Purification of Alkaline Phosphatase from Bovine Milk and Investigation of Inhibitory Effects Of Some Veterinary Drugs on Enzyme Activity

İnek Sütünden Alkalen Fosfataz Enziminin Saflaştırılması ve Bazı Veteriner İlaçların Enzim Aktivitesi Üzerine İnhibisyon Etkilerinin İncelenmesi

Research Article

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ABSTRACT

In this study, Alkaline Phosphatase (EC 3.1.3.1, ALP) which has a critical role in phosphate metabolism was purified via biochemical methods and the effects of some veterinary drugs on purified milk ALP were determined on account of the fact that using as a pasteurization indicator in milk. Enzyme was homogenized with different organic solvents, ammonium sulfate precipitation, dialysis and following Sephadex G-100 gel filtration chromatography. The enzyme product yielded bands, approximately 85 and 185-190 kDa on SDS-PAGE except casein fractions, while a single band, approximately 170-190 kDa interval. ALP is the most important enzyme in dairy industry as the check for the presence of residual ALP activity in milk is a good indication of proper pasteurization. Heat treatment of milk for adequate pasteurization, which kills pathogenic microorganisms, give rise to inactivate ALP. It seems as if milk is pasteurized before not to reach pasteurization temperature, in case of decreasing ALP activity notably by the veterinary drugs. Therefore, in vitro inhibitory effects of some drugs (furosemide, atropine sulfate, toldimfos sodium and levamisol/synonim: (-)- tetramisol HCl) were investigated in this study. While atropine sulfate didin't effect the enzyme activity, the IC_{50} values of drugs which have inhibitory effect were obtained 45.48 μM, 2.19 and 126.315 mM for tetramisol, furosemide and toldimfos sodium, respectively. When compared to Levamisol, which is a potent inhibitor of the enzyme, the inhibitory effects of the drugs on the enzyme activity are weak.

Key Words
Alkaline phosphatase, purification, inhibition, veterinary drugs.

ÖZET

Bu çalışmada, fosfat metabolizmasında önemli role sahip olan Alkalen Fosfatazın (ALP), sütte pastörizasyon belirteci olarak kullanılması gerekçesiyle sütte bulunan izoformu biyokimyasal yöntemlerle saflaştırılmış ve bazı veteriner ilaçların enzim aktivitesi üzerine etkileri araştırılmıştır. Enzim, çeşitli organik çözücülerle muamele edilerek, amonyum sülfat çöktürme aralıkları belirlenmiş ve elde edilen çökelek diyaliz edilerek, Sephadex G-100 jel filtrasyon kolonuna tatbik edilmiştir. Saflaştırılan enzim SDS poliakrilamid jel elektroforezine uygulanarak, elektroforezde gözlenen kazein fraksiyonları dışında, yaklaşık 85 kDa ve 185-190 kDa molekül ağırlığına sahip bandlar elde edilirken; doğal jel elektroforezinde, yaklaşık 170-190 kDa aralığında tek band gözlenmiştir. Enzim, pastörizasyon sıcaklığına gelindiğinde inaktive olacağından enzim aktivitesinin gözlenmemesi sütün pastörize olduğunu gösterir. Fakat, veteriner ilaçlar tarafından enzimin inaktivasyonu söz konusu olduğunda, pastörizasyon sıcaklığına ulaşılmadan enzim inhibe olacağından doğru bir pastörizasyon gerçekleştirilemeyecektir. Bu sebeple çalışmamızda, bazı ilaçların (furosemide, atropine sulfate, toldimfos sodium and levamisol/ (-)- tetramisol HCl) enzim aktivitesi üzerine inhibisyon etkileri incelenmiştir. Atropin sülfat enzim aktivitesini etkilemez iken, enzim aktivitesi üzerinde inhibisyon etki gösteren tetramisol, furosemid ve toldimfos sodyuma ait IC_{50} değerleri sırasıyla 45.48 μM, 2.19 ve 126.315 mM olarak hesaplanmıştır. ALP'nin bilinen inhibitörü olan Levamisol ile karşılaştırıldığında, enzim aktivitesini azaltan ilaçların zayıf inhibitör etki gösterdiği belirlenmiştir.

Anahtar Kelimeler
Alkalen fosfataz, saflaştırma, inhibisyon, veteriner ilaçlar.

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INTRODUCTION

Alkaline phosphatases (APs; EC 3.1.3.1) occur widely in nature, and are found in many organisms from bacteria to man. Each catalytic site of enzyme contains three metal ions, i.e., two Zn^{+2} and one Mg^{+2}, necessary for enzymatic activity [1] that two active-site Zn^{2+} ions coordinate the nucleophile and the leaving group, respectively and a nonbridging oxygen atom of the transferred phosphoryl group is coordinated between the two Zn^{2+} ions. A third metal ion site near the bimetallo site contains a Mg^{2+} ion and it has been suggested that a Mg^{2+}-bound hydroxide ion acts as a general base to deprotonate the Ser nucleophile [2]. ALP is a bi-metalloenzyme of potential applications in biotechnology, in which phosphate monoesters are nonspecifically hydrolysed under alkaline conditions to yield inorganic phosphate [3]. ALP is a membrane-bound glycoprotein, common in animal tissues and microorganisms [4] and contains sialic acid [5]. ALP is bound to the mammary cell and milk fat globule membranes via phosphotidyl inositol that is the common form of linkage of ALP to membranes [6] and dephosphorylate casein, phosphoprotein, under suitable conditions [7]. The enzyme had a molecular mass of 187 kDa and the isoelectric point ranged from 5.4 to 6.0 [5]. Kuzuya et al. reported that most of the purified microbial and mammalian alkaline phosphatases contain fucose, mannose, galactose, glucosamine, galactosamine and sialic acid in varying amounts [8].

ALP, native enzyme found in unpasteurized milk [5], is the most important enzyme in dairy industry. Activity of this enzyme is related to the quality of the process of pasteurization [4]. Heat treatment of milk at 62.8°C for 30 min or 71.7°C for 15 sec (minimum requirements for adequate pasteurization), which kills nonsporeforming pathogenic microorganisms, will inactivate ALP [5]. The check for the presence of residual ALP activity in milk is a good indication of proper pasteurization through a non-fluorescent aromatic monophosphoric ester substrate undergoes hydrolysis by ALP liberating a highly fluorescent product [9]. Besides spectrophotometric methods, immunochemical assays for ALP can be used to detect bovine milk ALP to determine proper pasteurization of milk for fermented products which could contain ALP from microbial sources as microbial and bovine milk ALP molecules may have distinctive structures that could be differentiated using antibodies [5]. Alkaline phosphatase is a protein that was first described by Suzuki et al. (1907) suggested that phosphatases constituted a separate class of eukaryotic enzymes [10]. Indigenous ALP in milk is similar to the enzyme in mammary tissue [6] and Rankin et al. reported that ALP is abundantly present in nature and found in many human body tissues, such as liver, kidney, bone, and blood cells [11]. There are at least four distinct but related alkaline phosphatases: intestinal (IAP), placental (PLAP), germ cell (GCAP) and tissue non-specific (TNAP) as the first three are located together on chromosome 2 while the tissue non-specific (liver, kidney, bone, stomach and colon) form is located on chromosome 1 [12].

Phosphatases are one of the most crucial enzymes for an organism's survival, which hydrolyze phosphate esters and provide inorganic phosphate (Pi) [13]. Zhu et al. reported that alkaline phosphatases (APases) activity serves a variety of essential functions which include nutrition, phosphate metabolism, intracellular signalling as well as modification and transport of metabolites across biological membranes [14]. Phosphate cannot be synthesized by microbes, makes APases all the more important for their survival and the cell obtains Pi from nucleic acids, phosphorylated sugars, proteins, etc. [13]. Moreover, for disease diagnostic purposes, where new tests based on ALP activity measurements are continuously being proposed in the literature [15]. Aminian et al. showed that increases in serum ALP levels have been associated with a variety of hepatic and bone diseases, such as cholestasis, hepatitis and infiltrative liver disease [16] and Abramowitz et al. indicated that higher serum levels of ALPase and phosphate are associated with increased all-cause and cardiovascular mortality [17]. In the light of this information, human placental alkaline phosphatase has gained new interest as a tumor marker now that highly specific monoclonal antibodies have become available [18]. Also it is possible that increased expression of placental alkaline phosphatase alters the growth of fetal

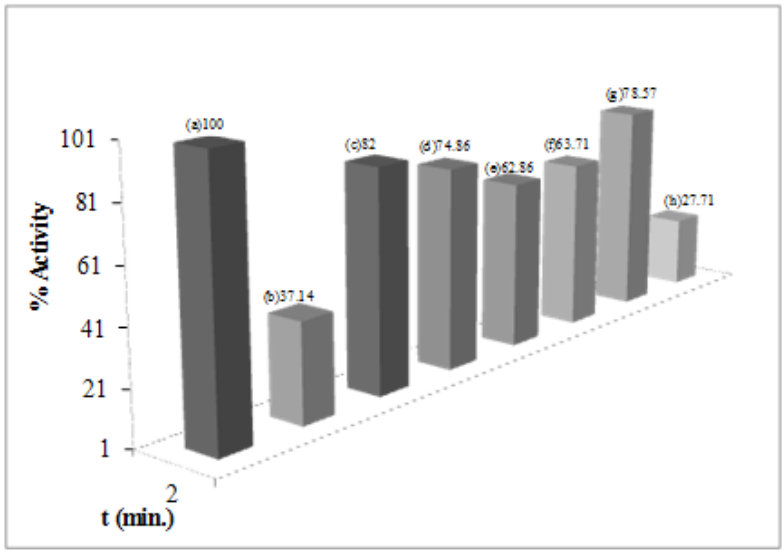

Figure 1. Initial purification steps of milk ALP; (a) raw milk, (b) milk and centrifuge, (c) milk and n-butanol %2 and centrifuge, (d) milk and EDTA % 0.2 and n-butanol %2 and centrifuge, (e) milk and toluene %3 and centrifuge, (f) milk and EDTA % 0.2 and toluene %3 and centrifuge, (g) milk and EDTA % 0.2 and centrifuge, (h) milk and casein precipitation.

and cancer tissues since the growth of fetus and tumors show many common features [19]. In addition to this, phosphatases have found increasing application in design of enzymatic biosensors and immunosensors, where they are used as labels in recent years [20].

In our study, we studied the optimization and characterization of milk alkaline phosphatase reaction according to different buffers and organic solvents with different ratios, following enzyme inhibition studies with some veterinary drugs, which effect onto milk alkaline phosphatase activity, were investigated because of accurate pasteurization process importance. No study is available on the veterinary drugs effects for purified milk ALP enzyme activity.

MATERIALS AND METHOD

Materials

The chemicals used in our study were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck.

Enzyme purification

Different Agents and Organic Solvent Treatment

Fresh bovine milk was cooled down to 4°C overnight without added preservative. For optimization of ALP purification procedure, the milk was churned with a blender at maximum speed for 40 min at cool media in organic solvent treatments. Fresh milk was homogenized with different solvents and agents: Casein precipitation with acidification [21] treatment in different diluted raw milk samples with distile water, (1/1) dilution with distile water, direct centrifuge of milk, toluene 3%, EDTA 2 mM, acetone [22] 2%, butanol 2%, butanol %2 after dilution and EDTA and toluene [23] were added to give final concentrations of 2 mM and 3% (v/v), respectively (Figure 1). Together with EDTA and butanol and EDTA and acetone (2 mM and 2% (v/v)) treatments, the most ALP enzyme activity observed with butanol extraction than others. Thereafter, raw fresh milk was homogenized with n-butanol at different volume ratios % v/v, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, respectively. Higher enzyme activity was obtained with 3% n-butanol proceeding as a result. The whole activity experiments were determined in buffers sodium carbonate and sodium sulfate till now. Much more enzyme activity was derived from sodium carbonate activity measurements. After each treatment the suspension was centrifuged at 15 000 rpm for 40 min and the precipitate formed was discarded.

Determination of Intervals of Ammonium Sulfate Precipitation

Discrepantly, ammonium sulfate precipitation intervals were determined (% w/v 0-10, 10-20, 20-30, ... ,90-100) respectively and salting precipitation interval was determined as % 20-40 for ALP after %3 (v/v) butanol treatment as a result. The sample was brought to 20% saturation by addition of solid ammonium sulphate. The suspension was centrifuged at 15 000 rpm for 40 min and the precipitate formed was discarded. The supernatant was brought to 40% saturation with solid ammonium sulphate. The precipitate formed was collected by centrifugation at 15 000 rpm for 40 min and dissolved 0.1 M Tris–Base (pH: 10) buffer.

Gel Filtration Chromatography

Gel filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes [24]. Molecules are separated according to differences in sizes as would be required in a purification or characterization protocol [25]. The pooled precipitate obtained from bovine milk by using ammonium sulfate precipitation was subjected to Sephadex G-100 gel filtration chromatography with the same buffer in salting precipitation. The fractions were collected and their absorbance measured at 280 nm.

Activity measurements

Alkaline phosphatase activity was determined by the modified method of Aschaffenburg and Mullen (1949) [26]. The conversion of p-nitro phenol was followed by monitoring the change in absorbance at 405 nm, using UV-Visible Spectrophotometer (ϵ_{405}=18.2 $L.cm^{-1}.mmol^{-1}$). The reaction mixture contained 0,15 M Na_2CO_3/0,1 M $NaHCO_3$ (pH: 10.5) buffer and 0.5 mM p-nitrophenyl phosphate as substrate at 37°C. The assay was initiated by the addition of the enzyme. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of p-nitrophenyl phosphate to p-nitro phenol per min under defined conditions [26,27].

Total Protein Determination

After elution step enzyme was determined spectrophotometrically at 280 nm and protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [28].

SDS and Native Polyacrylamide Gel Electrophoresis

The purity of enzyme ALP from using ammonium sulfate precipitation and gel filtration chromatography was assessed by SDS and native polyacrylamide gel electrophoresis according to the method of Laemmli [29].

RESULTS AND DISCUSSION

In our study, alkaline phosphatase was extracted from fresh bovine milk without added preservative using n-butanol, toluene and acetone besides EDTA and casein treatments. The most ALP enzyme activity was observed with butanol extraction as compared to other solvents (Figures 1,2,3). That's why n-butanol was used as organic solvent to seperate ALP from lipoprotein particules for further purification steps [6,30]. Cow milk was homogenized with n-butanol %3 (v/v) with respect to previous steps followed by ammonium sulfate precipitation, dialysis and Sephadex G-100 gel filtration. Total ALP isolation from milk is quite difficult as compared to other milk fat globular enzymes and whey proteins because of the identical isoelectric point for both gamma-casein (pI: 5.8-6.0) [31] and milk alkaline phosphatase (pI: 5.4-6.0) [5].

Approximately 30%-40% of ALP in milk is associated with the fat globule membrans, the rest of the enzyme is bound to the lipoproteins in skimmed milk fractions [4]. Therefore, the enzyme was successfully collected in the ratio of 3 percent of n-butanol at this step 69,81% yield purification was achieved (Table 1). ALP activity was observed in %3 (v/v) n-butanol treatment followed by ammonium sulfate precipitation, dialysis and gel filtration as 103.30 EU/ml. The dialysis sample, which was saturated by 0.1 mM Mg^{+2} following this, was applied to the Sephadex G-100 gel filtration column equilibrated with 0.1 M Tris-Base (pH 10.0) buffer. The gel was washed with 0.1 M Tris-Base (pH 10.0) and ALP was eluted by using the same buffer. Fractions of 2 mL were

Table 1. Summary of the purification of bovine milk alkaline phosphatase.

Step	Volume (ml)	Activity (EU/ml)	Total Activity (EU)	Protein Amount (mg/ml)	Total Protein (mg)	Specific Activity (EU/mg)	Overall Yield	Overall Purification (fold)
Milk	30	172.53	5175.82	0.64	19.13	270.49	100	-
3% n-butanol supernatant	24	150.55	3613.19	0.24	5.72	631.30	69.81	2.33
20-40% $(NH_4)_2SO_4$ precipitation	6,25	315.38	1971.15	0.42	2.61	755.89	38.08	2.79
Dialysis	6	196.15	1176.92	0.38	2.30	512.14	22.74	1.89
JFK	2	103.30	206.59	0.02	0.05	4053.55	3.99	14.99

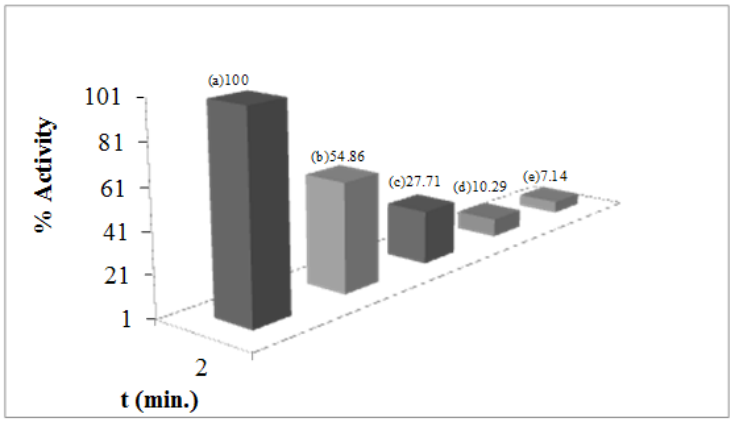

Figure 2. Initial purification steps of milk ALP. (a) raw milk, (b) milk and dilution with distile water (1/1), (c) milk and casein precipitation, (d) milk and dilution with distilated water (1/1) and casein precipitation, (e) milk and dilution with distile water (1/2) and casein precipitation.

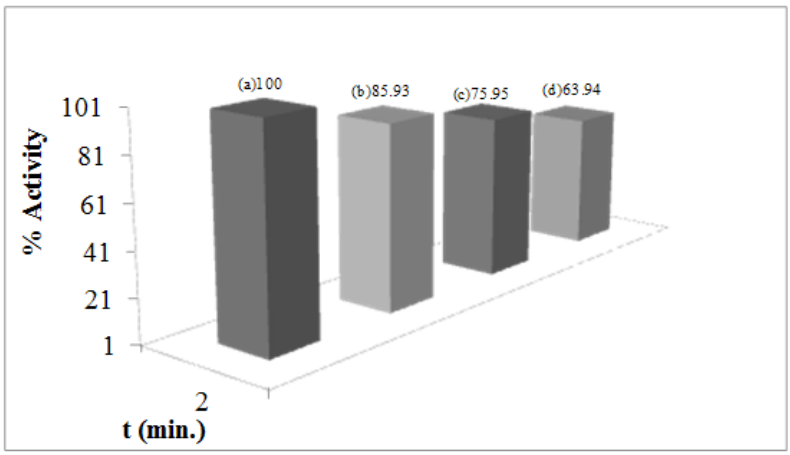

Figure 3. Initial purification steps of milk ALP. (a) raw milk, (b) milk and n-butanol 2% and centrifuge, (c) milk and acetone 2% and centrifuge, (d) milk and EDTA 0.2% and n-acetone 2% and centrifugation.

collected and their absorbance was measured at 280 nm. The eluates were characterized by protein determination at 280 nm and assaying ALP activity. Specific activity for ALP was calculated by using fresh raw milk, 3% n-butanol, ammonium sulfate precipitated sample, dialysis and Sephadex G-100 gel filtration. As a result, ALP was purified up to 14.99-fold with a recovery

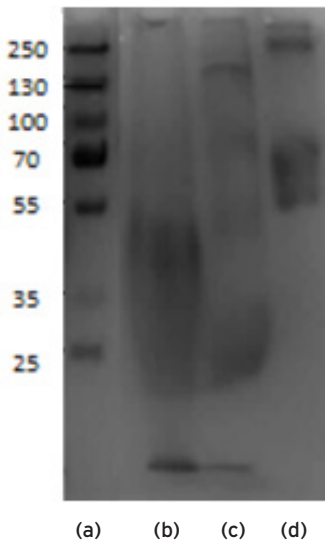

Figure 4. SDS-PAGE of bovine milk alkaline phosphatase. The purified ALP enzyme samples were analyzed by SDS-PAGE and revealed by Coomassie Blue staining. (a) marker, (b) ammonium sulfate precipitation (sample buffer/enzyme – 1/2), (c) dialysis sample which was applied to Sephadex G-100 (sample buffer/enzyme – 1/2), (d) pure bovine ALP from sigma (160 kDa).

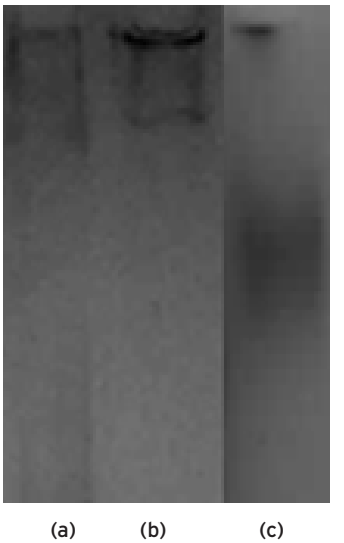

Figure 5. Native-PAGE of bovine milk alkaline phosphatase. The purified ALP enzyme samples were analyzed by Native-PAGE and revealed by Coomassie Blue staining.- (a) ammonium sulfate precipitation (sample buffer/enzyme – 1/1), (b) ammonium sulfate precipitated enzyme which was applied to Sephadex G-100 (sample buffer/enzyme – 1/1), (c) pure bovine ALP from sigma (160 kDa - sample buffer/enzyme – 1/2).

ratio of 3.99% (Table 1).

Milk ALP is a homo-dimer of two similar sub-units that each of molecular mass ~85kDa [6] in that the enzyme migrated apparently identical molecular masses showed between 72-95 kDa on SDS-PAGE except other fractions (Figure 4). On the other hand, milk isoenzyme of ALP had a molecular mass of 187 kDa [5] with unqualified two identical sub-units, comparably with other studies in the literature [32, 33]. This result also was similar between the range of 130-250 kDa (Figure 5). We used four steps for purification, i.e. n-butanol treatment, ammonium sulfate precipitation, dialysis and gel filtration chromatography. ALP purified using the Sephadex G-100 by using 0.1M Tris-Base buffer pH:10. The enzyme activities and total protein concentrations were obtained for all collected fractions of each purification step (Table 1).

In literature, ALP was carried out from bovine milk with overall purification of approximately 7.4-fold with Concanavalin A Agarose column and 8.5-fold with electroelution. The procedure included Macro-Prep High Q anion exchange chromatography, Sephacryl S-200 and Concanavalin A agarose affinity chromatography for column purification [5]. Moreover, ALP was purified from non-pasteurized milk with 17.67 purification fold. The procedure included separating of cream, butanol and acetone treatment, respectively [34]. With Concanavalin A Agarose column and electroelution, lower purification folds were obtained [5] by comparison with our study and similar with Upadhyay and Verma report [34].

There were no data for veterinary dugs effects on milk alkaline phosphatase activity. The enzyme application for industrial purposes depend on its activity to recognize adequate pasteurization [5]. It seems as if milk is pasteurized before not to reach pasteurization temperature, in case of decreasing ALP activity notably by the veterinary drugs used in most animal species before pasteurization process [35]. Therefore, inhibitory effects of some active ingredients (Figure 6) were determined.

Furosemide is one of the diuretics having ototoxic side-effects in clinical experience and in animal investigations [36]. Atropine sulfate is mostly used in veterinary preanesthetic agent to balance anesthesia and as a specific

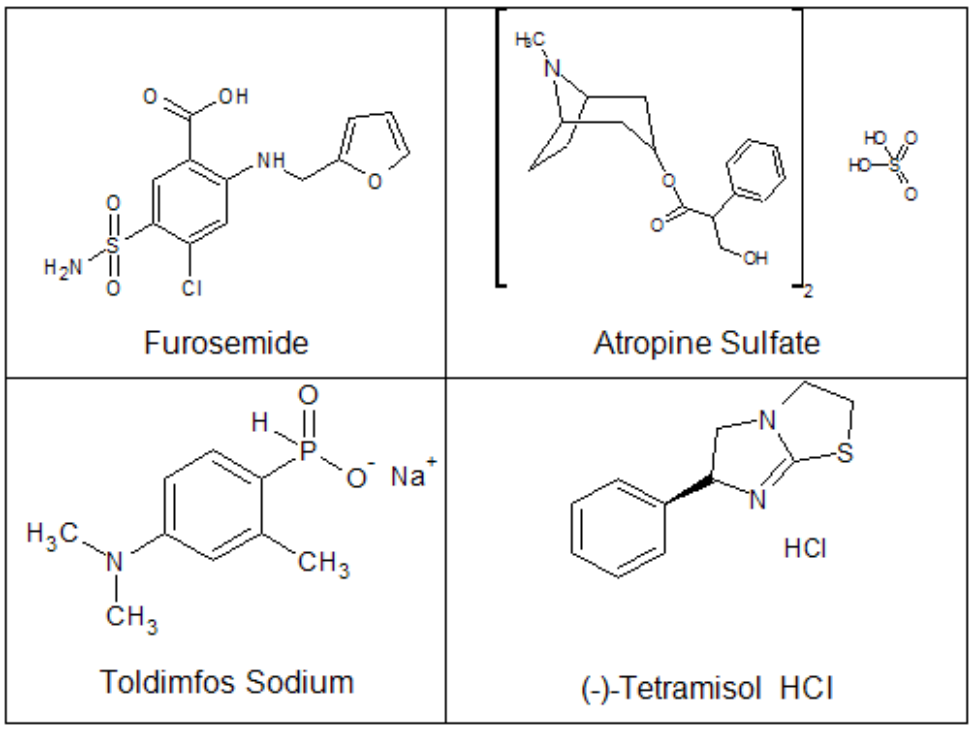

Figure 6. Molecular structure of active ingredients of some veterinary drugs.

antidote against organophosphate and carbamate insecticides (Plumb, 2002) which cause parasympathetic effects as a result of competitive acetylcholinesterase inhibition leading to acetylcholine accumulation (Marrs and Vale, 2006) at the nerve endings [37]. On the other hand, tea leaves, along with toldimfos sodium, were used to observe the recovery of the animals with red coloured urine [38]. Levamisole derivatives are well known anthelmintic drugs with immunomodulatory and anticancer activities [39]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in veterinary medicine for their ability to treat for bone and joint inflammation and mastitis [40].

The study found that the IC_{50} values of drugs which have inhibitory effect were obtained 45.48 μM, 2.19 and 126.315 mM for tetramisol, furosemide and toldimfos sodium, respectively.

Conlusions

In our study, differential effects of (furosemide, atropine sulfate, toldimfos sodium and tetramisol) veterinary drugs on to the milk ALP were determined in vitro for a better understanding of industrial importance of the enzyme as a pasteurization indicator and its relation to the enzyme activity. When compared to Levamisol, which is a potent inhibitor of the enzyme, the inhibitory effects of the drugs on the enzyme activity are weak. Moreover, the results of the current study suggest that an appropriate pasteurization procedure is considerably important through consumer health. Correspondingly, the check for the presence of ALP activity in milk is a good indication of proper pasteurization.

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