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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 IC_{50} 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.

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 $NaHCO_3$, 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), kaempferidinidin, 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% CO_2 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 1×10^4 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:

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

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 (IC_{50} values). Mean IC_{50} 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

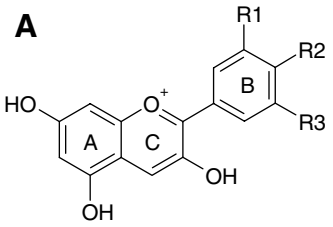
Table 1 summarizes IC_{50} values of all flavonoids under study (Fig. 1). Across all compounds investigated, inhibition of the proteasomal chymotrypsin-like activity differed by a factor of 4. Anthocyanins and their aglycons inhibited proteasome activity in a concentration-dependent manner and achieved IC_{50} values between 7.8 and 32.4 μM. Overall, the anthocyanidins kaempferidinidin, pelargonidin, and peonidin acted as powerful inhibitors with IC_{50} 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 IC_{50} values from 11.0 to 23.7 μM. The least potent inhibitors were identified as malvidin, pelargonidin-3,5-diglucoside, and delphinidin, featuring IC_{50} 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 IC_{50} values of anthocyanins (IC_{50} = 17.5 μM ± 6.6) and anthocyanidins (IC_{50} = 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 (IC_{50} = 7.8 μM), whereas pelargonidin-3,5-diglucoside showed more moderate inhibition (IC_{50} = 32.3 μM). For the second pair, however, inhibition by the aglycon delphinidin was less pronounced (IC_{50} = 32.4 μM) than that achieved by the corresponding delphinidin-3-glucoside (IC_{50} = 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)
Kaempferidinidin	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
p (*t*, df)	0.80 (0.28,15)	

For procyanidins B1 and B2, no inhibitory activity was observed. Only a marginal difference was noted in mean IC_{50} values of anthocyanins and anthocyanidins ($p = 0.78$). Data are based on three separate experiments, each performed in triplicate.

A



anthocyanidin	R1	R2	R3
cyanidin	OH	OH	H
delphinidin	OH	OH	OH
malvidin	O-CH_3	OH	O-CH_3
pelargonidin	H	OH	H
peonidin	O-CH_3	OH	H
petunidin	O-CH_3	OH	OH
kaempferidinidin	H	O-CH_3	H

B

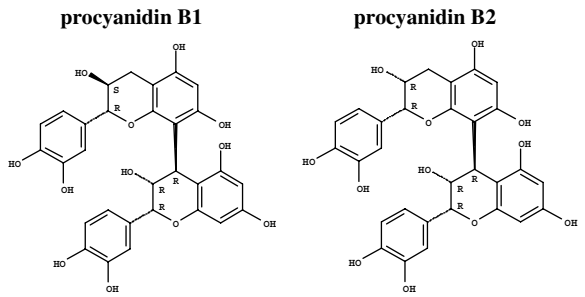


Fig. 1. Chemical structures of tested anthocyanidins (A) and the epicatechin–epicatechin dimers procyanidins B1 and B2 (B).

For the procyanidin dimers B1 and B2, no inhibitory effects on proteasome activity were observed at any of the concentrations tested.

Discussion

Anthocyanins' anti-inflammatory, anticarcinogenic, and neuroprotective potencies have been ascribed to radical scavenging effects [25], the interaction with signalling cascades [26], age-related changes in adrenergic and noradrenergic receptor function [27], the enhancement of dopamine release [14] and shifts in cerebrovascular permeability [28]. In continuation of earlier research that focused on the inhibition of proteasome activity by grape extract [18], the present study explored the effects of anthocyanins and procyanidins, as well as the anthocyanins' aglycons. Our data confirm that the proteasome inhibitory activity of grape extract is at least in part mediated by anthocyanins and suggest that anthocyanin concentration may aid in predicting the activity of other fruit and vegetable extracts. Using the chymotryptic site of the proteasome in HL-60 cells, a concentration-dependent inhibition was shown with IC_{50} values in the low micromolar range, with the exception of procyanidins which did not act as inhibitors. The anthocyanins and their aglycons achieve IC_{50} values comparable to those of other flavonoids. For the flavon apigenin, the flavonols quercetin, kaempferol, and myricetin [29], and the tea flavanol (−)-epigallocatechin-3-gallate (EGCG) [30], IC_{50} values range from 1 to 18 μM. Although the absence of sugar moieties from these flavonoids would appear to suggest a higher inhibitory potency of aglycons, this cannot be confirmed by our results (Table 1). Regarding the substitution-pattern of the anthocyanins' B-ring, mixed effects were noted on proteasome inhibition. Since the majority of effective inhibitors carry only one, or two, hydroxyl- or methoxyl-substituents on their B-ring, as opposed to three substituents in most of the remaining compounds, we assume that inhibitory potency may be sensitive to this structural feature.

For the dimeric procyanidins B1 and B2, the absence of effects in the present study does not imply that these substances cannot inhibit the proteasome. The chymotrypsin-like activity is generally considered the rate-limiting activity in protein breakdown [31], but dimeric procyanidins may inhibit other catalytically active sites of the proteasome, e.g. the "tryptic-like" or the "postglutamyl hydrolyzing" activity [32], or may exhibit proteolytical activities independent of the proteasome [33].

It should also be noted that cells were permeabilized to minimize any impact of transport parameters on measurements of proteasome inhibition. Only few data are currently available on cellular uptake of anthocyanins which limits extrapolations to in vivo effects [34].

Following oral intake and absorption in the intestine mostly as glycosides [35], up to 140 nmol/l of unconjugated anthocyanins are found in human plasma [36]. Anthocyanins and their corresponding aglycons are also found in animal brains within minutes after oral uptake [37,38]. In the present investigation, incubation time was kept short to avoid degradation of compounds.

Clinical applications of proteasome inhibition have focused on tumor suppression [39], a growing body of evidence supports additional roles in neuroprotection, e.g., dietary blueberry supplementation has been shown to elicit neuroplasticity in the hippocampus, and to improve cognitive performance [40]. Among the downstream effects already identified counts the upregulation of heat shock proteins (HSP) [41], and of enzymes involved in antioxidant defense [42], plus the suppression of the proinflammatory immune response [43]. Specifically, the expression levels of HSP22 and HSP70 are increased following proteasome inhibition [44], which is believed to prevent protein misfolding and the formation of protein aggregates. These cytoprotective qualities allow cells to survive under otherwise lethal conditions [45] and may slow the course of neurodegenerative disorders by refolding denatured proteins, as shown in models of Huntington's disease [46].

Furthermore, multiple antioxidant enzymes have been shown to be highly expressed following proteasome inhibition, including superoxide dismutase I [42], thioredoxin reductase I, peroxiredoxins I and VI, and metallothioneins I and II [44]. Moreover, it has been demonstrated that proteasomal inhibition affords cytoprotection against oxidative stress by inducing glutathione synthesis in animal models of Parkinson's disease [22]. Of all organs, the brain is most susceptible to oxidative damage due to its high oxygen demand [47]. Here, by protecting against oxidative and nitrosative stress [48,49], anthocyanins and anthocyanidins likely limit damage of brain cells at the protein, membrane lipid, and DNA levels. Finally, inhibitors of the proteasome affect the immune response by repressing antigen presentation on MHC class I receptors [50], by suppressing cytokine secretion, cell-cell-interaction, migration and chemotaxis of lymphocytes, and by inducing apoptosis in activated T-cells. Downregulation of cytokine secretion [51] and cell adhesion molecule expression [52] occurs via nuclear factor κB (NF-κB) inactivation [24]. It is suggested that activation of the NF-κB pathway may play a role in a number of acute and chronic diseases with an inflammatory component, such as atherosclerosis, asthma, rheumatoid arthritis, inflammatory bowel disease [53], and neurodegenerative disorders such as cerebrovascular disease, Parkinson's disease, and Alzheimer's disease. In line with this

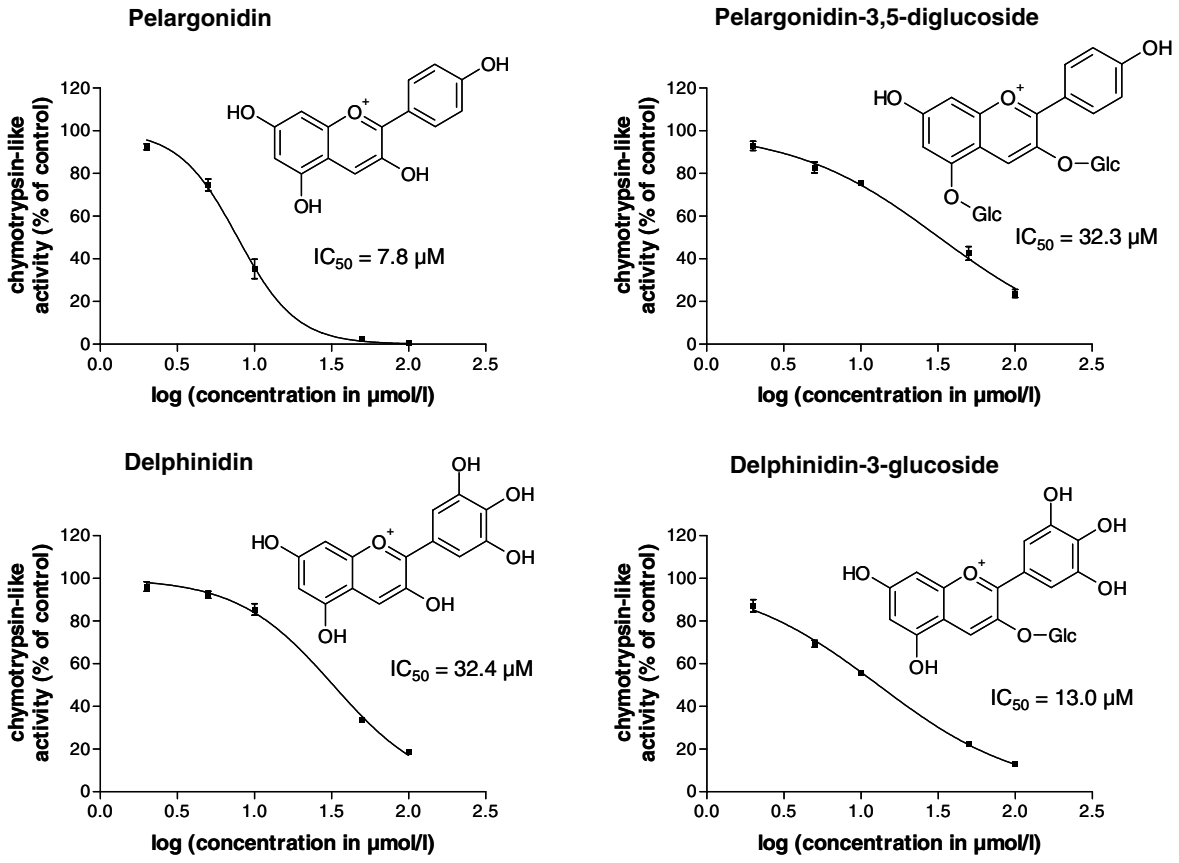


Fig. 2. Concentration-dependent inhibition of the chymotrypsin-like proteasome activity by pelargonidin, delphinidin, and the respective glucosides, pelargonidin-3,5-diglucoside, and delphinidin-3-glucoside: HL-60 cells were incubated with increasing amounts of test compounds for 7 min. Proteasome activity is expressed as relative residual activity of control cells treated with an equivalent volume of DMSO (100 %). Data points are means with standard deviation shown as vertical error bars of three independent experiments performed in triplicate. IC_{50} values were calculated from dose-response curves using non-linear regression.

assumption, rats fed a blueberry diet showed a decline in age-related cognitive deficits and a reduction in NF-κB expression compared to non-supplemented controls [13,54]. It appears that rapidly dividing cells are more sensitive to pro-apoptotic effects of proteasome inhibitors than differentiated or non-proliferating cells [55], which can be advantageous in tumor therapy. Thus dietary supplementation with anthocyanins may serve to prevent a number of common diseases by interfering with the proteasome pathway.

In summary, inhibition of the proteasome by anthocyanins and anthocyanidins adds to our understanding of cellular effectors that may control anti-inflammatory, immunomodulatory, and neuroprotective activities of these substances. Further research is invited to address the mechanisms further downstream, and specifically, the contribution made by anthocyanin metabolites to a healthy diet.

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