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PREPARATION AND CHARACTERIZATION OF HORSE SERUM BUTYRYLCHOLINESTERASE IMMOBILIZED ON PAPER-BASED CELLULOSE MATRIX

SONJA BAUK
Military Technical Institute, Belgrade, bauk.sonja@gmail.com

MAJA VITOROVIĆ-TODOROVIĆ
Military Technical Institute, Belgrade, majavitod@gmail.com

MARINA ILIĆ
Military Technical Institute, Belgrade, marinailic1970@gmail.com

TAMARA VUJATOVIĆ
Military Technical Institute, Belgrade, tamara.vujatovic@gmail.com

TATJANA MARKOVIĆ
Military Technical Institute, Belgrade, tanjin.mejl@gmail.com

Abstract: *The great importance of rapid detection and reliable quantification of toxic organophosphates (OPs) arose as a consequence of their widespread application, extreme toxicity and potential use as chemical warfare agents. The potency of OPs to inhibit enzymes of the cholinesterase family (ChEs) enabled the invention of various biosensors and disposable analytical devices able to provide simple, fast, sensitive, selective and low cost detection of OPs at low concentration. The effective immobilization of enzymes onto supporting material surfaces is an important step in design and fabrication of different bioassay devices, crucial for their performances. This work reports the immobilization of horse serum butyrylcholinesterase (BChE) on paper-based cellulose matrix and biochemical properties evaluation of the immobilized enzyme.*

Keywords: *butyrylcholinesterase, organophosphates, immobilization, cellulose, detection.*

1. INTRODUCTION

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two main and closely related representatives of enzymes from the cholinesterase (ChEs) family, which play important roles in human and animal function and health [1][2]. Characteristic feature of ChEs is their inhibition by organophosphates (OPs), a group of highly toxic chemicals commonly used for agricultural, industrial, household and medical purposes, but also as nerve agents with dominant position in chemical warfare.

OPs, also known as anti-ChEs, exert their toxic action by covalently binding to the active site of AChE, one of the most crucial enzymes responsible for the normal functioning of the central and peripheral nervous system. The inhibition of AChE by OPs prevents the enzymatic breakdown of neurotransmitter acetylcholine, causing the overstimulation of acetylcholine receptors in the brain, skeletal and muscular systems, which results in convulsion, paralysis and finally death for insects and mammals [3]. Although the inhibition of BChE does not lead to mortality, it has a great toxicological and pharmacological importance as endogenous target susceptible to inhibition with high sensitivity [4][5].

The irreversible nature of ChEs inhibition by OPs permitted the development of many enzymatic methods for analysis and determination of those toxic compounds. Many research papers are focused on the applications of ChEs in different biodetection techniques, since the existence of rapid detection methods and reliable quantification of toxic OPs are of great importance. The potency of OPs to inhibit ChEs enabled the invention of simple detectors and disposable analytical devices for OPs assay, which are especially suitable for military, environmental and agricultural purposes. Many efforts have been focused on the construction of various dipsticks and strips, able to operate without any instrumental device, based on visible color change and applicable for semi-quantitative OPs assay [6][7][8].

Recently, paper has attracted considerable attention as a matrix for fabrication of low-cost analytical devices, owing to its unique features of being inexpensive, biodegradable, biocompatible and hydrophilic. The immobilization of ChEs on paper surface led to the invention of various fast-responding tools for rapid screening and early diagnosis in health and environmental applications [9][10]. Paper-based biosensors provide rapid detection of OPs with minimal equipment requirements

and with advantages of cost effectiveness, simple fabrication, disposability and stability.

Bioactive paper can be produced by various techniques of enzymes immobilization which can be broadly divided into covalent and physical methods according to the molecular forces between enzymes and support matrix. Physical methods are simple, have a wide range of applications and include adsorption and entrapment, but a combination of adsorption, covalent attachment and entrapment can also be found in literature. Adsorption is the simplest and most cost-effective immobilization method that barely affects enzyme activity due to weak interaction with the carrier.

In this work, the immobilization of horse serum butyrylcholinesterase (BChE) on paper-based cellulose matrix using the simplest and the most cost-effective method was examined, as well as the influence of stabilizers, organic solvents and elevated temperature on the properties of the obtained immobilizates.

2. MATERIAL AND METHODS

2.1. Material

Horse plasma butyrylcholinesterase (BChE, liophilized powder, with the activity of ≥ 10 units/mg protein according to manufacturer's declaration), bovine serum albumin (BSA) and gelatin from porcine skin (high gel strength) were purchased from Sigma-Aldrich, as well as butyrylthiocholine iodide (≥ 99.0 %) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, ≥ 98 %). All other reagents were of analytical grade. Whatman No. 1 filter paper manufactured as circular disks with a diameter of 15 mm (paper circles) was used as a support matrix for enzyme immobilization.

The enzyme stock solution (53 U/mL) was prepared by dissolving liophilized BChE in phosphate buffer (PB, 50 mM, pH 7.4). Different volumes of enzyme stock solution were further diluted in PB to obtain working solutions with BChE concentrations in the range of 5-20 U/mL. Working solutions of BChE with albumin (1 - 5 %) or gelatin (0.5 - 1.5 %) were prepared according to the same procedure, by dilution of enzyme stock solution in PB containing the stabilizer in the amount required to achieve the final concentration.

2.2. Methods

2.2.1. Immobilization procedure

Working solutions of BChE were added to the paper circles in aliquots of 20 μL and let to dry at 25 ºC. The obtained immobilizates were stored in closed glass vessels and kept in the refrigerator until use.

The activity yield was calculated according to the following equation:

$$AY\,(\%) = \frac{A_i}{A_0} \times 100 \qquad (1)$$

where A_0 is the total number of BChE units added to the support for immobilization (offered activity) and A_i is the number of BChE units detected in the support after immobilization under the conditions defined by the assay method described below (observed activity).

2.2.2. Activity assay

The BChE activity measurements were performed essentially according to the method of Ellman [12], using butyrylthiocholine iodide (2 mM) as the substrate and DTNB (0.32 mM) as the indicator (Ellman's reagent). Free enzyme (20 μL) was added to a cuvette containing PB (2.64 mL) preincubated at 25 °C, followed by addition of DTNB (0.24 mL) and substrate (100 μL). The formation of the yellow anion obtained from the reaction between Ellman's reagent and the thiocholine generated by enzymatic hydrolysis of the substrate was monitored by measuring the increase in absorbance at 412 nm for 3 min at 15 s intervals. The measurement of the immobilized enzyme activity was carried out as described above, except that corresponding amount of the immobilizate (one paper circle) was taken instead of free enzyme and placed in a dry cuvette before all others reagents. All results were obtained from measurements performed in triplicate.

One unit of enzymatic activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of butyrylthiocholine per minute.

2.2.3. Thermal stability

Free enzyme (20 μL) or immobilizate (one paper circle) was incubated at 55 ºC for 4 h in a sealed cuvette with a portion of PB (1.5 mL). After incubation, the contents of the cuvette were diluted with another portion of cold PB from the refrigerator (1.14 mL) and the remaining activity in the cuvette was determined using a standard assay method. The contents of the cuvette were not previously equilibrated to a temperature of 25 ºC.

The percentage of the residual activity for each sample was calculated considering its initial activity before heat treatment as 100%.

2.2.4. The effect of organic solvents

Free enzyme (20 μL) or immobilizate (one paper circle) was incubated at 25 ºC for 15 min in a sealed cuvette with PB (2.64 mL) containing 5 % organic solvent. After incubation, the remaining activity in the cuvette was determined using a standard assay method. The percentage of the residual activity for each sample was calculated considering its activity in the absence of organic solvent as 100%.

3. RESULTS AND DISCUSSION

3.1. The effect of enzyme concentration

The activities of the obtained immobilizates and the activity yields achieved using working solutions of BChE

with different enzyme concentrations in accordance with the described immobilization procedure are shown in **Table 1**.

Table 1. The influence of the enzyme concentration in working solution on the activity exerted by the immobilizate and achieved activity yield (the mean ± SD)

Working solution (U/mL)	Offered activity (mU)	Observed activity (mU)	AY (%)
5.0 ± 0.2	100 ± 4	28 ± 2	28 ± 2
10.0 ± 0.3	200 ± 5	64 ± 8	32 ± 4
14.9 ± 0.3	299 ± 7	127 ± 7	42 ± 3
19.9 ± 0.3	398 ± 5	172 ± 8	43 ± 2

Over the range of concentrations tested, the activity of immobilized BChE gradually increased, while the activity yield reached a plateau for enzyme concentrations above approximately 15 U/mL (**Figure 1**).

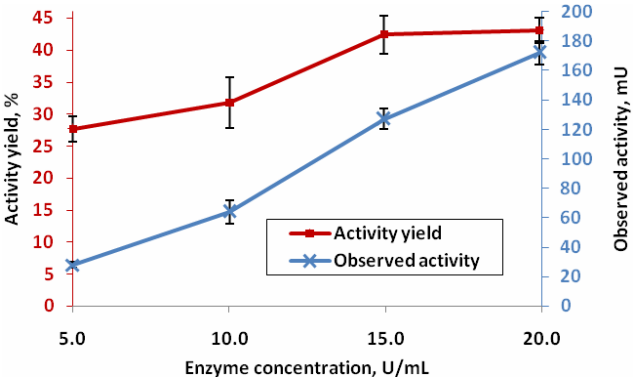

Figure 1. The observed activities of immobilized BChE and activity yields as a function of enzyme concentration

As the immobilization procedure did not include washing the immobilizates as an additional step that could remove unbound or loosely bound enzyme molecules from the support surface, enzyme leakage into the reaction solution during the activity assay was expected. However, control experiments in which the immobilizates were used for a second reaction cycle, after pre-washing traces of the reaction mixture from the first cycle, resulted in the activity drop below 5 % (data not presented) and revealed that the measured activity values, under the activity assay conditions, originated mostly from the action of enzyme molecules released from the surface into the reaction solution.

A working solution of BChE with the enzyme concentration of approximately 10 U/mL was chosen for further study.

3.2. The effect of stabilizers

Since BSA and gelatin were shown to have a stabilizing and protective effect on enzymes, working solutions of BChE with an enzyme concentration of approximately 10 U/mL and different concentrations of BSA or gelatin were used in the immobilization procedure. The activities of the obtained immobilizates and the achieved activity yields are shown in **Table 2**.

The immobilization of BChE with 1 % BSA in working solution led to a higher observed activity of immobilizate **Im-A1** compared to **Im**. Consequently, this also meant an increased activity yield given the slight fluctuation of the offered activity between samples, possibly due to changes in the viscosity of the prepared enzyme solutions. A further increase of BSA concentration did not correspond to an increase in the observed activity of immobilizates (**Im-A2**, **Im-A3**), while the highest concentration tested led to a slight decrease in the activity yield (**Figure 2**).

Table 2. The influence of the type and amount of stabilizer in the working solution of BChE on the activity exerted by the immobilizate and achieved activity yield

	BSA (%)	Gelatin (%)	Offered activity (mU)	Observed activity (mU)	AY (%)
Im	-	-	200 ± 5	64 ± 8	32 ± 4
Im-A1	1	-	212 ± 3	90 ± 6	42 ± 3
Im-A2	3	-	194 ± 2	82 ± 5	42 ± 3
Im-A3	5	-	215 ± 3	80 ± 6	37 ± 3
Im-G1	-	0.5	202 ± 4	55 ± 7	27 ± 4
Im-G2	-	1	221 ± 5	31 ± 5	14 ± 2
Im-G3	-	1.5	226 ± 5	20 ± 4	9 ± 2

The presence of gelatin in the working solution of BChE gradually reduced the observed activity of immobilizates, with the most pronounced decrease in the activity yield at the highest tested concentration.

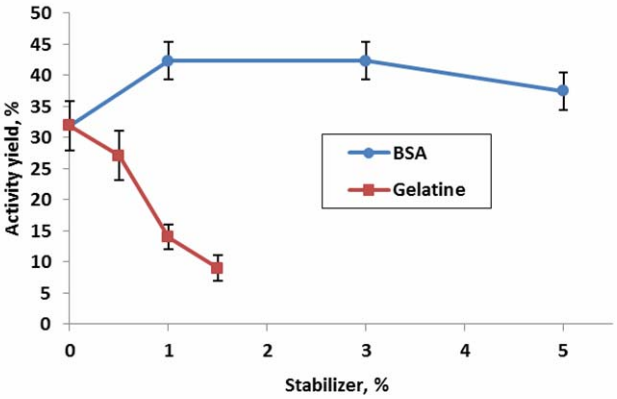

Figure 2. The activity yields as a function of the type and amount of stabilizer in the working solution of BChE used for immobilization

However, as the applied activity assay method hindered the true assessment of the overall immobilization efficiency and mainly reflected the amount of free enzyme in the solution, it is likely that both tested stabilizers had a different, but still beneficial effect on the immobilized BChE. A reasonable explanation for the diminishing effect of gelatin on the observed activities could relay on the fact that a higher concentration of

gelatin leads to a lower permeability and a higher diffusion resistance in immobilizates compared to the presence of BSA.

3.3. Thermal stability

The immobilizates prepared using working solutions of BChE with an enzyme concentration of approximately 10 U/mL without stabilizers or in the presence of three different BSA or gelatin amounts were subjected to thermal treatment at 55 ºC for 4 h, as well as the free enzyme in solution without any additives. The measured activities before and after thermal treatment and calculated residual activities of all tested samples are shown in **Table 3**.

It should be emphasized that the obtained results do not reflect the true degree of temperature-induced effects on individual samples due to differences in the temperature conditions of initial and residual activity assay, but reflect the relative ratio of these effects among different samples.

Table 3. The activities of free enzyme and immobilzates before and after incubation at 55 ºC for 4 h

	Initial activity (mU)	Activity after 4 h at 55 ºC (mU)	Residual activity (%)
Free	172 ± 5	87 ± 4	51 ± 3
Im	54 ± 5	28 ± 2	52 ± 6
Im-A1	95 ± 5	74 ± 9	78 ± 10
Im-A2	69 ± 9	76 ± 7	110 ± 17
Im-A3	68 ± 8	78 ± 6	115 ± 16
Im-G1	58 ± 3	43 ± 3	74 ± 6
Im-G2	26 ± 2	52 ± 3	200 ± 19
Im-G3	17 ± 2	58 ± 3	341 ± 43

Two main trends can be observed from the data presented in **Table 3**.

First, the decrease in activity after thermal treatment was characteristic of the free enzyme, immobilizate prepared without stabilizers (**Im**) and immobilizates prepared with the lowest stabilizers concentrations in the range tested (**Im-A1** with 1 % BSA and **Im-G1** with 0.5 % gelatin).

Second, the increase in activity after thermal treatment was effect exerted by immobilizates prepared with two higher concentrations of stabilizers in the tested range (**Im-A2** and **Im-A3** with 3 % and 5 % BSA, **Im-G2** and **Im-G3** with 1 % and 1,5 % gelatin, respectively).

Denoted observations are visually more noticeable at **Figure 3**, where all activities are presented in normalized form, with a value of 100% assigned to the initial activity of free enzyme in the working solution tested.

Thermal treatment reduced the activities of free enzyme and immobilizate **Im** (prepared in the absence of any stabilizer) by the same extent, while the presence of stabilizers attenuated the temperature-induced inactivation and brought about activity changes in the other direction. Higher activity values after thermal treatment in immobilizates prepared with stabilizers revealed higher enzyme loading and also leakage promoted under incubation conditions, the latter being more pronounced with gelatin. This is consistent with the fact that gelatin gels are not thermally and mechanically stable without additional cross-linking, since the gelation process is reversible with temperature.

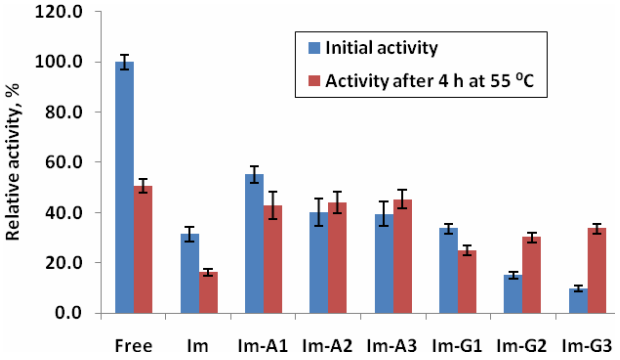

Figure 3. The activities of free enzyme and immobilzates before and after incubation at 55 ºC for 4 h, relative to the initial activity of the free enzyme

3.4. The effect of organic solvents

The immobilizates prepared using working solutions of BChE with an enzyme concentration of approximately 10 U/mL without stabilizers or in the presence of 5 % BSA or 1.5 % gelatin were incubated in PB with 5 % of organic solvent (2-propanol, acetonitrile, acetone or DMSO), as well as the free enzyme in solution without stabilizers. The calculated residual activities of all samples examined are shown in **Table 4.**

None of the organic solvents altered the activity of free enzyme by more than 25% at the tested concentration. Besides, the action of all tested solvents on the free enzyme was inhibitory, with the exception of 2-propanol which had an activating effect.

A similar behavior was observed with the immobilizate **Im**, with a milder inhibitory and more pronounced activating effect that led to a 60 % increase in the observed activity.

Table 4. The residual activities of free enzyme and immobilzates after 15 min incubation at 25 ºC in PB containing 5 % organic solvent

	Residual activity, %			
	2-propanol	**acetonitrile**	**acetone**	**DMSO**
Free	114 ± 6	81 ± 4	76 ± 5	94 ± 5
Im	160 ± 22	94 ± 13	91 ± 15	94 ± 14
Im-A3	174 ± 14	138 ± 11	135 ± 11	151 ± 15
Im-G3	301 ± 42	122 ± 22	154 ± 29	96 ± 21

Moreover, higher values of the observed activities after incubation in the presence of organic solvents relative to the initial activities were measured for immobilizates

prepared with stabilizers (**Im-A3** and **Im-G3**), with the exception of the slight decrease in the case of **Im-G3** in DMSO.

The results discussed are perceptible in the bar graph at the top of **Figure 4**. The significant increase in the activities of the immobilzates with stabilizers, simmilar as in the thermal stability test, again points to enzyme leakage during the incubation with organic solvent.

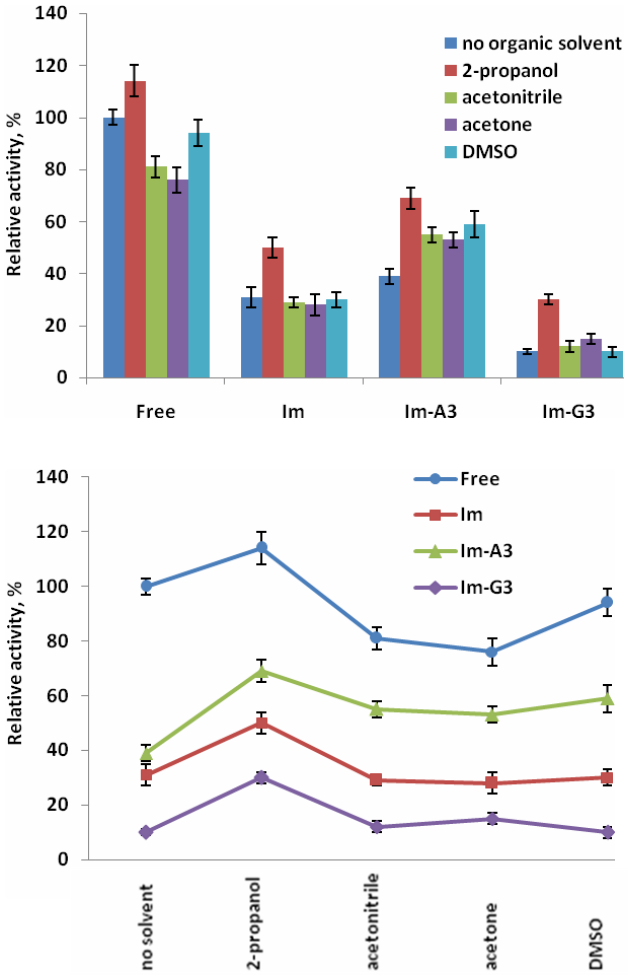

Figure 4. The effects of organic solvents on the activities of free and immobilized BChE relative to the initial activity of the free enzyme: graph in bar (top) and line form (bottom)

Apart from the fact that the applied treatment caused different changes in the magnitudes of the residual activities of different samples incubated in the presence of the same solvent, the spectrum of relative responses of free enzyme and imobilizates **Im** and **Im-A3** to the presence of different organic solvents have a similar profile, which corresponds well to the previous finding that the measured activity values in the activity assay conditions reflected particularly the action of the enzyme molecules released from the surface into the reaction solution. The similarity in the relative responses spectrum of free enzyme and immobilizates is more obvious from the line graph provided at the bottom of the **Figure 4**. A slightly different profile in the relative responses excreted by the immobilizate **Im-G3** implies the involvement of additional processes in the obtained results, which probably arose from the extent and nature of organic solvents interactions with the porous structure of the gelatin film on the paper surface.

4. CONCLUSION

The immobilization of BChE was carried out by simple method consisting of applying equal aliquots of different enzyme solutions onto the surfaces of Whatman No. 1 paper circles. The influence of changing the applied enzyme solutions in terms of BChE concentration, the presence of stabilizers and their type and content on the observed activity of the obtained immobilizates, achieved activity yields, thermal stability and the effect of organic solvents was investigated. The performance assessment of different immobilizates were based on the measurements of their catalytic activity in a solution containing butyrylthiocholine iodide (2 mM) as a substrate and Ellman's reagent (0.32 mM) as an indicator.

In the range of BChE concentrations tested (5 – 20 U/mL), the immobilizates showed a gradual increase in the observed activity, while the activity yield reached a plateau of just above 40 % at an enzyme concentration of 15 U/mL. The immobilizates prepared in the absence of stabilizers had a similar response to the effect of thermal treatment (4 h at 55 ºC) and organic solvents (5 %) as observed for the free enzyme. Immobilization in the presence of BSA or gelatin as stabilizers caused contrary effects on the observed activities of immobilizates and, consequently, the achieved activity yield, since BSA led to an increase, and gelatin to a significant and concentration-dependent decrease of these parameters. However, after thermal and organic solvents tests, both stabilizers contributed to higher values of the immobilizates residual activities that generally exceeded the initial ones, which revealed higher enzyme loading than obtained without stabilizers and also enzyme leakage from the carrier, the latter being in accordance with the used immobilization procedure.

It is known that the observed activity of an immobilizate is highly dependent on the activity assay used, which is mainly caused by diffusion limitations in the immobilization matrix. Accordingly, by examining the obtained immobilizates in a wider range of different conditions that allow a true assessment of their properties and overall immobilization efficiency, the possibilities and limitations of their application for the purposes of OPs detection would be more closely defined, which remains for further research.

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