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How does dilution affect the conductivity, the propensity to aggregate and the biological activity of enzymes?

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ABSTRACT

The current theories do not provide a convincing explanation for many of the protein' manifestations in solution. Therefore, to shed some light on this critical question, the present work analyzed the effect of dilution on the ability of proteins to catalyze chemical reactions, the so-called biological activity. As models enzymes, laccase from *Trametes versicolor* and chicken egg white lysozyme were used.

The results show that the enzymes' specific biological activity increases on dilution. Three explanations for the intriguing observation were advanced and submitted to experimental scrutiny. Amongst the three hypotheses, only one was corroborated by experiments. According to this explanation, when dissolved in water, proteins reveal two populations: one biologically active whose relative occurrence increases on dilution and another which is not active and whose molecular proportion varies in the opposite direction.

Therefore, the reported experimental facts strongly support the chemical behaviour of the proteins in the solution. According to the herein-advocated concepts, they could undergo a dissociation process similar to that found in electrolyte chemistry.

1. Introduction

Several investigations have shown that the proteins behave osmotically abnormally, increasing the osmotic pressure more rapidly than the protein concentration [1–5]. The works by Medda et al. [6] and Gaigalas et al. [7] have shown that the diffusion coefficient of bovine serum albumin (BSA) decreases with decreasing concentration of the protein, in apparent conflict with the Stokes–Einstein equation for the diffusion of bodies in solution [6]. Experiments undertaken in our laboratory [8–10] have shown that the precipitation of BSA at low pH and its aggregation behaviour under neutral conditions are influenced to a considerable extent by the salt ions holding the same charge as the protein macromolecular ions, raising some doubts about the existence of a double electrical layer surrounding the protein particles. Moreover, proteins are known to manifest in solution in distinct forms [10–11]. Recent work in our group [10] has shown that the relative occurrence of the aggregates, supposedly the energetically unfavourable protein populations, increases on decreasing the probability of the protein particles colliding. These are just a few experimental facts, to which many more could be added [8–10,12–15], for which the current theories attempting to explain the behaviour of proteins in solution do not provide a convincing explanation.

Recently [8–10], a suggestion has been made to explain the protein behaviour in solution under the auspices of the general chemical school of thought, in which dissociation and chemical equilibria concepts play essential roles [8–10]. Due to the relevance of the matter under consideration in the scientific domains where proteins are involved, it is justifiable to investigate the suggested conjecture in more detail.

One of the chief manifestations of the proteins in solution is their outstanding ability to catalyze chemical reactions, the so-called biological activity. Therefore, the catalytic action of laccase from *T. versicolor* and chicken egg white lysozyme has been addressed in the present work. The results brought to light intriguing facts, the clarification of which has been complemented by conductivity and dynamic light scattering experiments.

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2. Experimental

2.1. Materials

Reagents: Commercial laccase from *T. versicolor* (light brown powder), lots # BCCD0761 and BCCG6202, (Mw = 66 kDa) with an isoelectric point (pI) of ~ 4.7 used was from Sigma-Aldrich. The lysozyme from chicken egg white (Mw = 14.4 kDa) used was from Sigma-Aldrich, lot BCCC9899, with a pI of ~ 11.35. *Micrococcus lysodeikticus*, lot NO. SR03050 5 was acquired from MP Biomedicals, LLC. 2,2'-azino-bis3-ethylbenzathiazoline-6-sulfonic acid (ABTS), was acquired from Sigma-Aldrich. Methanol, HPLC grade, was obtained from Fisher Scientific. The water was ultra-pure, double distilled, passed by a reverse osmosis system, and further treated with a Mili-Q plus 185 water purification apparatus.

2.2. Methods

Preliminary experiments: Preliminary biological activity, conductivity, and dynamic light scattering experiments were undertaken in which the protein samples were submitted to purification steps. The purification procedure consisted of two filtrations with Vivaspin centrifugation tubes (from Cytiva, Sweden) of adequate pore sizes to remove small and large contaminants. The impurities were shown not to interfere with the trends herein reported.

Similar experiments to those reported here have also been undertaken in the background of standard phosphate buffer, pH 7.4. Analogous trends to those herein noted have been observed, eliminating the possibility that the observations were due to pH changes. Therefore, the present results were carried out in Mili-Q water, and the proteins were used without further purification, in which the concentration of the protein samples was found by UV–Vis spectroscopy using the extinction coefficients at 280 nm of 60520 $M^{-1}cm^{-1}$ and 37500 $M^{-1}cm^{-1}$ for laccase and lysozyme, respectively.

Biological activity of laccase: The concentration of laccase samples combined with ABTS mixtures varied from 0.00125 to 0.32 g. L^{-1}. Mixtures were incubated for 0.5 h at room temperature (RT), and samples were taken for laccase activity assays [16]. Aqueous ABTS (substrate) solutions with a concentration of 0.23 $g.L^{-1}$ were used to measure the enzyme activity. 50 μL of the sample (laccase solution at different concentrations) was added to 950 μL of ABTS solution. The increase in absorbance/min was recorded using a UV–Vis spectrophotometer (Shimadzu UV-1800 Spectrometer) with temperature control. Laccase activity is expressed in UL^{-1}, and specific activity in Ug^{-1}, where one unit (U) is the amount of enzyme that oxidizes 1 μmol of ABTS min^{-1} at 420 nm ($\varepsilon = 36.000\ M^{-1}\ cm^{-1}$). To calculate the laccase activity in enzyme units (EU) per L, the following equation was used[16]:

$$\frac{EU}{L} = \frac{\Delta Abs_{420nm} \times 60 \times f_{dil.} \times 10^6}{\varepsilon} \quad (1)$$

in which ΔAbs_{420nm} is the rate of change of absorbance with time, f_{dil}. is the dilution factor, ε the molar extinction coefficient, and the numbers are units conversion (from seconds to minutes and from μL to L).

Biological activity of lysozyme: Vigorously shaken Micrococcus lysodeikticus stock suspension (~0.3 % wt/wt) was diluted with water to have an A645 between 0.5 and 0.8. 950 mL of this diluted M. lysodeikticus suspension was taken into a spectrophotometer cell, and 50 μL of an appropriate lysozyme sample/blank was added. The rate of decrease of absorbance at A645 was monitored by a UV spectrophotometer (Shimadzu UV-1800 Spectrometer) with temperature control for 7 min at 25 °C. The enzyme activity was accessed by the method of Parry et al. [17], as the decrease in absorbance between 0.5 and 4.5 min. A unit of lysozyme activity is defined as the sample concentration causing a reduction in absorbance of 0.001/min at 645 nm [17].

Specific enzyme activity (EU_S): The amount of enzyme in samples was determined by UV–Vis spectrophotometer at 280 nm and was used for the calculation of specific enzyme activity (EU/mg) of both enzymes using the following equation:

$$\frac{EU}{mg\ of\ enzyme} = \frac{\frac{EU}{mL\ sample}}{\frac{mg\ enzyme}{mL\ sample}} \quad (2)$$

Conductivity experiments: The conductivity was measured with a Metter Toledo Seven Excellence conductivity meter in the following manner: Solutions with the required quantity of protein were prepared and left to equilibrate for 1 h at RT (~25 ± 2 °C). After calibration according to manufacturer instructions, the electrode was inserted in a sample for at least 5 min at RT (25 ± 2 °C), after which the conductivity was measured (at least three measurements have been undertaken). The error in the conductivity experiments using this approach was below 3 %, inferred by investigations undertaken with acetic acid and compared with literature data [18].

DLS measurements: A commercial instrument, Zetasizer Nano ZSP (Malvern instruments), was used for light scattering measurements with a He-Ne laser (633 nm, 4 mW) as a light source. Analysis was performed using the light scattering software DTS application. The scattering light was collected at a 173° backscattering angle. At least six scans of 15 s each were performed at the studied conditions. DLS is reported to overestimate the mean size of the clusters [11], rendering a qualitative interpretation of the same often doubtful. Aggregates of similar and reproducible radii were observed, which are herein reported. Slight corrections to the viscosity and refractive index values in the solutions containing methanol were made, whose values were obtained from the literature. [19–20].

3. Results

Fig. 1 shows the biological activity results of laccase and lysozyme at various enzyme concentrations. For a given ABTS quantity, the concentration of laccase, which displays measurable activity, could be varied 256 times. However, a saturation point is achieved for high protein concentrations (Fig. 1 left) in conformity with observations made by others [16], and only the linear portion of the curve has been considered for further analysis. On the other hand, for a given M. lysodeikticus quantity, the concentration of lysozyme, which displays measurable activity, could be varied roughly sixty-four times (Fig. 1 right).

It can be seen in Fig. 1 (left) that the catalytic oxidation of ABTS by laccase increases with increasing concentration of the enzyme. It can also be seen in Fig. 1 (right) that the lysis of the M. lysodeikticus cells by the catalytic action of lysozyme also increases with the gradual addition of the enzyme. Therefore, as expected, the results indicate that the enzymatic activity increases for both enzymes with the increase in protein concentration, consistent with the higher number of protein particles available to carry out its enzymatic activity.

The results shown in Fig. 2 concerning the specific enzymatic activity are relatively less intuitive. The results demonstrated in Fig. 2 are those of Fig. 1 expressed on a mass basis (see Eqs. 1–2). Since the enzymes display different activities, the data have been normalized for better visualization.

It can be seen in Fig. 2 that the specific enzymatic activity increases quite considerably on diluting the protein. Now, if all the protein particles were identical, the specific enzymatic activity would be expected to remain constant on varying the protein concentration.

The rather intriguing results shown in Fig. 2 can be explained if the proteins, when dissolved in water, *reveal two populations: one biologically active and another which does not, and the relative occurrence of the former increase on dilution and of the latter in the opposite direction.*

The presence of distinct forms of proteins, if existent, is experimentally challenging to investigate. However, the indirect evidence of these alleged forms can be gathered by conductivity data, as has been done in

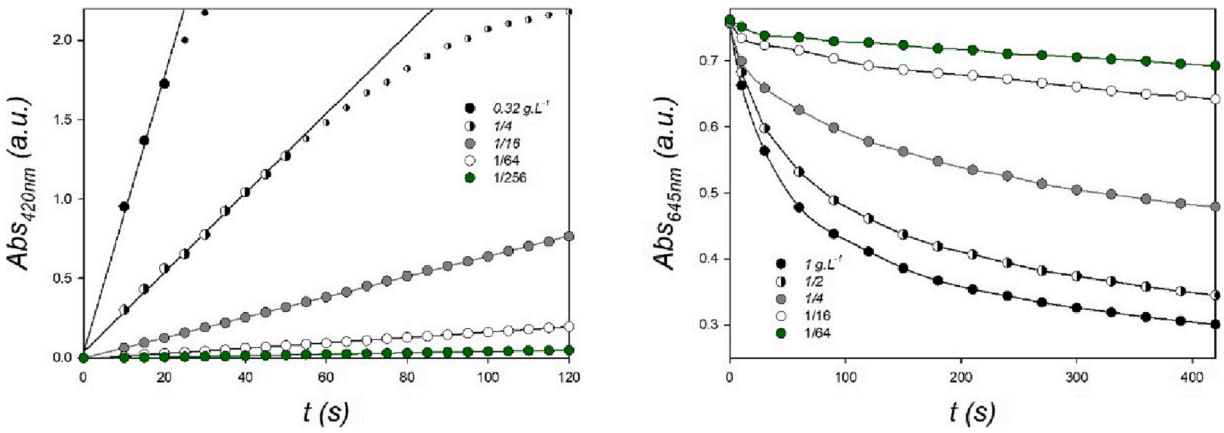

Fig. 1. Effect of enzyme dilution on the biological activity of laccase (left) and lysozyme (right) at 25 °C and neutral pH. The various protein concentrations shown are those of the solutions mixed with the respective substrates (ABTS for laccase and *Micrococcus lysodeikticus* for lysozyme). Laccase concentration varied from 0.32 to 1.25 × 10^{-3} g. L^{-1} (256 times diluted) and lysozyme concentration went from 1.56 × 10^{-2} (64 times diluted) to 1 g.L^{-1}. The enzyme activity was monitored by the increase in absorbance at 420 nm for laccase and by the decrease in absorbance at 645 nm for lysozyme.

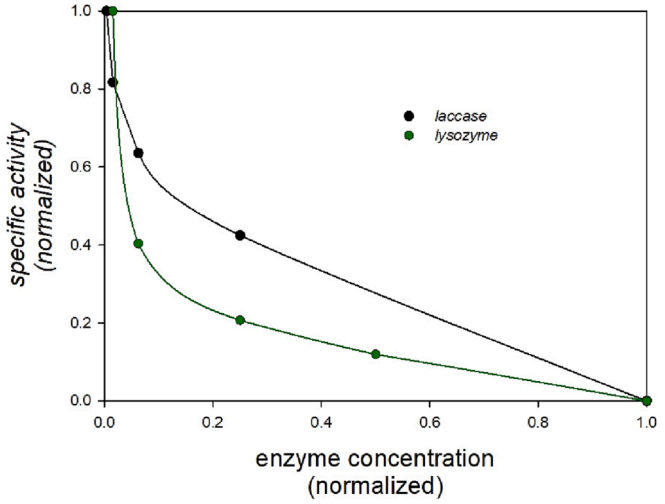

Fig. 2. Effect of enzyme dilution on normalized specific enzyme activity (EU/mg) of laccase (•) and lysozyme (●) at 25 °C and neutral pH4. The specific activity is expressed on enzyme mass basis. Laccase concentration varied from 0.32 to 1.25 × 10^{-3} g. L^{-1} (256 times diluted) and lysozyme concentration went from 1.56 × 10^{-2} (64 times diluted) to 1 g.L^{-1}.

the past for a distinct class of chemical compounds [21–22]. Therefore, conductivity experiments of aqueous solutions of both enzymes have been undertaken. Fig. 3 shows the conductivity results. For comparison purposes, the conductivity data for acetic acid is also shown. The data is expressed in units conventional in these circumstances.

Fig. 3 shows that the molar conductivity of the enzymes, similar to what has been observed for the specific enzymatic activity, increases on diluting the protein. It is to be emphasized the remarkable parallelism between the conductivity of the enzymes and that of a typical weak electrolyte (Fig. 3 right). The results shown in Fig. 3 are experimental support for the raised hypothesis, according to which proteins, when dissolved in water, seem to reveal the presence of two populations of proteins. Moreover, the results shown in Fig. 3 and those shown in Fig. 2 suggest that the enzyme forms, which are biologically active and conduct electricity, are the same.

Contrary to the prevailing ideas, the scientific method is more prone to reject than to demonstrate a hypothesis. In other words: if the experimental facts support a scientific theory, it doesn't necessarily mean the thesis holds. On the other hand, if the empirical facts contradict the hypothesis, and as long as the premises underlying the experiments have the logical value of truth, then the thesis is necessarily wrong. For that reason, rather than looking for further experimental support for the initially raised hypothesis, we attempted to find alternative explanations for the intriguing facts shown in Figs. 2 and 3 and put them to empirical scrutiny.

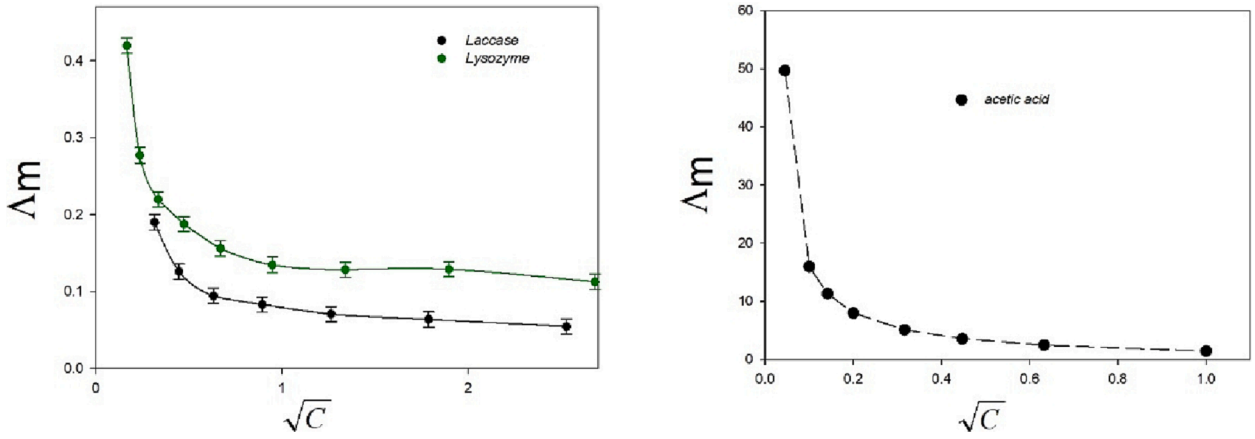

Fig. 3. Effect of enzyme dilution on molar conductivity (Λ_m) of laccase and lysozyme (left in $Sm^2.mol^{-1}$) and acetic acid (right in $Scm^2.mol^{-1}$). The protein concentration shown is in $(mol.L^{-1})^{1/2}$ × 1000 while that of acetic acid in $(mol.L^{-1})^{1/2}$.

Proteins are known to aggregate in solution [10]. Therefore, another possible explanation for the experimental facts under examination, that is, the specific biological activity and molar conductivity of the enzymes increases on diluting the protein, could be as follows. *On gradually increasing the protein concentration, the relative occurrence of the aggregates would also increase, rendering less available protein particles, on a comparable basis, to conduct electricity and catalyze chemical reactions*.

Dynamic light scattering (DLS) experiments were undertaken to study the enzymes' propensity to aggregate: Table 1 and Fig. S1 in the Supplementary Information show the results. In Table 1, the peak intensity corresponds to the area below the DLS curve (see Fig. S1) and provides an estimate of the frequency of protein particles with a specific size.

It is beyond the scope of the present considerations to discuss the slightly lower hydrodynamic radius (Rh) of the enzymes (c.a. 1.1 nm and 1.8 nm for lysozyme and laccase, respectively) observed in water when compared to those reported in salt solutions, or the fact that both enzymes displayed aggregates of distinct sizes (see the Supplementary Information Fig. S1). The interested reader can find helpful Information in both regards elsewhere [23–25].

Table 1 shows that, for both enzymes, the relative occurrence of the protein aggregates increases on dilution. A similar trend has been observed with bovine serum albumin [10], suggesting it to be a general characteristic of proteins. Therefore, the experimental facts undoubtedly do not corroborate the alternative explanation for the intriguing ones reported in Fig. 2.

Both the specific enzymatic activity and the molar conductivity increase in the same way as the propensity to aggregate, suggesting another possible explanation for the facts under scrutiny: *that the biological activity and conductivity could be due to the aggregates*.

The hypothesis was experimentally tested by adding methanol to the protein solutions. It was hoped and indeed observed that the frequency of the aggregates would likewise increase. Therefore, conductivity and enzymatic activity experiments were performed similarly in methanol (20 v/v %).

Fig. 4 shows the DLS, conductivity and biological activity results undertaken in aqueous methanol (20 v/v %). The data are expressed as a percentage of relative change to that observed in the absence of methanol. Further information can be found in the Supplementary Information (Figures S2-S4).

The results in Fig. 4 show that adding methanol at 20 % v/v increases the incidence of aggregates. At the same time, the conductivity decrease and the biological activity remains constant or decrease, indicating that the activity and the conductivity trends shown in Figs. 2 and 3, respectively, are not due to the aggregates. Although the conductivity and the enzymatic activity vary in distinct proportions in the presence of methanol at 20 % v/v, it can be safely concluded that the explanation for the intriguing experimental facts, according to which the biological activity and the conductivity are due to the aggregates, is likewise not corroborated by experiments.

Table 1
Influence of laccase and lysozyme concentrations upon the peak intensities of monomers and aggregates. Dynamic light scattering experiments were applied to determine the hydrodynamic radius of the enzymes' monomers and aggregates. The peak intensity (%) was determined using the light scattering software DTS application.

Enzyme concentration ($g.L^{-1}$)	*Peak intensity (%)*	
lysozyme	***Monomers***[a]	***Aggregates***[b]
0.1	15	85
0.8	38	62
laccase	***Monomers***[a]	***Aggregates***[b]
0.1	8	92
0.8	23	77

[a] Hydrodynamic radius of the monomers: lysozyme – 1.1 nm; laccase – 1.8 nm.
[b] Detected aggregates of distinct sizes (see Figure S1).

The only hypothesis experimentally supported is the former, according to which proteins, when dissolved in water, seem to reveal the presence of two populations. The relative molecular proportion in which they are found in solution varies with the protein concentration.

4. Discussion

It has been previously suggested [8–10] that proteins, when dissolved in water, undergo a dissociation process similar to that found in electrolyte chemistry, according to the following general and simplified Eq. 3:

$$P_U \leftrightarrow \sum P_M^{\pm} \tag{3}$$

in which PU represents undissociated protein molecules, which dissociate into active monomers. The symbol ± meant that the macromolecular ions have positive and negative charges on their backbone. The counter ion, H^+, was intentionally omitted since, depending on the pH and on the protein, the process carries the consumption or release of H^+. The charged macromolecular ions conduct electricity and are biologically active since all chemical reactions are ultimately phenomena of electrostatic nature.

Based on the suggested ideas, the hypothesis under scrutiny could be explained as follows. Proteins are non-active monomers (PU) that dissociate when dissolved in water in closely related but non-identical macromolecular ions ($P_M^{\pm}$). The protein populations are in chemical equilibria, which provides a rationale for the presence of two primary and distinct protein populations.

The degree of dissociation of proteins, like that of electrolytes, increases on dilution. Therefore, Eq. 3 is shifted in the direct direction by decreasing the protein concentration. As a result, the relative occurrence of the charged active form increases, which explains the intriguing facts reported in Figs. 2 and 3.

As is usually found in electrolyte chemistry, adding sufficient methanol suppresses the dissociation of the electrolytes. Similarly, on adding methanol to a protein solution, the chemical equilibrium illustrated by Eq. 3 is shifted in the reverse direction, which explains the decrease in the biological activity and conductivity of the protein particles in the presence of methanol at 20 w/w % (Fig. 4).

5. Conclusions

The present work analysed the behaviour of two enzymes: laccase from *T. versicolor* and chicken egg white lysozyme. It was shown that the enzymes' specific biological activity increases upon dilution. Three hypotheses were raised to explain the intriguing observation and submitted to experimental scrutiny.

Amongst the three hypotheses, only one, according to which proteins, when dissolved in water, apparently undergo a dissociation process similar to that found in electrolyte chemistry, was corroborated by experiments. Therefore, the reported facts strongly support the chemical behaviour of the proteins in the solution.

CRediT authorship contribution statement

Cecília Roque: Validation. **Érica Andrade:** Validation. **Luís Duarte:** Validation. **Margarida Costa:** Validation. **Ana Paula M. Tavares:** Writing – review & editing, Funding acquisition. **Pedro P. Madeira:** Conceptualization, Methodology, Supervision, Validation, Investigation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

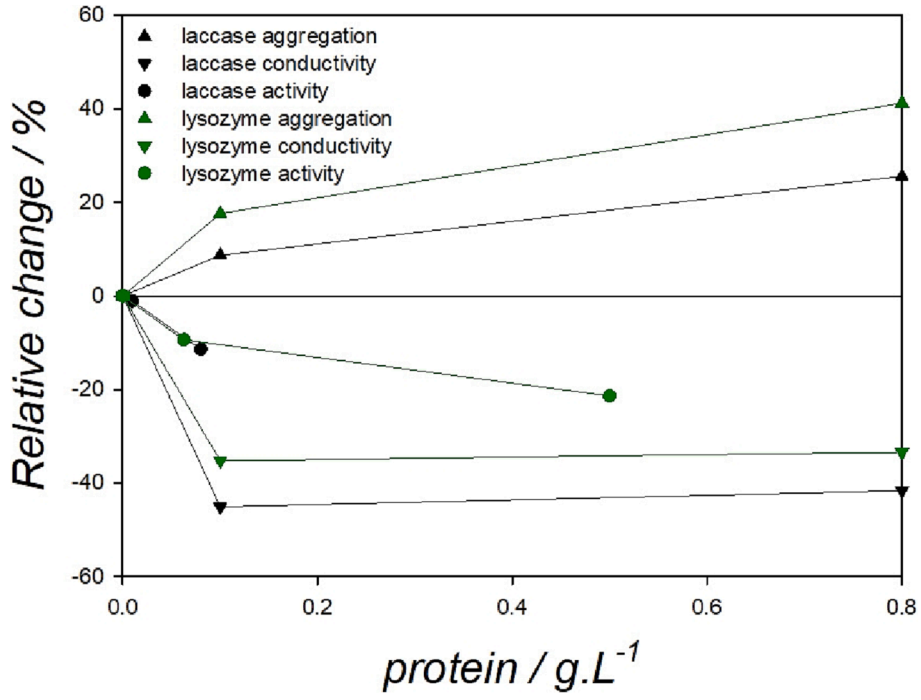

Fig. 4. Effect of laccase and lysozyme concentration on relative change (%) in conductivity, propensity to aggregate and biological activity of the enzymes in the presence of aqueous solution of methanol (20 % v/v). Data are expressed as a percentage of relative change to that observed in the absence of methanol.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2023.121926.

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