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Full Length Research Paper

Effect of sorbic acid and some other food preservatives on human serum cholinesterase activity

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The effect of some selected food preservatives on serum cholinesterase was determined. It was found that potassium metabisulphite, methyl parabene and propyl parabene caused a significant increase while sacharine, benzoic acid, salicylic acid, copper carbonate and sorbic acid caused a significant decrease in cholinesterase activity. The behavior of serum cholinesterase activity in response to a gradual change in the incubation time as well as the sorbic acid concentration was also studied. The graphical and statistical analysis of the data showed an exponential decrease in cholinesterase activity with an increase in the concentration of sorbic acid. The cholinesterase activity was also found to be decreased exponentially with an increase in the incubation time. The regression analysis showed a good agreement between the experimental and calculated values.

Key words: Sorbic acid, enzyme activity, cholinesterase, inhibition sensitivity.

INTRODUCTION

The use of preservatives in food industry has been increased with the advancement in the production technologies. These chemicals are principally used for the protection of the food from the microorganisms. Some of these chemicals are also used as additives to improve the texture, taste, flavor and color of the foods and beverages. However, excessive use of some of these chemicals in food materials may cause toxicity in human (Wakabayashi, 1990; Igoi and Hui, 1996; Simon, 2003). Sorbic acid (2,4-hexadienoic acid) is a natural organic compound and is used as a food preservative. Sorbic acid and its derivatives (calcium sorbate, potassium sorbate and sodium sorbate) have been found to possess antimicrobial activity. Owing to their antimicrobial effect, these substances have been used as preservatives in foods and drinks such as cheese products, pickles, certain fish products, carbonated beverages, margarine and certain fruit and vegetable products, including wines. Sorbates are generally used at concentrations of 0.025 to 0.10 % and the optimal pH for antimicrobial activity is below pH 6.5. The preservative action of sorbic acid is based on the evidences that it can inhibit certain enzymes responsible for the growth of microorganisms (Traller, 1965). It has been investigated earlier that the microbial growth would be stopped by the inhibition of one or more sulfhydryl enzymes by sorbic acid (Whitaker, 1959). Sorbic acid has been found to be an effective inhibitor of fumarase, aspartase and succinic dehydrogenase in microorganisms (George and Reese, 1964). The inhibitory effect of sorbic acid for malate dehydrogenase, α-ketoglutarate dehydrogenase and enolase in microorganisms has been also investigated (Rehm, 1967; Azukas et al., 1961). Due to its hydrolytic effects on the choline esters, Butyrylcholinesterase (BChE) also known as pseudocholinesterase, is one of many important enzymes needed for proper functioning of the nervous system of human, other vertebrates and insects. Although, BChE shows structural as well as functional similarity to acetylcholinesterase (AChE), yet it can be distinguished from AChE in that the excess amount of substrate inhibits AChE (Lee and Harpst, 1973; Tougu, 2001).

On the other hand, BChE exhibits substrate activation in excess substrate (Masson et al., 2001). BChE preferably acts on butyrylcholine, but it can also hydrolyze acetylcholine (Ekholm, 2001). BChE can also detoxify the natural compounds owing to its scavenging activity (Massoulie et al., 1993). The inhibition of cholinesterase (ChE) leads to the cholinergic over-stimulation due to

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the accumulation of acetylcholine at the nerve terminals and neuromuscular junctions (Wilson, 1998; Rusyniak and Nanagas, 2004). In previous studies, it has been reported that ChE is inhibited by the exposure to carbamates (Wilhelm and Reiner, 1973), organo-phosphates (Rusyniak and Nanagas, 2004) and other pesticides (Wilson et al., 2005). The orally dosed organophosphate insecticides have also been reported to decrease the ChE activity in chicks (Mohammad et al., 2008) while malathione has been investigated as the inhibitor of human plasma ChE (Pavelki et al., 2008).

It has been reported that sorbic acid reacts with cysteine residues present in ChE. Possibly, it is an addition reaction between sorbic acid and thiol group of cysteine (George and Reese, 1964) which is present away from the active site and may cause some confor-mational changes in the protein molecule. The modified cysteine residue may leads to an irreversible inhibition of the enzyme that follows pseudo first order kinetics (Odani et al., 1982; Ellman et al., 1961). Previously, the studies showed that the activity of ChE in the pig liver homogenate was enhanced by addition of potassium sorbate (Kreimer et al., 1994). The literature survey indicates that the inhibitory effect of sorbic acid on human serum ChE has not been studied and no guidelines are available regarding the modeling aspects of human serum ChE inhibition with sorbic acid. Therefore, in the present study we investigated the effect of some food preservatives on human serum ChE activity *in vitro*. This study was particularly focused on the effect of incubation time as well as the concentration of sorbic acid on human serum ChE activity.

MATERIALS AND METHODS

Acetylthiocholine iodide, 5,5'-dithiobis-(2-nitro) benzoic acid (DTNB), sorbic acid, disodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from Merck. All the chemicals were of analytical grade and used without further purification. Blood was collected from anticubital vein of a healthy adult and serum was analyzed for the effect of sorbic acid on human serum ChE activity. The percentage activity of the enzyme was measured by the colorimetric method using acetylethiocholine iodide as the substrate. The method is based upon the hydrolysis of cholinesterase. The yellow compound (2-nitro-5-mercaptobenzoate) formed by the reaction between thiocholine and 5,5-dithio-bis-(2-nitrobenzoate) (DTNB), can be measured at 410 nm (Steinberg et al., 1990).

The solutions of different food preservatives *viz.* potassium metabisulphite, methyl parabene, propyl parabene, sodium benzoate, sodium glutamate, sodium nitrite, sacharine, benzoic acid, salicylic acid, copper carbonate and sorbic acid were prepared by dissolving 0.1 g of each in small volume of deionized water and the final volume was made up to 100 mL. The effect of the selected food preservatives on serum ChE activity was determined by incubating serum (0.5 mL) with equal volume (0.5 ml) of each preservative at 37°C for 30 min followed by the addition of DTNB 0.27 mM/L in phosphate buffer of pH 7.7 (2.8 mL) and acetylthiocholine iodide 5 mM/L (0.1 mL). Immediately, after mixing the reaction mixture the absorbance was measured at 410 nm after successive intervals of time (1 min) for 3 min. The control experiment was also performed in which the inhibitor was replaced water and the activity of ChE (IU/L) by was calculated as:

$$\text{ChE activity} = 11.1 \times 10^3 \times \Delta A / \Delta t \text{ U/L}$$

Where, 11.1×10^3 is the factor for absorbance at 37°C; ΔA is the change in absorbance. The results were expressed as remaining activity (%) compared to control activity (100%). For the determination of the effect of different concentrations of sorbic acid on ChE activity, serum (0.1mL) was incubated with different concentrations (0.0073, 0.0146, 0.0219, 0.0292, 0.0365 and 0.0438 mM%) aqueous solution of sorbic acid (0.1mL) and at 37°C for 5 min followed by the addition of DTNB 0.27 mM/L in phosphate buffer of pH 7.7 (2.8 mL) and acetylthiocholine iodide 5 mM/L (0.1 mL). The incubation without sorbic acid addition was used as the control and the absorbance of each sample was recorded at 410 nm. To study the effect of incubation time (5, 10, 15, 20, 25 and 30 min) on inhibition of ChE by sorbic acid, serum (0.5 mL) was incubated with 0.0146 mM% sorbic acid (0.5 mL) at 37°C. After an interval of each 5 min, the incubated material (0.1 mL) was transferred to a glass cuvette containing DTNB 0.27 mM/L in phosphate buffer of pH 7.7 (2.8 mL) and acetylthiocholine iodide 5 mM/L (0.1 mL) and the absorbance was noted at 410 nm immediately after mixing the reaction mixture. The activity of the enzyme at 0 time incubation was taken as 100%. The ChE activity in each case was calculated as described earlier and the results were expressed as the remaining ChE activity (%) compared to the activity of control (100%).

Statistical analysis

The experimental data were analyzed by one way analysis of variance (ANOVA) and the means were differentiated at confidence level $P \leq 0.05$ using Tukey's multiple range test (SPSS version 12). The data for the determination of effect of incubation time and sorbic acid concentration on ChE activity was analyzed by applying statistical and mathematical methods.

RESULTS AND DISCUSSION

Effect of food preservatives on ChE activity

The effect of different preservatives on ChE activity as compared to the control is displayed in Figure 1. The statistical analysis of the data showed a significant increase in ChE activity after incubation with propyl parabene, methyl parabene and potassium meta-bisulphite. No significant change in ChE activity was observed after incubation with sodium benzoate, sodium glutamate and sodium nitrite. On the other hand, ChE activity was found to decreased significantly after incubation with saccharine, benzoic acid, salicylic acid, copper carbonate and particularly sorbic acid.

Effect of sorbic acid concentration on ChE activity

A number of experiments were carried out to see the effect of sorbic acid concentration on the activity of ChE at 37°C as shown in Figure 2. Depending on the process conditions, the results indicate that the activity of enzyme

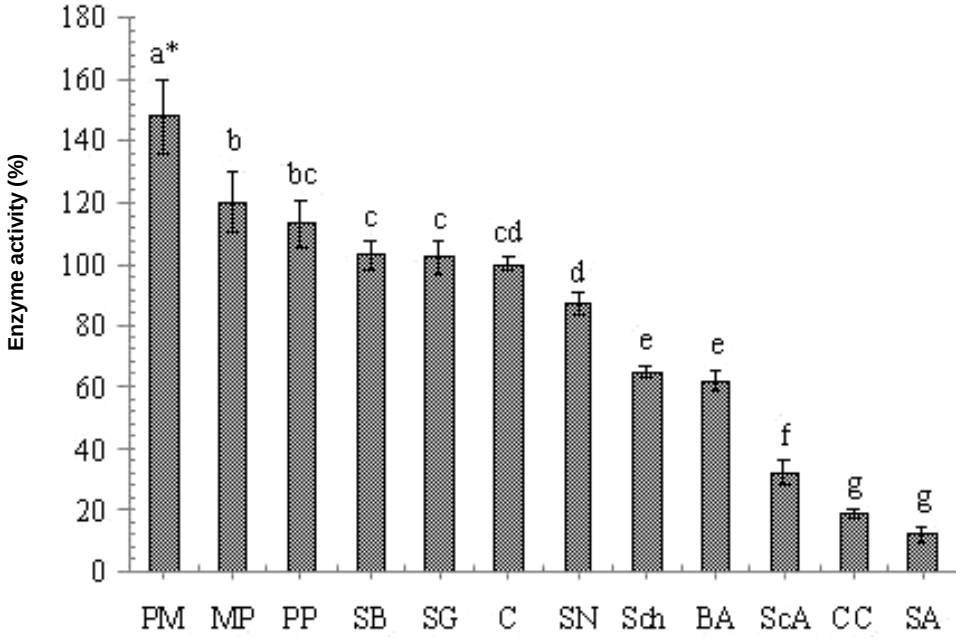

Figure 1. Comparative effect of some selected food preservatives on serum cholinesterase activity. PM: Potassium metabisulphite, MP: Methyl parabene, PP: Propyl parabene, SB: Sodium benzoate, SG: Sodium glutamate, C: Control SN: Sodium nitrite, Sch: Sacharine, BA: Benzoic acid, ScA: Salicylic acid, CC: Copper carbonate, SA: Sorbic acid. *The bars presented by the same letter are not significantly different at $P \leq 0.05$, using Tukey's multiple range tests.

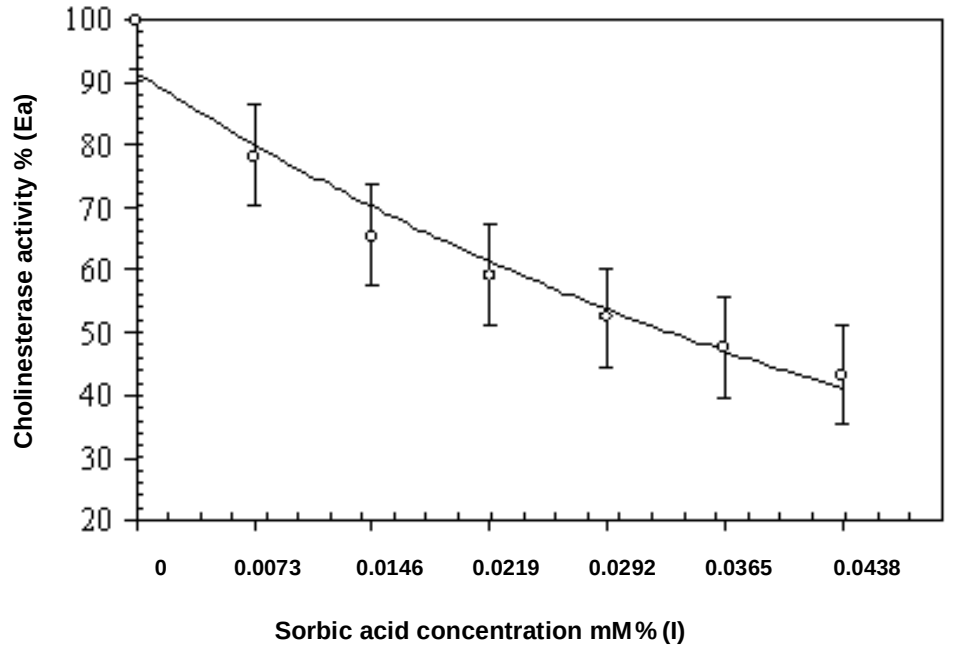

Figure 2. Effect of sorbic acid concentration on ChE activity at known time.

decreases with an increase in the acid concentration, which can be attributed to the interaction of sorbic acid with thiol group of cysteine residue in ChE. After a relatively high value of the acid concentration, the degree of inhibition of the enzyme is not much higher, a situation that can be attributed to the decrease in the free

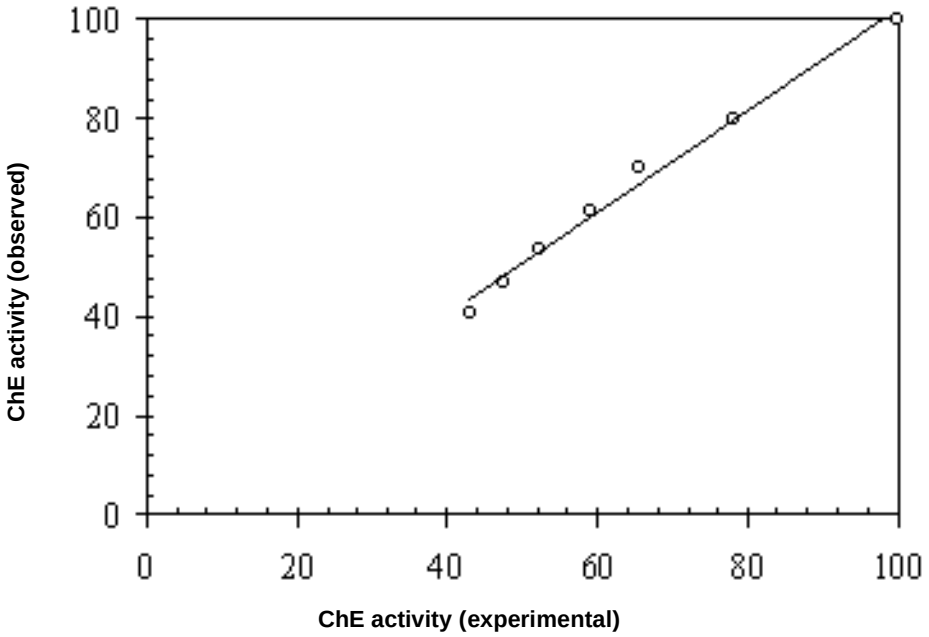

Figure 3. Agreement between experimental and calculated ChE activity values influenced by change in the concentration of sorbic acid.

availability of active sites/other sites on the enzyme molecule. Enzyme-inhibitors reactions are of great pharmaceutical and medicinal importance and have a number of applications in different chemical and biological processes. The experimental data were analyzed using statistical and graphical methods. The data analysis results show that the enzyme activity is a function of the acid concentration, following an exponential model:

$$E_a = E_{a_\circ} e^{I_{SC} C_{SA}} \quad (1)$$

Where, E_a is the enzyme activity; C_{SA} is the concentration of sorbic acid; $E_{a_\circ}(=91.522)$ is the pre-exponential factor and $I_{SC}(=-18.298)$ is the inhibition sensitivity coefficient. The pre-exponential factor and inhibition sensitivity coefficient may provide useful information about the inhibition process depending on the nature and type of enzyme-inhibition systems as well as inhibition conditions.

The pre-exponential factor indicates the degree of inhibition of the enzyme under the negligible concentration of inhibitor, whereas the inhibition sensitivity coefficient indicates the inhibition capacity of the enzyme with the change in the concentration of the acid used. Thus, using the values of $E_{a_\circ}(=91.522)$ and $I_{SC}(=-18.298)$ from the intercept and the slope of Figure 2, respectively, the Equation [1] can be written as:

$$E_a = 91.522\, e^{-18.298 C_{SA}} \quad (2)$$

The analysis of the results shows that applicability of the suggested model is good for the present enzyme-inhibition system and it can estimate the enzyme inhibition behavior using sorbic acid with standard error of estimate of ± 0.0564. To test the agreement between the experimental enzyme activity and the values calculated from the exponential model, the graph of $E_{a(exp)}$ versus $E_{a(cal)}$ was plotted as shown in Figure 3. It is observed that the agreement between the experimental and calculated values is good with correlation coefficient of 0.9895 and coefficient of determination of 0.9661. The data in the scatter diagram show a positive tendency to cluster around the regression line indicating a good relationship between the variables. The value of the standard deviation of regression or standard error of estimate of $E_{a(cal)}$ on $E_{a(exp)}$ indicates that the degree of scatter of the observed values about the regression line is small. Using the statistical analysis with 7 numbers of experimental data, a relative mean square of errors of 0.01084 has been calculated by Equation [3]:

$$ER = \left[\frac{1}{N} \sum_{i=1}^{N} \frac{\left(E_{a(cal)} - E_{a(\exp)}\right)^2}{\left(E_{a(cal)}\right)^2} \right]^{1/2} \quad (3)$$

The value of relative mean square of errors indicates that such a value of the random errors is not significant

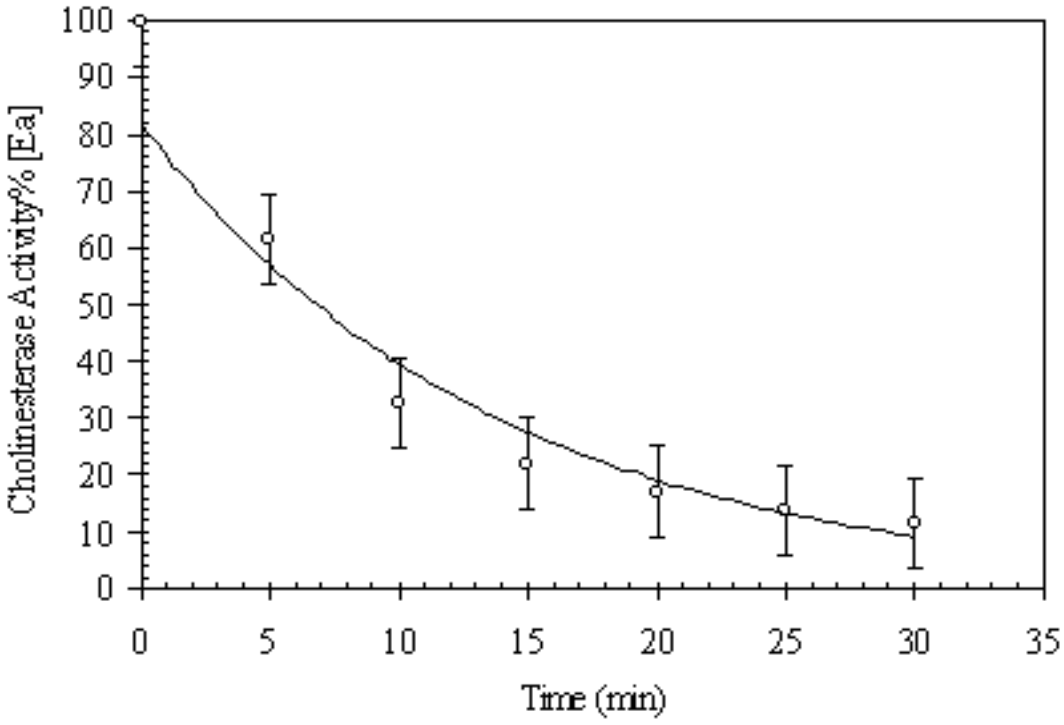

Figure 4. Effect of incubation time on ChE activity at specific concentration of sorbic acid.

regarding such probabilistic models. To check the reproducibility and applicability of the suggested model, I_{C50} can be calculated using Equation [2]. For 50% inactivation of the enzyme, I_{C50} was calculated to be about 0.03637 mM%.

Effect of incubation time on ChE activity

A number of experiments were also carried out to see the effect of incubation time on the activity of the enzyme using acid concentration of 0.0146 mM% at 37°C. The experimental data were analyzed using statistical and graphical methods. Depending on the experimental conditions, an exponential decrease in the activity of enzyme was observed in response to a gradual increase in the incubation time as shown in Figure 4. The analysis results show that the enzyme activity is a function of the incubation time following the model:

$$E_a = E_{a_\circ} e^{r_I t_I} \quad (4)$$

Where, E_a is the enzyme activity; $E_{a_\circ} (= 81.79)$ is the pre-exponential factor and $r_I (= -0.0727)$ is the inhibition rate. Thus, using the values of $E_{a_\circ}$ and r_I from the intercept and the slope of Figure 4 respectively, the Equation [4] can be written as:

$$E_a = 81.79 e^{-0.0727 t_I} \quad (5)$$

The analysis of the results shows that applicability of the above model is relatively low as compared to the suggested model Equation [2], regarding the effect of the acid concentration on the enzyme inactivation. However, for the present enzyme-inhibition system, it can estimate the enzyme inhibition rate with coefficient of determination of 0.9518 using sorbic acid concentration of 0.0146 mM%. To test the agreement between the experimental enzyme activity and the values calculated from the model, the graph of $E_{a(exp)}$ versus $E_{a(cal)}$ was plotted as shown in Figure 5. It is observed that the agreement between the experimental and calculated values is fair with correlation coefficient of 0.9847. The data in the scatter diagram show positive tendency to cluster around the regression line. The data in the scatter diagram show a positive tendency to cluster around the regression line indicating an existence of fair relationship between the variables.

The value of the standard deviation of regression or standard errors of estimate of $E_{a(cal)}$ on $E_{a(exp)}$ indicates that the degree of scatter of the observed values about the regression line is small. Although the regression parameters may need further studies using different concentrations of the acid at various incubation times,

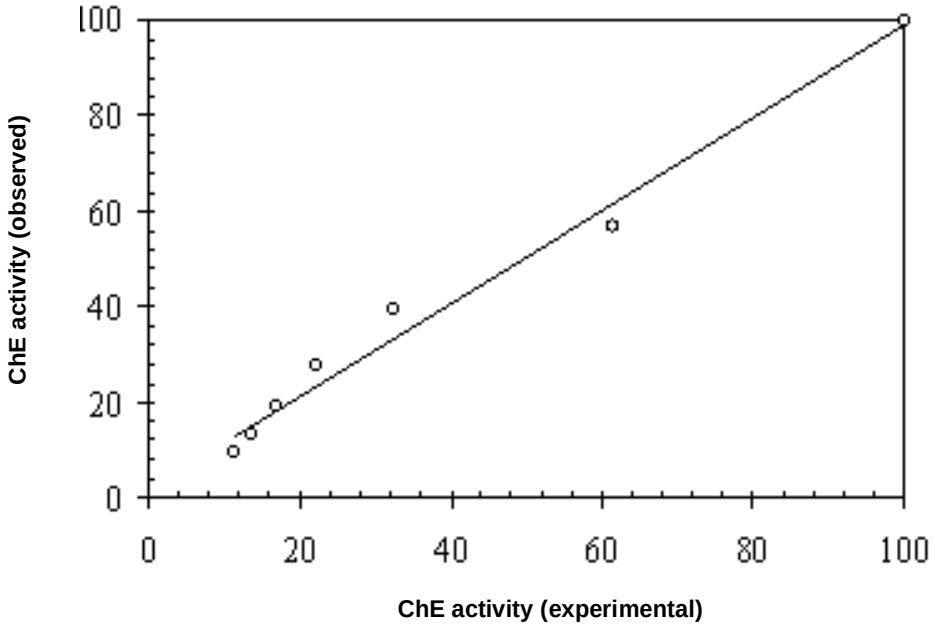

Figure 5. Agreement between experimental and calculated ChE activity values influenced by change in the incubation time.

however, the present study is expected to provide some valuable guidelines for researches confronted with the need to use such typical enzyme-inhibition systems.

The present results show that among the investigated food preservatives, potassium metabisulphate, methyl parabene, propyl parabene, sacharine, benzoic acid, salicylic acid, copper carbonate and sorbic acid cause a significant change in normal ChE activity and, in this regard, the use of these preservatives in food materials may be harmful to human health. Sorbic acid may decrease the serum ChE activity due to the possible binding with the thiol group of cysteine present at the site other than the active site of the enzyme. The present work can be extended further more by carrying out *in vivo* studies for finding the relationship between the model and the toxicity evaluation using the laboratory animals.

The enzyme activity showed a fair concentration-dependent behavior shifting from 100 to 45% at 37°C. The resultant enzyme inhibition curve was analyzed by graphical and statistical methods and it has been found that the enzyme inhibition exponentially increases with an increase in the acid concentration. The experimental results also show that the enzyme inhibition increases by increasing the incubation time. The regression analysis results show that the agreement between the experimental and calculated values is good. The regression parameters may need some further investigations using different conditions of the acid concentration to investigate the mechanistic aspect of the present enzyme-inhibitor system. However, the present study is expected to be of some value to others confronted with the need to use such typical enzyme-inhibition systems.

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