

Comparative studies on ability of *N*-acetylated chitooligosaccharides to scavenge reactive oxygen species and protect DNA from oxidative damage

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Antioxidants have key role in scavenging free radicals and have been considered as dietary part of health conscious population for reducing the risk of many diseases. In the present study, commercially available *N*-acetylated chitooligosaccharides [(GlcNAc)_n; n=2, 3, 5, 6] were studied for their antioxidant ability against superoxide, hydroxyl, DPPH radicals and H₂O₂ by various radical scavenging assays. The *N*-acetylated chitooligosaccharides exhibited radical scavenging activities in the range of 49.1-63% for hydroxyl radical and 41-53.3% for superoxide radical. Their antioxidant ability was at par or better than standard antioxidants like butylated hydroxyanisole and mannitol. These *N*-acetylated chitooligosaccharides were also evaluated for their ability to prevent oxidative DNA damage in peripheral blood mononuclear cells exposed to H₂O₂. (GlcNAc)₂, (GlcNAc)₃, (GlcNAc)₅ and (GlcNAc)₆ decreased the oxidative DNA damage due to H₂O₂ by 59.5, 55.5, 22.9 and 4.9%, respectively. This antioxidative potential of *N*-acetylated chitooligosaccharides can be extended to beneficial health applications.

Keywords: Antioxidant, *N*-acetylated chitooligosaccharides, comet assay, oxidative DNA damage, oxidative stress, reactive oxygen species

Introduction

The identification of low cost natural bioactive resources with antioxidant potential is gaining an increasing interest in the improvement of food quality and human health¹. Further, value added compounds from seafood waste are becoming an innovative alternative to synthetic dietary antioxidants in the global market. Less focused *N*-acetylated chitooligosaccharides (COS) have been prioritized in the present research since an extensive attention to the chitosan oligosaccharides has so far been given in many publications. COS are partially hydrolyzed products of chitin, one of the most abundant natural renewable bioresource materials from aquatic environment². Besides being water soluble, they are biodegradable, biocompatible and non-toxic³. They also possess antioxidant^{4,5}, anti-microbial⁶, anti-inflammatory⁷, anti-angiogenic and immune-stimulating properties⁸. Thus, to enhance the nutritional quality of food and human health, there is a great need to understand the antioxidant potential of COS to scavenge free radicals, especially reactive oxygen species (ROS) by various radical scavenging

assays and the protective effects of COS against oxidative DNA damage, which is the root cause of a number of diseases.

Recent studies have focused on ROS, which play a vital role in day to day life style activities. Oxidative stress (OS) is a redox status as a result of imbalance between pro-oxidant and antioxidant⁹. 'OS' is initiated by free radicals, which are highly reactive, causing injury to the healthy human cells and finally damaging the necessary biomolecules, such as, DNA, proteins, carbohydrates and lipids^{10,11}. The variety of diseases, viz., cancer¹², diabetes mellitus¹³, cardiovascular disease¹⁴, neurodegenerative disorders¹⁵ and aging process¹⁶ are caused due to 'OS'. The popularity of antioxidants, which prevent 'OS' and provide value added nutrition to the food by antioxidant therapy, is increasing day by day¹⁷. The antioxidant defense mechanism by scavenging free radicals is offered by many dietary antioxidants (α -tocopherol, β -carotenes, ascorbic acid, uric acid, etc.) and endogenous enzymes (catalase, superoxide dismutase, glutathione peroxidase/reductase etc.). However, synthetic antioxidants, such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have adverse side effects on human health¹⁸.

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Thus, attempts have been made to compare the prospective antioxidant potential of *N*-acetylated chitoooligosaccharides, which would be further beneficial as functional food. Current study was aimed to find out the *in vitro* free radical scavenging ability of commercially available COS [(GlcNAc)_n with degree of polymerization (n=2, 3, 5, 6)] against superoxide, hydroxyl, 2,2-diphenyl-2-picrylhydrazyl (DPPH) radicals and hydrogen peroxide (H₂O₂), which are normally found in the body. Protective effects of COS against oxidative DNA damage induced by H₂O₂ were also investigated by single cell gel electrophoresis (SCGE) or comet assay, which is a well known molecular technique for evaluating DNA damage at individual cell level.

Materials and Methods

Materials

COS [(GlcNAc)_n] with degree of polymerization (n=2, 3, 5, 6), phenazine methosulfate (PMS), NADH, nitroblue tetrazolium (NBT), deoxyribose and thiobarbituric acid, and low melting point agarose were purchased from Sigma Aldrich (St. Louis, MO, USA). (GlcNAc)₄ was discontinued by Sigma Company, hence was not included in the comparative studies and was not replaced with same product from other company to maintain uniformity of the preparations. Butylated hydroxyanisole (BHA) and mannitol was procured from HiMedia Laboratories Pvt. Ltd., (Mumbai, India). H₂O₂ (50% v/v) and all other chemicals used for comet assay were of highest analytical grade obtained from Thermo Fischer Scientific (Mumbai, India).

Evaluation of *In Vitro* Free Radical Scavenging Activity of COS

Superoxide Radical Scavenging Assay

Antioxidant ability of COS was assayed by superoxide radical scavenging activity by the method of Fernandes *et al*¹⁹ and Valentao *et al*²⁰. This was primarily evaluated by a non-enzymatic method, which was based on PMS/NADH (nicotinamide adenine dinucleotide) system for the generation of superoxide anion and reduction of NBT. The reaction mixture contained NADH (160 µM), NBT (25 µM) in sodium phosphate buffer, pH 7.4 (100 mM) and 200 µL of test sample (COS). The reaction started by addition of PMS (20 µM) followed by incubation at room temperature for 5 min and the absorbance was read at 560 nm against blank without COS and PMS. In control, COS was replaced with buffer. The percent

inhibition was determined by comparing the results of control and test samples by formula 1.

Superoxide radical scavenging activity (%) =

$$A_C - A_T / A_T \times 100 \quad \dots (1)$$

Where A_C is absorbance of control and A_T is absorbance of test sample at 560 nm.

Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging assay was performed as described by Elizabeth and Rao²¹. The assay is based on the generation of hydroxyl radicals by the Fe³⁺-EDTA-ascorbate-H₂O₂ system (Fenton reaction), which further attack 2-deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS). The degradation product of 2-deoxyribose, malonaldehyde is quantified by its condensation with thiobarbituric acid (TBA). The reaction mixture contained 2-deoxyribose (2.8 mM), FeCl₃ (100 µM), KH₂PO₄-KOH buffer, pH 7.4 (20 mM), EDTA (100 µM), H₂O₂ (1.0 mM), ascorbic acid (100 µM) and 200 µL test sample (COS) in a final volume of 1 mL. The mixture was incubated at 37°C for 1 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 0.6% aqueous solution of thiobarbituric acid (TBA) were added to 0.5 mL of reaction mixture mentioned above. This was heated at 95°C for 15 min, cooled and the absorbance of TBARS formed was measured at 532 nm against reagent blank without deoxyribose. Control sample was devoid of COS. The percent hydroxyl radical scavenging ability was calculated as a result of deoxyribose degradation, which was measured as TBARS. Percentage scavenging of hydroxyl radical was determined by the formula 2.

Hydroxyl radical scavenging activity (%) =

$$A_C - A_T / A_T \times 100 \quad \dots (2)$$

Where A_C is absorbance of control and A_T is absorbance of test sample at 532 nm.

H₂O₂ Scavenging Assay

This assay was performed according to the method of Wei *et al*²². The reaction mixture contained 200 µM phenol red, 10 µM H₂O₂ and 200 µL of test compounds (COS), which was incubated at room temperature for 5 min. Then 10 µL of horseradish peroxidase (10 U/mL) was added and further incubated for 5 min for oxidation of phenol red. This reaction was terminated by addition of 10 N NaOH.

The purple colour formed due to oxidation of phenol red was measured at 610 nm by keeping blank without horseradish peroxidase. Control was devoid of COS. The percent scavenging activity of H_2O_2 was calculated as described for superoxide radical scavenging assay by formula 2.

DPPH Radical Scavenging Assay

This assay is based on the reaction with purple coloured stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical, which is determined by the assay of Nagai *et al*²³. The reaction mixture contained 0.1 mL of 1 mM DPPH, 0.8 mL of 99% ethanol and 0.1 mL of the test compound (COS). The solutions were mixed rapidly and the scavenging capacity was measured by monitoring the absorbance at 517 nm. The bleaching of DPPH absorption is representative of the radical scavenging capacity of the test compound. The DPPH radical scavenging activity was estimated by formula 3.

DPPH radical scavenging activity (%) =

$$\frac{A_C - A_S}{A_S} \times 100 \quad \dots (3)$$

Where A_C is absorbance of control and A_S is absorbance of test sample at 517 nm.

Assessment of Protection of DNA Damage

The alkaline comet assay was carried out according to the method described by Singh *et al*²⁴ as previously carried out by Holz *et al*²⁵ with minor modifications. Briefly, the comet slides were prepared by sandwich method where frosted area of microscopic slides were half pre-coated with 400 μ L of 1.5% normal-melting point agarose to enhance the attachment of subsequent layers. A thin layer was obtained with the help of cover glass (22 $\times$ 50 mm²; No.1) and air dried for 15 min. Peripheral blood mononuclear cells (PBMC) were treated with 100 μ M of H_2O_2 (oxidant) for 10 min at 4°C to induce DNA damage. After treatment, the cells were centrifuged at 3000 g for 5 min and the pellet washed with PBS. 2×10^6 cells/mL were then embedded in 1:10 (v/v) proportion in 1% low melting point agarose and 85 μ L of this suspension was placed on the top of first agarose layer, covered with the coverglass and kept at 4°C for 5 min to solidify. The cover glass was removed after solidification and 80 μ L of 1% low melting point agarose was added as third layer, covered with coverglass and kept at 4°C for another 10 min. After solidification, the cells were lysed for 1 h in prechilled lysis buffer (2.5 M NaCl, 100 mM

Na_2 -EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO & 1% Sarcosine, pH 10.0) at 4°C without coverglass. DNA unwinding was carried out by placing the slides for 20 min in alkaline electrophoresis buffer (300 mM NaOH & 1 mM Na_2 -EDTA, pH > 13). The electrophoresis was run at 4°C for 25 min (25 V, 300 mA). The slides were gently washed 3 times in neutralization solution (0.4 M Tris, pH 7.5) for 5 min each and then stained with 50 μ L of propidium iodide solution (10 μ g/mL). All the above mentioned steps were performed in the dark to minimize additional DNA damage. The slides were observed immediately after 15 min of staining under a fluorescent microscope at 100 $\times$ magnification (Olympus CX41, Olympus optical Co., GmbH, Hamburg, Germany) attached to a ProgRes C3 camera connected to a computer.

To determine the sublethal concentration of H_2O_2 , a dose response experiment was carried out where PBMC were exposed to various concentrations (25, 50, 100 & 200 μ M) of H_2O_2 as described above. The control contained cells with phosphate buffered saline (PBS) throughout the experiments to check the endogenous DNA damage. The probable genotoxic effect of COS at 100 μ M was also determined. To evaluate the protective effects of COS against oxidative damage induced by H_2O_2 , cells were initially treated with COS for 10 min at 4°C, centrifuged at 3000 g for 5 min, washed with PBS and then treated with 100 μ M of H_2O_2 for 10 min at 4°C. The cells were washed again with PBS and then processed for comet assay. Cell viability for each treatment was determined by trypan blue dye exclusion assay.

All the experiments were carried out in triplicates. Single cells were analyzed by ImageJ 1.48v analysis software with OpenComet 1.3 as plug-in²⁶. The calculations were done on the basis of random selection of 50 individual cells in triplicates. The induced DNA damage was expressed by one of the most common comet parameters, % DNA in tail²⁷ by formula 4.

$$\% \text{ DNA in tail} = \frac{\text{Intensity of tail DNA}}{\text{Intensity of cell DNA}} \times 100 \quad \dots (4)$$

Statistical Analysis

All *in vitro* radical scavenging tests were performed in triplicates. The results from the assays were expressed as mean $\pm$ standard deviation (SD) of three measurements (n=3). The data of the comet

assay was subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test by using GraphPad Prism 6.0 (GraphPad Prism software, USA). The test criterion for statistical significance was $p < 0.05$. The graphs were plotted using Origin 6.0 statistical software.

Results and Discussion

In Vitro Free Radical Scavenging Activity of COS

Table 1 illustrates the free radical scavenging activity of the COS [(GlcNAc)_n with n=2, 3, 5, 6] using various radical scavenging assays against hydroxyl and superoxide radicals and H₂O₂, and their antioxidant ability was compared with the standard antioxidants, BHA and mannitol. The hydroxyl radical being highly potent oxidant reacts very fast with almost all biological macromolecules. The initiation of chain reaction occurs when it reacts with cell membrane leading to formation of lipid peroxides, which then further decompose to malondialdehyde (MDA) and cause DNA damage²⁰. In the present study, (GlcNAc)₅ exhibited the highest hydroxyl radical scavenging ability (63%) at 10 mM that was approx 3.6-fold higher as compared to that of mannitol (17.4%). COS [(GlcNAc)_n, n=2, 3, 6] have shown hydroxyl radical scavenging activity in the order of (GlcNAc)₆ > (GlcNAc)₂ > (GlcNAc)₃. The hydroxyl radical scavenging activity for (GlcNAc)₂ reported here is comparable to values reported by Azam *et al*²⁸ who demonstrated 51.56% for (GlcNAc)₂ produced by exolytic chitinase from *Bacillus cereus* EW5.

The scavenging of superoxide radicals was in the range of 41-53.3% in the order (GlcNAc)₂ > (GlcNAc)₅ > (GlcNAc)₆ > (GlcNAc)₃. These values were higher as compared to standard BHA (29.3%). H₂O₂ has the capacity to penetrate into the cell membrane and generates the hydroxyl radicals through Fenton reaction, thereby causing damage to

the cell²². We observed that (GlcNAc)₂ and (GlcNAc)₃ had very low hydrogen peroxide scavenging activity, whereas (GlcNAc)₅ and (GlcNAc)₆ did not exhibit this activity as compared to BHA (84.8%).

Thus all the COS analyzed were effective in the scavenging of free radicals in order of hydroxyl radical > superoxide radical > hydrogen peroxide. However, DPPH radical scavenging activity was not observed for any COS. It has been observed that most functions of the COS are clearly dependent on their mol wt²⁹. This suggests that protection against hydroxyl and superoxide radicals by COS is of great significance for their possible use as dietary antioxidants and functional foods contributing to the health benefits.

Effect of COS on Antioxidative DNA Damage

The protective effect of COS on PBMC against oxidative stress induced by H₂O₂ was investigated by comet assay. A linear increase was seen in DNA damage of PBMC with increase in concentration of H₂O₂ as shown in Fig. 1. The cell viability, determined by trypan blue dye exclusion assay, was above 90% for all the concentrations; except for 200 μM H₂O₂,

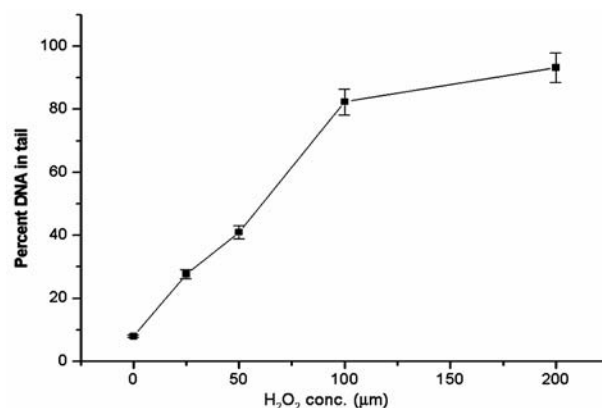


Fig. 1—DNA damage of PBMC exposed to H₂O₂. [Cells were treated with different concentrations of H₂O₂ for 10 min at 4°C in dark (n=3×50 PBMC, $p < 0.05$)]

Table 1—*In vitro* free radical scavenging activity of N-acetylated chitoooligosaccharides

Assay	Antioxidant activity (%)*					
	BHA	Mannitol	(GlcNAc) ₂	(GlcNAc) ₃	(GlcNAc) ₅	(GlcNAc) ₆
Hydroxyl radical scavenging activity	ND	17.40±0.40	57.10±0.20	49.10±0.15	63.00±0.35	61.20±0.30
Superoxide radical scavenging activity	29.30±0.20	ND	53.30±0.35	41.00±0.61	51.40±0.30	44.20±0.25
Hydrogen peroxide scavenging activity	84.80±0.15	ND	0.91±0.10	1.54±0.08	Nil	Nil

*% values were calculated from mean±SD of 3 replicates, Nil: No scavenging activity, ND: Not done

for which it was 72%. The cell viability was limited to $\geq 75\%$ according to Kiskinis *et al*³⁰. Based on the results, 100 μM was used as sublethal dose of H_2O_2 (82.32% DNA in tail) throughout the experiments. Fig. 2 shows the DNA damage due to COS where $(\text{GlcNAc})_2$, $(\text{GlcNAc})_3$, $(\text{GlcNAc})_5$ and $(\text{GlcNAc})_6$ did not show any significant oxidative damage to the cells as compared to the control (13.25, 11, 17.6 & 8.95% DNA in tail respectively). Furthermore, the COS $[(\text{GlcNAc})_n, n=2, 3, 5, 6]$ were tested at 100 μM for their DNA protective ability as shown in Figs 3 and 4. Our results indicate that $(\text{GlcNAc})_2$ and

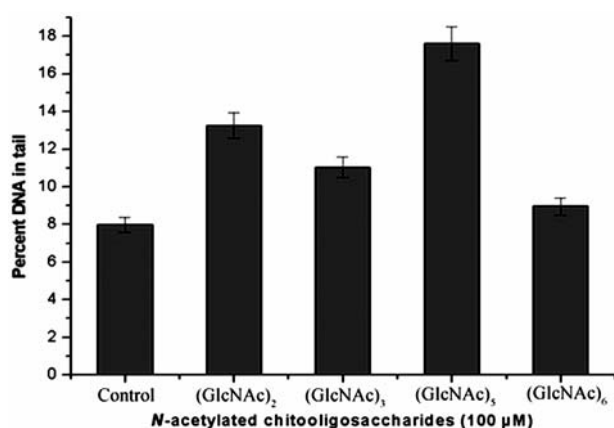


Fig. 2—Effect of $[(\text{GlcNAc})_n, n=2, 3, 5, 6]$ on PBMC treated with COS. [Cells were treated with 100 μM COS $[(\text{GlcNAc})_n, n=2, 3, 5, 6]$ for 10 min at 4°C in dark ($n=3 \times 50$ PBMC, $p < 0.05$)]

$(\text{GlcNAc})_3$ could significantly prevent DNA damage. DNA damage decreased to 24.51 and 28.58% for $(\text{GlcNAc})_2$ and $(\text{GlcNAc})_3$, respectively as compared to 84.1% damage induced by H_2O_2 . A relatively lesser protection was given by $(\text{GlcNAc})_5$ and $(\text{GlcNAc})_6$ where DNA damage was 61.24 and 79.25%, respectively. ANOVA followed by Tukey's multiple comparison test showed significant protection was offered by $(\text{GlcNAc})_2$, $(\text{GlcNAc})_3$ and $(\text{GlcNAc})_5$ compared to $(\text{GlcNAc})_6$ (correlation coefficient

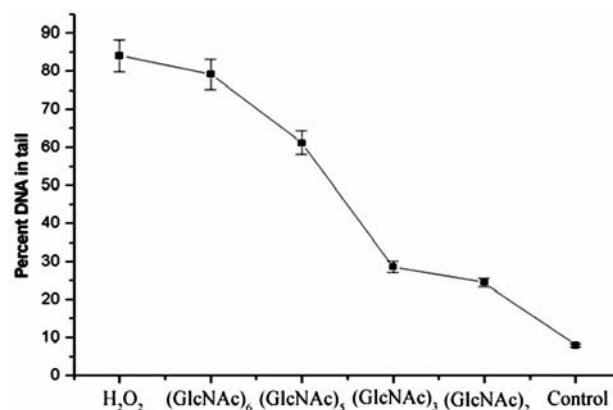


Fig. 3—Protection given by COS $[(\text{GlcNAc})_n, n=2, 3, 5, 6]$ towards induced DNA damage in PBMC exposed to 100 μM H_2O_2 . [PBMC were pretreated with 100 μM COS $[(\text{GlcNAc})_n, n=2, 3, 5, 6]$ for 10 min at 4°C in dark and further exposed to H_2O_2 at 100 μM concentration for 10 min at 4°C in dark ($n=3 \times 50$ PBMC, $p < 0.05$)]

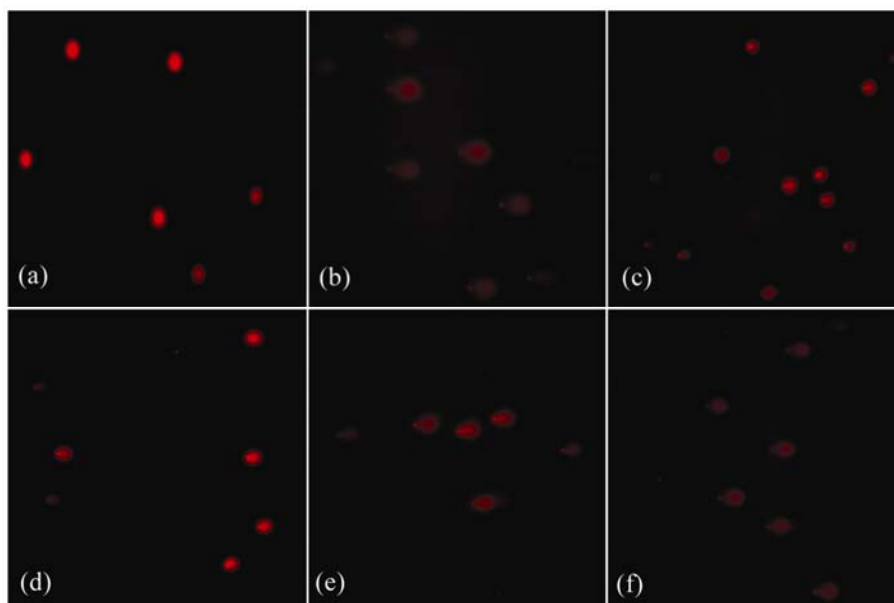


Fig. 4—Representative comets (indicating DNA damage) in (a) PBMC, (b) PBMC treated with 100 μM H_2O_2 , (c) PBMC pretreated with 100 μM $(\text{GlcNAc})_2$ and then 100 μM H_2O_2 , (d) PBMC pretreated with 100 μM $(\text{GlcNAc})_3$ and then 100 μM H_2O_2 , (e) PBMC pretreated with 100 μM $(\text{GlcNAc})_5$ and then 100 μM H_2O_2 , & (f) PBMC pretreated with 100 μM $(\text{GlcNAc})_6$ and then 100 μM H_2O_2 .

0.99 & $p < 0.05$). The inhibitory effect of low mol wt (229.21-593.12 Da) *N*-acetylated chitoooligosaccharides on oxidative DNA damage of human lymphoma U937 was shown by Ngo *et al*³¹, which correlates with our study. Our attempts to check the protective effects of COS clearly indicate that (GlcNAc)₂ and (GlcNAc)₃ offer better protection to DNA against oxidative damage as compared to (GlcNAc)₅ and (GlcNAc)₆. The protective effects of glucosamine and *N*-acetyl glucosamine have been studied by Jamialahmadi *et al*³² where higher dose of GlcNAc (40 mM) was used. In this study, we could observe the protective effects of COS at relatively lower concentrations (100 μ M). This dose would suffice in preventive manner against the oxidative DNA damage by reducing their risk of being pro-oxidants.

Conclusion

N-acetylated chitoooligosaccharides [(GlcNAc)_n, n=2, 3, 5, 6] can be used as potent antioxidants and in future can serve as nutraceuticals. The protective function of *N*-acetylated chitoooligosaccharides against oxidative DNA damage and their *in vitro* free radical scavenging ability holds promise for their demand in functional foods. *N*-acetylated chitin oligosaccharides may be useful to replace the synthetic dietary antioxidants in near future.

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