In vitro effects of 2, 4-Dichlorophenoxy acetic acid dimethylamine salt and enrofloxacin on BTH

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ABSTRACT

In this study, the in vitro effects of enrofloxacin, a broad spectrum antibiotic, and 2, 4-Dichlorophenoxy acetic acid dimethylamine salt that, a herbicide, on the bovine testicular hyaluronidase (BTH) were analyzed. BTH was purified using the method of ammonium sulfate precipitation and using affinity gel (Sepharose-4B-L-tyrosine-m-anisidine). Then the inhibiting effects of enrofloxacin and 2, 4-Dichlorophenoxy acetic acid dimethylamine salt on the purified BTH enzyme were identified. IC$_{50}$ values of these chemical substances were found to be 0.3101 and 0.4276 mM, respectively.

Key words: Antibiotic, Herbicide, Hyaluronidase, IC$_{50}$, Inhibition.

INTRODUCTION

Hyaluronic acid (HA), which is also known as hyaluronate or hyaluronan, is a nonsulfated linear glycosaminoglycan with a high molecular weight. Hyaluronidases have been found in numerous organisms such as certain bacteria (bacteriophage-bacterial hyaluronidase), pathogenic fungi (Candida, Streptomycetes), and invertebrates (crustaceans, insects) or tissues or the venoms of lizards and snakes (Kreil, 1995; Frost et al., 1996; Csoka et al., 1997; Meyer, 1971).

It is known that the testicle extracts of the mammals contain hyaluronidase activity (Chain and Duthie, 1939). Bovine and ovine testicular hyaluronidase preparations have been applied in therapeutic and other medical fields as a spreading factor for many years (Pavan et al., 2016). It was also determined that the main soluble hyaluronidase was found in bull testicle extracts and found to be a part of the PH-20 enzyme, which is bonded to the membrane (Meyer et al., 1997). BTH is an endo-glucanohydrolase capable of cleaving HA from the β-1, 4-glycosidic bond (EC 3.2.1.35). BTH can also degrade chondroitin, chondroitin-4 and chondroitin-6-sulfate and, to a lesser extent, dermatan sulfate, in addition to HA. Due to the broad substrate specificity of BTH, chain lengths have great importance in glycotecnological applications such as the preparation of varying glycosaminoglycan oligosaccharides (Takagaki et al., 1994). In addition, since it is known that the BTH enzyme is a part of the PH-20 protein, the enzyme is considerably important in penetration and artificial insemination due to its activity (Cherr et al., 2001; Kumar et al., 2015).

Enrofloxacin is synthesized among the 2nd generation of the fluoroquinolones, which is a class of three types of veterinary antibiotics that are frequently used to fight the animal diseases and can be obtained by synthetic means. Enrofloxacin is a light yellow, slightly soluble, and crystallized substance that has a bactericidal effect on Gram (-) and Gram (+) microorganisms, anaerobic bacteria, and mycoplasmas. Fluoroquinolones provide the death of bacteria by inhibiting bacterial gyrase enzymes, which play a role in the production of DNA in bacteria. Therefore, enrofloxacin is effective against bacterial infections, especially in poultry of cattle, pigs, dogs, and cats and used for the treatment of respiratory system diseases caused by mycoplasma in cattle (Ceylan, 2004).

The pesticides, which are used to destroy unwanted plants and weeds, are called herbicides. 2, 4-Dichlorophenoxyacetic acid (2, 4-D), a kind of herbicide from the chlorophenoxy acetic group, used for the first time in the mid-1940s, and known to be one of the most common herbicides used to increase agricultural production for the time (IARC, 1977). Although 2, 4-D is slightly soluble in water, the amine salt of the 2, 4-D form is soluble in water (Koca, 2001). 2, 4-D, which is in the form of a white powder, is generally not used commercially in its acid form. Instead, amine and rather ester salts are preferred. Because of the fact that the ester form of 2, 4-D can be lost by evaporation, the use of amine salts is more common. Moreover, 2, 4-D effective herbicides has similar biological activity of hormones and found as weed killers and selective against broad-leaved weeds (Ecevit et al., 1999).

MATERIALS AND METHODS

Sepharose-4B, L-tyrosine, m-anisidine (3-methoxysaniline), hyaluronic acid sodium salt from...
Streptococcus equi, protein assay reagents and electrophoresis chemicals were provided by Sigma Chem.Co (Milan, Italy), and other chemicals were provided by Merck & Co (Darmstadt, Germany).

The testis tissue of the bovine was fragmented into small pieces and it was homogenized in 100 mM sodium acetate buffer (pH 5.4), which contained 150 mM NaCl and 0.25 mM phenylmethane sulfonyl fluoride (PMSF). The homogenate that was prepared and was centrifuged at 26,916 g for 75 min at +4°C, and afterward the supernatant was recovered. The crude BTH was isolated through the precipitation of the supernatant with ammonium sulfate (40–60%). The precipitate was obtained through centrifuging it at 26916 g for 45 min, and then, it was dissolved again in 50mM sodium phosphate buffer (pH 7.0) Kaya et al. (2014).

Later, affinity chromatography was applied to the re-dissolved precipitate, which was derived from the precipitate of the ammonium sulfate. It was then charged to an affinity column that was composed of sepharose-4B-L-tyrosine-m-anisidine. This affinity column was prepared in the way as described by Kaya et al. (2014). By using 50mM sodium phosphate buffer (pH 7.0), the affinity column was brought into the equilibrated status, and afterwards, the crude BTH enzyme preparation was inserted into the same buffer. The BTH was eluted with 25 mM sodium phosphate buffer (pH 4.0) that contained 250 mM Na₂SO₄ and 50 mM m-anisidine, after thoroughly washing it with buffer. The BTH enzyme was purified and was kept at +4°C.

In accordance with the definitions of the International Union of Biochemistry, the Hyaluronidase activity may be quantified. For example, under specified conditions, 1 unit (U) enzyme catalyzes the liberation of 1 mmol of reducing terminal N-acetylhexosamine, per minute. By using Greiling’s method, the hyaluronidase activity towards HA has been quantified spectrophotometrically (Greiling, 1957). The reaction was monitored at 37°C for 1 min by tracking the view of HA at 232 nm in Biotek automated recording spectrophotometer (Bad Friedrichshall, Germany). The final concentration of the substrate (12.3 mM) was utilized during the analyses of the enzyme. The speed of the enzymatic reaction was calculated via the following equation:

\[
\frac{\Delta A}{\Delta t} = \varepsilon \left( \frac{\Delta \varepsilon}{v} \right)
\]

Various concentrations of enrofloxacin and 2, 4-Dichlorophenoxy acetic acid dimethylamine salt were included in the enzyme, for analyzing the inhibition. Regression analysis was performed to determine the proportional values (%) of BTH activity with six different inhibitor concentrations. Without using any of the inhibitors, BTH activity was accepted as 100%. The concentrations of the inhibitors causing 50% inhibition (IC₅₀ value) were identified through the graphs as shown in Figure 1.

![Figure 1: The graph of 2, 4-Dichlorophenoxy acetic acid dimethylamine salt and enrofloxacin activity (%) on BTH.](image-url)
RESULTS AND DISCUSSION

Ammonium sulfate precipitation was taken as 40-60% in this study according to the relevant literature (Hoechstetter, 2005). After precipitation of the sulfate, an affinity gel that has the chemical structure of Sepharose-4B-L-tyrosine-m-anisidine was utilized for enzyme purification. Then the inhibiting effects of enrofloxacin and 2, 4-Dichlorophenoxy acetic acid dimethylamine salt on the purified BTH enzyme were identified. As presented in Table 1, IC$_{50}$ values of these chemicals were identified as 0.3101 and 0.4276 mM, respectively.

Because of the importance of the BTH enzyme in the field of preparative organic chemistry and its broad substrate specificity, this enzyme’s ability to be purified both economically and rapidly may be regarded as a great advantage. Given its advantages, more practical purification methods of the BTH enzyme have been utilized in clinical, artificial insemination, and cancer studies, as well as in the industrial field (Takagaki et al., 1994).

In this study, fresh testis samples were obtained from slaughterhouses of Siirt region from commercially slaughtered cows as an enzyme source for few reasons such as due to their easy and wide availability in mammalian testes and the advantage of working on them when they are fresh, etc. Ammonium sulfate precipitation, which is a very easy to apply as a pretreatment method, was applied in the study. The cattle are fed with the plant containing 2, 4-D (EPA., 1989). 2, 4-Dichlorophenoxy acetic acid dimethylamine salt, which is the active ingredient of the pesticide (EPA., 1989), is an extremely important element in our study. Since no study on the effects of 2, 4-Dichlorophenoxy acetic acid dimethylamine salt on BTH enzyme has been seen in the literature, 2, 4-Dichlorophenoxy acetic acid dimethylamine salt used as a potential inhibitor. In addition, the enzyme’s relationship with enrofloxacin, a fluoroquinolone group antibiotic widely used in clinical practice for the prevention of pathological conditions in artificial insemination and for the treatment of diseases (Ceylan, 2004), was investigated.

Although there are some studies on the effects of hyaluronidases obtained from the seeds on mobility and bacteriological attacks (Tanyildizi and Bozkurt, 2003), still there is lack of information in the literature about the effects of some antibiotics on the BTH enzyme, which increases the originality of this study.

2, 4-D is known to have a high water solubility rate and its excretion is made through urine in all species (Keller et al., 1994), which may show a high distribution in the body (Wang et al., 1994). While 2, 4-D is absorbed well by the gastrointestinal regions, it is absorbed at a lower level in the lungs and at a minimum level in the skin (Arnold and Beasley, 1989).

It has been reported in the literature that 17% of the single dose of 2, 4-D herbicide in pregnant mammals was able to reach the embryo through the placenta (Lindquist and Ulberg, 1971). When applied at a dose of 1 mg/kg, accumulation in blood, liver, kidneys, and lungs of the pigs and in the spleens of the rats were also observed. However, it was detected at a lower level in muscles and in the brain. It is not found in the tissues after the application of the medicine (Extoxnet, 1996).

The amount of 2, 4-D binding to the serum proteins in the blood is an important factor in determining the distribution in the body (Erne, 1966). There is a close relationship between the concentration of 2, 4-D in blood and tissues and its binding affinities to proteins in an in vitro environment (Blair et al., 2000).

Ozdas et al. (2006) investigated the effect of 2, 4-D on testicular tissues of rats and found atrophy in seminiferous tubules, moreover, deterioration in the order of spermatogenic cells and testicular damage in high dose groups among the experimental groups is directly proportional to the dose. In addition, histopathologic effects such as a decrease in tunica albuginea and in the diameter of the seminiferous tubule epithelium and the effusion of the spermatogenic cells into the lumen were determined (Ozdas et al., 2006). In another study, it was reported that some animals, e.g., dogs, have greater sensitivity to 2, 4-D than humans and rats, and malignant cancers form in dogs that have contact with grasslands that are exposed to 2, 4-D (Ibrahim et al., 1991).

There have been two alternative binding modes in the enzyme to the date in the literature. One of the modes is the hydrophobic channel formed by the most conserved amino acids Ala-84, Leu-91, Tyr-93, Try-220, and Leu-344 in BTH and the other is the hydrophilic interactions (Botzki et al., 2004). A hydrophobic region is more or less opposite the catalytic site and is evidenced at the surface of the bovine testicular hyaluronidase molecule. This region could be responsible for the amphiphilic properties of the protein, but the modeling did not involve all of the amino acids of the protein. Indeed the non-modeled C-terminal peptide part that is not significantly enriched in hydrophobic amino acids could mask the hydrophobic region (Belem-Gonçalves et al., 2006).

Table 1: IC$_{50}$ values of 2, 4-Dichlorophenoxy acetic acid dimethylamine salt and enrofloxacin

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ Values (mM)</th>
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<tbody>
<tr>
<td>2, 4-Dichlorophenoxy acetic acid dimethylamine salt</td>
<td>0.3101</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.4276</td>
</tr>
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Rigden et al. (2006) show that sulfamoyloxy group forms a network of hydrogen bonding interactions with protein and water. The interactions associated with the charge-assisted bidentate H bonds between Arg466 and the two-sulfamoyl oxygen atoms. Both the oxygen atoms and nitrogen atoms are additionally linked to the protein by through–water interactions. Moreover, there is only a single direct H bond of a sulfamoyl oxygen with the indole NH of Trp292. In this case, a water molecule mediates additional interactions with the carboxylate of Asp352 (Rigden et al., 2006).

The results showed that the compounds inhibited the enzyme activity that is 0.3101 mM for 2,4-Dichlorophenoxy acetic acid dimethylamine salt and 0.4276 mM for enrofloxacin. The compound (2,4-Dichlorophenoxy acetic acid dimethylamine salt) has ester and ether groups, and has capacity of forming hydrogen bond interaction with the enzyme active sites. We assume that ester and ether oxygen on the compound form an H-bond with Asn290, Trp291, Arg462, Tyr408, and His399. In addition to this, chlorine atoms on the phenyl group make the compound more lipophilic.

Enrofloxacin has lower inhibitory effect than the other compound because it has alkyl and cyclopropyl groups, which decrease lipophilic properties, and besides, the compound is larger than the other so we could assume that the molecule did not fit the active site of the enzyme. Same kind of effect was mentioned by Kaessler et al. (2008). Therefore, these effects increase or decrease enzyme inhibition activity. The results show that they are in agreement with the literature (Olgen et al., 2007; Olgen et al., 2010).

Declaration of Interest: This research was presented at the “3rd International Conference on Engineering and Natural Sciences (ICENS)” (3-7 May 2017, Budapest/HUNGARY) as an oral presentation.

REFERENCES


