Title: Red-beet betalain pigments inhibit amyloid-beta aggregation and prevent the progression of Alzheimer's disease in a *Caenorhabditis elegans* model

Author names and affiliations:

Tomohiro Imamura[1], Hironori Koga[2], Yasuki Higashimura[3], Kenji Matsumoto[3], and Masashi Mori[1*]

[1]Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 308-1, Nonoichi, Ishikawa 921-8836, Japan

[2]Department of Bioproduction Science, Ishikawa Prefectural University, 308-1, Nonoichi, Ishikawa 921-8836, Japan

[3]Department of Food Science, Ishikawa Prefectural University, 308-1 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

***Corresponding authors:** Masashi Mori

Phone: +81-76-227-7527

Fax: +81-76-227-7557

E-mail: mori@ishikawa-pu.ac.jp

Abstract

Betalain pigments are mainly produced by plants in the order Caryophyllales. The biological functions of betalain pigments have gained recent interest; antioxidant, anti-inflammatory, and anticancer activities have been reported. To explore the biological effects of betalain pigments, we investigated the effects of betalain pigments derived from red-beet on amyloid-β (Aβ) aggregation, which is one of the causes of Alzheimer's disease (AD). We conducted a ThT fluorescence assay, which revealed that red-beet betalain extract significantly suppressed the increase in fluorescence derived from Aβ aggregation compared the control. Observations using transmission electron microscopy confirmed that Aβ fibers and amorphous aggregation were reduced in the betalain pigment treatment. Furthermore, we performed a trait investigation using a nematode model of AD and found that the progression of symptoms was significantly suppressed in the group that ingested betalain pigment. These results suggest that betalain pigment may suppress the progression of AD.

Keywords: amyloid beta aggregation, betalain, *Caenorhabditis elegans*, red beet betanin

extract, transmission electron microscopy (TEM), Thioflavin T fluorescence assay

1. Introduction

Betalains are tyrosine-derived pigments found exclusively in plants of the order Caryophyllales [1], some *Basidiomycota* fungi [2], and one bacterial species, *Gluconacetobacter diazotrophicus* [3]. Betalain pigments are divided into two groups, betacyanins (red and purple) and betaxanthins (yellow and orange). Unlike other plant pigments, e.g., flavonoids and carotenoids, betalains contain nitrogen. Previous studies have demonstrated that betalains exhibit strong antioxidant activity [4] and are involved in plant responses to environmental stimuli and stress [5, 6]. Betalains also have commercial applications; they are often used as food additives because of their vivid colors. For example, beetroot extract, which is designated by the E162 label, is approved by the United States Food and Drug Administration and European Union regulatory agencies for use as a natural food colorant.

The biological activities of betalain pigments have been reported. Betalain-rich extracts possess anti-inflammatory, anticancer, and anti-diabetic properties [7-11]. Furthermore, Betanin, a major red pigment of beetroot extract, has been demonstrated to inhibit

low-density lipoprotein oxidation [12,13]. In our recent study, we found that amaranthin betalain pigment, which accumulates in amaranth and quinoa, inhibits HIV-1 protease activity [14]. Although various physiological functions of the betalain pigment have been reported, it is expected that many physiological activities have yet to be discovered.

Alzheimer's disease (AD) is an irreversible, progressive brain disorder in which memory and thinking ability become impaired over time [15]. From a neuropathological viewpoint, AD is characterized by the aggregation of amyloid-β (Aβ) protein in senile plaques and hyperphosphorylated tau protein in brain neurofibrillary tangles [16]. Thus, inhibiting the aggregation and accumulation of Aβ and phosphorylated tau protein in the brain is the key to treating AD. However, all currently FDA-approved anti-AD drugs are only symptomatic treatments, which do not cure the disease. Recent studies have reported the efficacy of plant-derived polyphenolic compounds as candidate substances for AD treatment [17,18]. Polyphenolic compounds such as resveratrol, curcumin, quercetin, genistein, morin, and epigallocatechin gallate (EGCG) have been shown to inhibit Aβ production and aggregation [19,20].

In this study, we evaluated the physiological activity of betacyanin against Aβ using red-beet betanin extract. First, we performed a Thioflavin T (ThT) fluorescence assay to evaluate the effect of betanin extract on Aβ, using transmission electron microscopy (TEM) for observation. We found that red-beet betanin extract suppresses the aggregation of Aβ. Next, we evaluated the biological activity of red-beet betanin extract using a *Caenorhabditis elegans* AD model. The progression of AD symptoms in the *C. elegans* model was attenuated in the betanin treatment group. These results indicate that betanin has potential as a treatment to suppress the progression of AD.

2. Material and methods

2.1 Materials

Lyophilized human amyloid-β 1-40 (Aβ40) was obtained from Peptide Institute Inc. (Osaka, Japan). Red-beet betanin extract (red-beet extract diluted with Dextrin) was obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). Morin hydrate (TCI) was used as a control for the ThT fluorescence assay. Dextrin (TCI) was used as a control in

experiments using model the *C. elegans* for AD.

2.2 Sample preparation

To obtain pure red-beet betanin, open column chromatography was performed to remove dextrin form the red-beet betanin extract. First, an aqueous solution of red-beet betanin extract was prepared and added to DEAE Sephacel (Pharmacia, Uppsala, Sweden). The solution was washed with water, before elution with 100 mM NaCl. Next, the eluate was added to reversed-phase Cosmosil 140C_{18}-OPN (Nacalai tesque, Kyoto, Japan), washed with water, and then eluted with 10% acetonitrile. The eluate was allowed to evaporate to dryness and then the residues were dissolved in water and stored at −20 °C until needed. This purified red-beet betanin was used for the ThT fluorescence assay and Aβ aggregation experiments, and the unpurified red-beet betanin containing dextrin was used for the evaluation of biological activity in *C. elegans*.

2.3 High-performance liquid chromatography (HPLC)

A Shimadzu LC-20AD system (Kyoto, Japan) was used for analytical HPLC separations.

Samples were separated on a Shim-pack GWS C18 column (5 μm; 200 × 4.6 mm i.d.; Shimadzu GLC, Tokyo, Japan), and linear gradients were run from 0% B to 45% B over 45 min using 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). Elution was monitored by absorbance at 536 nm at a flow rate of 0.5 mL min^{-1} at 25 °C.

2.4 UV−Vis spectroscopy

A UV-2450 (Shimadzu) spectrophotometer was used for UV-Vis spectroscopy. Betalain pigment excluding dextrin concentration was determined using molar extinction coefficients of ε = 65,000 M^{-1} cm^{-1} at 536 nm for the purified red-beet betanin [21, 22].

2.5 ThT fluorescence assay

A ThT fluorescence assay was performed using the SensoLyte Thioflavin T β-Amyloid (1-40) Aggregation Kit (ANASPEC, Fremont, CA, USA). Aβ40 solution and ThT solutions at final concentrations of 50 μM and 200 μM, respectively, were used in the evaluation system according to the manufacturer's protocol. Red-beet betalain extract

was evaluated at final concentrations of 6.5, 12.5, 25, and 50 μM. Morin, which inhibits Aβ aggregation [23], was used as an inhibitor control at a final concentration of 50 μM. The sample with water, instead of inhibitor red-beet betalain extract or morin, was used as a positive control. The sample without inhibitor and Aβ40 was used as a reference control. ThT fluorescence was monitored at 37 °C using a spectrofluorometer (VarioskanLUX; Thermo Fisher scientific, Waltham, MA, USA) at Ex/Em = 440/484 nm; reading were taken every 5 minutes and was shaken for 15 s just before the reading. Fluorescence data were analyzed using Skanlt software (Thermo Fisher scientific). The reported values are the average of five wells.

2.6 TEM observation of Aβ40 aggregates

Aβ40 was solubilized in dimethylsulfoxide (DMSO) to prepare a 1 mM stock solution. Aβ40 stock solutions were prepared with and without the addition of 50 μM red-beet betanin extract and diluted to 25 μM with PBS (pH 7.4). The Aβ40 mixtures were incubated at 37 ºC for 5 days before TEM observation.

To prepare the samples for imaging, approximately 2 μL of solution stained with 2.0 % (w/v) uranyl acetate solution was placed on 150-mesh copper grid covered with formvar. The solution was allowed to dry on the grid before imaging. TEM images of the Aβ aggregates were obtained using a Hitachi 7650 TEM (Hitachi Co., Ltd., Tokyo, Japan) with an acceleration voltage of 80 kV.

2.7 C. elegans maintenance and synchronization

C. elegans were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 (OP50) as a food source at 15 °C unless otherwise indicated. The transgenic strain CL2006 [dvIs (unc-54::human β-amyloid 1-42; pRF4)] was obtained from the Caenorhabditis Genetics Center (CGC; Minnesota, USA). In CL2006, the human Aβ42 protein is expressed intracellularly within the body wall muscle. The expression and subsequent aggregation of Aβ in the muscle result in progressive paralysis [24].

For synchronization, the nematodes were cultured on fresh NGM plates for 2–3

generations without starvation. Young adult nematodes were collected and cleaned, then disintegrated using a lysis solution (0.6% sodium hypochlorite and 200 mM sodium hydroxide). After 12–14 h at 20 °C, the isolated eggs were hatched to obtain the synchronized L1 larvae.

2.8 Paralysis assay

The synchronized L1 larvae were fed OP50 and grown to young adults at 20 °C; 5-fluorodeoxyuridine (0.5 mg/ml) was added to prevent progeny production. The resultant synchronized hermaphrodites were transferred to OP50-seeded NGM plates containing various concentrations of dextrin or betanin (20 nematodes per plate). Dextrin was used as a control because the betanin extract used in this study contained dextrin. The nematodes were tested for paralysis by tapping their noses with a platinum wire. According to previous reports, nematodes that moved their noses in response but failed to move their bodies were considered paralyzed [25].

3. Results

3.1. Pigment analysis of red-beet betanin extracts

We confirmed the betalain pigment composition of the red-beet betanin extract used in the experiment by HPLC. The red-beet betanin extract was composed of equal amounts of betanin and isobetanin (Fig. 1). The red-beet betanin extract used in this study was a mixture of dextrin and betalain, containing 3.08 ± 0.09 μmol of betalain pigment per gram of extract. However, betalain pigment purified from the red-beet betanin extract was used in the *in vitro* ThT fluorescence assay and Aβ aggregation experiments to eliminate the influence of dextrin.

3.2. Red-beet betalain extracts inhibit the aggregation of Aβ40

To identify new betanin bioactivity, we investigated the effect of red-beet betanin extract on Aβ40 aggregation using a ThT fluorescence assay. The effect of red-beet betanin extract at concentration of 6.25, 12.5, 25, and 50 μM on Aβ40 aggregation was evaluated. The 4-h ThT fluorescence assay revealed that all treatment concentrations of red-beet betanin extract inhibited Aβ40 aggregation compared to Aβ40 alone (control (Fig. 2). Furthermore, the red-beet betanin extract showed higher Aβ40 aggregation inhibitory

activity than morin, the inhibitor control (Fig. 2). The inhibitory effects were similar between 6.25 µM red-beet betanin extract and 50 µM morin.

Next, the inhibitory effect of red-beet betanin extract on Aβ aggregation was observed using TEM. Two 25 mM Aβ40 solutions, one with 50 µM red-beet betanin extract added, were incubated at 37 °C for 5 days, after which Aβ40 aggregation was observed. As expected, Aβ fibrosis and amorphous aggregation were observed in the Aβ40 solution. However, Aβ fibrosis and amorphous aggregation were significantly inhibited in the Aβ40 solution with red-beet betanin extract (Fig. 3).

3.3. Betanin delayed Aβ-induced paralysis in transgenic C. elegans strain CL2006

We used *C. elegans* strain CL2006 to determine the effects of betanin on Aβ-induced toxicity in the whole animal. The nematodes were treated with dextrin or red-beet betanin extract at concentrations of 2, 10, and 50 µM. The highest concentration of betanin (50 µM), delayed Aβ-induced paralysis in the transgenic nematodes, while the 2 and 10 µM treatments did not (Fig. 4). Additionally, dextrin alone (2, 10, and 50 µM) did not delay Aβ-induced paralysis (Fig. 4). Microscopic observation confirmed that betanin but not

dextrin protected morphological changes of the transgenic nematodes (Fig. 4).

4. Discussion

In this study, we demonstrated that a red-beet betanin extract composed of betanin and isobetanin inhibits Aβ aggregation. Furthermore, in feeding experiments using Aβ42-overexpressing nematodes, the ingestion of red-beet betanin extract delayed the onset of paralytic symptoms derived from Aβ42 aggregation. Together, these results strongly suggest that betanin and isobetanin, which are the main components of the red-beet betanin extract inhibit the aggregation of Aβ. Previous studies have reported that the biological effects of betanin include suppression of cancer cell proliferation [11, 14] and low-density lipoprotein oxidation [12, 13].

In this study, we discovered a new biological effect of betanin, suppression of Aβ aggregation. Nematode feeding experiments have shown that the ingestion of specific betalain pigments produce lifespan-prolonging effects [26, 27]. In our recent investigation, we confirmed that amaranthin, a betanin glucuronide, inhibits HIV-1

protease activity; however, betanin did not exhibit significant inhibitory effects [14]. These findings suggest that the bioactivity retained by each betalain molecular species is different. Therefore, the Aβ aggregation inhibitory effect may also differ depending on the individual betalain molecular species. Through the examination of the activities of other betalain molecular species, we can clarify whether the Aβ aggregation inhibitory action of betanin and isobetanin molecule is a common biological activity among betalain pigments.

Experiments using nematodes are simpler to evaluate and require less labor for breeding compared to mice. Moreover, if the sample amount can be prepared, the activity of various substances can be easily evaluated. The evaluation system using this nematode is useful for the initial investigation of the biological activity of a substance; the physiological activity of several phytochemicals has been examined in such a system [26-28]. In this study, feeding experiments with Aβ42-overexpressing nematodes demonstrated that long-term ingestion of red-beet betanin extract effectively delays the onset of symptoms caused by Aβ aggregation. From this result, we can postulate that

long-term intake of betanin in humans may also delay the progression of AD. In addition, it has been reported that the betalain pigment indicaxanthin is able to cross the blood-brain barrier in rats [29]. Therefore, it supports the possibility that red-beet betanin extract may also reach and act directly on the brain. Further analysis using model organisms such as mice and eventually humans are necessary to confirm the bioactive inhibitory effects of betanin on Aβ aggregation. With future investigation, it can be expected that red-beet betanin extract or purified betanin and isobetanin can be developed as a preventive drug or supplement for AD.

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Contributions

MM, KM, and HK conceived the study. TI and MM designed the experiments. TI performed the ThT assay. YH conducted the *C.elegans* assay. H.K. performed TEM observation. TI and MM wrote the manuscript. All authors have read and approved the final manuscript.

Competing of interests

The authors declare no competing interests.

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Figure legends

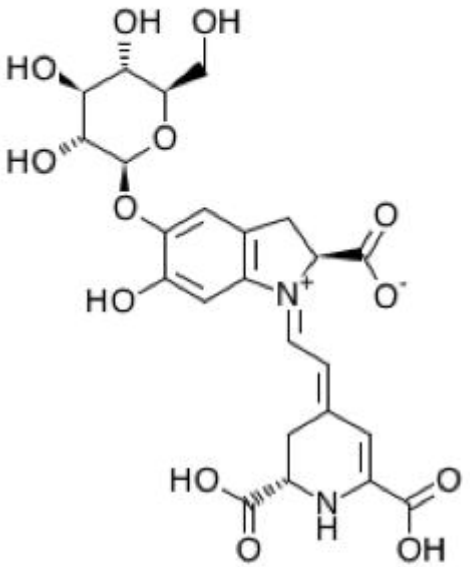
Fig. 1 Composition of red-beet betanin extracts (A) Chemical structure of betanin and isobetanin (B) HPLC chromatogram of red-beet betanin extract. HPLC elution sample peaks at 23.5 min and 24.6 min indicate betanin and isobetanin, respectively.

Fig. 2 Effects of red-beet betanin extracts on the aggregation of human amyloid-β 1-40 (Aβ40). Thioflavin T (ThT) fluorescence changes for Aβ40 incubated in the absence and presence of red-beet betanin extract. Aβ40 (25 μM) incubated with 6.25, 12.5, 25, 50 μM red-beet betanin extracts at 37 ºC for 4 h. Error bars represent the means ± SD; *p < 0.05 compared to 50 μM morin at the same treatment time. ThT relative fluorescence was expressed as a percentage of the fluorescence for Aβ40 alone, whose maximum value was taken as 100%.

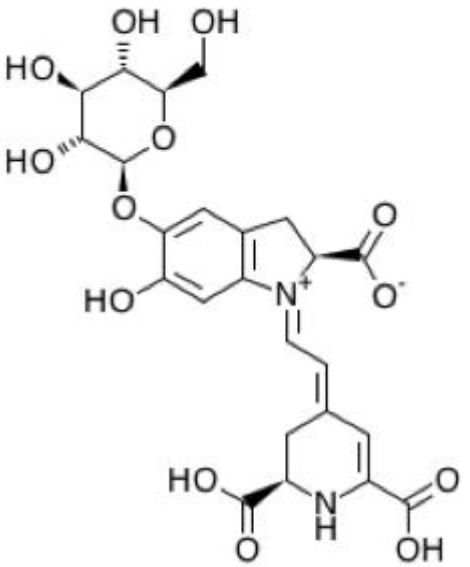
Fig. 3 Transmission electron microscope images of human amyloid-β 1-40 (Aβ40) aggregates. (A), (B) Aβ40 alone; (C), (D) Aβ40 + 50 μM red-beet betanin extract. Scale bars 200 μm; incubation time, 5 d.

Fig. 4 Effect of betanin on amyloid-β-induced paralysis in *Caenorhabditis elegans* transgenic strain CL2006. The synchronized hermaphrodites were exposed to 2, 10, and 50 μM of dextrin (A) and betanin (B). The paralysis rate was scored three times per week and is expressed as the percentage of not-paralyzed nematodes. (n = 109–119 nematodes/group). Differences in paralysis rates were tested for significance using the log-rank test ($^{*}p < 0.05$). Results are representative of two independent experiments. (C) Morphological assessment of transgenic nematodes in the presence and absence of betanin using a light microscope.

A

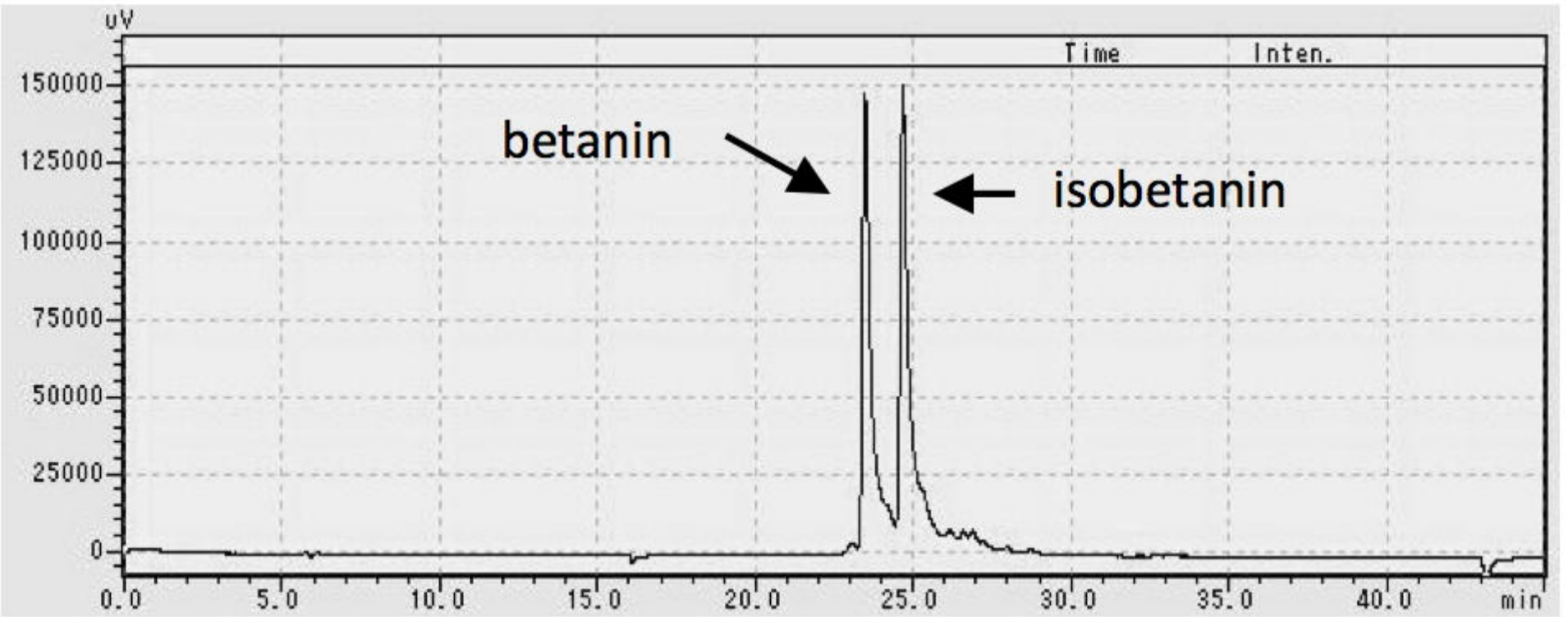


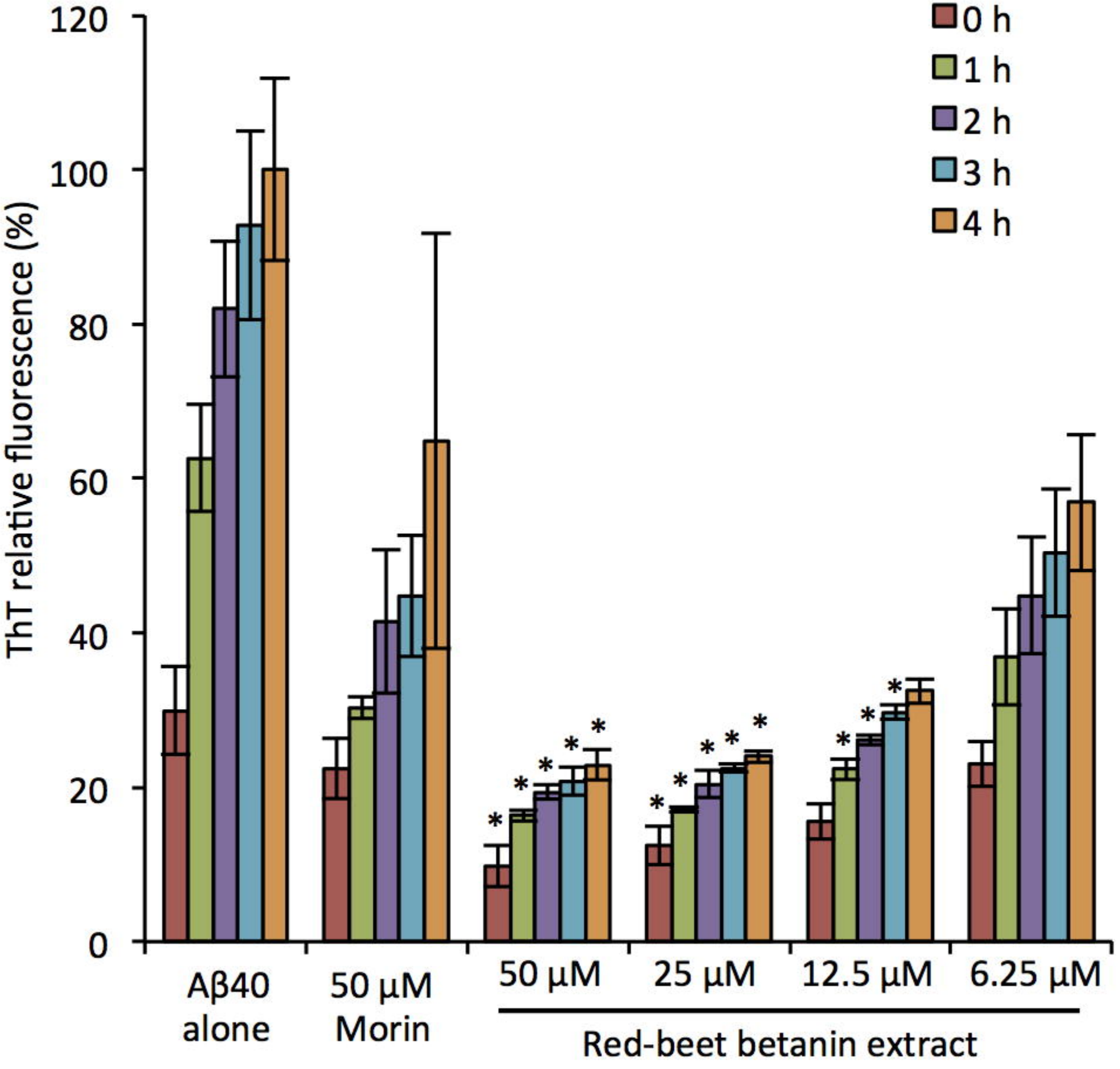
betanin



isobetanin

B

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1 h
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ThT relative fluorescence (%)
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100
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60
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12.5 μM
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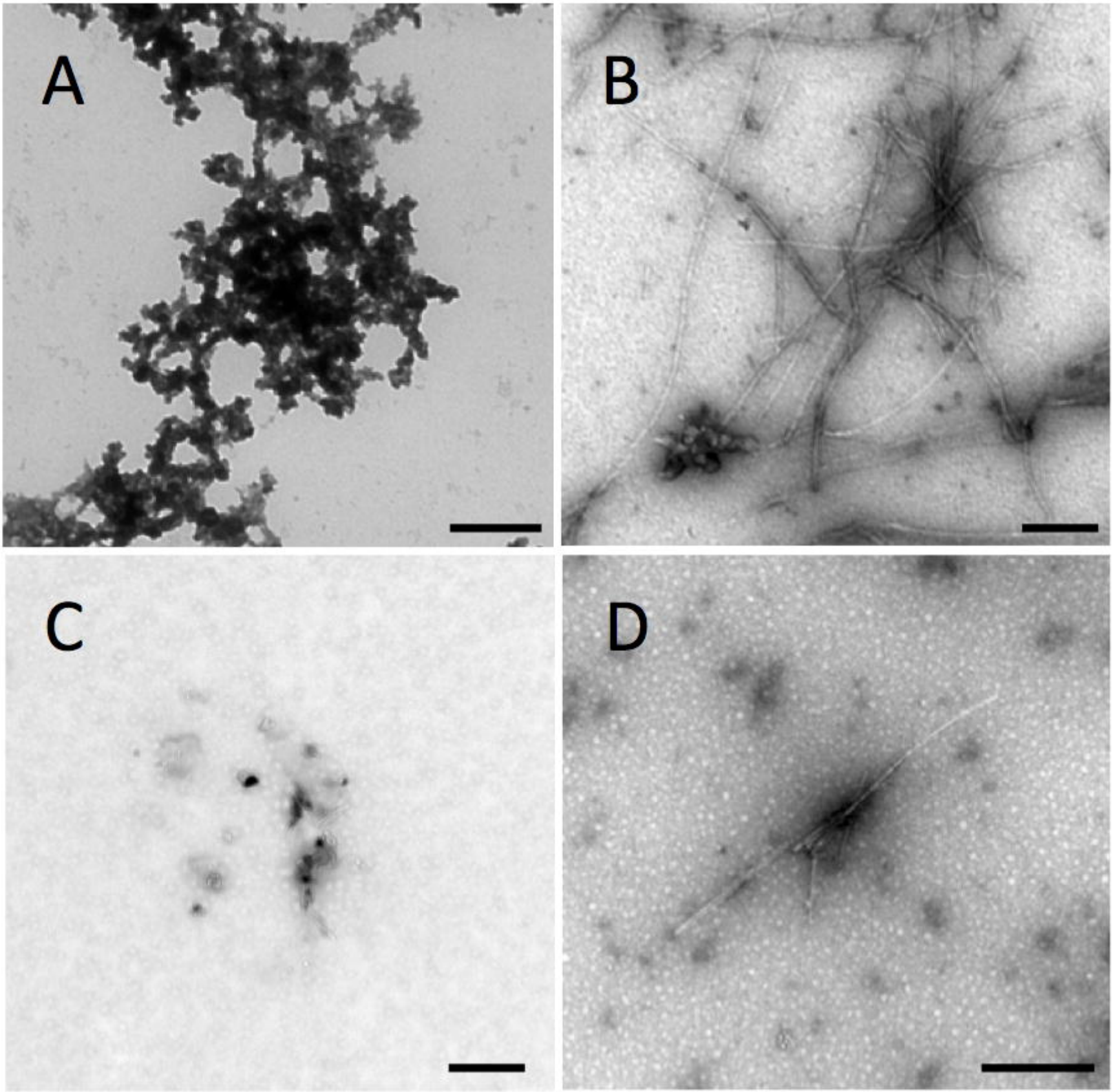
Red-beet betanin extract


A
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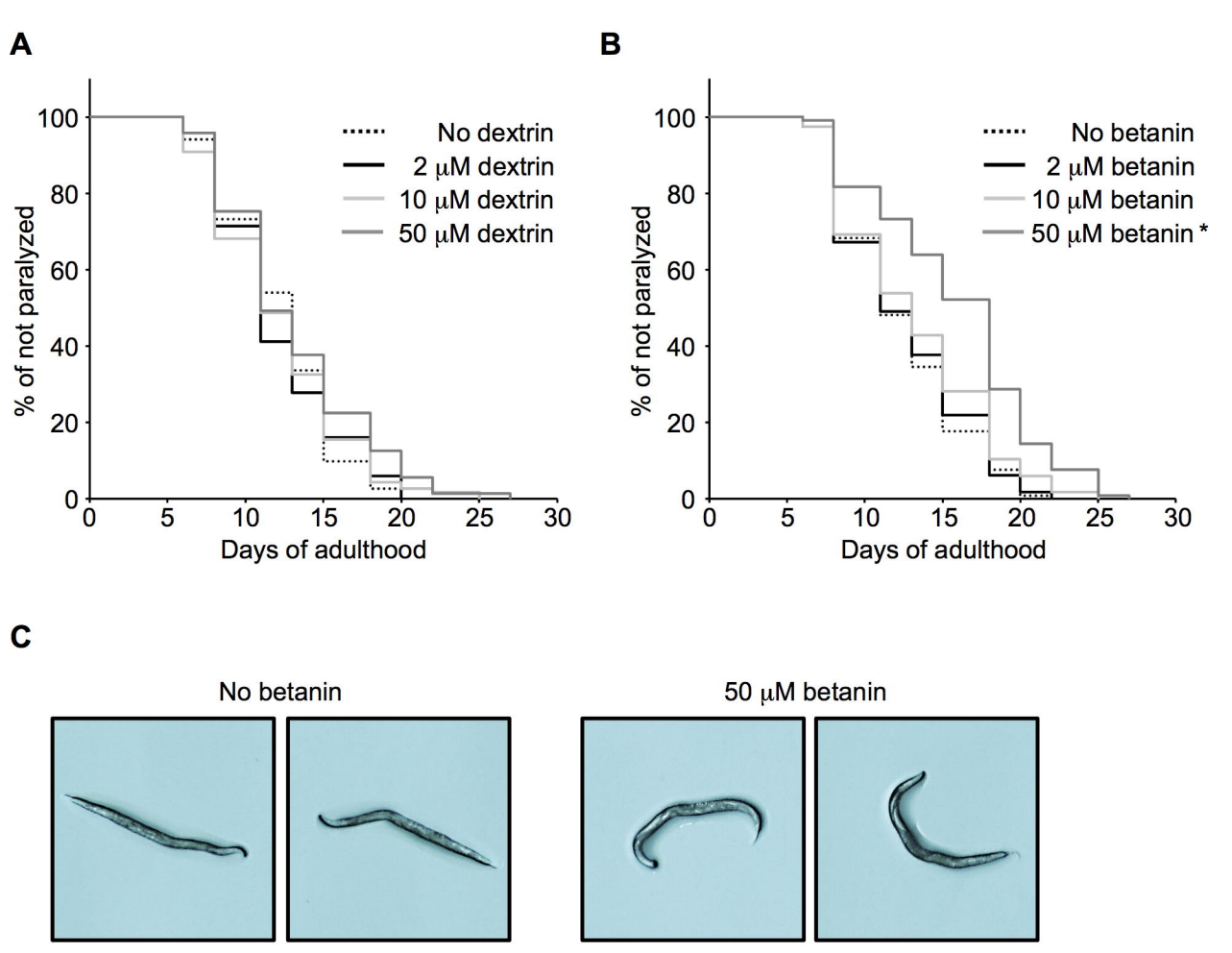
A
B
C
% of not paralyzed
Days of adulthood
No dextrin
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