# BEHAVIOR OF ALKALINE PHOSPHATASE-POLY(ETHYLENEIMINE) COMPLEXES WITH TEMPERATURE AND pH CHANGES

**F. Tristán-López[1], F. Alarcón-Oseguera[2], J. L. Cuellar-Camacho[3], E. Pérez[3]***

[1]*Centro de Investigación y Estudios de Posgrado, Manuel Nava 6, C.P. 78290, San Luis Potosí, S.L.P., México*
[2]*Facultad de Ciencias Físico-Matemáticas de la Universidad Michoacana de San Nicolás de Hidalgo, Edificio B, Ciudad Universitaria, C.P. 58030, Morelia, Michoacán, México*
[3*]*Instituto de Física, Universidad Autónoma de San Luis Potosí, Alvaro Obregón 64, C.P. 78000, San Luis Potosí, SLP, México. elias@dec1.ifisica.uaslp.mx*

Abstract.- One of the main requirements needed to apply protein molecules in different fields is the preservation of their activity which is strongly related to their structure. In order to protect the structure of the protein molecule against denaturalization agents like temperature and pH variation one of the most effective strategies is the formation of complexes with polyelectrolytes. The aim of this work is to study complexes formed by alkaline phosphatase and cationic poly(ethyleneimine). These complexes are characterized by fluorescence spectroscopy and circular dichroism spectroscopy. Both techniques give information about structural changes of the alkaline phosphatase contained in the complexes as a consequence of the changes in the temperature or pH of the sample. These results are compared against the structural changes obtained for the alkaline phosphatase in its native state in solution using the same variables.

## Introduction

In the field of polymeric materials, the polyelectrolyte complexes (PEC) have a great relevance because they offer the possibility to combine the physicochemical properties of at least two polyelectrolytes (PEL). The driving forces that lead to PECs formation are the Coulumbic forces originated between two PELs of opposite charge allowing the interpolymer ionic condensation with the corresponding counterion liberation. There are secondary interactions involved in PECs formation like hydrogen bonds, hydrophobic interactions, van der Waals interactions or the charge-dipole transfer.[1] The PECs formed by PEL and proteins play an important role in a wide variety of chemical and biological processes like the protein separations,[2,3,4,5] enzymatic stability[6,7,8,9] and controlled drug release. [10] A very important and related topic is the formation of complexes between polycations and polynucleotides or oligonucleotides that found application as vectors in gene therapy.[11]

Since the initial work proposed by Morawetz and Hugues, it was corroborated that globular proteins can form complexes with PEL obtaining soluble species, coacervates of complexes, precipitates or gels. [12]

The formation of PECs from PELs and enzymes is also due to electrostatic forces through the stoichiometric formation of ionic pairs between opposite charged groups.[13,14] These ionic pairs between PELs and enzymes are very sensitive to the pH and small ions or polyions addition. However after PECs formation it appears that enzyme retains its biological activity,[15,16] therefore conformational changes in the three-dimensional structure of the enzyme caused during the PECs formation process are not important to allow the lost of its original function.[17] According to the results reported for several enzymes, the native structure of the enzyme seems to be protected.[15,18,19,20,21] The impact

of this characteristic in applications is evident, because beside the enzyme stabilization it allows the development of novel and selective enzyme separation methods in food or pharmaceutical industry.[18,22] It also makes easy the stabilization of enzymes in surfaces making possible the design of new biosensors.[23]

Alkaline phosphatase is a very important enzyme related to the production of mineralized tissue in several living organisms. The aim of this paper is to study the structural behavior of alkaline phosphatase as a model enzyme in PECs formed with poly(ethylenimine) when they are exposed to pH and temperature changes. The results are compared to those obtained for the enzyme in its native state in solution exposed at the same variables. Although there are evidences of retention for the native structure of some other enzymes, the obtained results demonstrate that complexation appears to modify strongly the structure of the alkaline phosphatase.

## Experimental Section

Substances: Cationic poly(ethyleneimine) (PEI, Mw ~ 50-60 000) in 50% aqueous solution (ACROS Organics), and alkaline phosphatase (Ortophosphoric-monoester phosphohydrolase), Type I-S from bovine intestinal mucose (AF, Mw ~ 140 000) (SIGMA).

Both enzyme and PEL solutions are prepared in phosphate buffer 8.3 mmol/l and pH 7.2 (mixture of sodium and potassium phosphates in deionized water). PEL concentration is 1 mg/ml and enzyme concentration is 0.8 mg/ml.

PECs are prepared mixing volumes of PEL and enzyme solutions to obtain the previously designated charge ratios. Temperature tests are done in a range between 25 ºC and 85 ºC. pH tests involve a titration procedure with 0.01 M HCl solution in a range between 6 and 7.2.

Equipment: Fluorescence spectroscopy for tryptophan residues is done in a commercial spectrometer (C-700 TimeMaster PTI). This equipment uses chromatic light produced by a 75 Watts xenon arc lamp. Two monochromators make the selection of the excitation and emission wavlenght. For tryptophan residues emission is detected at 350 nm while excitation occurs at 295 nm. Emission scans were made between 315 and 400 nm.

Circular dichroism is made in a commercial spectropolarimeter JASCO J-810. The spectra are obtained following the next conditions: scan from 190 to 280, cell length of 10 mm, resolution of 0.5 nm and 5 accumulative scans.

## Results and Discussion

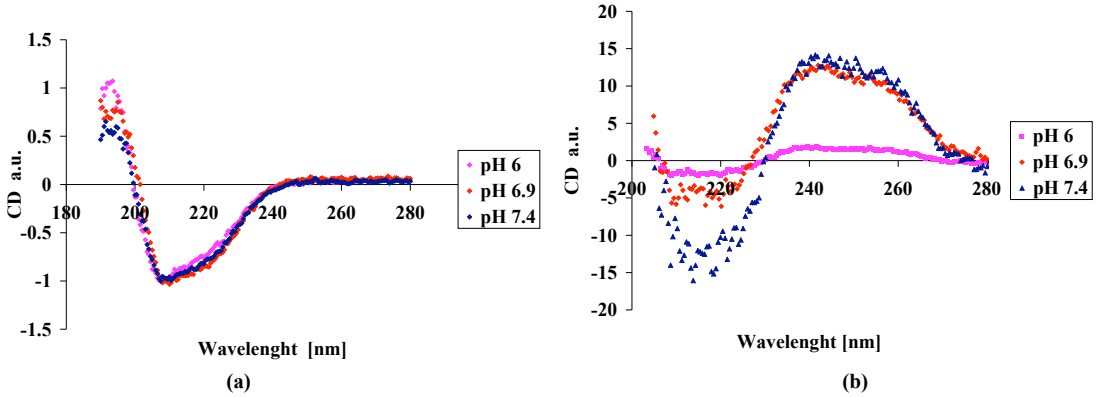

**Figure 1.-** Circular dichroism spectra at different pH values for (a) the alkaline phosphatase in solution and (b) in the complex PEI v25.

In order to have a reference, alkaline phosphatase in solution is exposed to changes in temperature and pH. Structural information is collected qualitatively using circular dichroism spectroscopy and fluorescence spectroscopy. The obtained circular dichroism spectra in solution show a change in the alkaline phosphatase structure from α-helix to β-sheet as a consequence of a pH increasing (see **Figure 1-a**). This structural transition change when pH is increased is identified by a decreasing and shifting to a higher wavelength for the band located in the 190-200 nm range. However the conformational changes are not clear in the 200-240 nm range, where all the spectra are almost without differences. This means that alkaline phosphatase in solution is slightly affected by pH changes in the 6-7.4 range. On the other hand, when the alkaline phosphatase is forming complexes with PEI there are evident structural changes caused by the complexation process. A big band appears in the spectra at the 230-270 range (see **Figure 1-b**). This band increases when the pH value also increases. The meaning of this band could be related to the denaturalization of the enzyme. Moreover the band located at the 210-230 nm range increases when the pH increases. This behavior could also be related to a denaturalization process.

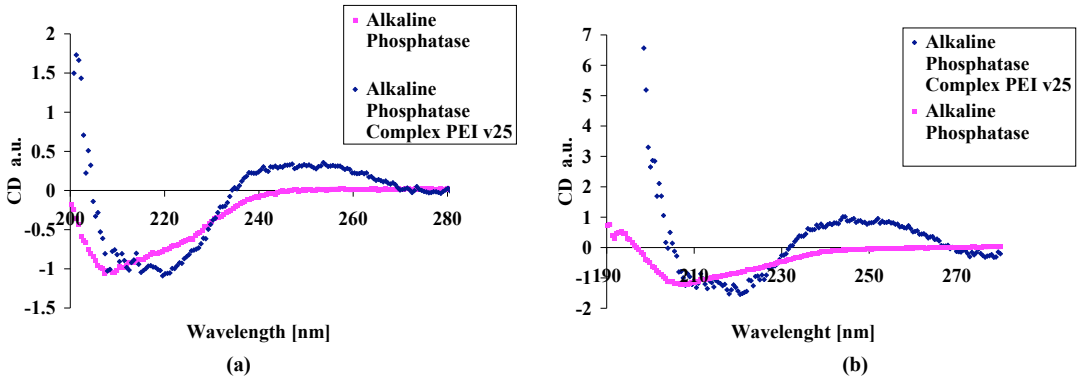


**Figure 2.-** Circular dichroism spectra at different temperatures for the alkaline phosphatase in solution and in the complex PEI v25: (a) T = 25 ºC and (b) T = 85 ºC.

A similar behavior is observed for the alkaline phosphatase when the temperature is increased. While the alkaline phosphatase in solution is resistant to the temperature changes, once the enzyme is forming complexes with PEI a denaturalization process occurs. No difference is found in the circular dichroism spectrum for the alkaline phosphatase complex at 25 ºC or 85 ºC while the native alkaline phosphatase spectrum has small structural changes as a consequence of the temperature, however these structural changes are more important to those caused by the pH value changes (see **Figure 2-a** and **2-b**).

Both processes, temperature and pH effects are also followed using fluorescence spectroscopy. The reference is provided by the triptophan molecules contained in the enzyme molecule. Using fluorescence is possible to study the environment around the triptophan molecules in the enzyme. These molecules are excited at 295 nm and their emission is detected at 350 nm. The intensity of this band is reported to understand the effect of the pH and temperature on the enzyme molecule. In the case of the effect caused by pH it is detected that only when there is excess of PEI during the complex formation

the triptophan molecules appears to be more exposed and the fluorescence emission is higher (see **Figure 3-a**). On the other hand when the temperature is increased the triptophan molecules exhibit a quenching process indicating that these molecules are no longer available to emit (see **Figure 3-b**). These results and those obtained by circular dichroism indicate the possible existence of two denaturalization mechanisms. One of them involves a complete destruction of the globular shape of the protein caused by the interactions with PEI, explaining why the triptophan molecules are more exposed to emit when an excess of PEI is used to prepare the complexes. The other mechanism probably implies only the unfolding of the protein as consequence of the changes in pH or temperature.

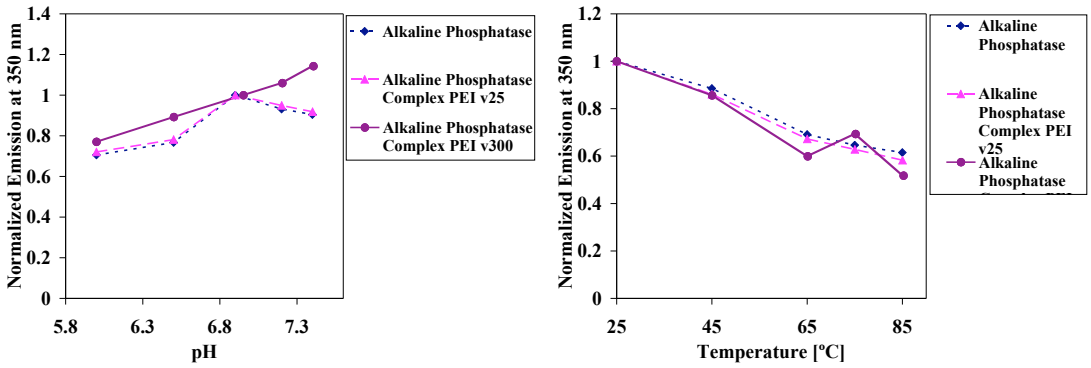


**Figure 3.-** Normalized fluorescence emission at different (a) pH values and (b) different temperatures for the alkaline phosphatase in solution and in the complexes PEI v25 and PEIv300

## Conclusions

Although further information about the behavior of this alkaline phophatase-PEI system is required, it appears that PEI is not an ideal polyelectrolyte to protect the native structure of the alkaline phosphatase. Moreover PEI seems to act against the structure of the enzyme molecule causing denaturalization during the complex formation and destroying its globular structure.

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