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Water Soluble *Pleurotus ostreatus* Polysaccharide Down-Regulates the Expression of MMP-2 and MMP-9 in Caco-2 Cells

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Abstract

Many polysaccharides and polysaccharide-protein complexes isolated from mushrooms have immunomodulatory and anti-cancer effects. Our aim was to study the regulatory mechanisms of Caco-2 cell response to water soluble *P. ostreatus* polysaccharide extract up to 72 hours. Specific enzymatic activities were assessed by kinetic measurements. The reduced glutathione content and the lipid peroxidation level were also analyzed. Protein expression of several heat shock proteins, Bcl-2 and metalloproteinases 2 and 9 were revealed by Western blot. Gelatin zymography assay was used to evaluate the MMP-2 and MMP-9 activities. Until the third day of exposure the total SOD activity decreased continuously by 30%, whereas GST and GR ones diminished by 17% respectively 30.5% compared to control. No significant changes were observed in CAT and G6PDH specific activities as well as in GSH and MDA concentration. After the third day of exposure a significant up-regulation of Hsp60 and Hsp90 expression and a down-regulation of Hsp70 one were registered. Bcl-2 protein levels were down-regulated by 50% in the first day of treatment but increased after 3 days. MMP-2 and 9 secretion in the culture medium was significantly reduced suggesting a diminished ability of invasion of colon cancer cells. Our data revealed that in vitro treatment with *P. ostreatus* aqueous polysaccharide extract does not induce apoptosis in Caco-2 cell line but it could inhibit the invasion of colon cancer cells through the basement membrane.

Keywords: colorectal cancer cells, MMP 2 and 9, Hsp60, Hsp70, Hsp90, mushroom extract

Introduction

The colorectal cancer is the third most commonly diagnosed cancer and third leading cause of cancer death in both men and women all over the world. In order to control the cancer growth or relieve symptoms, chemotherapy is used but this has short as well as long term effects. The discovery and development of new safe drugs without side effects has become one of the most important goals of the biomedical science. Traditional therapies which used various indigenous plants in order to cure cancer could be a source of new therapeutic agents. Different types of natural compounds such as: flavonoids, polysaccharides, omega-3 fatty acids, non-flavonoid phenolic compounds, terpenes are efficient in this respect.

A large amount of fungal compounds like polysaccharides and polysaccharide-protein complexes with immunomodulatory and anti-cancer effects have been isolated from mushrooms (Wang *et al*., 2002; Wasser and Weis, 1999). There are approximately 650 representatives of higher *Basidiomycetes* that have been found to possess antitumor activity, among them species from genera like *Ganoderma*, *Grifola*, *Lentinus*, *Trametes* and *Pleurotus*. These are rich in β-glucans (Wasser, 2002).

The beneficial health properties of *Pleurotus ostreatus* are attributed to a wide range of bioactive components such as lovastatin (Gunde-Cimerman and Cimerman, 1995), amino-acids such as: cysteine, methionine and aspartic acid) (Mattila *et al*., 2002), proteins, carbohydrates, minerals (as calcium, iron and phosphorus) and vitamins (thiamin, riboflavin and niacin) (Wolff *et al*., 2008).

There are some reports concerning the antioxidant properties of *P. ostreatus* petroleum ether and acetone (Iwalokun *et al*., 2007) and methanolic (Kim *et al*., 2009) extracts as well of its polysaccharides (Vamanu, 2012).

The aim of this study was to investigate the regulatory mechanisms of Caco-2 cell response to water soluble *P. ostreatus* polysaccharide.

Materials and methods

Chemicals

Trypsin, ethylenediaminetetraacetic acid (EDTA), Bradford reagent, Tris, thiobarbituric acid (TBA) and malondialdehyde tetraethylacetal (MDA) were from Sigma–Aldrich (GmbH, Steinheim, Germany). Sodium bicarbonate ($NaHCO_3$), trichloroacetic acid (TCA) and hydrochloric acid (HCl) were from Riedel-de Haen (Sigma-Aldrich, Seelze, Germany). Nicotinamide adenine dinucleotide phosphate disodium salt (NADP+), nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) and hydrogen peroxide (H_2O_2) were supplied by Merck (Darmstadt, Germany). The Detect X® Glutathione Colorimetric Detection Kit was purchased from Arbor Assay (Michigan, USA). Chromogenic Western Blot Immunodetection Kit anti-mouse was from Invitrogen (Carlsbad, California, USA). Primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals used were of analytical grade. Modified Eagle's Medium (MEM), fetal bovine serum (FBS), antibiotic mix and phosphate buffer saline (PBS) purchased from Gibco-Invitrogen (Grand Island, NY, USA). For the preparation of all the solutions Milli-Q ultrapure water was used.

Cell culture and treatment

The human epithelial colorectal adenocarcinoma cells (Caco-2) were obtained from American Type Culture Collection (ATCC HTB-37) and were grown in MEM medium (containing non-essential amino acids, Earle's salts, 2 mM L-glutamine, 1 g/L glucose, 1500 mg/L sodium bicarbonate, 1 mM sodium pyruvate) supplemented with 20% fetal bovine serum and 1% antibiotic mix (penicillin, streptomycin, amphotericin) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. For treatment, 5 x 104 cells/mL were seeded onto 75 cm^2 culture flasks and allowed to adhere for 48 hours. At approximately 70% confluence, Caco-2 cells were synchronized in G0 by their incubation for 18 h in FBS-free medium. After cell synchronization, they were treated with *P. ostreatus* extract at a final concentration of 0.5 mg% polysaccharides for one and 3 days. Untreated Caco-2 cells were used as controls.

Preparation of the Pleurotus ostreatus polysaccharide extract

The *P. ostreatus* extract was obtained according to the method of Lavi *et al*. (2006). An amount of 3.5 kg of *P. ostreatus* purchased from the market was dried for 48 hours at 75 °C and then grinded. The second step consisted in refluxing the powder for 3 times for 4 hours each at 60 °C with a mixture of chloroform and methanol (2:1 v/v). After the vacuum filtration and the reflux, the sediment was recovered and then refluxed 3 times for 3 hours each at 60 °C with a mixture of methanol and Milli-Q water. The next step involved the drying of the obtained extract at 50 °C overnight with ventilation. Another extraction in water at 75 °-95 °C was performed the next day and the solution was concentrated by evaporation. The extract was centrifuged at 1500 rpm and the sediment was washed in absolute ethanol at 40 °C. Centrifugation, sediment collection, water suspending and precipitation with absolute ethanol was repeated until the color of the sediment became lighter. Finally, the sediment was dried at 85 °C for 24 hours and grinded.

The concentration of total hexoses was determined by phenol sulfuric method (Dubois *et al*., 1956) using carboxymethyl-cellulose (CM-cellulose) as a standard.

Biochemical assays

a. Reduced glutathione determination

Reduced glutathione (GSH) level was assayed using a Glutathione Detection Kit (Arbor Assays DetectX) and following manufacturer's instructions. The cellular lysate, deproteinized with 5% sulfosalicylic acid, was analyzed spectrophotometrically for total and oxidized glutathione (GSSG) at 405 nm.

b. Malondialdehyde assay

MDA was assayed by a method described by Del Rio *et al*. (2003). A volume of 200 µL of sample properly diluted was mixed with 0.1 N HCl and incubated for 20 min at room temperature. Then, 0.025 M TBA was added, and the total volume was incubated for 65 min at 37 °C. Finally, a suitable volume of 10 mM PBS was added. The fluorescence of MDA was recorded using a 520/549 (excitation/emission) filter. A calibration curve with MDA in the range 0.05–5 µM was used to calculate the MDA concentration.

c. Enzymatic assays

The superoxide dismutase (SOD) (EC 1.15.1.1.) activity was measured by the method of Paoletti and Mocali (1990). One unit of SOD activity was defined as half-maximal inhibition.

The catalase (CAT) (EC 1.11.1.6) activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm, according to the method of Aebi (1974). The CAT activity was calculated in terms of U/mg protein, where one unit is the amount of enzyme that catalyzed the conversion of one µmole H_2O_2 in a minute.

The glutathione reductase (GR) (EC 1.6.4.2.) activity was determined according to the method of Goldberg and Spooner (1983). One unit of GR activity was calculated as one µmole of NADPH consumed per minute.

The glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49.) activity was assayed by the Lohr's (1974) method. The rate of NADPH formation was a measure of G6PDH activity and was followed at 340 nm.

The glutathione-S-transferase (GST) (EC 2.5.1.18.) activity was determined spectrophotometrically at 340 nm according to the method of Habig *et al*. (1974) by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH. One unit of GST activity was defined as the formation of one μmole of conjugated product per minute.

All the enzymatic activities, calculated as specific activities (units/mg of protein) were expressed as percents from controls.

d. Protein concentration

The protein content expressed as mg/mL was determined using Bradford's method with bovine serum albumin as standard (Bradford, 1976).

e. Western blotting

Sample extracts mixed with loading buffer containing 0.25M Tris-HCl, pH 6.8, 15% sodium dodecyl sulfate (SDS), 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue were boiled for 5 minutes. A quantity of 25 μg proteins from each sample was loaded onto SDS-polyacrylamide gels (10%) using the mini-cell system Mini Protean 3, Bio-Rad. The electrophoretic analysis was performed using a Tris 0.05 M -glycine 0.05 M with SDS 0.1% running buffer at 70V-90V, until adequate separation of the protein molecular weight markers (See Blue Plus2 Prestained Standard 1X) was achieved. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane using a wet-blot (Bio-Rad) transfer system at 350 mA for 3 hours. Standard transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol was used. The membranes were exposed overnight to mouse antibodies for human Bcl-2, heat shock proteins (Hsp60, Hsp70, Hsp90) and matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9) and then with alkaline phosphatase-conjugated anti-mouse secondary antibody for 30 min at room temperature. The detection was performed using the chromogenic substrate. Beta-actin was used as internal control. The immunoreactive bands were visualized and captured with BioCapt 12.6 softwared from Vilbert Lourmat and quantified with ImageJ software. All values of samples were referred to the respective β-actin values. Data were calculated from three independent experiments.

f. Gelatin zymography assay

The gelatinolytic activities of MMP-2 and MMP-9 secreted in culture medium were assayed by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml gelatin at 4 °C. Gels were run at 120 V, washed in 2.5% Triton X-100 for 1 h, and then incubated for 16 h at 37 °C in activation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$). After staining with Coomassie brilliant blue (10% glacial acetic acid, 30% methanol and 1.5% Coomassie brilliant blue) for 2–3 hours, the gels were de-stained with a solution of 10% glacial acetic acid, and 30% methanol for one hour.

g. Statistical Analysis

The values were expressed as means with standard deviations. The differences between controls and *P. ostreatus* extract treated samples were compared with the Student t-test using standard statistical packages. The results were considered significant if the p value was less than 0.05.

Results and discussions

Antioxidant enzymes

The exact mechanism of intestinal absorption of orally administrated β-glucan is not exactly known. This could be: nonspecific intestinal absorption, passage of β-glucans through the gap junction in the intestinal epithelial membrane, absorption through intestinal M cells (Kurcharzik *et al*., 2000), or absorption after binding to Toll-like receptor proteins in the intestinal lumen, and dendritic cell probing (Pamer, 2007).

Tab. 1 shows the effects of this polysaccharide on the activities of SOD, CAT, GR, GST and G6PDH in Caco-2 cells. The SOD activity decreased by 17% and 30%, after one and 3 days , whereas, for the same exposure time, the activity of CAT was not significantly modified.

Tab. 1. Specific activities of SOD, CAT, GST, GR and G6PDH in Caco-2 cells treated with 0.5 mg% polysaccharide up to 3 days of exposure. Activities were calculated as means ± SD (n = 3), *P0.05, **P0.01, ***P0.001 vs. control of each time interval

Time (days)	Sample	SOD (U/mg protein)	CAT (U/mg protein)	GST (U/mg protein)	GR (U/mg protein)	G6PDH (U/mg protein)
1	Control	0.367 ± 0.03	0.048 ± 0.003	0.294 ± 0.001	0.023 ± 0.0009	0.06 ± 0.006
	P. ostreatus	0.305 ± 0.004*	0.05 ± 0.003	0.332 ± 0.001	0.02 ± 0.0009*	0.063 ± 0.001
3	Control	0.397 ± 0.03	0.045 ± 0.002	0.731 ± 0.0007	0.024 ± 0.0007	0.057 ± 0.001
	P. ostreatus	0.281 ± 0.05	0.044 ± 0.001	0.608 ± 0.001*	0.016 ± 0.002*	0.055 ± 0.0004

The production of superoxide in the colon could be due to leukocyte invasion (Bedard and Krause, 2007) or to the presence of commensal microorganisms such as *Enterococcus faecalis* (Huycke *et al*., 2002). This anionic radical generates H_2O_2 and hydroxyl radical (Moore *et al*., 2004). H_2O_2 in the presence of Fenton metals, such as ferrous ions produces hydroxyl radicals. However, colon epithelial cells are also capable of generating superoxide (Perner *et al*., 2003) due to highly expressed NOX1, an important isoform of NADPH oxidases, which is highly expressed in the distal colon (Szanto *et al*., 2005). This anion would enter the cells through an anion channel (Bedard and Krause, 2007) inducing SOD activity (Niwa *et*

al., 1990). In our experiment, the total SOD activity decreased continuously in the three day interval. This suggests that level of superoxide diminished, possibly, due to the free radical scavenging and ferrous ion chelation abilities of the polysaccharide fraction (Vamanu, 2012; Xia *et al*., 2010). As a result, the quantity of generated H_2O_2 was probably low and no significant change in CAT specific activity was registered.

In the case of GST activity an important increase in the three day control cells compared with one day ones, could be noticed. This could happened due to the fact that Caco-2 cells express in a time-dependent manner several GST isoforms as previously was proved by Peters and Roelofs (1989).

Polysaccharide treatment generated a slight increase of about 13% of GST activity, after one day of exposure, and decreased after 3 days by 17% compared with control for each time interval (Tab. 1). GR specific activities decreased by 12% and 30.5% after one respectively 3 day treatment by *P. ostreatus* polysaccharide. At the same time, G6PDH activity did not vary significantly.

As GR activity decreased in a time dependent manner, probably the level of GSH was maintained by the activity of γ-glutamyl cycle and not by the reduction of the GSSG. Taking into account that the oxidative branch of pentose phosphate shunt generated almost the same quantity of NADPH it appears that this coenzyme was used in GSH de novo synthesis and not in GSH regeneration reaction.

Lipid peroxidation and GSH level

Our data revealed that MDA level, a marker of lipid peroxidation, did not vary significantly after exposure of Caco-2 cells to a dose of 0.5 mg% of *P. ostreatus* polysaccharide for one and 3 days (Tab. 2). After one day of treatment an insignificant decrease with 10% of MDA level occurred whereas after 3 days, the level of MDA returned to the control value.

Also, the results shown in Tab. 2, indicate that Caco-2 cells exposure to *P. ostreatus* water soluble polysaccharide resulted in an insignificant variation of GSH level after one respectively three days compared to controls.

Tab. 2. Lipid peroxidation and GSH concentrations in Caco-2 cells after treatment with *P. ostreatus* polysaccharide extract. Values are means ± S.D. (n = 3)

Time (days)	Sample	GSH (nmoles/mg of proteins)	Lipid peroxidation (nmoles MDA/ mg of proteins)
1	Control	9.19 ± 0.45	3.33 ± 0.18
	P. ostreatus	11.41 ± 0.22	3.01 ± 0.003
3	Control	9.67 ± 0.13	3.65 ± 0.09
	P. ostreatus	8.72 ± 0.29	3.59 ± 0.42

It seems that the slight increase of GSH in these cells, after the first day of exposure by *P. ostreatus* polysaccharide was noticed previously for other polysaccharides, i.e. the sulfated one extracted from algae (Silva *et al*., 2012) and *Sargassum* one (Zhang *et al*., 2011). In our case, possibly the lower level of MDA after the first day of exposure compared to control could be correlated with the slight increase of GSH concentration and GST activity. At three days post-exposure, the recover of MDA concentration might be due to the diminution of GST specific activity. Our data are different compared to previous findings which highlighted that the water-soluble polysaccharides from radix *A. sinensis* (Yang *et al*., 2007) and *H. cumingii* (Hu *et al*., 2010) lowered the lipid peroxidation in murine peritoneal macrophages and in rat brain.

Hsp60, Hsp70 and Hsp90 expression

Caco-2 cells have different levels of expression for Toll like receptor-2, and -4, which could recognize the *P. ostreatus* polysaccharide and activate signalling pathways in which Hsp60, Hsp70 and Hsp90 are involved (Furrie *et al*., 2005).

The analysis of expression of Hsp60, Hsp70 and Hsp90 in Caco-2 cells treated with 0.5 mg% of *P. ostreatus* polysaccharide revealed an insignificant variation after one day whereas after the third day of exposure a significant up-regulation of Hsp60 and Hsp90 expression and a down-regulation of Hsp70 were registered (Fig. 1).

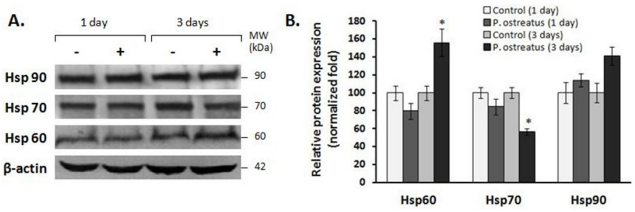

Fig. 1. Western blot analysis. (A) Detection of human Hsp60, Hsp70 and Hsp90 in Caco-2 cells untreated (control – lines 1and 3) and treated with 0.5 mg % P. ostreatus extract (lines 2 and 4) after 1 and 3 days of exposure (B) Quantitative analysis of the bands. Values are calculated as means ± SD (n = 3) and expressed as % from controls. *p < 0.05, vs. controls

The heat shock proteins are required for cell survival during stress. The intracelular reactions catalyzed by them are orientated to protein holding and protein folding (Buchner, 1999; Wegele *et al*., 2004).

The principal holding proteins belong to the Hsp70 and Hsp90 families, which bind to unfolded sequences in polypeptide substrates and show preference for hydrofobic regions. Hsp90 has a principal role in regulating mitogenesis and cell-cycle progression and Hsp70 is closely involved in protection from programmed cell death.

Hsp90 is also involved in the stabilization of mutant proteins as well as in activity of protooncogene HER2 and its downstream proteins, including the protein kinases Akt, c-Src and Raf-1, which have key roles in cell growth and survival (Calderwood *et al*., 2006). Furthermore,

it is necessary for induction and stability of vascular endothelial growth factor and nitric oxide synthase, which interact in a complex Hsp90-dependent signalling network generating new capillaries in tumors (Neckers and Ivy, 2003). On the other hand, Ghosh *et al*. (2008), using high throughtput proteomics screening, identified a close interaction between Hsp60 and survivin, a cell cycle regulator and apoptosis inhibitor. Previous studies revealed that Hsp60 is up-regulated in human cancers in vivo and determines the stabilization of survivin by the diminution of p53 function. Survivin is actively imported from cytosol in mitochondria. In cytosol this protein is associated with Hsp90 (Fortugno *et al*., 2003), which together with Hsp70 is implicated in mitochondrial pre-protein import (Young *et al*., 2003). The final result of these events is the cytoprotection of cancer cells.

The decrease of Hsp70 protein level in Caco-2 cells could be also due to its secretion through an unknown mechanism to exert functions in signal transduction (Broquet *et al*., 2003).

Determination of Bcl-2 expression

Bcl-2 protects cells from apoptosis induced by a variety of apoptotic stimuli. It also has other important functions including regulation of the cell cycle, modulation of cell differentiation, and the regulation of gene expression (Tsujimoto, 2003).

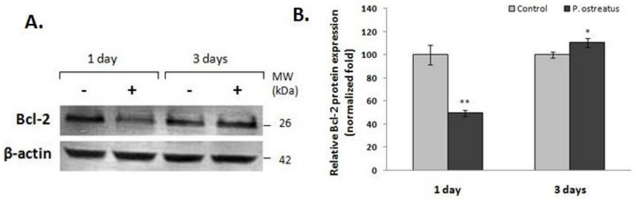

Fig. 2. Human Bcl-2 expression in Caco-2 cells untreated (control) and treated with 0.5 mg % *P. ostreatus* extract after 1 and 3 days of exposure. (A) Detection of Bcl-2 protein levels by western blot, (B) Quantitative analysis of the bands. Values are calculated as means ± SD (n = 3) and expressed as % from controls. *P < 0.05, **P < 0.01 compared with control

In our case, Western blot analysis has demonstrated that mushroom polysaccharide extract significantly down-regulated Bcl-2 protein levels by 50% in the first day of treatment (Fig. 2) that could suggest an apoptotic initiation in colon cancer cells. But after 3 days, the level of Bcl-2 expression increased by 10% compared to control, which means that the survival of Caco-2 cells was not affected at the highest exposure interval.

Previous studies revealed that HSPs inhibit apoptosis by selectively interfering with mitochondrial death signaling pathway in various cell types (Parcellier *et al*. 2003). For instance, Hsp90 plays an important anti-apoptotic role via regulation of the Bcl-2 expression (Dias *et al*. 2002). The increasing of Bcl-2 levels after 3 days could be possibly due to the increased expression of Hsp90 at the same time interval.

MMP 2 and MMP 9 levels

Matrix metalloproteinases (MMPs) are zinc–dependent endopeptidases which degrade the extracellular matrix and therefore play an important role in tumor invasion and metastasis (Westermarck and Kähäri, 1999). Among the members of the MMP family, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are particularly up-regulated in malignant tumors and contribute to the invasion and metastatic spread of cancer cells by degrading type IV collagen, a major component of the basement membrane (Vihinen and Kähäri, 2002). Caco-2 cells secrete MMP-2 and MMP-9, which degrade extracellular matrix, permitting cell migration (Kermogant *et al*., 2001).

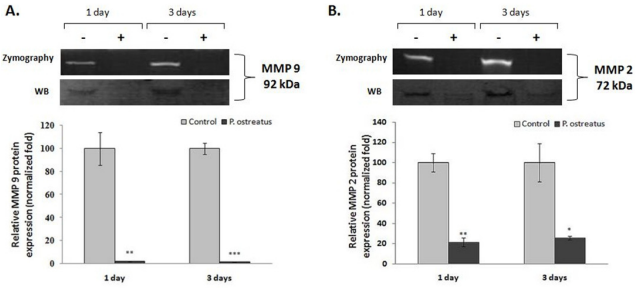

Fig. 3. Gelatin zymography and Western blot analysis of (A) MMP-9 and (B) MMP-2 in Caco-2 cells untreated (control) and treated with 0.5 mg % *P. ostreatus* extract after 1 and 3 days of exposure. Values are calculated as means ± SD (n = 3) and expressed as % from controls. *P < 0.05, **P < 0.01

Our study showed that treatment with *P. ostreatus* polysaccharide extract on human Caco-2 cells reduced the secretion of MMP-2 and MMP-9. Western blot analysis revealed that it down-regulates their protein expression as well. As a consequence, the invasion ability of colon cancer cells after one and 3 days of exposure could be diminished. Although, Bcl-2 was reported to activate MMPs (Choi *et al*., 2005), the up-regulation of Bcl-2 level after 3 days had no effect on MMP-2 and -9 expression.

Conclusions

In the case of our experiments, it appeared that exposure to the aqueous polysaccharide extract from *P. ostreatus* did not induce apoptosis in Caco-2 cell line as was highlighted previously by Lavi *et al*. (2006) in the case of HT-29 cells. But it seems that this extract could inhibit the invasion of Caco-2 cells through the basement membrane.

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