05
	9	$CH(CH_3)_2$	–	–	–	–	37.9	1.87
	10	$C(CH_3)_3$	–	–	–	–	37.5	0.29
	**11**	–	–	–	–	–	36.2	0.37

[a] Mean from at least three determinations. Errors in the range of 5–10% of the reported value (data not shown).

ative control in our experiments, and for comparison reasons. The previous reports by Innocenti et al.[6] investigated other phenol derivatives (including salicylic acid and paracetamol) by using a stopped flow, CO_2 hydration assay for monitoring CA inhibition. Data of Table 2 show the following regarding inhibition of hCA I and II with phenols **1-10**, by an esterase assay,[14] with 4-nitrophenylacetate (4-NPA) as substrate:

(i) Against the slow cytosolic isozyme hCA I, compounds **1–9** behave as weak inhibitors, with K_i values in the range of 37.5–274.5 μM. 2,6-Dimethylphenol **1** was an ineffective hCA I inhibitor (K_I of 198.3 μM), similarly to the structurally related compounds **3** and **4** (K_Is of 245.2–274.4 μM). A second group of derivatives, including **2** and **5–10**, showed better inhibitory activity as compared to the previously mentioned phenols, with K_I values of 37.5–92.8 μM, (Table 2). Thus, the nature of the groups in *ortho*- and *ortho*′- to the phenolic OH moiety strongly influences hCA I inhibitory activity. It is also interesting to note that the biphenyl derivatives **8–10** were much better hCA I inhibitors as compared to the corresponding monophenols **1–3** from which they were prepared. Acetazolamide **11** is also a medium-weak CAI with this assay and substrate against hCA I (K_I of 36.2 μM). Kinetic investigations (Lineweaver Burke plots, data not shown) indicate that similarly to sulfonamides and inorganic anions,[4–6,18–21] all the investigated phenols act as non-competitive inhibitors with 4-NPA as substrate, that is, they bind in different regions of the active site cavity as compared to the substrate. However the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme.[10]

(ii) A better inhibitory activity has been observed with compounds **1–10** investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 2). Thus, two derivatives, that is, **1**, **2**, showed moderate hCA II inhibitory activity with K_I-s in the range of 19.5–113.5 μM, Table 2), whereas the remaining eight derivatives were quite effective hCA II inhibitors, with K_i-s in the range of 0.29–4.05 μM, (Table 2). Structure–activity relationship (SAR) is thus quite sharp for this small series of phenols: the 2,6-dimethyl- and 2,6-di-isopropyl-phenols **1** and **2** are ineffective leads, but already the bulkier **3**, with two *tert*-butyl moieties in *ortho* and *ortho*′ is already a submicromolar hCA II inhibitor. This trend is maintained when different groups are present in the *para* position to the phenol OH moiety, such as in compounds **4** and **5**. 2-Methoxy-phenol **6** and vanilline **7** are also effective hCA II inhibitors, whereas the biphenyl diphenols **8–10** showed increasing hCA II inhibitory activity with the increase of the volume of substituents in the 2 and 6 positions of both phenyl rings. The best hCA II inhibitor in this series of derivatives was the bulky, tetra-*tert*-butyl biphenyl derivative **10**, which with a K_I of 0.29 μM, is a better inhibitor than acetazolamide, a clinically used sulfonamide. It must be stressed that K_Is measured with the esterase method are always in the micromolar range because hCA I and II are weak esterases.[22]

In a recent study it was reported that thioxolone,[23] a simple compound lacking the sulfonamide, sulfamate, or related functional groups that are typically found in all known CA inhibitors, acts as a CAI inhibitor, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies (thioxolone acts as a prodrug, being hydrolyzed in situ with formation of a mercapto phenol derivative which is the real enzyme inhibitor).[23] However Innocenti et al.[24] showed that compared to sulfonamides thioxolone was inefficient for generating isozyme-selective inhibitors, since except for hCA I which was inhibited in the nanomolar range (K_i of 91 nM), the remaining 12 mammalian CA isoforms (CA II–CA XV) were inhibited with a very flat profile by this compound (K_i-s in the range of only 4.93–9.04 μM). In contrast to thioxolone, 3,5-dichloro-4-hydroxybenzenesulfonamide as well as the clinically used heterocyclic sulfonamide acetazolamide showed K_i-s in the range of 58 nM–78.6 μM and 2.5 nM–200 μM, respectively, against the 13 investigated mammalian CAs. The sulfonamide zinc-binding group is thus superior to the thiol one (from the thioxolone hydrolysis product) for generating CA inhibitors with a varied and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

3. Conclusions

Antioxidant phenolic compounds **1–10** used in this study affect the activity of CA isozymes due to the presence of the different functional groups (OH, OCH_3 and CHO) present in their aromatic scaffold. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Indeed, some antioxidant phenolic compounds investigated here showed effective hCA I and II inhibitory activity, in the low micromolar range, by the esterase method which usually gives K_I-s an order of magnitude higher as compared to the CO_2 hydrase assay.[24] These findings point out that substituted phenolic compounds may be used as leads for generating potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

4. Experimental

4.1. Chemicals

2,6-Dimethylphenol (DMP), 2,6-diisopropylphenol (propofol, DIP) and 2,6-di-*t*-butylphenol (DTP), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Sepharose 6B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma–Aldrich Co. (Sigma–Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter strasse 250, D 64293 Darmstadt Germany).

4.2. Synthesis of dimeric phenols

The compounds used in this study Di-DMP, Di-DIP and Di-DTP were synthesized as described in the literature.[7]

4.2.1. Synthesis of di(2,6-dimethylphenol) (Di-DMP) 8

DMP **1** (5.0 g, 40.93 mmol) was dissolved in CH_2Cl_2 (100 mL) and mixed with CuCl(OH)·TMEDA (160 mg, 0.34 mmol) at room temperature at 24 h. The reaction product was extracted with AcOEt and evaporated. The reactant was dissolved in ethanol, $Na_2S_2O_4$ (10.0 g, 57.47 mmol) was added and the mixture was heated for 2 h. The precipitate was collected in a filter and crystallized from hexane to give di(2,6-dimethylphenol) (3.62 g, 73%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.16 (s, 4H, H_{aryl}), 4.60 (s, 2H, OH), 2.30 (s, 12H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 151.47, 133.55, 127.22, 123.36, 16.30. Anal. Calcd for $C_{16}H_{18}O_2$: C, 79.31; H, 7.49. Found: C, 79.17; H, 7.67.

4.2.2. Synthesis of di(2,6-diisopropylphenol) (Di-DIP, Dipropofol) 9

Propofol **2** (4.0 g, 22.47 mmol) was dissolved in CH_2Cl_2 (40 mL) and stirred with CuCl(OH)·TMEDA (64 mg, 0.14 mmol) for 24 h at room temperature. The reaction product was extracted with AcOEt and the solvent was evaporated to afford a resultant residue which

was dissolved in ethanol, and heated with $Na_2S_2O_4$ (6.0 g, 34.48 mmol) for 20 min. The separated precipitate was collected on a filter and was crystallized from hexane to give the dimeric propofol, dipropofol (2.94 g, 74%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.33 (s, 4H, H_{aryl}), 4.99 (m, 2H, OH), 3.35 (h, J = 6.8 Hz, 4H, CH), 1.46 (d, J = 6.8 Hz, 12H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 149.46, 135.13, 134.19, 122.74, 27.77, 23.21. Anal. Calcd for $C_{24}H_{34}O_2$: C, 81.31; H, 9.67. Found: C, 79.62; H, 9.70.

4.2.3. Synthesis of di(2,6-di-*t*-butylphenol) (Di-DTP) 10

DTP **3** (5.0 g, 24.27 mg) was dissolved in CH_2Cl_2 (100 mL) and stirred with CuCl(OH)·TMEDA (160 mg, 0.34 mmol) for 24 h at room temperature. The reaction product was extracted with AcOEt. After removal of solvent, the reactant was dissolved in ethanol, and heated with $Na_2S_2O_4$ (6.0 g, 34.48 mmol) for 2 h. The precipitate was collected on a filter and crystallized from hexane to give di(2,6-di-*t*-butylphenol) (3.58 g, 72%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm**):** 7.33 (s, 4H, H_{aryl}), 5.22 (s, 2H, OH), 1.52 (s, 36H, CH_3), ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 153.06, 136.15, 134.18, 124.38, 34.70, 30.61. Anal. Calcd for $C_{28}H_{42}O_2$: C, 81.90; H, 10.31. Found: C, 81.50; H, 10.27.

4.3. Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Centre of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20,000 rpm for 30 min at 4 °C. The pH of the hemolysate was adjusted to 8.7 with solid Tris.[9a] Firstly, Sepharose-6B oxidized by $KMnO_4$ and subsequently activated by $SOCl_2$. After that, aniline attached to the activated gel as a spacer arm and finally diazotized sulfanilamide clamped to the *para* position of aniline molecule as ligant. The hemolysate was applied to the prepared Sepharose 6B-aniline-sulfanylamide affinity column equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4 °C.[12]

4.4. Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[13] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_o - t_c/t_c)$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

4.5. Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV–vis) according to the method described by Verpoorte et al.[14] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris–SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of 2,6-dimethylphenol (DMP), 2,6-diisopropylphenol (DIP, Propofol), 2,6-di-*t*butylphenol (DTP), butylated hydroxytoluene(BHT), butylated hydroxyanisole (BHA), vanillin, guaiacol, di(2,6-dimethylphenol) (Di-DMP), di(2,6-diisopropylphenol) (Di-DIP, dipropofol), di(2,6-di-*t*butylphenol) (Di- DTP), and acetazolamide were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. HCA-I enzyme activities were measured for 2,6-dimethylphenol (DMP)(19.95–199.5 μM), 2,6-diisopropylphenol (DIP, Propofol) (13.66–95.6 μM), 2,6-di-tbutylphenol (DTP) (0.05–0.2 μM), butylated hydroxytoluene(BHT) (0.047–0.18 μM), butylated hydroxyanisole (BHA) (10.3–51.5 μM), vanillin (5.1–51.2 μM), guaiacol (5.48–43.9 μM), di(2,6-dimethylphenol) (Di-DMP) (10–50 μM), di(2,6-diisopropylphenol) (Di-DIP, dipropofol) (6.87–68.78 μM), di(2,6-di-t-butylphenol) (Di-DTP)(2.96–29.63 μM) and acetazolamide (2.6–13.4 μM) at cuvette concentrations and HCA-II enzyme activities were measured for 2,6-dimethylphenol (DMP)(9.97–99.75 μM), 2,6-diisopropylphenol (DIP, propofol) (3.03–21.24 μM), 2,6-di-tbutylphenol (DTP) (0.09–0.36 μM), butylated hydroxytoluene(BHT) (0.049–0.396 μM), butylated hydroxyanisole (BHA) (0.078–0.39 μM), vanillin (0.17–1.7 μM), guaiacol (0.27–2.68 μM), di(2,6-dimethylphenol) (Di-DMP) (0.37–1.85 μM), di(2,6-diisopropylphenol) (Di-DIP, dipropofol) (0.163–1.637 μM), di(2,6-di-tbutylphenol) (Di-DTP)(0.027–0.269 μM) and acetazolamide (0.065–0.33 μM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity (%)-[Inhibitor] graphs were drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver–Burk curves were drawn.[17]

4.6. Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard.[15]

4.7. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.[16] The electrophoretic pattern was photographed (data not shown).

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