The Effects of Bisphenol A and Di-Isononyl Phthalate on Conformational Stability and Activity of Lysozyme

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Received: 1 September 2018
Accepted: 28 September 2018
Version of Record Online: 24 October 2018

Abstract

The purpose of this study was to investigate the effect of Bisphenol A (BPA) and Di-Isononyl Phthalate (DINP) on conformational stability and biological activity of a model protein lysozyme. Lysozyme was incubated to various concentrations (2-100 µM) of bisphenol A and phthalate for 6 weeks. The conformational stability of lysozyme was figured out by using Differential Scanning Calorimeter (DSC) and Fourier Transform Infrared (FTIR) spectrophotometer. Biological activity of the samples was determined by an enzyme activity assay using Micrococcus lysodeikticus (M. luteus) as a specific substrate to lysozyme. A rate of change of 0.001 in absorbance at 450 nm (A450 nm) was used to define 1 unit of biologically active lysozyme. The DSC data showed significant (p<0.05) decrease in Tm and H values for lysozyme samples treated with BPA or DINP with increase in exposure time. The H value of BPA treated 7 days’ sample was negative which may indicate complete reversal of conformational integrity. In 7 days old DINP treated sample, no measurable peak was observed which may be due to complete loss of conformational structure. DSC data were further corroborated by FTIR and biological activity data. FTIR spectra of all the treated lysozyme samples showed the splitting of β sheet secondary structure which was completely lost in 7 days DINP treated samples which also showed the minimal biological activity. BPA and DINP (constituents of plastic bottles) may disrupt the conformational stability of protein which is expected to be more severe in human where they can remain longer than the 7 days’ exposure period used in this study.

Keywords

Bisphenol A; Conformational Stability; Di-Isononyl Phthalate; Lysozyme

Introduction

Plastics are incredible materials; they are easy to manufacture, impervious to water, inexpensive, lightweight, strong, durable, and corrosion-resistant with high thermal insulation properties. A range of chemicals are added to plastics during manufacture, to enhance the performance of plastics. These additives can be referred to as plasticizers and include flame retardants, stabilizers, softeners, coupling agents, pigments, extenders, and lubricants that give each type of plastic its unique properties [1]. Huge concern has been raised over these potentially harmful chemical additives including phthalates, Bisphenol A (BPA) and Polybrominated Diphenyl Ethers (PBDE) which improve the properties of plastics yet leak into the environment, food and beverages [2,3]. These chemicals could be transferred to humans either
Isodecyl Phthalate (DIDP), and the Di-Isononyl Phthalate phthalates are Di-2-Ethyl Hexyl Phthalate (DEHP), the Di-
applications and medical devices. The most widely-used
of flexible vinyl plastic which, in turn, is used in food contact
Phthalates are primarily used as plasticizers in the manufacture
fibroids and breast cancer [12]. Bisphenol-A (BPA) is a key component
reproductive capacity, reduction in semen quality, infertility or possibly increased risk of testicular or prostate cancer [2]. Bisphenol-A (BPA) is a key component used to make epoxy resins and polycarbonate plastic which is used to make consumer goods. Polycarbonate plastic is clear, strong, lightweight, and heat resistant which makes it ideal for use in water bottles, food containers and medical equipment's. Likewise, epoxy resin coatings are durable, adhere well to metal, and chemical resistant thus they are ideal for use as a protective coating on food cans (www.fda.gov).

BPA is found to leach out in discerning amounts as evident from the detectable amounts found in food cans, microwave containers, polycarbonate bottles, and in human saliva after treatment with dental sealants [9]. Recent studies on human exposure to BPA, have shown a two-third increase in the urinary concentration of the BPA molecule following the use of polycarbonate drinking bottles for 1 week [10]. Effects of BPA are a function of the species, strain, dose, and time of exposure. Adult animals have shown reversible effects in response to BPA exposure. In contrast to this, irreversible organizational effects were produced in Sprague-Dawley rats after neonatal and perinatal exposure [11]. BPA at high and low doses causes long-term adverse reproductive and carcinogenic effects if exposure occurs during critical periods of differentiation, causing precocious hemicranial-putitary maturation and precocious puberty [12,13]. Prenatal exposure to BPA is associated with an increase in the risk for breast cancer [14]. Rodent studies have shown that prenatal and neonatal exposure to BPA results in early onset of sexual maturation, reproductive tract lesions, and altered development of the mammary gland [15]. Recent studies have shown that hormonal perturbations during fetal or neonatal development may predispose individuals to reproductive problems including infertility/sub fertility; and increased tumors such as uterine fibroids and breast cancer [12].

Phthalates are primarily used as plasticizers in the manufacture of flexible vinyl plastic which, in turn, is used in food contact applications and medical devices. The most widely-used phthalates are Di-2-Ethyl Hexyl Phthalate (DEHP), the Di-Isodecyl Phthalate (DIDP), and the Di-Isononyl Phthalate (DINP). Recent studies indicate that certain phthalate diesters and their metabolites are measurable in human breast milk, blood and other pregnancy-related specimens. In general, children’s exposure to phthalates is greater than that of adults. Baby care products having phthalates are a source of exposure for infants [16]. A study showed that reported use of infant lotions, powders, and shampoos were associated with increased urine concentrations of phthalate metabolites, and this association is strongest in younger infants [17]. These findings suggest that dermal exposures may contribute significantly [18] to phthalate body burden in this population. Animal studies have documented an association between phthalate exposure and reproductive or developmental toxicity [19,20]. There have been a few human studies which suggest an indirect anti-androgenic action of phthalates in the perinatal period. Even, low-dose phthalate exposure may affect several markers of human male genital development [2,21,22]. In rats, phthalate exposure produced neurobehavioral, hepatic, immunologic and hepatotoxicity [23]. BPA and phthalates have shown substantial reproductive and developmental toxicity as evident from rodent studies. BPA and phthalates both function by formation of ROS and superoxide [24]. The oxidative stress produced as a result of the ROS formation, adversely affects the brain, testis, and kidney. BPA has been shown to cause oxidative damage in the brain of rats through ROS formation [25].

Thus, there are many toxicity data available in literature which are related with the long-term exposure of constituents of plastic containers [26-28]. However, there are no data on their influence on stability of therapeutic proteins. The instability and conformational changes in proteins may result in many pathological conditions many of which are chronic and life-debilitating [29].

Lysozyme is a129 amino acid residues enzyme which catalyzes hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan present in the bacterial cell walls [30]. This property has been used for determining its biological activity which would be reduced drastically due to the exposure of any unfavorable/destabilizing environments. Moreover, lysozyme is cheap, well investigated, and characterized protein. Its biochemical and biophysical characteristics are well reported in literature. Therefore, in this study we investigated the influence of exposure of BPA and DINP on the conformation stability and biological activity of lysozyme which would further corroborate the deleterious effect of plastic constituents by providing some additional new information at the conformation level of protein. A conformational change in biological protein can result in a complete loss of its activity or a gain of mutated/altered activity, resulting in the development of a disease in both conditions [31-33].
Materials and Methods

Materials

Bisphenol A (≥ 99%) and DINP technical grade (ester content ≥99 %) were purchased from Sigma Aldrich, USA. Lysozyme (EC 3.2.1.17) from chicken egg white and Micrococcus Lysodeikticus (Micrococcus luteus) was purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals bought were of analytical reagent grade and used as obtained. Anhydrous dibasic sodium phosphate and citric acid needed to prepare Citrate-Phosphate (CP) buffer were purchased from Fisher Scientific, USA and used as received.

Methods

Preparation of citrate-phosphate buffer and lysozyme solution: Citrate Phosphate (CP) buffers of different pH were prepared by mixing the different proportions of 100 mM citric acid and 200 mM dibasic sodium phosphate stock solutions (Stoll and Blanchard, 1990). Lysozyme was dissolved at a concentration of 2 mg/mL (0.14 mM) in the CP buffer (pH 4.4, 72.2 mM) and centrifuged (4229 × g) for 20 minutes to remove any insoluble material. Supernatant was filtered through 0.1 μm Polytetrafluoroethylene (PTFE) filter (Millipore Corp., Bedford, MA) before using for exposure, thermodynamic and conformational evaluation studies. All pH measurements were done using a VWR Scientific model 8010 pH meter (VWR Scientific Products, Batavia, IL). Lysozyme was incubated to various concentrations (2-100 μM) of bisphenol A and DINP for 7 days. Samples (3 ml) were withdrawn at intervals of 1, 3, and 7 days which were for studies using DSC and FTIR.

Thermodynamic evaluation of lysozyme sample: Conformational stability of lysozyme samples exposed to BPA/DINP was evaluated by using a Differential Scanning Calorimeter (DSC) (VP-DSC, MicroCal, Northampton, MA). All the lysozyme samples were centrifuged and supernatants were filtered through a 0.1 μm filter. These filtered supernatants and CP buffer were degassed by stirring using a magnetic bar under vacuum before loading into the DSC sample and reference cells, respectively. The heat flow needed to keep the sample cell and reference cell at the same temperature was recorded at a temperature range of 15°C to 95°C and a scan rate of 1.5°C/min. At the beginning, the CP buffer was loaded in both sample cell and reference cells to ensure that the heat transition during protein conformational alterations is the only source of thermal difference between these two cells. This resulted in a baseline thermogram which was subtracted from the sample thermogram during data analysis. Midpoint transition temperature (Tm) and calorimetric enthalpy (ΔH) were used as conformational stability indicating thermodynamic parameters. A decrease in ΔH and Tm of the lysozyme was interpreted as an indicator of deleterious effect of BPA/DINP on conformational integrity of lysozyme. All data manipulations were performed by using Origin software (MicroCal) provided with the DSC.

Conformational characterization of lysozyme sample: The changes in the conformational integrity of the secondary structures of lysozyme solution exposed to BPA/DINP were further investigated by using Fourier Transform Infrared (FTIR) spectrophotometer. Forty microliters of the lysozyme samples exposed to BAP/DINP for 1, 3, and 7 days was placed in the sample compartment of the IR Prestige FTIR (Shimadzu, Kyoto, Japan). Spectra were obtained in the frequency range of 4000-1000 cm⁻¹ in absorbance mode. The parameters selected for running the scans were resolution of 4 cm⁻¹, average of 15 scans, and no apodization. Forty microliters of CP buffer was used to obtain the background spectra which was subtracted automatically from each sample spectrum. We used second derivative spectra for calculation of peaks in the region of 1600-1700 cm⁻¹ wavenumber as indicators of various secondary structural components of lysozyme [34].

Determination of enzyme activity of lysozyme sample: Biological activity of the lysozyme samples exposed to BPA/DINP was figured out by an enzyme activity assay using Micrococcus lysodeikticus (M. luteus) as a substrate specific to the ability of the native lysozyme to lyse cellular membrane [35]. Briefly, 100 mL sample of the bacteria μ (0.0155 g) in CP buffer (pH 4.4) was prepared so that its absorbance at 450 nm was in the range of 1.0-2.0. Specific amounts of BPA/DINP exposed lysozyme samples were added to one mL M. luteus sample contained in a spectrophotometric cuvette and rate of decrease in absorbance was measured by operating the spectrophotometer (UV 1700, Shimanzu, Kyoto, Japan) in rate measurement mode using the parameters: wavelength = 450.0 nm, measure Time = 180 seconds with (δ) = 0.2, lag/ rate = 5 seconds/175 seconds, and temperature = 25°C. The amount of lysozyme sample was optimized so that change in absorbance was about 0.2 unit. The blank CP buffer was used as the reference for the study. A rate of change of 0.001 in absorbance at 450 nm (A450 nm) was used to define 1 unit of biologically active lysozyme and was calculated by using following formula [36].

Units of lysozyme/ml sample = \( \frac{(\Delta A_{\text{enzyme}}/ \text{min Test} - \Delta A_{\text{enzyme}}/ \text{Black}) \cdot (\text{df})}{(0.001) \cdot (0.1)} \)

where, df = dilution factor

0.001 = Change in absorbance at A per the unit definition,

0.1 = Volume (in ml) of sample/standard used.
Data analysis: Statistical comparisons were made using student’s t-test and Analysis of Variance (ANOVA). The level of significance was used as p<0.05.

Results and Discussion

In this study, CP buffer was used because it covers a sufficiently wide pH range and has a small enthalpy of ionization which minimizes heat effects due to protonation of the protein. Figure 1 is the typical DSC thermogram of lysozyme which shows that folded state of lysozyme gets converted into the unfolded state at a specific temperature, $T_m$, due to absorption of heat indicated by H. Generally, a higher value of $T_m$ and H indicate a greater conformational stability. Table 1 shows significant decrease in $T_m$ and H values for lysozyme samples exposed to BPA or DINP with increase in exposure time indicating destabilization of conformational structure of lysozyme. This is important to note that the H value of BPA treated 7-day sample was negative which may indicate some drastic conformational change. In 7 days old DINP exposed samples, no measurable peak was observed which may be due to complete loss of conformational structure. DSC is ideally suited to the evaluation of the thermodynamic parameters of a protein during thermal denaturation since it measures the forces stabilizing the conformational structure directly as it is model independent. The biophysical methods for the characterization of protein unfolding indicate that a loss in compact structure of protein resulting in non-native conformational change has a dramatic effect on aggregation, deamidation, and oxidation. Biophysical studies have provided information about the relationship between protein unfolding and degree of stability on the basis of $T_m$ and $\Delta H$ values reported in a DSC thermogram.

These DSC data were further corroborated by FTIR spectroscopic investigation of lysozyme samples. This is one of the spectroscopic techniques which has been used regularly to evaluate the secondary structures of therapeutic proteins. Figure 2A shows the FTIR spectra of 7 days old
lysozyme sample and figure 2B of 1-day old lysozyme containing BPA where decrease in various peak intensity is evident. FTIR spectra of all the lysozyme samples exposed to BPA/DINP showed the splitting of β sheet secondary structure (Table 2). Furthermore, a complete loss of β sheet structure was found in the 7-day DINP exposed samples (Table 2) which also

| Sample           | Exposure Time (Day) | Location (1/cm) |
|------------------|---------------------|-----------------|
| Lysozyme         | 1                   | 1624            |
|                  | 3                   | 1624            |
|                  | 7                   | 1624            |
| Lysozyme + BPA   | 1                   | 1630            |
|                  | 3                   | 1630            |
|                  | 7                   | 1628            |
| Lysozyme + DINP  | 1                   | 1631            |
|                  | 3                   | 1633            |
|                  | 7                   | No peak         |

Table 2: Splitting of β sheet secondary structure.
showed the minimal biological activity (Figure 3). *M. Luteus* is a specific substrate for lysozyme. It is able to break down bacterial cell membrane only if its native three-dimensional secondary structural conformation is intact. When *M. luteus* is lysed by lysozyme its absorbance decreases. Therefore, a rate of decrease of such absorbance is an estimation of lysozyme activity [37]. This approach has been used by other investigators too in various studies involving lysozyme [35,47-49]. It is clear from figure 3 that lysozyme samples exposed to BPA/DINP are containing significantly less (p<0.5) enzyme activity than the control samples not exposed to these chemicals at all-time points studied.

European Chemical Bureau’s 2003 Risk Assessment Report ([https://www.greenfacts.org/en/dinp-didp/figtableboxes/tableq4-3.htm](https://www.greenfacts.org/en/dinp-didp/figtableboxes/tableq4-3.htm)) [50], indicates that exposure of phthalate in infants with toys can be up to 250 μg/kg. Another study investigated the metabolites of phthalate in urine in the range of 132-44.3 μg/L [51]. Thus, the concentrations (0-100 μM) used in this study is in the lower range of possible amount of phthalate in humans. Therefore, the protein conformational disruption could be even greater due to real life burden of these chemicals.

**Conclusion**

We conclude that BPA and DINP (which are constituents of plastic bottles) disrupts the conformational stability of the model protein lysozyme which is expected to be more severe in real life because the greatest exposure period used in this study was only 7 days. Conformational changes in protein may cause many disease conditions such as Alzheimer and Parkinson, therefore, it would be better not to use plastic items containing BPA and DINP.

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