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Research paper

Wheat sprout extract induces changes on 20S proteasomes functionality

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Abstract

Wheat sprouts contain a very high level of organic phosphates and a powerful cocktail of different molecules such as enzymes, reducing glycosides and polyphenols.

The antioxidant properties of wheat sprouts have been widely documented and it has been shown that they are able to protect DNA against free-radicals mediated oxidative damage. Furthermore, we have recently reported on the effects of several polyphenols on 20S proteasomes, underlying the dual role of epigallocatechin-3-gallate as an antioxidant and a proteasome effector in cancer cells.

The aim of this study was to investigate the effects of wheat sprout extracts on 20S proteasome functionality. Wheat sprout extracts have been analysed and characterized for their polyphenolic content using the Folin-Ciocalteau reagent and RP-HPLC technique. Comparing our data with a polyphenol standard mixture we identified five different polyphenols: gallic acid, epigallocatechin-3-gallate, epigallocatechin, epicatechin and catechin.

The treatment of isolated 20S proteasomes with the extract induced a gradual inhibition of all the tested components, ChT-L, T-L, PGPH and BrAAP, in both the complexes. At low extract concentration a slight activation of the enzyme was evident only for the BrAAP component of the constitutive enzyme and the ChT-L activity of the immunoproteasome. β-casein degradation rate decreased, particularly with the immunoproteasome. Human Colon adenocarcinoma (Caco) cells, stimulated with 12-*O*-tetradecanoylphorbol-13-acetate, showed activation of the 20S proteasome activities at short incubation times and an increase in intracellular oxidative proteins. Cells treatment with wheat sprout extract led to proteasome inhibition in unstimulated cells and attenuated the effects mediated by TPA. Finally, exposure to the extract affected the expression levels of pro-apoptotic proteins.

Keywords: 20S proteasome; Wheat sprout extract; Antioxidant; Polyphenol; Caco cells; 12-*O*-tetradecanoylphorbol-13-acetate

1. Introduction

Wheat sprouts contain a very high level of organic phosphates and a powerful cocktail of antioxidant molecules such as enzymes, reducing glycosides and polyphenols that show a remarkable reducing and radical scavenging activity [1,2]. It has been reported that wheat sprout antioxidant activity is able to protect DNA against oxidative stress induced by

Abbreviations: AE, ellagic acid; AG, gallic acid; AMC, 7-amino-4-methyl-coumarin; AP-N, aminopeptidase-N; BrAAP, branched chain amino acid preferring; C, (+)-catechin; Caco, human colon adenocarcinoma cell line; CG, catechins-3-gallate; ChT-L, chymotrypsin-like; EC, (−)-epicatechin; ECG, epicatechin-3-gallate; ECL, Enhanced ChemiLuminescence; EGCG, epigallocatechin-3-gallate; GAE, gallic acid equivalents; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-gamma; L, luteolin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pAB, 4-aminobenzoate; PABA, 4-aminobenzoic acid; PARP, poly ADP ribose polymerase; PGPH, peptidyl-glutamyl peptide hydrolyzing; pNA, *p*-aminobenzoate; Q, quercetin; RDS, relative standard deviation; SNAAP, small neutral amino acid preferring; Suc, succinyl; T-L, trypsin-like; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TPC, Total Phenolic Content; Z, benzyloxycarbonyl.

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reactive oxygen species [3]. Studies conducted on old mice treated with wheat sprouts extracts showed a recovery of hepatocyte DNA synthesis levels when compared with the old untreated ones. After the treatment, the increase in DNA and protein contents observed in aged animals was comparable to that present in young mice hepatocytes [4]. Moreover, the oral assumption of wheat sprout powder induces in old dogs a significant reduction of senile cataract confirming that the wheat sprout biologically active substances can be at least partially absorbed during the digestion process [2].

In the last years, an increasing interest has been focused on the antioxidant activity of natural products and on their action as enzymes effectors.

We have recently reported on the ability of several polyphenols to interact with 20S proteasomes and on the dual role of epigallocatechin-3-gallate as an antioxidant and a proteasome effector on cancer cells [5].

The aim of the present work is to investigate the effects of hydroalcolic wheat sprout extract on the 20S proteasome functionality.

The 26S proteasome is a multi-subunit protease complex present in the nucleus and cytoplasm of eukaryotic cells and is responsible for most of the extralysosomal degradation of ubiquitin-tagged proteins. The proteasome is composed by a central catalytic core, referred to as the 20S proteasome, showing distinct proteolytic activities associated with N-terminal threonine residues at the catalytic sites: chymotrypsin-like (ChT-L), trypsin-like (T-L), peptidylglutamyl-peptide hydrolysing (PGPH), branched-chain amino acid preferring (BrAAP) and small neutral amino acid preferring (SNAAP) components [6–9]. Proteasome mediated degradation of proteins is a crucial event for the regulation of cellular homeostasis and cellular functions such as cell cycle progression [10,11], apoptosis, oncogenesis, transcription, selective elimination of abnormal and oxidized proteins [12–17]. Moreover, under the influence of cytokines, such as interferon-γ (IFNγ), the 20S proteasome is converted into the immunoproteasome with its constitutive catalytic subunits, β1, β2, and β5, being replaced by the inducible subunits β1i, β2i, and β5i [18]. This specialized form of the proteasome is responsible for the generation of immunogenic peptides [19].

It has been shown that the regulation of proteasome functionality by natural or synthetic compounds could play a role in cancer therapy: proteasome inhibition, in fact, leads to growth arrest in the G1 phase of the cell cycle and induction of apoptosis in cancer cells [20].

Several studies have previously demonstrated that natural extracts and flavonoids can act as inhibitors of proteasome activity. Catechin-3-gallate (CG), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG) are the most abundant polyphenols in green tea and they have been well characterized as potent proteasome effectors inducing, primarily, the inhibition of the ChT-L activity and apoptosis of cancer cells [21,22]. Furthermore, Dou Q.P. et al. have shown that the proteasome is a cancer-related molecular target for some vegetable extracts such as apple, grape, onion and strawberry extracts, and that the inhibition of proteasome activity by these fruits or vegetables may contribute to their cancer-preventive effects [23].

The goal of this study is to analyse the effects of wheat sprout extracts on 20S proteasome functionality, both in isolated systems and in cell lysates. Additionally, considering that the tumor promoter TPA, besides its role in activating protein kinase C, is also responsible for an increase of oxidative stress and an upregulation of the proteasome proteolytic pathway [24], we wanted to check whether treating the cells with the extract could reverse the effects induced by TPA.

Our results demonstrate that wheat sprout extract is able to inhibit the ChT-L and T-L of the purified constitutive proteasome and the BrAAP activity of the immunoproteasome, whereas in human Colon adenocarcinoma (Caco) cells it exerts a modulatory role on proteasome activities.

2. Materials and methods

2.1. Materials

Wheat sprouts hydroalcolic extract was obtained as previously reported [25]. Methanol, acetonitrile and phosphoric acid were HPLC-grade and purchased from J.T Baker (Milan, Italy). Folin-Ciocalteau reagent and highly purified polyphenols as (+)-catechin (C), (−)-epicatechin (EC), epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), ellagic acid (AE), quercetin (Q), luteolin (L) were purchased from Sigma-Aldrich S.r.L., Italy. Gallic acid (AG) was purchased from J.T Baker (Netherlands). Other used chemicals and solvents were of the highest analytical grade available.

Substrates for assaying the ChT-L, T-L, PGPH, (Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC) were purchased from Sigma-Aldrich S.r.L., Italy. The substrate Z-Gly-Pro-Ala-Phe-Gly-pAB to test the BrAAP activity and the inhibitor Z-Gly-Pro-Phe-Leu-CHO were the kind gift of Prof. Orlowski (Department of Pharmacology, Mount Sinai School of Medicine, New York). Aminopeptidase N (EC3.4.11.2), used for the coupled assay utilized for detecting the BrAAP activity [26] was purified from pig kidney, as reported elsewhere [27,28]. Lactacystin was purchased from Sigma-Aldrich S.r.L., Italy. Caco cells were purchased from Istituto Zooprofilattico Sperimentale – Lombardia ed Emilia Romagna, Italy.

The 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) was purchased from Sigma-Aldrich S.r.L., Italy.

The membranes for Western blot analyses were acquired from Millipore. Immobilized proteins on the film were detected with the Enhanced ChemiLuminescence (ECL) system (Amersham Pharmacia, Biotech). The anti-$p27^{kip-1}$ human and mouse (Mouse) monoclonal antibody was obtained from Oncogene Research products (Germany). The anti-PARP antibody was purchased from CALBIOCHEM (Germany). The Oxidized Protein Detection Kit was purchased from Appligene-Oncor (Strasbourg, France).

2.2. Determination of total phenolic content

Total Phenolic Content (TPC) was estimated by Folin-Ciocalteau assay [29,30]. A gallic acid solution has been used as

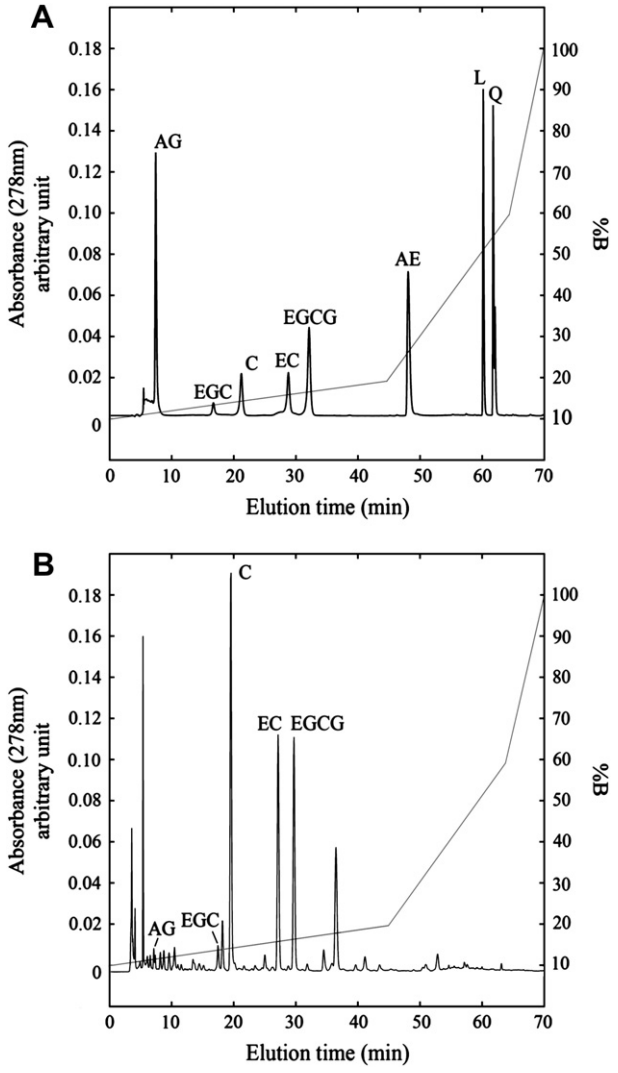

Fig. 1. HPLC analysis. Panel A: elution profile ($\lambda = 278$ nm) of a standard mixture of polyphenols: catechin (C), epicatechin (EC), epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), gallic acid (AG), ellagic acid (AE), luteolin (L) and quercetin (Q). Panel B: elution profile ($\lambda = 278$ nm) of the wheat sprout hydroalcolic extract. Chromatographic analysis was performed on a Beckmann Gold HPLC system equipped with a C18 Phenomenex Luna column (2.5 μm porosity, 250 × 4 mm, with an UltraSep ES RP18 pre-column) with a three-step linear gradients of solvent B (100% CH_3CN) in a solvent A (0.3% H_3PO_4).

standard and data are expressed as milligrams of gallic acid equivalents (GAE)/ml of extract.

2.3. RP-HPLC qualitative and quantitative analysis of polyphenols amount

The HPLC analysis of wheat sprout extract was performed on a Beckmann Gold HPLC system equipped with a C18 Phenomenex Luna column (2.5 μm porosity, 250 × 4 mm, with an UltraSep ES RP18 pre-column). A three-step linear gradient successfully separates all six polyphenols under study (Fig. 1). The mobile phase composition used is (A) 0.3% phosphoric acid and (B) CH_3CN with the following gradient: from 10% to 20% of B in 45 min, from 20% to 60% of B in 20 min, then 60%–90% of B in 20 min (flow rate 0.7 ml/min, temperature 20 °C). The elution pattern was monitored with a UV-VIS detector at 278 nm.

The extract was dissolved in methanol, sonicated for 5 min and then injected in a 10 μl loop.

Each marker was identified by comparison with the retention time of the reference standard and by internal standard. Quantitative analysis was performed by calibration curves using the reference standards of ellagic acid, gallic acid, catechin, epicatechin, epigallocatechin and epigallocatechingallate, luteolin and quercetin, and the linearity was investigated in the range of 0–5 μg at five increasing concentrations. Even levels of concentration tested were performed in triplicate. Intra-day analyses of the same solution containing all phenolic compounds tested were used to validate the precision of the chromatographic system. The precision of the method was estimated by $n = 7$ replicates: the relative standard deviation (RSD) of the retention time was <0.5% and the RSD of the peak areas was <1%.

2.4. Effect of wheat sprout extract on 20S proteasomes functionality

To evaluate the effects of the extract on 20S constitutive and immuno-proteasome peptidase activities, *in vitro* assays were carried out with fluorogenic peptides.

Suc-Leu-Leu-Val-Tyr-AMC was used for chymotrypsin-like activity (ChT-L), Z-Leu-Ser-Thr-Arg-AMC for trypsin-like activity (T-L), Z-Leu-Leu-Glu-AMC for peptidylglutamyl-peptide hydrolase activity (PGPH), and Z-Gly-Pro-Ala-Phe-Gly-pAB for branched chain amino acid preferring activity (BrAAP) [8,31,32].

Isolation and purification of the 20S proteasomes from bovine brain and thymus were carried out as previously reported [31,33].

The incubation mixture contained the extract, 5 μg of isolated 20S proteasome, the appropriate substrate and 50 mM Tris–HCl pH 8.0 up to a final volume of 500 μl.

Incubation was carried out at 37 °C and, after 60 min, the measurements of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (PABA) were detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA: $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm) on a spectrofluorimeter Shimadzu RF5301.

Control assays were also performed in order to evaluate a possible effect of the extract on the proteolytic activity of AP-N: incubation mixtures containing the extract, 8 μg of AP-N, the substrate L-Leu-pNA and 50 mM Tris–HCl pH 8.0 up to a final volume of 500 μl were incubated at 37 °C. After 10 min incubation the activity was measured ($\lambda =$ 405 nm) on a spectrophotometer Varian Cary 100Bio.

2.5. Determination of casein degradation by the 20S proteasomes

β-casein was used as proteasomal substrate in order to test the proteolytic activity of isolated 20S proteasomal complexes in the presence of wheat sprout extract.

20S proteasomes were preincubated for 30 min at room temperature in 50 mM Tris–HCl pH 8.0 with 40 μg of GAE/ml of extract. Then 25.5 μg of treated proteasome were incubated with 100 μg of β-casein and 50 mm Tris–HCl, 20 mM $MgCl_2$, pH 7.5 up to a final volume of 100 μl. The mixture was kept at 37 °C, and 20 μl aliquots were withdrawn at different times (from 0 to 120 min), acidified with 2 μl TCA 10%, and subjected to HPLC on a Hamilton PRP-3 column (4.1 × 150 mm) by an Äkta HPLC system (GE Healthcare Biosciences, Sweden).

Samples elution and the measurement of casein degradation were carried out as reported previously [33,34].

The rate of casein degradation was determined by measuring the peak height of casein ($\lambda = 210$ nm). Control experiments were performed in the absence of extract. Each experimental set was repeated three times and relative mean values and standard errors calculated.

2.6. Cell treatment with wheat sprout extract

Human colon adenocarcinoma (Caco) cell line was cultured in DMEM supplemented with 15% fetal calf serum, 1% non-essential amino acids, 0.4% L-glutammine, penicillin and streptomycin. Cells were grown in 100 mm tissue culture dishes and maintained in a 5% CO_2 atmosphere at 37 °C. After 24 h of starvation they were treated with the extract (0 and 0.137 mg of GAE/ml of medium) for 4, 8, 12, 24 and 48 h. The experiment was performed in the presence and absence of 0.1 μM 12-*O*-tetra-decanoylphorbol-13-acetate (TPA). After removing the medium, cells were harvested in PBS, centrifuged and the pellet was resuspended in a lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12,000 × *g* for 15 min and the supernatants were stocked at −80 °C. Protein concentration in cell lysates was determined by the method of Bradford [35] using bovine serum albumine as a standard.

2.7. Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [36,37]. After experimental treatment, MTT was added to culture medium at a final concentration of 0.5 mg/ml and incubated for 2 h at 37 °C. The medium was replaced with 100 μl of DMSO and the optical density was measured at 550 nm in a microtiter plate reader. At least six cultures were utilized for each time point.

2.8. Proteasomal activities on Caco cell lysates

Activity assays with cell lysates (5 μg of total proteins in the mixture) were performed using the substrates listed above; control experiments were done in the presence of specific inhibitors: Z-Gly-Pro-Phe-Leu-CHO and lactacystin (5 μM in the reaction mixture). Tests for the BrAAP activity were performed in the presence of AP-N (EC 3.4.11.2) [9].

Incubation was carried out at 37 °C for 60 min, then the measurements of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (PABA) were detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA: $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm) on a spectrofluorimeter Shimadzu RF5301.

2.9. Western blotting analyses

Intracellular levels of p27 and poly (ADP)-ribose polymerase (PARP) proteins after extract treatment were analyzed through western blotting assays.

Cell lysates (30 μg) were loaded on 12% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes. After incubation with anti p27 and anti full length PARP antibodies, the immunoblot detections were carried out with Enhanced ChemiLuminescence western blotting analysis system (Amersham-Pharmacia-Biotech). Every gel was loaded with molecular weight markers including proteins with MW from 6.5 to 205 kDa (SigmaMarker – Wide Molecular Weight Range, Sigma-Aldrich S.r.l., Milano, Italy). As a control for equal protein loading glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized: membranes were stripped and reprobed for GAPDH using a monoclonal antibody diluted 1:500. A densitometric algorithm has been developed to quantitate the Western Blot results. Each Western Blot film has been scanned (16 bits greyscale) and the obtained digital data were processed to calculate the background mean value and its standard deviation. The background-free image was then obtained subtracting the background intensity mean value from the original digital data. The integrated densitometric value associated to each band was then calculated as the sum of the density values over all the pixels belonging to the considered band having a density value higher than the background standard deviation. The band densitometric value was then normalized to the relative GAPDH signal intensity. The ratios of band intensities were calculated within the same Western Blot. All the calculations were carried out using the Matlab environment (The MathWorks Inc., MA, USA) [38].

2.10. Determination of protein carbonyl content

Immunoblot detection of carbonyl groups was performed with the OxyBlot oxidized protein detection kit (Appligene-Oncor, Strasbourg, France), according to the manufacturer. Briefly, 15 μg of cell lysates were incubated for 15 min at room temperature with 2,4-dinitrophenylhydrazine to form the dinitrophenylhydrazone carbonyl derivate and separated on a 12% SDS/PAGE. The modified proteins were separated by SDS-PAGE (12%), transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA) and revealed by anti-dinitrophenylhydrazone antibodies as specified in the OxyBlot data sheet.

2.11. Statistical analysis

Values are expressed as mean values and standard error of results obtained from separate experiments. Student's *t*-test was used to compare differences of means between control

Table 1
Polyphenol concentrations present in the wheat sprout hydroalcolic extract

Polyphenol compound	mg/ml of extract
Catechin	4.375
Epicatechin	3.425
Epigallocatechin	1.8375
Epigallocatechin gallate	1.340
Gallic acid	0.0125

Quantitative analysis was performed by calibration curves using the reference standards of ellagic acid, gallic acid, catechin, epicatechin, epigallocatechin and epigallocatechingallate, luteolin and quercetin, as described in Section 2.

and treated groups in the densitometric analyses of western blottings.

Analysis of variance (ANOVA), followed by the Holm-Sidak test for multiple comparisons, was used to assess differences among multiple sets of data obtained with cells untreated and treated with the extract in the presence and absence of TPA. Statistical tests were performed with Sigma-Stat 3.1 software (SPSS, Chicago, IL). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Analysis of polyphenol content

Quantitative measurement of the TPC was performed with the Folin-Ciocalteau reagent [29,30].

The TPC wheat sprout extract was 4.7 mg of gallic acid equivalent (GAE)/ml of extract (1.0 ml of extract corresponds to 1.0 g of dehydrated wheat sprouts).

Moreover, molecular polyphenol identification was determined by RP-HPLC analysis comparing the retention time of reference standards. The reference standard mixture (panel A) and the extract (panel B) elution profiles are shown in Fig. 1.

Comparing the retention time of the peaks obtained from the wheat sprout hydroalcolic extract with the peaks obtained from the standard mixture, it was possible to identify five

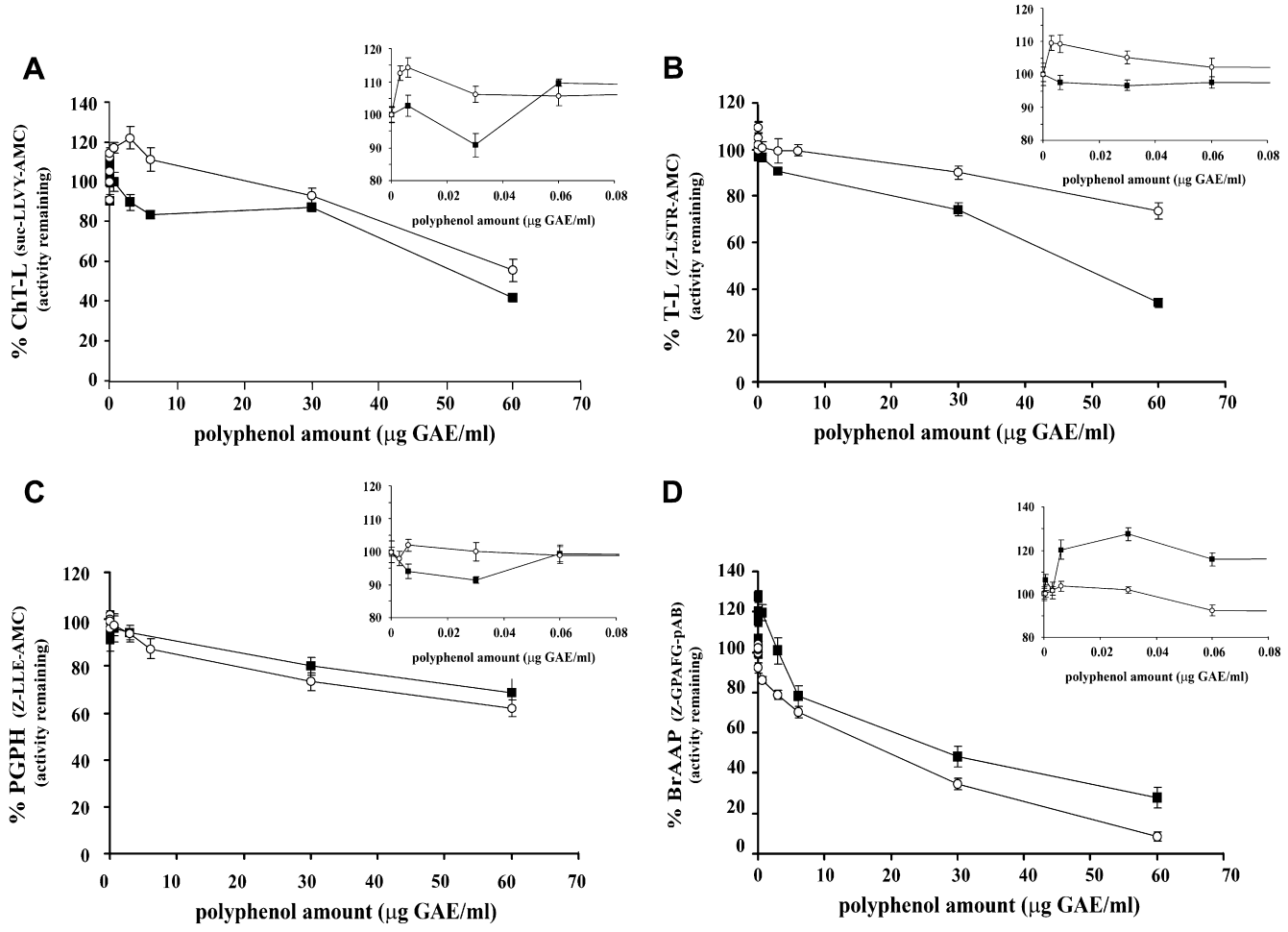

Fig. 2. Effect of wheat sprout extract on the activity of isolated 20S proteasomes. Effect of increasing amounts of wheat sprouts extract (from 0 to 60 μg GAE/ml) on the ChT-L (panel A), T-L (panel B), PGPH (panel C) and BrAAP (panel D) proteolytic components of constitutive (■) and immuno (○) 20S proteasomes isolated from bovine brain and thymus. The graph inserts represent proteasome activities in the presence of low concentrations of extract. The incubation mixture contained the extract, 5 μg of isolated 20S proteasome, the appropriate fluorogenic substrate and 50 mM Tris–HCl pH 8.0 up to a final volume of 500 μl. Incubation was carried out at 37 °C and, after 60 min, the measurements of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (PABA) were detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA: $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm). The data shown are mean values ± SD from six distinct determinations.

components: gallic acid, epigallocatechin, catechin, epicatechin and epigallocatechin gallate. The differences observed between the elution times related to the extract and the standard mixture (Fig. 1) could be due to the matrix effect. This effect depends upon variations of ionization efficiency in the presence of coeluting substances and causes analytes to elute at different retention times, considering the same polyphenolic compound dissolved either in a specific buffer or in a whole vegetable extract.

The amounts of each identified polyphenol were determined by RP-HPLC analysis using as standards the peak area values obtained by serial dilutions of the polyphenols standard mixture. Data reported in Table 1 show the polyphenol concentrations present in the wheat sprout hydroalcolic extract. The extract has a high catechins content, in particular catechin, epicatechin and epigallocatechin gallate.

3.2. Effect of wheat sprout extract on isolated 20S proteasomes functionality

Earlier studies have suggested that ester bond-containing tea polyphenols potently and selectively inhibit the chymotrypsin-like activity of the 20S proteasome isolated from *Methanosarcina thermophile* [21,22].

Moreover, we have previously demonstrated the effects of polyphenolic compounds on the functionality of isolated 20S proteasomes from bovine brain and thymus showing a relevant inhibitory action on the ChT-L component of EGCG and gallic acid [5]. Additionally, in this work and in previous reported data, the antioxidant characteristics of EGCG have been well addressed [5,39–42].

Taking into account the high content in polyphenols, mostly catechin and epicatechin, of the wheat sprout extract we have determined whether the extract could be effective on influencing the proteasome functionality. With this aim, we have tested the effect of increasing amount of wheat sprout extract on the proteolytic activities of constitutive and interferon-γ inducible 20S proteasomes isolated from bovine brain and thymus, respectively.

Fig. 2 shows the inhibitory effect of increasing amounts of wheat sprouts extract (from 0 to 60 μg GAE/ml) on the ChT-L (panel A), T-L (panel B), PGPH (panel C) and BrAAP (panel D) proteolytic components of constitutive (solid square) and immuno (circle) 20S proteasome.

All assayed activities were inhibited by the extract, with the BrAAP component being the most affected whereas the PGPH the least influenced. The constitutive and the immunoproteasome were both affected by the action of the extract showing some differences in the response to the treatment. At low

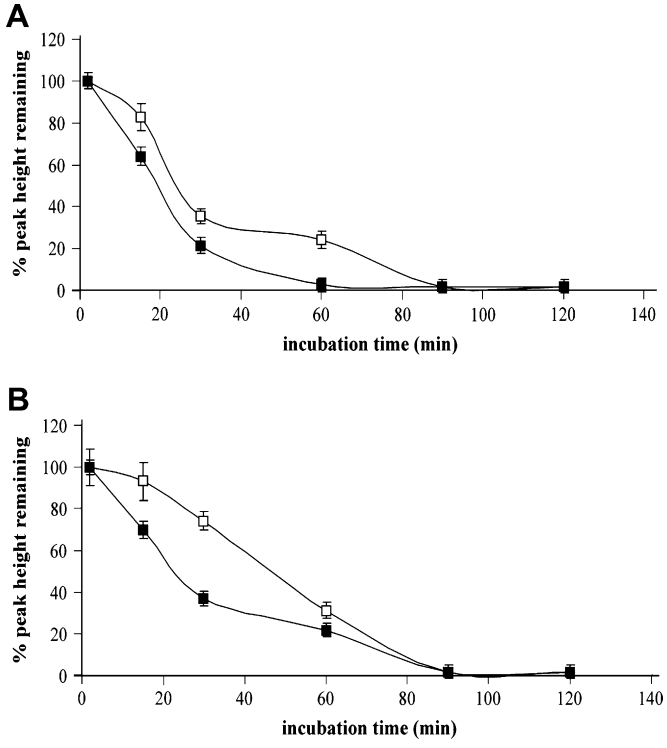

Fig. 3. Proteasomal degradation of β-casein. Effect of wheat sprouts extract on β-casein degradation by constitutive (panel A) and immuno (panel B) 20S proteasomes isolated from bovine brain and thymus. 20S proteasomes were pre-incubated for 30 min at room temperature with 40 μg of GAE/ml of extract. The treated proteasome was incubated with the β-casein, kept at 37 °C, and an aliquot was withdrawn at different times (from 0 to 120 min), acidified with 2 μl TCA 10%, and subjected to HPLC. The rate of casein degradation was determined by measuring the peak height of casein ($\lambda = 210$ nm) and it is represented as percent value of the control (0 min). Control experiments were performed in the absence of extract (■). Each experimental set was repeated three times and relative mean values and SD calculated.

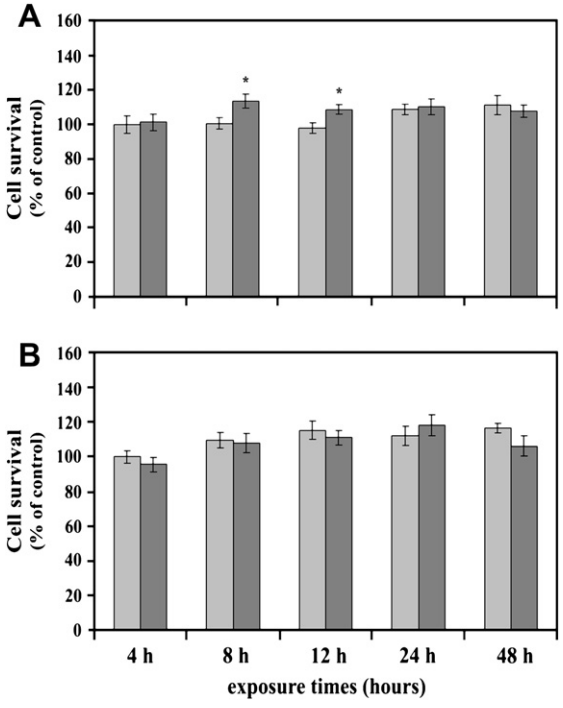

Fig. 4. Cell survival analysed with the MTT assay. Graphs related to cells viability of Caco cells treated with wheat sprout extract (0.137 mg of GAE) over times (4, 8, 12, 24 and 48 h) in the absence (panel A) and presence (panel B) of TPA 0.1 μM. Data are reported as % of cell viability (dark grey: treated cells; light grey: controls). Results are representative of three distinct experiments. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells (*$p < 0.05$).

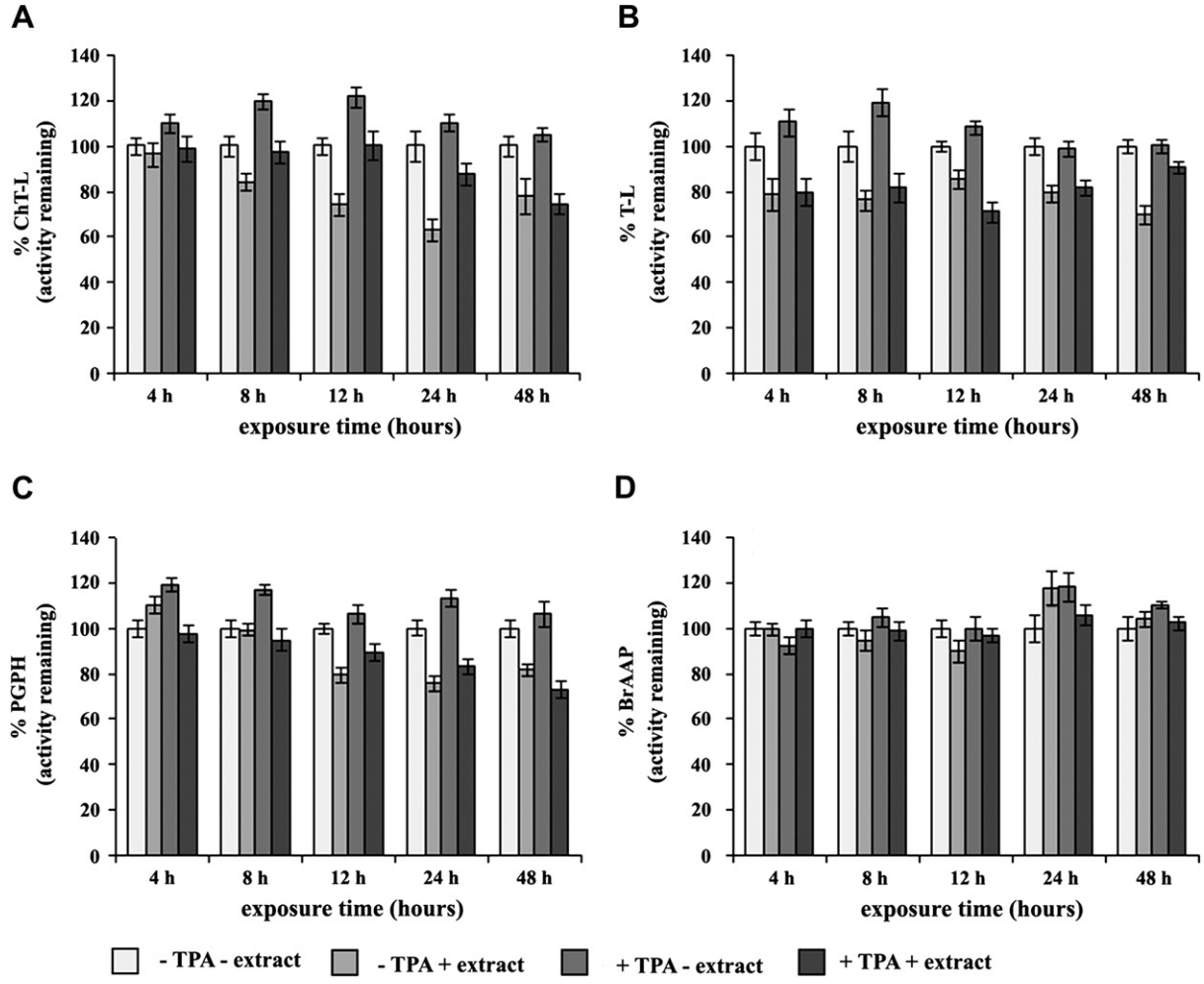

Fig. 5. Proteasomal activities measured in cell lysates upon treatment with wheat sprout extract. Caco cells were cultured in the presence and in the absence of wheat sprout extract and 0.1 μM TPA for 4, 8, 12, 24, and 48 h. Lysates were tested for the ChT-L (panel A), T-L (panel B), PGPH (panel C) and BrAAP (panel D) activities, using fluorescent substrates (suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC, Z-Gly-Pro-Ala-Leu-Ala-pAB respectively). Data are expressed as % activity remaining towards control in each time set. Fluorescence units were subtracted of the values of control assays in the presence of specific inhibitors; results derived from six independent experiments. Values represent mean ± SD ($n = 6$). $p < 0.05$ by ANOVA with post-hoc Holm-Sidak test.

extract concentrations the ChT-L and T-L components of the constitutive proteasome and the BrAAP activity of the immunoproteasome were weakly activated. This feeble activation could be due to a rearrangement of proteasome structure induced by low extract concentration that facilitates substrate entry into the proteasome active sites [43].

Furthermore, the 20S proteasomes proteolytic activity towards β-casein was examined upon treatment with 40 μg GAE/ml of extract. Both 20S proteasomes degrade β-casein within 90 min incubation (Fig. 3, panels A and B). The presence of extract slows down the degradation rate at short incubation times, especially with the immunoproteasome (Fig. 3, panel B), inducing no changes on the degradation pattern as evidenced by the elution profiles (data not shown).

3.3. Cell treatment with wheat sprout extract

Treatment of Caco cell line with wheat sprout extract over times (4, 8, 12, 24 and 48 h) did not induce changes in cell survival towards control cell, both in the presence and absence of TPA (100 nM), (Fig. 4). In fact, even if two data points show a statistically significant variation between control and treated cells, the differences are always below 6% and therefore cannot be considered as biologically relevant.

Being the tumor promoter TPA also responsible for an increase of oxidative stress and an upregulation of the proteasome proteolytic pathway [24] we wanted to check whether treating the cells with the extract could reverse the effects induced by TPA.

Caco cell lysates were assayed for proteasomal ChT-L, T-L, PGPH and BrAAP activities (Fig. 5). The extract induced inhibition of the proteolytic components, mainly of the ChT-L (40% inhibition at 24 h) and T-L activities (around 30% at 48 h) (Fig. 5, panels A and B). TPA effects were particularly evident for the ChT-L, T-L and PGPH activities. Besides that, the overall activation at short incubation times produced by TPA was interfered with the wheat sprout extract treatment; in detail, co-exposing the cells to TPA and extract resulted in a decrease of the ChT-L, T-L and PGPH components (Fig. 5, panels A–C). For the BrAAP activity, control assays were

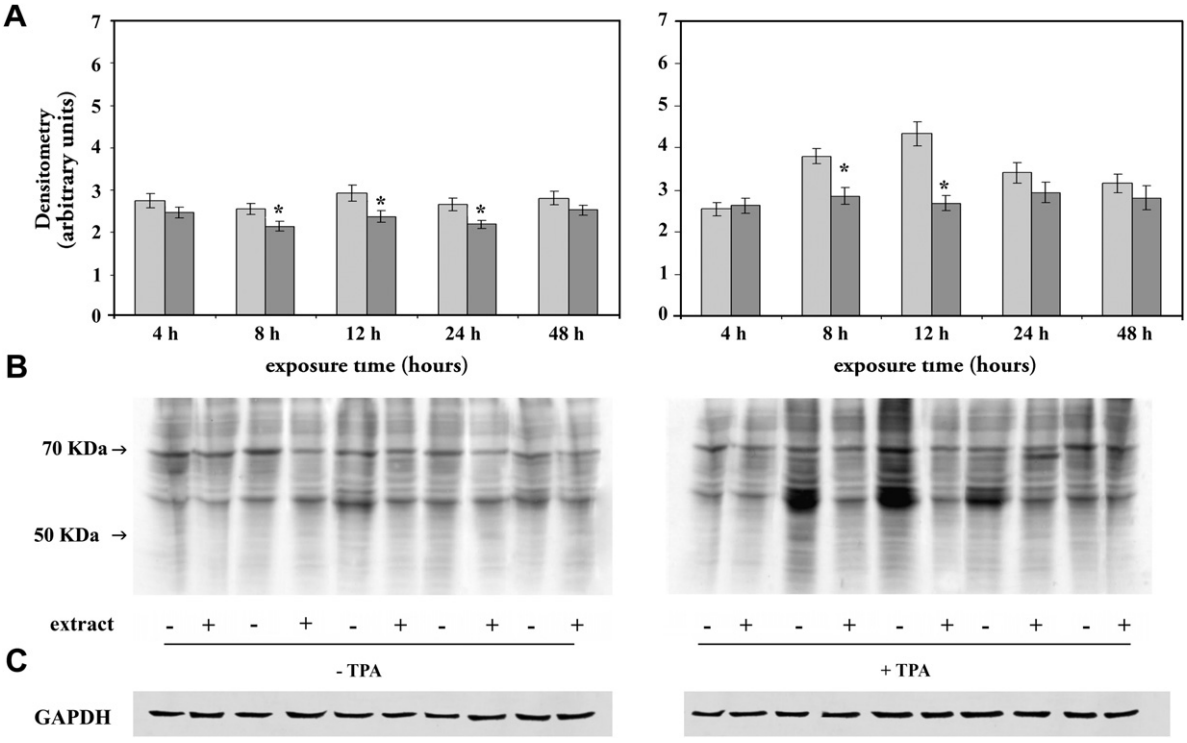

Fig. 6. Effect of wheat sprout extract treatment on protein carbonyl groups levels. Measure of protein carbonyl groups levels in Caco cells treated with the extract. The densitometric analysis from six separate blots provided for quantitative analysis is presented (panel A) and a representative Western blot is shown (panel B). Equal protein loading was verified by using an antibody directed against GAPDH (panel C). Caco cells were cultured in the presence and in the absence of wheat sprout extract and 0.1 μM TPA for 4, 8, 12, 24 and 48 h. After harvesting and lysing the cells, samples were subjected to SDS-PAGE and electroblotted on a polyvinylidene fluoride membrane. The immunostaining was performed using antibodies against derivatized protein carbonyl groups and the detection was executed by Enhanced ChemiLuminescence western blotting analysis system. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells (*$p < 0.05$).

performed in the presence of the specific inhibitor Z-GPFL-CHO in order to verify the effective contribution to the proteolysis of this component. The ability of this compound to interact with the proteasome has been already demonstrated, suggesting a strong inhibitory effect of Z-GPFL-CHO specifically toward the BrAAP component [34,44]. A 80% decrease in BrAAP activity was obtained in the presence of the inhibitor (data not shown).

In order to evaluate if the TPA-associated upregulation of the proteasome system was caused by an increase in oxidized proteins, due to an enhancement of oxidative conditions induced by phorbol esters, cellular protein carbonyls content was measured performing immunoblot analyses using antibodies against derivatized protein carbonyl groups (Fig. 6).

Wheat sprout extract, clearly, had an antioxidant effect on Caco cells. In fact, the densitometric analysis of the blotting show an evident decrease of signal in the presence of wheat sprout extract. TPA induced, essentially, an increase in the intracellular level of oxidized proteins and the extract was able to attenuate this effect.

With the aim of estimating the proteasome functionality we also determined the intracellular levels of p27, whose degradation is known to be mediated by the proteasome [45]. p27 is involved in the apoptotic cascade; its increase, in fact, leads to the activation of pro-apoptotic mechanisms [46]. Treatment with the extract induced an increase of p27 levels (Fig. 7). TPA, promoting a stimulation of the proteasome activity, reduced p27 intracellular levels and its effect was lowered after adding wheat sprout extract.

To check whether, upon wheat sprout hydroalcolic extract treatment, in Caco cells the apoptotic pathway is triggered, we measured the intracellular level of Poly (ADP-ribose) polymerase (PARP) using an antibody that recognizes the full length molecule (116 kDa). PARP is a nuclear enzyme that signals the presence of DNA damage by catalyzing the addition of ADP-ribose units to itself, DNA, histones, and various DNA repair enzymes and by facilitating DNA repair [47]. Furthermore, PARP is a substrate of caspase-3 and its fragmentation is regarded as a hallmark of apoptosis [48].

As it is shown in Fig. 8, in unstimulated cells PARP was not affected by the presence of the extract.

TPA stimulated PARP fragmentation, in fact, bands related to full length PARP are darker in the absence of phorbol ester. The extract did modulate the expression level of full length PARP, inhibiting the TPA-mediated decrease of PARP at 8 and 12 h.

4. Discussion

Wheat sprout extract is a rich source of antioxidants [1], such as reducing glycosides and polyphenols, especially catechin, epicatechin and epigallocatechin gallate.

In recent years, health-promoting effects of wheat sprout extract are being examined. In fact antimutagenic properties

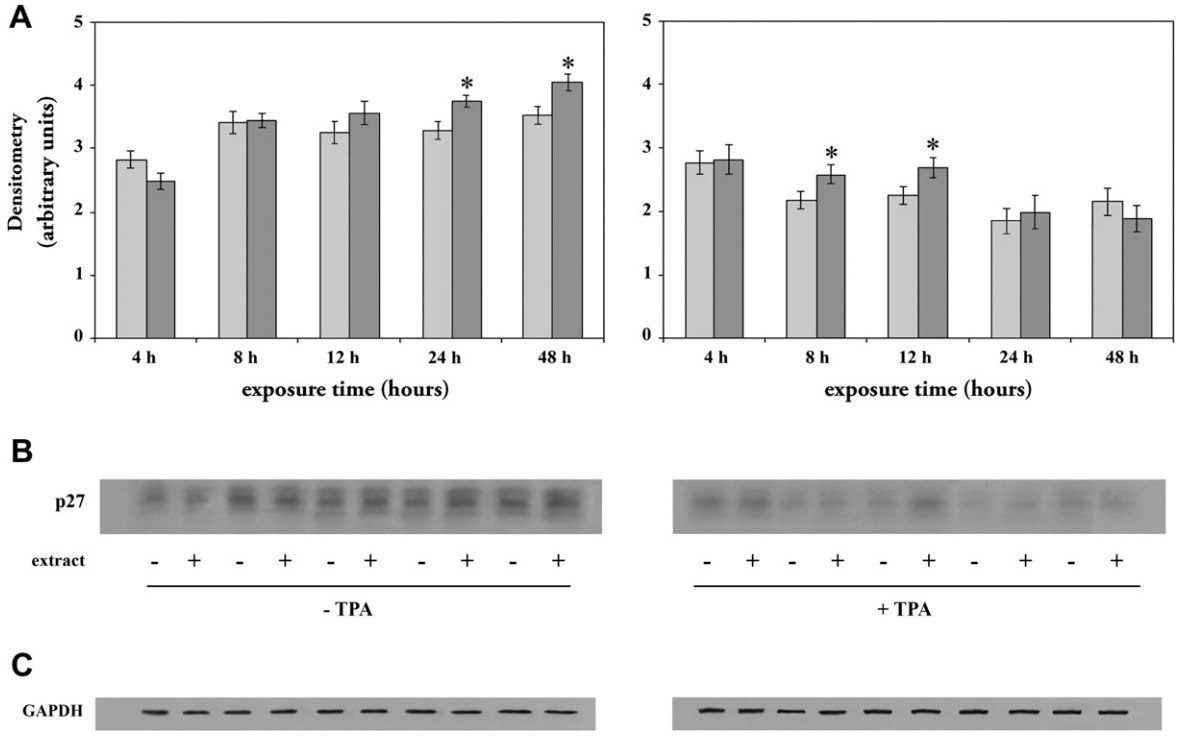

Fig. 7. Effect of wheat sprout extract treatment on p27 levels. Autoradiographs of p27 expression in Caco cells exposed to the extract. The densitometric analysis from six separate blots provided for quantitative analysis is presented (panel A) and a representative Western blot is shown (panel B). Equal protein loading was verified by using an antibody directed against GAPDH (panel C). Caco cells were cultured in the presence and in the absence of wheat sprout extract and 0.1 μM TPA for 4, 8, 12, 24, 48 and 72 h. After harvesting and lysing the cells, samples were subjected to SDS-PAGE and electroblotted on a polyvinylidene fluoride membrane. The immunostaining was performed using anti-p27 antibodies and the detection was executed by Enhanced ChemiLuminescence western blotting analysis system. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells (*$p < 0.05$).

have been demonstrated [3,49] as well as the recovery of age-related alterations, such as senile cataract, and all the changes caused by an imbalance between free radical production and antioxidant defences [2,4].

Lately, we have reported that several antioxidant molecules affect 20S proteasomal systems, pointing at the strong inhibitory effect of EGCG on the 20S proteasome functionality depending on the complex subunit composition. In cell lysates, besides its role in the modulation of proteasome activities, especially the ChT-L, EGCG presents also antioxidant properties [5].

In the current study, we have characterized the polyphenolic components of wheat sprout extract, evidencing a high catechins content, in particular catechin, epicatechin and epigallocatechin gallate.

Furthermore, we have explored the potential effects of the extract on the functionality of isolated 20S proteasomes: constitutive and immuno 20S proteasomes undergo an overall inhibition but they are differently influenced by the extract; in detail, the BrAAP component, in both the proteasomes, is the most depressed activity, followed by the ChT-L and T-L.

Although EGCG is not the most abundant polyphenol found in the extract, its inhibitory concentration (IC) towards proteasome components is severely lower than the IC of the other polyphenols found in wheat sprout [5]. The diminished proteasome activity can be therefore considered the result of a global action of all the extract components, with the EGCG playing a major role among the different polyphenols.

The protein degradation rate, measured using β-casein as substrate for the proteasomal systems, has been also affected by the extract: a decrease of casein degradation rate has been detected at short incubation times, more evident for the immunoproteasome.

In Caco cell lysates, the extract has induced inhibition of the proteolytic components of the proteasome, in particular the ChT-L and T-L activities, confirming the inhibition obtained in *in vitro* experiments for these two components. Exposing the cells to TPA, an increase in protein carbonyl content and a matching upregulation of the proteasome activity have been determined; in fact, the increase in proteasome functionality measured at 8 and 12 h corresponds to an enhancement of oxidized proteins. This regulation of the proteasomal complex during and after oxidative stress is a complicated issue and several studies have been conducted on this field investigating proteasome response to oxidative stimuli. Previous works have demonstrated an enhancement of proteasome-mediated protein degradation after oxidative stress in different cellular systems [50–52]. The increase in proteasome proteolytic activity could be due to a stress-mediated rearrangement of the oxidized proteins which is responsible for the exposure of hydrophobic residues generally hidden in the normal structure. This conformational change allows the

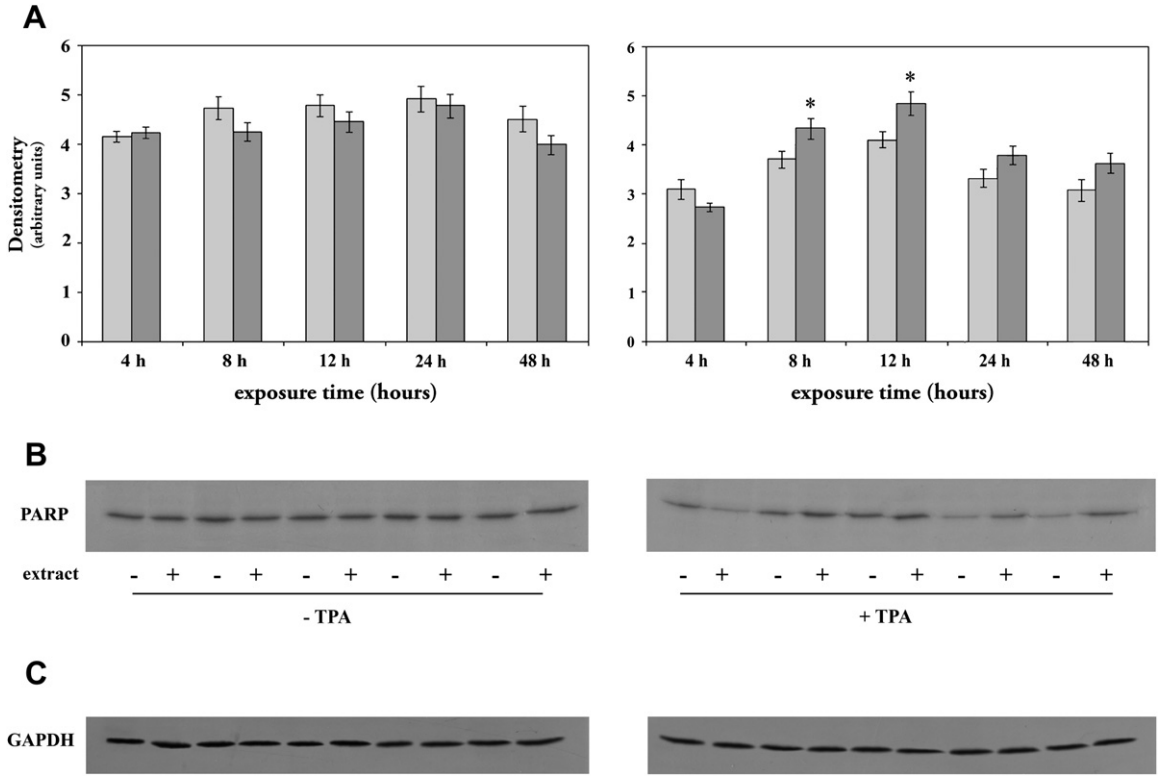

Fig. 8. Effect of wheat sprout extract treatment on Poly (ADP)-ribose polymerase (PARP) levels. Autoradiographs of PARP expression in Caco cells exposed to the extract. The densitometric analysis from six separate blots provided for quantitative analysis is presented (panel A) and a representative Western blot is shown (panel B). Equal protein loading was verified by using an antibody directed against GAPDH (panel C). Caco cells were cultured in the presence and in the absence of wheat sprout extract and 0.1 μM TPA for 4, 8, 12, 24, 48 and 72 h. After harvesting and lysing the cells, samples were subjected to SDS-PAGE and electroblotted on a polyvinylidene fluoride membrane. The immunostaining was performed using anti-PARP antibodies and the detection was executed by Enhanced ChemiLuminescence western blotting analysis system. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells ($*p < 0.05$).

proteasome to recognize and bind its substrate, and start the degradation process [53].

p27 cellular content has varied following the trend of the enzyme activities, further demonstrating the inhibition of the proteasome upon wheat sprout extract exposure. Interestingly, wheat sprout extract treatment has been able to abrogate the oxidative stimulus caused by TPA, restoring the proteasome functionality to a basal level.

We also checked whether the accumulation of p27 was able to initiate the apoptotic cascade, estimating the fragmentation of PARP: we found that TPA-induced PARP degradation, the trigger for apoptosis, was contrasted by the extract.

In conclusion, wheat sprout hydroalcolic extract does modulate the proteasomal systems functionality, in a different way depending on the proteasome subunit composition, producing an overall inhibition, both in isolated and in cellular complexes.

A related change in intracellular levels of proapoptotic proteins, such as p27 and PARP, has been detected. Worth to be highlighted is that treating the cells with TPA induces an increase in intracellular oxidative proteins which, in turn, could be responsible for stimulating the proteasome activities. Wheat sprout extract, besides its antioxidant effect, has been shown to be able to abrogate the TPA-mediated proteasome activation and the mechanisms related to it, bringing back cellular homeostasis. Therefore, the proteasome upregulation is not only dependent on NF-κB activation, as Wyke et al. have published [24,54], but also on the cellular oxidative protein levels. The proteasome inhibition induced by wheat sprout extract, here documented, may be a novel mechanism for the previously observed anticarcinogenic activity.

Further experiments will be performed in order to better characterize the wheat sprout extract composition. In addition, the effect of separated extract components on the proteasome functionality will be investigated in order to verify an alteration in the levels of cell cycle regulatory proteins. Moreover, our future investigations will deal with the treatment of transformed and normal cells with the wheat sprout extract to determine a potential different sensitivity of the two cell lines. The aim of this analysis will be to evaluate the possible chemopreventive activity of the extract for a potential application as an anticancer drug.

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