



Inhibition of proteasome activity by anthocyanins and anthocyanidins

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ABSTRACT

Recent reports have demonstrated multiple benefits associated with the consumption of berry fruits, including a decreased vulnerability to oxidative stress, reduced ischemic brain damage, protection of neurons from stroke-induced damage and the reversal of age-related changes in brain and behaviour. Berry fruits contain high amounts of anthocyanins, which play a major role as free radical scavengers. The present study addresses proteasome inhibition as a further mechanism by which anthocyanins and their aglycons, the anthocyanidins, may exert health-promoting effects. HL-60 cells were incubated with 19 test substances and inhibition of the chymotrypsin-like enzyme activity was determined in a chemiluminescent assay. Anthocyanins and their aglycons achieved IC₅₀ values ranging from 7.8 μM for kaempferidinidin and pelargonidin, to 32.4 μM for delphinidin. Thus proteasome inhibitory properties of anthocyanins may contribute to their known anticarcinogenic, antioxidative, anti-inflammatory and neuroprotective activities, rationalizing dietary supplementations with anthocyanins in the prevention and treatment of chronic diseases, including neurodegenerative disorders.

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Over the past decade, awareness has increased of multiple health-promoting effects of anthocyanins, powerful antioxidants that confer the characteristic red, blue, and purple colours to many dietary plants [1]. Anthocyanins are water-soluble, glycosidic polyhydroxyl, and polymethoxyl derivatives of flavylium salts and are most abundant in berries, grapes, and red cabbage, among other foodstuffs. When expressed per 100 g fresh weight, approximately 1480 mg of anthocyanins may be obtained from chokeberries [2], 588 mg from bilberries [3], 476 mg from black currant and 322 mg from red cabbage [2]. The average dietary per capita and day consumption of anthocyanins was originally estimated at 180–215 mg in Western societies [4]. More recent calculations from U.S. American surveys have concluded to a daily intake of only 12.5 mg [2], but the actual amount has been shown to vary considerably with sociodemographic and life-style factors [5], plus the seasonal availability of anthocyanin-rich fruits and vegetables [2].

Health benefits associated with diets rich in anthocyanins are ascribed to multi-level biological activities including antioxidative

[6], anti-inflammatory [7], antiviral [8], vasoprotective [9], antiangiogenic [10], and anticarcinogenic effects [11]. In animals, blueberry-enriched diets protect neurons from ischemia-induced damage, decrease the vulnerability to oxidative stress and improve motor function [12]. It has been proposed that consumption of fruits rich in anthocyanins may protect against age-related deficits and may mitigate existing deficits with regard to central nervous system function [13,14]. In humans, epidemiological findings and placebo-controlled trials indicate that anthocyanins can reduce mortality from cardiovascular and coronary heart disease [15], can aid in the management of diabetic retinopathy [16] and can improve dark adaptation [17]. However, despite the spectrum of pharmacological effects elicited by anthocyanins, information on the underlying cellular mechanisms is scarce. We hypothesized that the proteasome may be targeted by anthocyanins based on data from apple and grape extracts [18]. Proteasome activity controls the degradation of cellular proteins and is closely implicated in signal transduction, development, cell cycle progression [19], antigen processing, immune response [20], and inflammation [21] plus protection from oxidative stress [22] relevant to neurodegenerative diseases [23,24]. The present study was conceived to examine anthocyanins', anthocyanidins', and proanthocyanidins' in vitro impact on the chymotrypsin-like (ChT-L) proteasome activity.

Abbreviations: ChT-L, chymotrypsin-like; DMEM, Dulbecco's modified Eagle Medium; HSP, heat shock protein; NF-κB, nuclear factor κB.

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Experimental methods

Chemicals. L-glutamine (200 mM) and Dulbecco's modified Eagle Medium (DMEM, 4.5 g/l glucose, sodium pyruvate, 3.7 g/l NaHCO₃, without L-glutamine) were purchased from Pan Biotech GmbH (Aidenbach, Germany), FCS from Gibco BRL Life Technologies (Eggenstein, Germany) and penicillin-streptomycin (10,000 units/10 ml) from PAA Laboratories GmbH (Coelbe, Germany). Proteasome Glo Cell-Based Assay was obtained from Promega (Mannheim, Germany). Cyanidin, cyanidin-3,5-diglucoside (cyanin), cyanidin-3-galactoside (ideain), cyanidin-3-glucoside (kuromanin), cyanidin-3-rutinoside (keracyanin), delphinidin, delphinidin-3-glucoside (myrtillin), kaempferidin, malvidin, malvidin-3,5-diglucoside (malvin), malvidin-3-galactoside, malvidin-3-glucoside (oenin), peonidin, peonidin-3-glucoside, pelargonidin, pelargonidin-3,5-diglucoside (pelargonin), petunidin, procyanidin B1, and procyanidin B2 were purchased from Extrasynthese (Genay, France). All test substances were dissolved and diluted in DMSO.

Cell culture. HL-60 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in DMEM supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin plus 100 µg/ml streptomycin, and were maintained in an atmosphere of 5% CO₂ at a temperature of 37 °C. Passages were carried out every three days. For the assay, an aliquot of the cell suspension was drawn from the cell culture flask and centrifuged at 1200 rpm for five minutes. The supernatant was removed and the cell pellet was resuspended in the culturing medium.

Inhibition of chymotrypsin-like activity in human cells by flavonoids. Effects of the test substances on the proteasomal chymotrypsin-like activity were determined using a chemiluminescent assay (Proteasome Glo Cell-Based Assay, Promega). HL-60 cells were dispensed into white opaque 96-well microtiter plates, at a density of 1 × 10⁴ cells in 100 µl culturing medium per well. Instantly, 1 µl of flavonoid, diluted in DMSO, was added to the culturing medium. An equivalent volume of DMSO was used as a negative control. After incubation at room temperature for 7 min, 100 µl assay buffer, containing the luminogenic proteasome substrate succinyl-leucine-leucine-valine-tyrosine-aminoluciferin and a recombinant firefly luciferase were added. Subsequent to further incubation at room temperature for 7 min, chemiluminescence, expressed as relative light units (RLU), was measured on an Anthos Lucy 1 microplate luminometer (Anthos Labtech, Salzburg, Austria) with a measuring time of 12 s for each well. Anthocyanins, anthocyanidins, and procyanidins were diluted with DMSO to yield final concentrations of 2, 5, 10, 50, and 100 µmol/l in the assay.

Data analysis. To quantify proteasome inhibition by our test substances, the chymotrypsin-like (ChT-L) activity of the proteasome was obtained using a coupled-enzyme system, with proteasome cleavage of the substrate releasing aminoluciferin and a luciferase reaction generating a chemiluminescence signal. Chymotrypsin-like activity was calculated according to the following equation:

$$\%A = 100 \times \left(1 - \frac{A_i}{A_{\text{DMSO}}} \right)$$

Where %A is the percentage of the ChT-L activity of the proteasome remaining after treatment with test substances, A_i is the activity of the chymotryptic site in the presence of an inhibitor, and A_{DMSO} is the activity of the proteasome in the absence of inhibitors.

For each substance tested, mean values from three separate experiments performed in triplicate at five concentrations were analyzed with Prism v. 2.01 (GraphPad Software, CA, USA) using a non-linear regression model to determine the concentrations

inhibiting 50% of the chymotrypsin-like proteasome activity (IC₅₀ values). Mean IC₅₀ values for anthocyanins and anthocyanidins were compared using *t*-tests. Statistical significance was set at *p* = 0.05. ISIS/Draw v. 2.1.4 (MDL Information Systems, CA, USA) served to create structures of anthocyanins and anthocyanidins.

Results

Table 1 summarizes IC₅₀ values of all flavonoids under study (Fig. 1). Across all compounds investigated, inhibition of the proteasomal chymotrypsin-like activity differed by a factor of 4. Anthocyanins and their aglycons inhibited proteasome activity in a concentration-dependent manner and achieved IC₅₀ values between 7.8 and 32.4 µM. Overall, the anthocyanidins kaempferidin, pelargonidin, and peonidin acted as powerful inhibitors with IC₅₀ values of 7.8, 7.8, and 9.0 µM, respectively. In the order of decreasing potency, cyanidin-3,5-diglucoside, cyanidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-galactoside, malvidin-3,5-diglucoside, cyanidin-3-rutinosid, cyanidin, malvidin-3-galactoside, malvidin-3-glucoside, and petunidin gave IC₅₀ values from 11.0 to 23.7 µM. The least potent inhibitors were identified as malvidin, pelargonidin-3,5-diglucoside, and delphinidin, featuring IC₅₀ values above 30 µM. When compounds were grouped according to the presence or the absence of a sugar moiety, no trend was seen with regard to proteasome inhibition. Mean IC₅₀ values of anthocyanins (IC₅₀ = 17.5 µM ± 6.6) and anthocyanidins (IC₅₀ = 18.7 µM ± 11.0) showed only a marginal difference (*p* = 0.78, *t* = 0.28, Table 1). This finding is further illustrated by the inhibitory profiles of the two anthocyanins, pelargonidin-3,5-diglucoside, and delphinidin-3-glucoside, and their corresponding aglycons (Fig. 2). For the first pair of compounds, pelargonidin, the aglycon, acted as a powerful proteasome inhibitor (IC₅₀ = 7.8 µM), whereas pelargonidin-3,5-diglucoside showed more moderate inhibition (IC₅₀ = 32.3 µM). For the second pair, however, inhibition by the aglycon delphinidin was less pronounced (IC₅₀ = 32.4 µM) than that achieved by the corresponding delphinidin-3-glucoside (IC₅₀ = 13.0 µM).

Table 1

Inhibition of proteasomal chymotrypsin-like activity in HL-60 cells by anthocyanins and anthocyanidins

Anthocyanin/anthocyanidin	IC ₅₀ in µM (anthocyanidins)	IC ₅₀ in µM (anthocyanins)
Kaempferidin	7.8	
Pelargonidin	7.8	
Peonidin	9.0	
Cyanidin-3,5-diglucoside (cyanin)		11.0
Cyanidin-3-glucoside (kuromanin)		12.6
Peonidin-3-glucoside		12.8
Delphinidin-3-glucoside (myrtillin)		13.0
Cyanidin-3-galactoside (ideain)		13.5
Malvidin-3,5-diglucoside (malvin)		17.1
Cyanidin-3-rutinoside (keracyanin)		18.1
Cyanidin	18.4	
Malvidin-3-galactoside		21.7
Malvidin-3-glucoside (oenin)		23.2
Petunidin	23.7	
Malvidin	32.0	
Pelargonidin-3,5-diglucoside (pelargonin)		32.3
Delphinidin	32.4	
Mean IC ₅₀ value ± SD	18.7 +/- 11.0	17.5 +/- 6.6
<i>p</i> (<i>t</i> , <i>df</i>)	0.80 (0.28, 15)	

For procyanidins B1 and B2, no inhibitory activity was observed. Only a marginal difference was noted in mean IC₅₀ values of anthocyanins and anthocyanidins (*p* = 0.78). Data are based on three separate experiments, each performed in triplicate.

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