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Short communication

# A novel nanoprotein particle synthesis: Nanolipase

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ABSTRACT

Lipases (triacylglycerol acylhydrolases; E.C. 3.1.1.3) constitute a group of enzymes defined as carboxylesterases that catalyse the hydrolysis (and synthesis) of long-chain acylglycerols at the lipid–water interface. In this study, a novel method has been developed to prepare nanoprotein particles carrying lipase using a photosensitive microemulsion polymerization process. The nanostructured lipases with photosensitive features have been characterized by transmission electron microscopy (TEM) and Zeta Sizer. The average particle size of nanolipases was found to be about 100 nm. Lipase nanoparticles were used in hydrolysis of paranitrophenyl palmitate (p-NPP) and the results were compared with free lipase. The parameters like pH, temperature and hydrolytic activity that affect p-NPP hydrolysis were investigated by using lipase nanoparticles and compared to free lipase. Lastly, reusability of lipase nanoparticles was investigated and according to our results, this novel lipase nanoparticles showed admirable potential in reusable catalyst.



## 1. Introduction

Lipases (glycerol ester hydrolase, E.C. 3.1.1.3) have been widely used for enzymatic conversion in the various biotechnological applications in dairy industry, manufacturing of specialty chemicals, organic synthesis, and preparation of enantiomerically pure pharmaceuticals as they combine a broad range of substrate specificity with a high regio- and enantio-selectivity for many reactions [1–3]. In most cases, especially in industrial applications, enzymes are preferably used in their immobilized states owing to many advantages, such as improved activity and stability of the immobilized enzyme, ready reutilization of the catalyst and possibility of continuous process operation. To date, numerous efforts have been towards the development of enzyme immobilization techniques, e.g. covalent attachment [4,5], physical adsorption [6] and encapsulation [7–15]. Adsorption techniques are easy to perform, but the interactions between the enzyme and the support is relatively weak. Such biocatalysts are usually not stable enough for a long-term utilization. Covalent attachment normally leads to improved enzyme stability. However, partial deactivation is often obtained due to the conformational restrictions by the covalent bonding of enzyme moieties to the support [16].

Chemical conjugation of enzymes with synthetic polymers enables their colloidal stability or thermal stability to be enhanced, and their enzyme activity to be controlled [17–20]. However, chemical conjugation has the difficulty that the synthesis is not always easy, and that conjugation sometimes induces a decrease in the enzyme activity [20]. Bioconjugation is a useful technique for modifying the function of enzymes for pharmaceutical and biotechnological applications. The biconjugation approach entails the advantages of easy preparation and versatility for various proteins [21–23].

In this study, nanostructured lipase was prepared by the photosensitive cross-linking microemulsion polymerization technique. This technique is a new technique which allows the synthesis of nanoparticles with narrow size distribution [24]. Microemulsion polymerization is a complex heterogeneous process where transport of monomers, free radicals and other species (such as chain transfer agent, co-surfactant and inhibitors) between the aqueous and organic phases, takes place [25]. The polymerization rate is controlled by monomer partitioning between the phases, particle nucleation, adsorption and desorption of radicals. The particle stability is affected by the amount and type of surfactant and the pH of the dispersing medium [26]. Although microemulsion polymerization is a complex heterogeneous process, but in this study, photosensitive cross-linking technique become more easy and efficient by using the particles which were synthesized according to ANADOLUCA (**A**mi**N**o**A**cid (monomer) **D**ecorated and **L**ight **U**nderpinning **C**onjugation **A**pproach) method. The proteinous nanoparticles prepared by photosensitive cross-linking were

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easily prepared at room temperature, in day light and under nitrogen atmosphere. A nano-protein is a new generation polymeric material prepared in ANADOLUCA concept. This nano-protein (lipase in current study) can be used directly as a monomer (enzymes, antibodies or similar proteins) without using any other platforms. The ANADOLUCA method is used while preparing photosensitive nanostructured lipase and provides a strategy for the preparation of photosensitive ruthenium based amino acid monomers and oligomers, amino acid monomer-protein cross-linking using photosensitation and conjugation approach on micro and nanostructures by ruthenium-chelate based monomers [27].

In this study, the nanostructured lipase enzyme with photosensitive features gained lipase was synthesized by the microemulsion polymerization technique and characterized with fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy (TEM) and Zeta-Sizer. After that, the activity of lipase nanoparticles and parameters that affect paranitrophenyl palmitate (p-NPP) hydrolysis such as pH, temperature and hydrolytic activity by using lipase nanoparticles were investigated compared with free lipase. Lastly, the reusability of lipase nanoparticles was determined using the p-NPP hydrolysis reaction.

## 2. Experimental

### 2.1. Chemicals

Lipase (E.C. 3.1.1.3 from *Candida rugosa*), p-nitrophenyl palmitate, isopropanol, p-nitrophenol, Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Ethyleneglycol dimethacrylate (EDMA) was purchased from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4 °C until use. 2,2-Dimethoxy-2-phenyl acetophenone was supplied from Aldrich Chem. Co. (Milwaukee, WI, USA). Poly(vinyl alcohol) (Mw 27,000) was purchased from Fluka Chemica, Dimethylsulfoxide was purchased from Sigma. Ammonium persulfate (APS) was purchased from local sources. All glassware was extensively washed with dilute $HNO_3$ before use. All other chemicals were of analytical grade purity and purchased from Merck AG (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANO pure® organic/colloid removal and ion exchange packed-bed system.

### 2.2. ANADOLUCA method for the preparation of nanoenzyme

#### 2.2.1. Synthesis of N-methacryloyl-(L)-tyrosine

The N-Methacryloyl-(L)-Tyrosine (MATyr) was selected as the functional monomer for lipase proteinous nanoparticles. 3-(4-hydroxyphenyl)-2-[(2-methylacryloyl)amino]propanoic acid (methacryloyl tyrosine, (MATyr)), was prepared according to the previously published method [28]: 5.0 g of L-tyrosine methylester and 0.2 g of hydroquinone were dissolved in 100 mL of dichloromethane solution. This solution was cooled down to 0 °C and 12.7 g triethylamine was added into the solution. 5.0 mL of methacryloyl chloride was poured slowly into this solution and the solution was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, hydroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MATyr) was crystallized in an ether–cyclohexane mixture and then dissolved in ethyl alcohol.

#### 2.2.2. Chemical synthesis of photosensitive ruthenium based aminoacid-monomer bis (2-2′-bipyridyl) MATyr-MATyr-ruthenium (II)

A dichlorobis(2-2′-bipyridyl)ruthenium(II) ($RuCl_2(bipyr)_2$) was synthesized according to previously published method [29]: $RuCl_2(bipyr)_2$ (0.1 g, 1 eq.) was dissolved in water and the solution was cooled to 0 °C. Then, triethylamine and the aqueous solution of MATyr (0.1 g, 2 eq.) were added dropwise into that solution and the solution stirred at room temperature for 30 min. The mixture was heated to 80 °C for refluxing ca. 24 h. The brown complex was filtered off, washed with ether and dried under vacuum. M.p.: >200 °C.

Anal. for $C_{46}H_{44}N_6O_8Ru$: found: C 61.54%, H 4.63%, N 10.56%, calcd.: C 60.72%, H 4.87%, N 9.24%.

$^1H$ NMR (500 MHz, $CD_3OD$), ppm: 9.78 (1H, s), 7.8 (1H, d, $J$ = 8.15 Hz), 7.73 (t, 1H, $J$ = 4.1 Hz), 7.64 (t, 2H, J= 4.11 Hz), 7.47–7.41 (q, 2H), 7.3–7.11 (m, 11H), 7.05 (t, 3H, $J$ = 8.18 Hz), 6.95 (d, 2H, $J$ = 8.18 Hz), 6.78 (s, 9H), 6.69 (d, 1H, $J$ = 12.75 Hz), 6.34 (s, 1H), 5.84 (s, 1H), 4.5 (s, 1H), 4.25 (s, 2H), 2.06 (s, 3H), 1.91 (s, 3H).

MALDI-TOF-MS: the ion peaks at 79, 128 and 155 $m/z$ relating to bipyridyl. $m/z$ 101, 413 peaks show Ru and $Ru(bpy)_2$, respectively. $m/z$ 250, 599 and 755 data show MATyr monomer, Ru-$(MATyr)_2$ and -Ru(bpy)- MATyr complex.

#### 2.2.3. Synthesis of the photosensitive poly(bis (2-2′-bipyridyl) MATyr-MATyr-ruthenium(II))

The phosensitive polymer was prepared by a conventional polymerization reaction. The necessary amount of bis (2-2′-bipyridyl)-MATyr-MATyr ruthenium(II) monomer in dimethyl sulfoxide was mixed to obtain a photosensitive oligomer. The polymerization reaction was taken place in the presence of 2-2′-azobisisobutyronitrile (AIBN) as an initiator. For the MALDI-TOF MS analysis, 2 μL of ($[Ru(bpy)_2MATyr\text{-}MATyr]_n$) solution was mixed with 23 μL of a 10 mg mL$^{-1}$ solution of α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/% 0.3 TFA. The acceleration voltage was set to 20 kV, the delay time is 400 ns, grid voltage 70%, laser intensity 2092 and at reflector mode [27].

#### 2.2.4. Synthesis of lipase proteinous nanoparticles

Lipase nanoparticles were synthesized according to microemulsion polymerization technique. Microemulsion system was prepared by dispersing 0.5 g of poly(vinyl alcohol) (PVA) in 45 mL of deionized water. On the other hand, 0.5 g of MATyr was dissolved in 5 mL of dimethylsulfoxide and added into the lipase solution prepared by dissolving 10 mg of lipase in 5 mL of deionized water. Then, 0.5 mL of rutenium based polymer p(MATyr-Ru $(bipyr)_2$-MATyr) was added into the mixture and mixed for 1 h. This mixture was added into 25 mL of PVA dispersing medium. Beside this, 20 mL of initiator solution prepared by dissolving 0.02 g of APS in 45 mL of deionized water was added into reaction mixture. Finally, 0.3 mL of EDMA was added and mixed for 48 h under nitrogen atmosphere at room temperature, at daylight. Lipase nanoparticles were separated from the reaction solution by centrifugation at 6000 rpm for 20 min and washed with deionized water to remove unreacted substances.

### 2.3. Characterization of lipase proteinous nanoparticles

The average particle size and size distribution lipase nanoparticles were determined by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England) and a FEI 120 kV transmission electron microscope (TEM).

### 2.4. Activity of lipase proteinous nanoparticles

The activity of lipase nanoparticles was measured by the spectrophotometric method using paranitrophenyl palmitate (p-NPP) as a substrate. The activity was assayed by measuring the absorbance of released p-nitrophenol at 405 nm. For this purpose, firstly stock solution of substrate containing 20 mM p-NPP in isopropanol was prepared. Then, working substrate was prepared by diluting the p-NPP stock solution (1:20) using 20 mM Tris HCl buffer (pH 8) and synthetic lipase activity was measured by mixing 0.9 mL of working substrate and 0.25 mg mL$^{-1}$ of lipase proteinous nanoparticles.

## 3. Results and discussion

### 3.1. Properties of lipase proteinous nanoparticles

As seen in Fig. 1A, the average particle size and size distribution of the nanolipase were measured by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England) and about 100 nm. The average particle size was an average of minimum 30 measurements, and the size distribution (Fig. 1A) was of these repeated measurements recorded automatically by the software. The average particle size of the lipase nanoparticles was measured as about 100 nm. The average particle size was an average of minimum 4 measurements, and the size distribution (Fig. 1A) was of these repeated measurements recorded automatically by the software. Also, lipase nanoparticles were investigated by TEM analysis (Fig. 1B). As seen in Fig. 1, nanoparticles have regular distribution.

### 3.2. Effect of pH on paranitrophenyl palmitate (p-NPP) hydrolysis

The pH is one of the important parameters that has the ability of changing enzymatic activities in aqueous solutions [30]. In this study, the optimum pH was determined applying different pH values in the range of 6.0–10.0 using 20 mM p-NPP. The effect of pH on the time course of p-NPP hydrolysis is shown in Fig. 2. As seen from the figure, the maximum hydrolytic activity was observed at pH 8.0. All fat-digesting enzymes act in an alkaline media and the optimal pH varies slightly from species to species. This situation can be explained as proteinous nanoparticles are mimic the natural lipase.

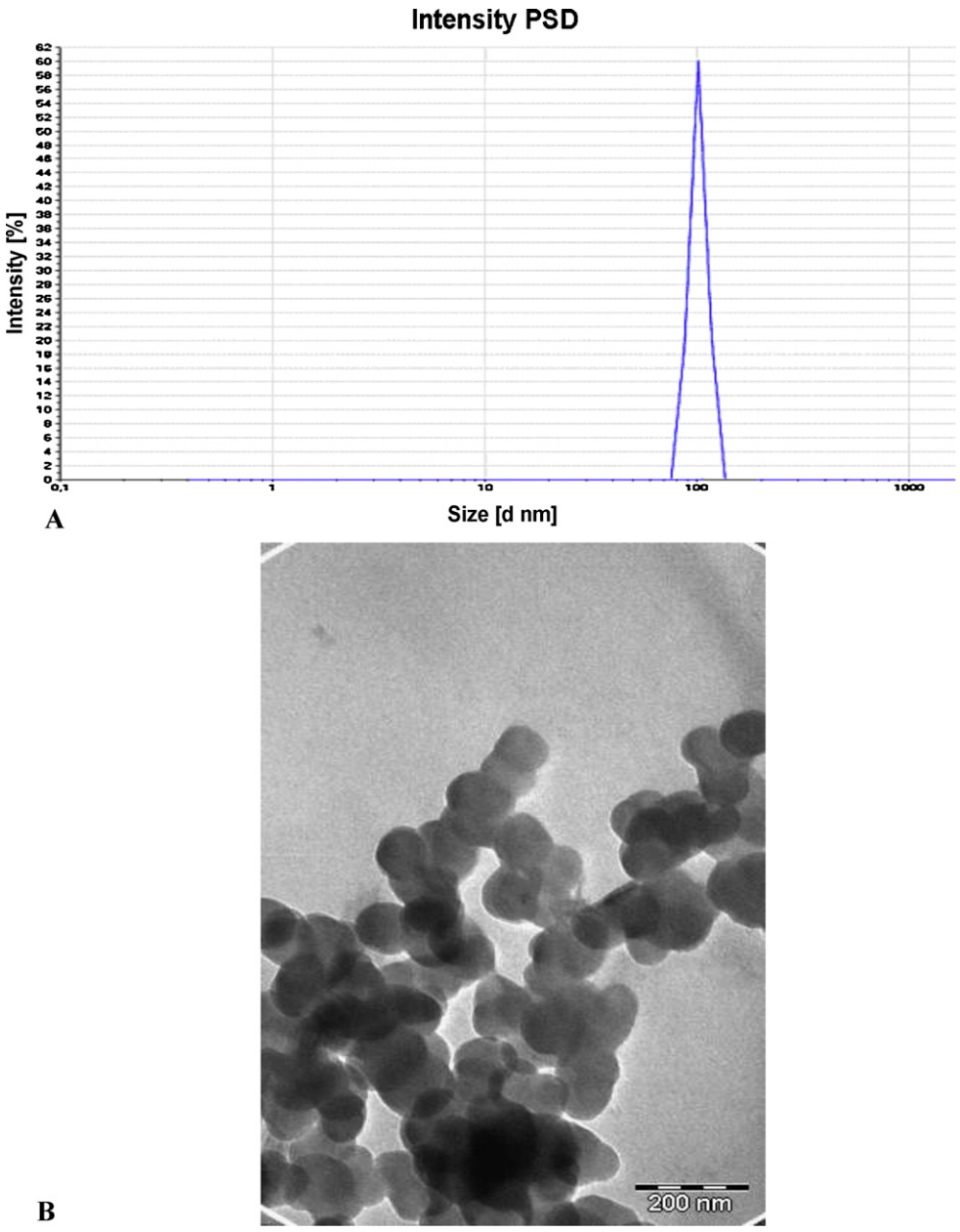


**Fig. 1.** (A) Size distribution of the lipase nanoparticles distrubution result (mean/area) = 100.2 d nm/100.0% Zeta potential = −31 mV, conductivity: 0.0344 mS $cm^{-1}$. (B) TEM image of the lipase nanoparticles (the average particle size of nanolipases was about 94 ± 9 nm).

### 3.3. *Effect of temperature on paranitrophenyl palmitate (p-NPP) hydrolysis*

Fig. 3 shows the effect of temperature on p-NPP hydrolysis. The optimum temperature was determined applying different temperature values ranging from 20 °C to 50 °C. As can be seen from Fig. 3, the optimum temperature is approximately 40 °C. This situation can be explained as free lipase and lipase nanoparticles have shown similar active site and this is an indication of the

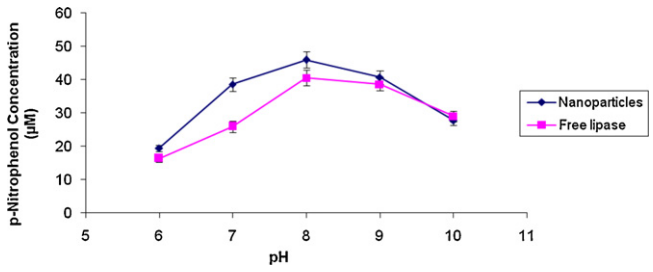


**Fig. 2.** The effect of pH on p-NPP hydrolysis using free lipase and lipase nanoparticles.

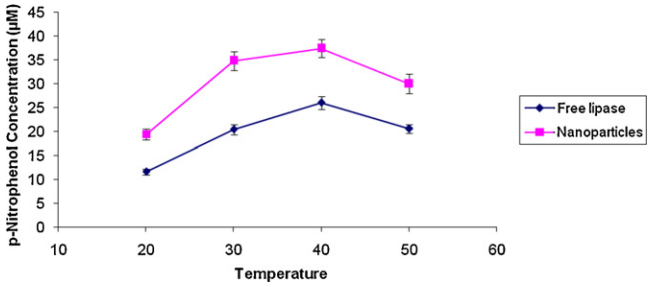


**Fig. 3.** The effect of temperature on p-NPP hydrolysis using free lipase and lipase nanoparticles.

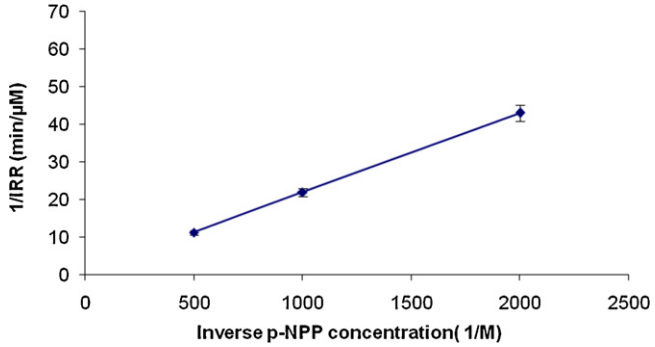


**Fig. 4.** Lineweaver–Burk Plot of kinetics data of hydrolysis of paranitrophenyl palmitate.

lipase enzyme was represented in the structure of the polymeric matrix.

### 3.4. Hydrolytic activity of lipase nanoparticles

The kinetic constants of p-NPP hydrolysis were obtained fitting the data in Eq. (1).

$$V = \frac{V_m \cdot S}{K_m + S} \quad (1)$$

where $V$ is the initial rate, $V_m$ is the maximum rate, $S$ is the concentration of substrate, $K_m$ is the Michealis–Menten constant. Hydrolytic activity of free lipase and nanolipase proteinous particles was evaluated in the framework of Michealis–Menten kinetics. For this purpose, p-NPP substrate concentrations in the range of 2.0–20 mM were used in each case and initial reaction rates (IRR) of hydrolysis were determined. Then, 1/IRR versus reciprocal of the substrate concentration ($1/S$) for nanoparticles was plotted that is called Lineweaver–Burk plot (Fig. 4) and $V_m$ and $K_m$ values were found to be 0.72 mM $min^{-1}$ and 9.3 mM, respectively.

In Michealis–Menten kinetics, $K_m$ reflects the affinity of an enzyme for a particular substrate (the lower value of $K_m$ the higher affinity). In the present case, $K_m$ represents the affinity of functional groups of lipase proteinous nanoparticles for substrate p-NPP. In studying the enzyme kinetics of substrate hydrolysis, the $K_m$ value determined for the nanolipase is lower than free enzyme (10.5 mM) indicating that the affinity of nanolipase towards the substrate is higher than that of free lipase. On the other hand, the $V_{max}$ of the free enzyme (1.7 mM $min^{-1}$) was higher than that of the nanolipase, thus suggesting that the because of the crosslinking of nanolipase, the number of active site that the substrate binds decreases. At the same time, the regeneration of active sites of the nanolipase is possible without loosing the affinity towards the substrate. This situtation brings with the reusability advantage of nanolipase.

### 3.5. Reusability of lipase nanoparticles

In order to show the stability and reusability of the lipase proteinous nanoparticles, the adsorption–desorption cycle was repeated ten times using the same lipase proteinous nanoparticles (0.25 mg $mL^{-1}$). For sterilization (after one cycle), the particles were washed with 1 M NaOH solution and then, washed with distilled water. As seen in Fig. 5, the nanoparticles are very stable even after ten cycles and actually, enzymes are for single use only, this also increases costs. In this study, it was aimed to create a large number of active sites and reusable synthetic enzymes and was also aimed to reach high enzyme activity using nanoenzyme conceptual approach. As a result, the lipase proteinous nanoparticles can be used many times without decreasing their capacities significantly.

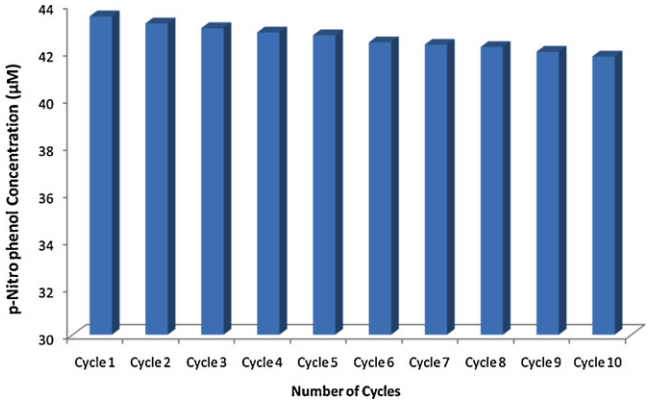


**Fig. 5.** Reusabilities of lipase nanoparticles (0.25 mg $mL^{-1}$).

## 4. Conclusion

A novel photosensitive nanolipase selective for p-NPP palmitate was prepared by a microemulsion polymerization technique. The microemulsion polymerization by using photosensitive crosslinking conjugation of the protein on nano-structure without affecting conformation and function of protein through the aminoacid-monomer and/or ruthenium-chelate based aminoacid monomers is not reported in the literature. Prepared lipase nanoparticles showed high level of activity over a wider range of pH between 6.0 and 10.0, especially at the optimum pH 8.0 than that of free form and could be removed from a reaction mixture easily and used repeatedly. The nanoparticles are very stable even after 10 cycle usage. Thermal stability was considerably enhanced by the photosensitive crosslinking polymerization. Since, the proteinous nanoparticle preserves high enzyme loading, activity with enhanced stability and reusability, this simple and effective nanoparticle synthesis method is useful in enzyme engineering or biotechnology.

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