Comparative studies on the interaction between biogenic polyamines and bovine intestinal alkaline phosphatases: spectroscopic and theoretical approaches

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Abstract

In this work, the effect of two organic polyamines (spermine and spermidine) on the fluorescence intensity and activity of bovine intestinal alkaline phosphatase (BIALP) are investigated. The interaction of BIALP with spermine and spermidine was studied in a diethanolamine buffer with 0.5 mM magnesium chloride (pH 9.8) and at two temperatures by using the fluorescence quenching method. Furthermore, the activity of enzyme was studied using UV–Vis spectrophotometry in a diethanolamine buffer with 0.5 mM magnesium chloride, at 37 °C, in the absence and presence of different concentrations of each polyamine (0–5 mM). It was demonstrated that both polyamines quenched the intrinsic fluorescence of BIALP by the static quenching process. Based on these results, the values of the binding site for both polyamines were close to each other and decreased by increasing the temperature. The calculated thermodynamic parameters ($\Delta H^\circ < 0$ and $\Delta S^\circ < 0$) also showed that the acting forces in the formation of the complex between BIALP and polyamines were hydrogen bonds and van der Waals forces with an overall favorable Gibbs free energy change ($\Delta G^\circ < 0$). In addition, kinetic studies revealed that these polyamines enhanced the enzyme activity of BIALP in a concentration-dependent manner. This result also indicated that spermine had more of an effect on BIALP activity in the same condition. Also, molecular docking as well as thermodynamic parameters showed that hydrogen bonds and van der Waals forces played an important role in the stabilization of BIALP–polyamine complexes.

Keywords Bovine intestinal alkaline phosphatase · Polyamines · Enzyme activity · Intrinsic fluorescence · Molecular docking

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1 Introduction

Polyamines are low molecular weight aliphatic compounds. They have several amine groups in their structures (shown in Fig. 1); therefore, they are present as polycations form at the physiological pH and can interact with negatively charged molecules such as amides, nucleic acids, lignins, and proteins. Hence, polyamines play a significant role in gene therapy. They help DNA with negative charge to condense into nanoparticles of ~ 100 nm and they comfort DNA transport to the cell membrane [1]. Biogenic polyamines include putrescine ($C_4H_{12}N_2$), spermidine ($C_7H_{19}N_3$), and spermine ($C_{10}H_{26}N_4$), which are involved in many biological processes; they are also essential for cell growth, differentiation, proliferation processes, and nucleic acid biosynthesis in eukaryotic and prokaryotic cells [2].

The review of the literature shows that biogenic polyamines can keep biomacromolecules such as proteins against thermal aggregation and inactivation of enzymes. Therefore, polyamines have been used widely as an additive to keep enzymes from thermal aggregation and inactivation. The high content of various polyamines in hyperthermophilous creatures can exhibit the protective role of biogenic polyamines in protecting proteins in high temperature conditions [3–5].

Alkaline phosphatases (ALPs) or alkaline phosphomonoesterases (E.C. 3.1.3.1) are enzymes that catalyze the hydrolysis of phosphate monoesters in high pH regions [6]. These enzymes have been found in many sources ranging from bacteria to human and play an important role in biochemical processes [7]. Mammalian ALPs have been isolated from several organs including liver, kidney, placenta, intestinal mucosa, etc. Bovine Intestinal mucosa Alkaline phosphatases (BIALP) is a dimeric metalloenzyme having two similar subunits with 530 amino acid residues in each polypeptide chain and a molecular weight of about 160 kDa. At termination, a number of helices and sheets create an active site located in a hydrophobic pocket that can have access to media [8]. Each subunit contains two zinc and one magnesium ion and one phosphorylable serine residue per active site. These divalent ions are necessary for the catalysis and stability of the enzyme. The magnesium ion does not directly take part in the mechanism but it is important for enzyme stability; in contrast, both zinc ions directly participate in catalysis. Zinc ions activate molecules of water and stabilize the deprotonated form of the serine residue that is required for nucleophilic attacks. Furthermore, in the active site, the magnesium ion can facilitate the transfer of the phosphoryl group of the substrate *via* a water molecule [8, 9]. ALPs have five cysteine residues forming two disulfide bonds, which cause more stability of the

Fig. 1 Spermine (a) and spermidine (b) structures

enzyme [10]. Among mammalian ALPs, BIALP, with the highest specific activity, has been widely applied in molecular biology for such purposes as conducting enzyme immunoassay (EIA), dephosphorylating the termini of nucleic acids in nucleic acid hybridization assays and employing the polymerase chain reaction; it also plays an important role in diagnosis [11–13].

Recently, the use of small molecules as cosolvents, which can increase enzyme activity and stability, has received more attention owing to the practical uses required [14, 15]. Literature review shows that the *in vitro* use of polyamines as a solution additive can involve stabilization and enzyme hyperactivation [4, 16–18]. This research study aims to investigate the interaction between polyamines (spermine and spermidine) as cosolvents and BIALP by using the fluorescence quenching method. Furthermore, the main binding force in the combination of BIALP and polyamines is illustrated by using thermodynamic parameters (ΔH° , ΔS° , and ΔG°). In addition, the effect of these polyamines on enzyme activity is evaluated through kinetic studies.

2 Results and discussion

2.1 The effect of polyamines on the fluorescence intensity of BIALP

Fluorescence spectroscopy and its applications in life and physical sciences have evolved rapidly during the past decade. Fluorescence intensity can be decreased by a wide variety of processes called quenching reactions; the compounds causing them are called quenchers [19]. Quenching reactions are facile to carry out, need only a small sample, are nondestructive, and can be applied to almost any system that has an intrinsic or extrinsic fluorescence probe (fluorophores) [20].

Aromatic amino acids (tryptophan, tyrosine, and phenylalanine) are intrinsic fluorophores responsible for the fluorescence of proteins. The fluorescence properties of these fluorophores are dependent on the protein's structure as well as the surrounding environment [21, 22]. Thus, the quenching reaction can be applied to probe the topographical features of a macromolecular assembly and to unveil any alterations in protein structures that can occur by various conditions or the addition of reagents. In addition, quenching reactions can provide information about conformational fluctuations [23, 24]. In proteins, tryptophan fluorescence dominates [25]. Zero or weak tyrosine and phenylalanine fluorescence results from energy transfer to tryptophan residues and/or neighboring amino acid residues [20, 26]. BIALP is a homodimeric enzyme with four tryptophan residues in each polypeptide chain. These fluorophores are sensitive to their microenvironmental properties. Their position in the structure of the protein exposed to the solvent in different degrees determines the intrinsic fluorescence intensity of BIALP [8, 27].

Fluorescence spectral plots generally present the fluorescence intensity versus wavelength (nanometers). The emission spectra of BIALP were plotted in the range of 290–450 nm, in the absence and presence of various concentrations of polyamines (spermine and spermidine), at two temperatures, as shown in Figs. 2 and 3, respectively. It became clear that increasing the concentration of polyamines reduced the fluorescence intensity of the enzyme without any red or blue shift, with the maximum emission being at 333 nm. It was inferred that spermine and spermidine could interact with BIALP by changing the enzyme's conformation and the

Fig. 2 Fluorescence intensity quenching of BIALP in the absence and presence of different concentrations of spermine (0–6 mM) at 25 °C (a) and the change in the maximum fluorescences of BIALP in the absence and presence of different concentrations of spermine at 25 °C and 35 °C (b)

microenvironment of Trp residue(s), which could act as a quencher. The accessibility of these residues was in the order: Trp-12 > Trp-248 > Trp-168 > Trp-237 [28].

2.2 Mechanism of fluorescence quenching reactions and binding parameters

Fluorescence quenching can be created by different mechanisms that usually consist of static quenching, dynamic quenching, or both simultaneously [29]. Collisional or dynamic quenching is caused by collisional encounters between the fluorophore and quencher, whereas in static quenching, a non-fluorescent complex is formed between the fluorophore and the quencher in a ground state [30]. Both the static and dynamic types of quenching

Fig. 3 Fluorescence intensity quenching of BIALP in the absence and presence of different concentrations of spermidine (0–6 mM) at 25 °C (a) and the change in the maximum fluorescence of BIALP in the absence and presence of different concentrations of spermidine at 25 °C and 35 °C (b)

require some molecular contact between the fluorophore and the quencher [31]. Static and dynamic quenching can be distinguished by their differing dependence on temperature and viscosity. In dynamic quenching, higher temperatures result in faster diffusion and larger amounts of collisional quenching, while in the static one, it leads to the dissociation of weakly bound complexes and smaller amounts of quenching [32]. Thus, increasing the quenching constant values in higher temperatures can indicate dynamic quenching; conversely, in static quenching, higher temperatures result in lower quenching constant values. The Stern–Volmer equation is a relationship explaining the effect of the quencher on the steady-state fluorescence of a sample. Equation (1) [33, 34] is:

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

In this equation, F_0/F are the fluorescence intensities of BIALP in the absence and presence of the quencher (spermine or spermidine), respectively; Q is the concentration of the quencher and K_{sv} is the Stern–Volmer quenching constant. The data of quenching are reported as Stern–Volmer plots (F_0/F against $[Q]$), with the interception of one being on the y -axis and their slopes being equal to K_{sv} [35]. The Stern–Volmer plots for different concentrations of spermine and spermidine at 25 °C and 35 °C are shown in Fig. 4a, b; also, the obtained values for K_{sv} are presented in Table 1. As shown in Fig. 4, the plots were liner, so a single class of the quenching mechanism of fluorophore could be represented in the BIALP-polyamines complex formation.

The slopes of plots or K_{sv} were decreased by increasing the temperature; as a result, the quenching reaction of BIALP in the presence of both polyamines was a static one. Therefore, it is possible to compute the binding parameters. These parameters, binding constant (K_b) and number of binding sites (n), can be calculated using the following Eq. (2) [24, 36]:

$$\log(F_0 - F)/F = \log K_b + n \log [Q] \quad (2)$$

The plots of $\log(F_0 - F)/F$ versus $\log [Q]$ at two temperatures (25 °C and 35 °C) are shown in Fig. 5; also, the computed amounts of K_b and n are presented in Table 1. According to Eq. (2), the number of binding sites can be obtained from the slopes of these plots [34].

These results indicated the closeness of the binding site values for both polyamines at the two temperatures. In addition, the binding constant of both polyamines on BIALP was decreased by increasing the temperature, indicating that combinations between BIALP and polyamines depended on the temperature. As depicted in Table 1, the values of K_b were decreased at higher temperatures, once more confirming the static mechanism of quenching. Also, the higher values of the binding constants of spermine showed its higher tendency for binding on BIALP under the same conditions. This could be due to more amino groups of spermine, as compared to spermidine [37].

2.3 Analysis of thermodynamic parameters and binding forces

In general, the weak interaction forces between proteins and ligands play a significant role in complex formations. There are four main noncovalent forces that are classified on the basis of the sign and amount of thermodynamic parameters, enthalpy change (ΔH°) and entropy change (ΔS°). Hence, according to previous studies, these forces consist of

Fig. 4 Stern–Volmer plots of the interaction between BIALP with spermine (**a**) and spermidine (**b**) at two temperatures (25 °C and 35 °C)

Table 1 Stern–Volmer quenching constants, bimolecular quenching rate constants and binding parameters for BIALP-polyamines complexes

Compound	<i>T</i> (°C)	<i>K_{sv}</i> (M ⁻¹)	<i>n</i>	<i>K_b</i> (M ⁻¹)
Spermine	25	101.2	1.03	93.4
	35	64.1	1.01	63.1
Spermidine	25	63.5	0.9	76
	35	43.8	0.9	52.4

hydrophobic interactions ($\Delta H^\circ > 0$ and $\Delta S^\circ > 0$), electrostatic forces ($\Delta H^\circ \approx 0$ and $\Delta S^\circ > 0$), hydrogen bonds and van der Waals forces ($\Delta H^\circ < 0$ and $\Delta S^\circ < 0$) [38, 39]. Since the enthalpy and entropy were almost constant in the temperature range studied, the values of these parameters and the dominant forces can be calculated by the van't Hoff equation (Eq. 3) [25, 40]:

$$\ln K_b = -\Delta H^\circ / RT + \Delta S^\circ / R \quad (3)$$

where K_b is the binding constant at the related temperature, R is the gas constant and T is the temperature. Also, the change in The Gibbs free energy (ΔG°) can be calculated by using the following relationships (Eq. 4) [29, 34]:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_b \quad (4)$$

The computed thermodynamic parameters, in the presence of spermine and spermidine, at two temperatures are summarized in Table 2. The values of ΔH° and ΔS° for spermine and spermidine were negative, indicating that the dominant force in the binding of BIALP with both polyamines was driven by hydrogen bonds and van der Waals forces. Also, the values of ΔG° at 25 °C and 35 °C were negative, showing that the interactions between enzyme and polyamines were spontaneous [41].

2.4 The effect of polyamines on the kinetics of BIALP

Enzymes are also employed for industrial, medical and biotechnological purposes [42]. They may often be subject to conditions very different from those in their native environment, leading to their inactivation and unfolding. To understand the enzymes function and determine their potential for use in different applications, a kinetic description of their activity is needed. The quantities V_{max} and K_m are often referred to as the kinetic parameters of an enzyme and their determination is an important part of the characterization of an enzyme. The Michaelis–Menten equation (Eq. 5) describes the initial reaction rate of a single substrate with an enzyme under steady-state conditions [43].

Fig. 5 Plots of $\log(F_0 - F)/F$ against $\log[\text{Spermine}]$ (a) and $\log[\text{Spermidine}]$ (b) at two temperatures (25°C and 35°C)

Table 2 Thermodynamic parameters of interactions between BIALP and polyamines

Compound	<i>T</i> (°C)	ΔH° (kJ.mol ⁻¹)	ΔS° (J.mol ⁻¹ .K ⁻¹)	ΔG° (kJ.mol ⁻¹)
Spermine	25	– 29.63	– 61.65	– 11.25
	35			– 10.6
Spermidine	25	– 27.97	– 57.8	– 10.73
	35			– 10.15

$$V = (V_{\max} [S]) / (K_m + [S]) \quad (5)$$

The Michaelis–Menten equation is not satisfactory for the determination of K_m and $V_{\max}$. The values of K_m and $V_{\max}$ from experimental rate data can be obtained from a double reciprocal Lineweaver–Burk plot (the linear form of Michaelis–Menten). A Lineweaver–Burk plot ($1/V$ against $1/[S]$) is described by Eq. (6). Hence, the intercept on the vertical axis is $1/V_{\max}$, the slope is $K_m/V_{\max}$ and the intercept on the horizontal axis equals $-1/K_m$ [44].

$$1/V = 1/V_{\max} + K_m/V_{\max} \cdot 1/[S] \quad (6)$$

The BIALP activity depends on pH [45], temperature and solution of the reaction [46]. Therefore, a small change in these factors can influence the enzyme's activity. In this study, BIALP activity was measured in the absence and presence of various concentrations of polyamines at pH=9.8 and a temperature of 37 °C by using a UV-Vis spectrophotometer, with a wavelength of 405 nm [47]. The results are presented in Fig. 6(a,b), as well as Tables 3 and 4 for spermine and spermidine, respectively.

The Lineweaver–Burk plots of BIALP in the absence and presence of polyamines (Fig. 6) unveiled that by increasing the concentration of spermine and spermidine, the maximum velocity of enzymes was increased, too. It was possible that the addition of polyamines led to further exposure of the catalytic center of BIALP to *p*-nitrophenyl phosphate [48]. As shown in Tables 3 and 4, it was also found that the affinity of BIALP for the substrate was decreased (K_m was increased) along with an increase in the polyamines concentration. The catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of the enzyme were increased as the concentration of polyamines was raised.

According to these calculated kinetic parameters, polyamines enhanced the enzyme activity of BIALP; so they could be considered in the hyperactivation of this enzyme. We also reported similar results on the activation of proteinase K due to the favorable interaction with these polyamines [49, 50]. In addition, the comparison of the effect of each polyamine on enzyme activity is shown in Fig. 7.

According to this diagram, spermine had more effect on enzyme activity; it could be due to more amino groups of spermine and a larger number of hydrogen bonds. These results were consistent with the results obtained in fluorescence studies. Interestingly, our investigations revealed that spermine and spermidine changed the local environment of Trp residues of BIALP due to binding between these polyamines and the enzyme. Furthermore, these structural changes can increase the catalytic activities of BIALP.

Fig. 6 Lineweaver–Burk plots of BIALP in the hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and temperature 37 °C in the absence and presence of different concentrations of spermine (a) and spermidine (b)

Table 3 Kinetic parameters of BIALP for the hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and temperature 37 °C in the absence and presence of different concentrations of spermine

Spermine (mM)	K_m (mM)	V_{max} (mM/min)	$K_{cat} \times 10^4$ (1/min)	K_{cat}/K_m (1/min.mM)
0	0.201	0.440	23.158	115.213
1	0.204	0.480	25.000	122.549
3	0.210	0.520	27.368	130.326
5	0.217	0.550	28.947	133.398

2.5 Molecular docking results

Docking studies provide information on the binding sites of ligands on proteins [51]. The best binding site with the lowest free energy between BIALP and spermine and spermidine is shown in Figs. 8 and 9, respectively. Docking results for the spermidine and spermine-alkaline phosphatase system are shown in Table 5. As represented, there were some hydrogen interactions between the nitrogen atoms of spermine or spermidine with five residues of BIALP (Asp, Glu, Tyr with N₁, N₂, N₄ of spermine and Asp, Glu, and Phe with N₂, N₃ of spermidine).

These results were consistent with the calculation of the thermodynamic parameters, showing that hydrogen bonds contributed to (BIALP-spermine or BIALP-spermidine) complex formation. Asp 233 and Asp 300 residues could participate in both types of complexation. Furthermore, it was seen that spermine and spermidine could interact with BIALP by hydrophobic forces. The values of the Gibbs free energy (ΔG°), as computed from docking, were negative (results not shown) for both complex formations, showing that the combination process was spontaneous and complied to fluorescence measurements.

3 Materials and methods

3.1 Materials

Alkaline phosphatase was obtained from bovine intestinal mucosa and *p*-nitrophenyl phosphate and spermine tetrahydrochloride were purchased from Sigma-Aldrich. Spermidine trihydrochloride was obtained from Applichem Company, Darmstadt, Germany. All enzyme solutions were made on the same day in a 1 M diethanolamine buffer with 0.5 mM magnesium chloride and at pH 9.8. Other solvents used for this study were prepared in double-distilled water.

Table 4 Kinetic parameters of BIALP for the hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and temperature 37 °C in the absence and presence of different concentrations of spermidine

Spermidine (mM)	K_m (mM)	V_{max} (mM/min)	$K_{cat} \times 10^4$ (1/min)	K_{cat}/K_m (1/min.mM)
0	0.201	0.440	23.158	115.213
1	0.212	0.470	24.74	116.68
3	0.216	0.490	25.79	119.39
5	0.220	0.510	26.84	122.01

Fig. 7 Comparison of alkaline phosphatase maximum velocity in the presence of different concentrations of spermine and spermidine at pH 9.8 and temperature 37 °C

3.2 Methods

3.2.1 Intrinsic fluorescence measurements

The fluorescence spectra were studied in the absence and presence of different concentrations of polyamines (spermine and spermidine) at different temperatures (25 °C and 35 °C). The emission spectrum was recorded in the range of 290–450 nm and the excitation wavelength was set at 280 nm. The slit widths for excitation and emission scans were set at 3 nm and 10 nm, respectively. At first, the fluorescence intensity of BIALP in the diethanolamine buffer was investigated under different temperatures; then the effects of different concentrations of polyamines were studied. In all measurements, each sample was incubated for 3 min before measurement.

3.2.2 Enzyme kinetic studies

The effect of the addition of polyamines on BIALP activity was investigated by using a UV-Vis spectrophotometer at 37 °C and pH = 9.8. The kinetic parameters (K_m and V_{max}) of BIALP in diethanolamine 1 M as the reaction buffer (pH = 9.8) with 0.5 mM $MgCl_2 \cdot 6H_2O$ were determined in the absence and presence of polyamines (0–5 mM) for steady-state kinetics. In these studies, p -nitrophenyl phosphate was used as a substrate; during this reaction, the produced p -nitrophenol with yellow color was absorbed at 405 nm; it quantified via the molar absorption coefficient of the product at 405 nm ($\epsilon = 18,800 \text{ M}^{-1} \text{ cm}^{-1}$) [52]. All the kinetics studies were performed in 1-cm-path glass cells.

3.2.3 Molecular docking studies

The sequence of bovine intestinal alkaline phosphatase (BIALP) was identified at the NCBI public database (<http://www.ncbi.nlm.nih.gov/proteins>) and saved in FASTA format. The

Fig. 8 Hydrogen bonds (black) and hydrophobic bonds (red) of docking poses by the Ligplot plus tool for spermine ligands in BIALP

BLAST program (<http://www.ncbi.nlm.nih.gov/blast>, [53]) was used for the identification of similar sequences with the Protein Data Bank (<http://rcsb.org>).

PDB code 1EW2 from human placental alkaline phosphatase [max score; 79.9%, total score; 77.9%, query coverage; 93%, E value: 0.0%] was used as the template and Modeller 9.15 was employed for the homology modeling of the 3D structure of target protein [54]. Among the generated models, the best one corresponding to the lowest value of the design of a novel metal binding peptide probability density function (pdf) and the fewest restraints violations was selected for further analysis. At the end, different efficient tools such as Ramachandran were used for the evaluation and validation of the 3D structure.

The three-dimensional structure of spermine and spermidine (as ligands) was retrieved from the PubChem compound database (<http://pubchem.ncbi.nlm.nih.gov/>) as

Fig. 9 Hydrogen bonds (black) and hydrophobic bonds (red) of docking poses by the Ligplot plus tool for spermidine ligands in BIALP

Structure Data File (SDF) format and converted to the Protein Data Bank (PDB) format using Open Babel converter (<http://openbabel.org>) [55]. Ligand preparation was then carried out by adding hydrogen bonds and lowering energy using HyperChem software and MM⁺ force field.

Docking simulations were carried out with the prepared ligands. BIALP was considered as rigid and spermine and spermidine were taken as fully flexible during docking. For the ligand, random starting positions, random orientations, and torsions were used. The Lamarckian Genetic Algorithm (LGA) was applied to the defined parameters for determining the docking performance and looking for the best binding site of spermidine and spermine on BIALP [56]. Docking energy in the largest cluster was selected as the

Table 5 Docking results for the spermidine and spermine-alkaline phosphatase system

Complex	Lowest binding energy (kcal/mol)	Estimated inhibition constant, Ki	vdW+ Hbond + desolvation energy (kcal/mol)	Interaction bonds	
				Hydrogen bonding	Hydrophobic bonding
Spermine-protein	− 9.07	224.78	− 3.81	Glu289 Asp300 Glu235 Tyr236 Asp233	Asp238 Asp300
Spermidine-protein	− 9.47	113.79	− 3.46	phe288 Asp300 Glu235 Glu289 Asp233	Asp292 Leu307 Tyr236

representative binding pose [51]. Ligplot plus was used to analyze the docking poses for hydrogen bonding and hydrophobic bonding.

4 Conclusion

In this paper, the effects of spermine and spermidine on BIALP as a model enzyme were investigated by employing fluorescence emission studies, kinetic measurements, and molecular docking. The results of fluorescence studies indicated that both polyamines acted as a quencher on BIALP and quenched the fluorescence intensity of enzymes by the static quenching mechanism. Furthermore, the higher value of the binding constant in the presence of spermine, as compared to spermidine, under the same conditions, demonstrated that spermine had a higher tendency to binding of BIALP, which could be due to higher groups of spermine. In addition, based on the signs and magnitudes of calculated thermodynamic parameters ($\Delta G^\circ < 0$, $\Delta H^\circ < 0$ and $\Delta S^\circ < 0$), interactions between polyamines and enzyme were spontaneous and van der Waals as well as hydrogen bonds acted as dominant forces between polyamines and BIALP. Kinetic studies also revealed that by increasing the concentration of polyamines, enzyme activity was also enhanced. The comparison of kinetic parameters showed the larger effect of spermine on the enzyme hyperactivation of BIALP. The results of docking, like thermodynamic parameters, suggested that hydrogen bonds and van der Waals forces had major effects on stabilizing the polyamines–BIALP complex formation.

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Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest The authors declare that they have no conflicts of interest.

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