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Immobilization of plant peroxidases in cellulose–ionic liquid films

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S1 Materials. The solid preparations of horseradish peroxidase, HRP (M=44173.9 g mol^{-1}), RZ=3.1, 256 purpurogallin units per mg solid; soybean peroxidase, SBP (M=44000 g mol^{-1}), RZ=0.5, 90 purpurogallin units per mg solid (both enzymes were purchased from Sigma, USA), and cationic peanut peroxidase from the cultural medium of *Arachis hypogea* cells, PNP, RZ=2.0 (supplied by Prof. Robert van Huystee, University of Western Ontario, Canada); and bovine serum hemoglobin, Hb (M=64458 g mol^{-1}) (Sigma, USA) were used in the work. The catalytic activity of HRP, SBP, and Hb calculated in TMB units (the indicator substance with λ_{max}=374 nm) at 25°C was found to be 859, 433, and 31 units per mg solid, respectively.

Stock solutions of HRP, SBP, and Hb (10 μmol dm^{-3}) were obtained by dissolving precisely weighed portions of their solid preparations in 0.1 mol dm^{-3} phosphate buffer solution, pH 7.0. The concentrations of HRP, SBP, and Hb stock solutions were determined by spectrophotometric method (*ε:* 94[1]; 140[2] and 178[3] $mmol^{-1}$ dm^3 cm^{-1} at 403, 402 and 405 nm, respectively; *l*=1 cm). Phosphate buffer solution was prepared by mixing 0.1 M solutions of K_2HPO_4 and KH_2PO_4 (both samples were purchased from Merck, Germany). The solid preparations and solutions of the proteins were refrigerated at +4°C.

The solution of H_2O_2 with the concentration of 10.2 mol dm^{-3} (Sigma, USA) was used. The exact concentration of this solution was determined by permanganometry.[4] Working solutions of H_2O_2 (with concentration of 0.1 mol dm^{-3} and lower) were prepared daily by the dilution of the stock solution with water. Stock solutions of aryldiamines (0.1 mmol dm^{-3}) were prepared by dissolving accurately weighed amounts of the solid preparations of TMB и *o*-DA (Merck, Germany and Sigma, USA, respectively) in ethanol, and *o*-PDA (Sigma, USA) in water. Working solutions (0.1 mmol dm^{-3}) of catecholamines were obtained by dissolving precisely weighed portions of the solid preparations of dobutamine, dopamine hydrochloride, adrenaline hydrotartrate, and α-methyldopa sesquihydrate (Sigma, USA) in water in amber-glass bottles and prepared daily. The solutions with lower concentrations of substrates were prepared by successive dilution of their stock solutions with water or ethanol.

Microcrystalline cellulose was purchased from Sigma, USA. Commercial preparation of [bmim][Cl] (Sigma, USA) was dried according to the recommendations.[5]

Doubly distilled water (resistivity at 25°C 18 MΩ cm) purified with a Simplicity Proto system (Millipore, France) was used throughout.

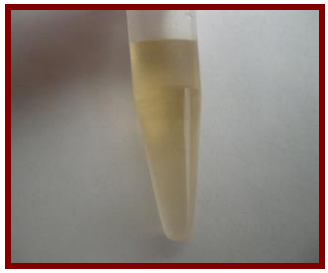
Apparatus. Kinetic measurements were performed using a Shimadzu UV mini-1240A spectrophotometer (Japan, accuracy ±0.0003 absorbance units) using a microcuvette with *l*=1 cm. The pH of aqueous solutions was measured with an accuracy of ±0.005 using a potentiometer in a pH-meter mode (Econics-Expert-001, Russia). Reaction mixtures were thermostated using a Model Thermit solid state thermostat (Russia, ±1°C precision). Micro-dosage devices produced by Biohit (Denmark) and Eppendorf (Finland) were used for sampling. Mettler Toledo MX5 Microbalance (Switzerland; ±0.005 mg precision) and Ohaus Adventurer Pro balance (USA, ±0.0005 g precision) were used for weighing the enzymes and others reagents, respectively. A stop watch (Russia, ±0.2 s precision) was used for fixing the time of the appearance of the color of a cellulose film (see S6).

S2 Dissolution of cellulose and HRP in [bmim][Cl]

Photos of the mixture of the lyophilized preparation of HRP with microcrystalline cellulose in melted [bmim][Cl] as a function of time (melt temperature: 85°C, thermostat control)

3 min

3 h



6 h

In the absence and in the presence of cellulose the solutions of HRP and SBP in [bmim][Cl] were yellow but not brown. Thus, cellulose destruction was not observed.

S3 Spectrophotometric determination of the concentration of HRP (SBP, Hb) in wash waters using the reaction of TMB oxidation by H_2O_2.

First, a 504 μl portion of a 0.1 mmol dm^{-3} phosphate buffer solution (pH 7.0), a 6 μl portion of the standard solution of the enzyme/protein (or wash water used for removing an excess of [bmim][Cl]; a 8 ml portion of collected wash

waters was diluted to 25.0 ml in a volumetric flask), a 30 μl portion of 0.5 mmol dm^{-3} TMB solution and a 60 μl portion of 0.01 mmol dm^{-3} solution of H_2O_2 were successively placed into a 1-ml plastic test-tube with a stopper. The total volume of the reaction mixture was 600 μl. A timer was turned on at the moment when the H_2O_2 solution was added to the reaction solution and mixed. The reaction solution was poured into a microcuvette and its absorbance at 374 nm was measured against water with 5 s intervals during 2 min on the Shimadzu UV mini-1240A spectrophotometer (l=1 cm). For a blank experiment, a 6 μl portion of wash water obtained after washing the biocatalyst-free film was added into the reaction mixture instead of the protein. Note the IL from wash water slightly accelerated the nonenzymatic reaction of TMB oxidation by H_2O_2.

The rate of the reaction of TMB oxidation by H_2O_2 with the participation of native biocatalysts was calculated using the tangent method.[6] According to the obtained data, the kinetic curves were plotted as absorbance at 374 nm (A) *vs.* time (t, s) (this wavelength corresponds to maximum absorbance of the indicator substance, the intermediate product of TMB oxidation with ε=23.7 $mmol^{-1}$ dm^3 cm^{-1})[7], and the slope of the initial part of the kinetic curve (tanα) was calculated. The quantity of the biocatalysts in wash waters was determined using the calibration curve plotted in the coordinates: tanα *vs.* the biocatalyst quantity, nmol (Table S1).

Table S1 Biocatalyst leaching from a cellulose film during washing.

Biocatalyst	Calibration curve equation: $y = ax + b$*	Quantity of the biocatalyst in wash waters (n=3, P=0.95)	
		mol×10^{11}	% of its initial content in the film (0.2 mg)**
HRP	$y = 3.72x + 0.14$	1.26±0.01	0.31±0.02
SBP	$y = 0.45x + 0.03$	2.06±0.02	0.51±0.02
Hb	$y = 0.02x + 0.01$	4.55±0.02	1.63±0.03

*y – tanα·10^2, x – quantity of a biocatalyst in the film, nmol.

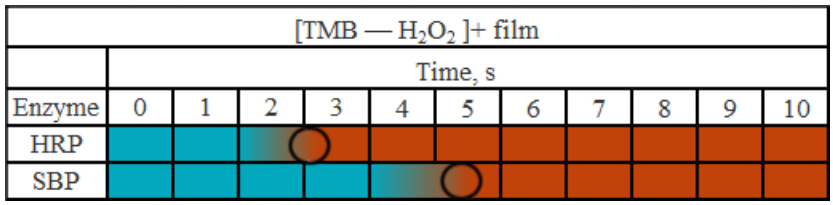
**to prepare one cellulose film (11.7 cm^2), we used only a 300 μl portion (one-third of the initial volume) of the melt {cellulose–IL–enzyme} which contained 0.2 mg of a biocatalyst.

S4 Color scales for monitoring the catalytic activity of plant peroxidases in the cellulose film

by the reactions of aryldiamines (TMB (1), *o*-DA (2), and *o*-PDA (3)) and catecholamines (α-methyldopa (4), dopamine (5), adrenaline (6), and dobutamine (7)) oxidation by H_2O_2. To compare the catalytic activity of the immobilized peroxidases in the same indicator reaction, the developing color in the films in the course of each reaction was videotaped and the time was

determined when the intensities of colors in the films with HRP and SBP (marked with circles on the scales) became equal. Note that cellulose films with immobilized SBP were colorless in the reactions of adrenaline and dobutamine oxidation. That can be related to a lower specificity of SBP towards these substrates in comparison with HRP. In the blank experiments the films prepared without the biocatalyst were used.

1



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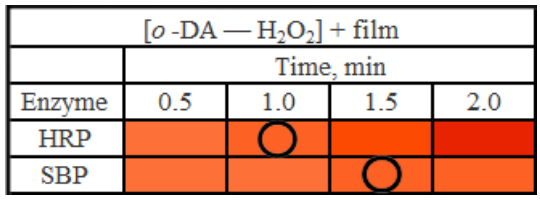

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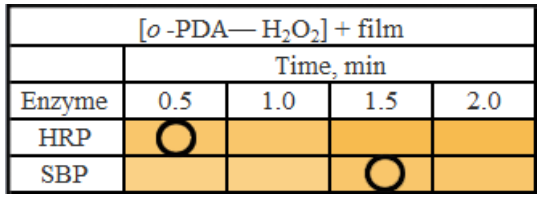

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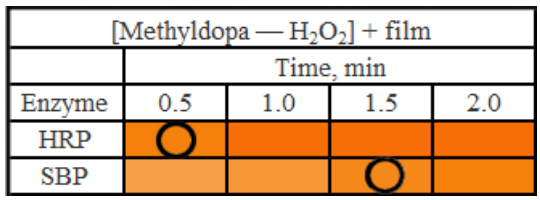

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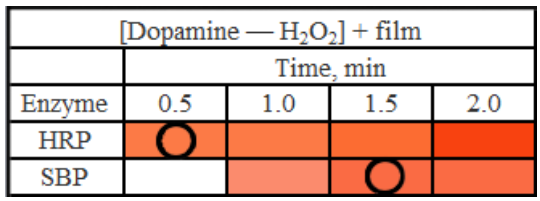

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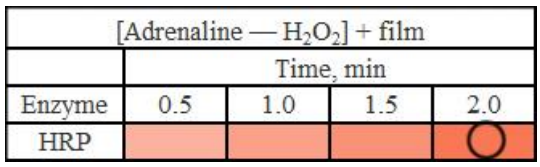

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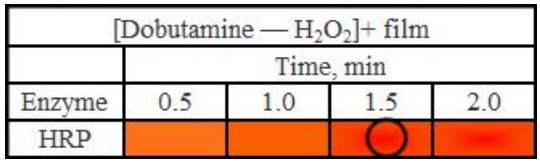
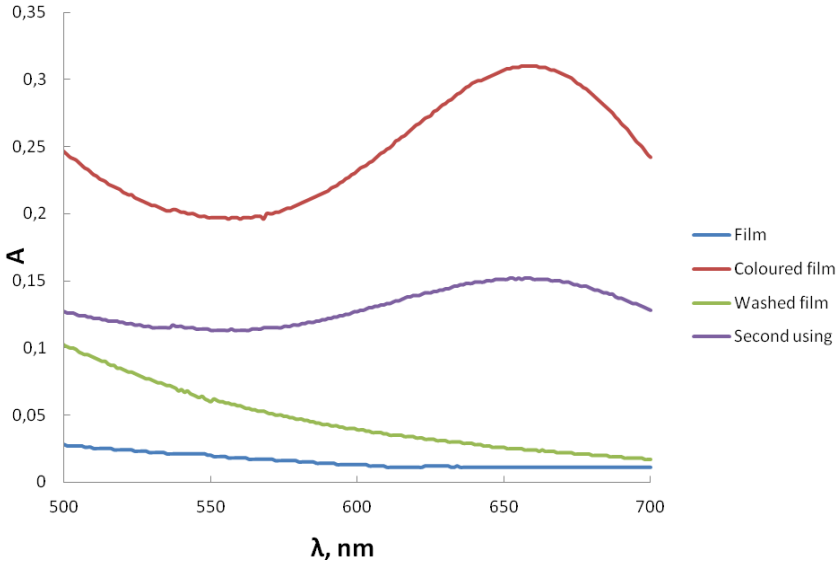


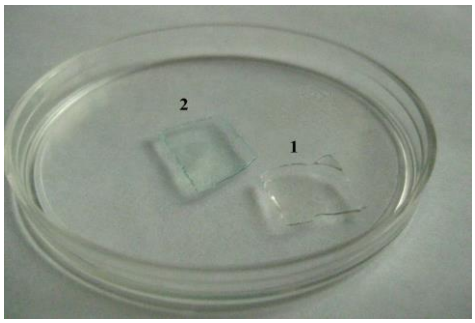
S5 Absorption spectra of the films that prove the possibility of film reuse.



To prove the possibility of a film reuse, a 20 μl portion of 0.1 mmol dm^{-3} solution of TMB and a 10 μl portion of 0.1 mmol dm^{-3} solution of H_2O_2 were successively added to the same point of a film. Timer was turned on at the moment when H_2O_2 was added. A swollen film was placed rapidly on a glass slide surface where it was kept by adhesive forces. The absorption spectrum of the colored film was registered (red curve). Then the colored film was washed in water for 15 min and the absorption spectrum was registered again (green curve). Further the oxidation reaction of TMB was carried out on the surface of the film once more and its spectrum was registered (violet curve), as it was done in the beginning. It should be noted that it was more convenient to control the degree of film washing by the absorption peak of the intermediate product of TMB oxidation at 652 nm.[8]

S6 Oxidase activity of plant peroxidases in cellulose films in the reactions of aryldiamines oxidation. Developing color in the cellulose film with immobilized HRP and SBP in the indicator systems aryldiamine–O_2 in time is shown on the left; a photo of the cellulose film prepared without the enzyme (1) and the cellulose film with HRP (2) in 2 min after dropping a 20 μl portion of 0.1 mmol dm^{-3} solution of TMB solution and a 10 μl portion of H_2O is given on the right.

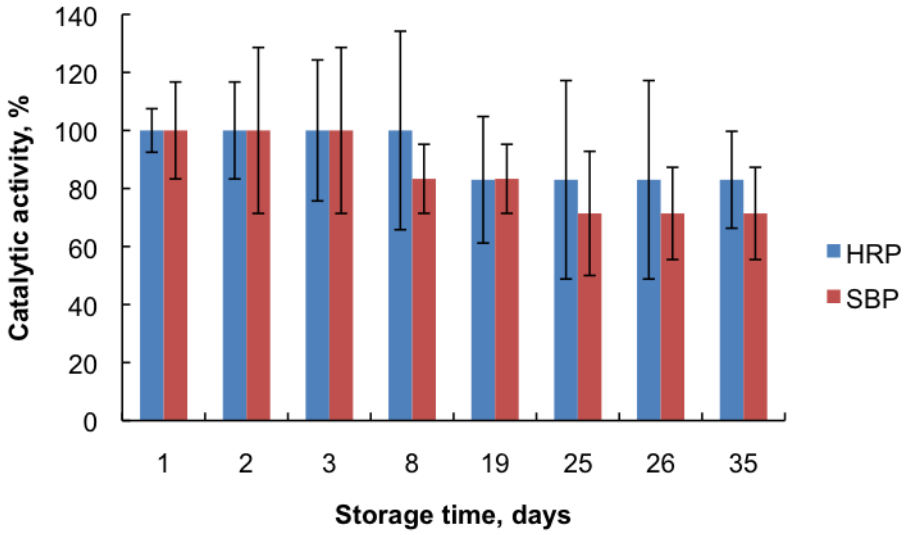
Time, min											
	0	1	2	3	4	5	6	7	8	9	10
Enzyme	TMB + film										
HRP											
SBP											
	o -DA + film										
HRP											
SBP											
	o -PDA + film										
HRP											
SBP											



S7 Optimization of the storage conditions of enzyme-containing cellulose films, which was obtained by the procedure described in S2. Three ways to store films were: 1) in water; 2) in a dry state on air; 3) in a swollen state in a sealed container. During storage of the films in water for a week, both peroxidases were leached from the film, and more seriously from that surface contacting with water. If a film was stored for one day on air, it lost its permeability towards the substrates, became crisp and useless for further practical application (see photo).

The film in a swollen state in a sealed container did not dry out during the whole storage period and retained its penetrability to the components of the indicator reaction. The films in a swollen state were stored in a refrigerator at +4°C and at room temperature. HRP and SBP in the cellulose films kept at +4°C lost their catalytic activity 1.3 times faster than the enzyme in the films stored at room temperature (20–25°C). Thus, the best condition of storing the cellulose films is in a swollen state in a sealed container at a room temperature.

S8 The dependence of the catalytic activity of HRP and SBP immobilized into the films {cellulose–[bmim][Cl]} *vs.* storage time. The residual catalytic activity of peroxidases was calculated as a ratio of time of the appearance of solid brown color (S6, scale 1) in the reaction of TMB oxidation by H_2O_2 on the tested day of the storage of the film and on the day of the film formation (n=3, P=0.95). The indicator reaction was carried out as described in S5.



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