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Effect of some inhibitors on hyaluronidase production by *Streptococcus mitis* MTCC *2695 and S*treptococcus equi* SED 9 species

S. SAHOO*, P.K. PANDA, S.R. MISHRA, A. TRIPATHY and P. ELLAIAH[1]

University Department of Pharmaceutical Sciences, Utkal University, Bhubaneswar -751 004 (India)
[1]Pharmaceutical Biotechnology Division, Department of Pharmaceutical Sciences,
Andhra University, Visakhapatnam - 530 003 (India)

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ABSTRACT

The inhibitory effects of some metal ions, some synthesized compounds and their derivatives with 2-aryl acenaphthimidazoles and their N-Mannich bases on optimum production of hyaluronidase was evaluated employing *Streptococcus mitis* MTCC *2695 and *Streptococcus equi* SED 9 by submerged fermentation. The maximum enzymatic activity was obtained with an initial pH (6 and 5.5), incubation temperature 37º, incubation time for 48 h and inoculum level (6 and 10 %) with inoculum age 24 h by *Streptococcus mitis* and *Streptococcus equi* SED-9 respectively. The results indicated that metals like Fe^{3+}, Zn^{2+} Mn^{2+}, Ag^{+}, Ni^{2+}, Sr^{2+} and Cu^{2+} acted as strong inhibitors of the enzyme. N-Mannich bases of 2-aryl acenaphthimidazoles exhibited promising inhibitory activity.

Key words: Hyaluronidase production, *Streptococcus* sp.

INTRODUCTION

Hyaluronidase (Hyase) enzymes are of three main classes: (i) hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase, EC 3.2.1.35), e.g. testicular hyaluronidases[1], (ii) hyaluronate 3- glycanohydrolase (EC 3.2.1.36), e.g. leech hyaluronidases[2] and (iii) hyaluronate lyase (EC 4.2.99.1), e.g. bacterial hyaluronidases[3]. The enzyme increases absorption and dispersion of injected drugs, fluids, resorption of radiopaque agents, dyes and toxins[4]. Hyases are widely used in many fields like orthopaedics[5], ophthalmology[6], surgery, dermatology, dentistry, fertilization and oncology[7].

Two microbial strains *Streptococcus mitis* MTCC *2695 (procured from IMTECH, Chandigarh) and a local pathological isolate *Streptococcus equi* SED 9 (isolated from gingival crevices of diseased periodontium) producing an extracellular hyaluronidase enzyme within 48 h were used in this study. After rejuvenation of both cultures, they were preserved and transferred onto in nutrient agar slants. The growth content of each slant was suspended in 5 mL of sterile water and the optical density (OD) of the pooled suspension was measured at 675 nm resulting 0.580 OD that constitutes the inoculum of *S. mitis and S. equi* respectively. A 5 % level of each inoculum was transferred into 250 mL Erlenmeyer flask containing 50 mL of modified nutrient broth with composition (g/L) peptic digest of animal tissue, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5; casein enzyme hydrolysate (type-1), 4; KH_2PO_4, 3; $MgSO_4$, 3; hyaluronic acid (HA), 0.001 % with pH 7. After inoculation, the flasks were incubated at 37° on a rotary shaker at 150 rpm for 48 h. At the end of fermentation 5 ml broth was aseptically withdrawn and centrifuged at 10000 x g for 30 min at 4°.

The clear supernatant obtained was subjected to enzyme assay. Hyase activity was measured spectrophotometrically by turbidity reduction assay[8] using HA sodium salt (Sigma Aldrich, USA) as a substrate. The enzymatic assay is based on Dorfmans method[9] in which the enzymatic reduction in turbidity, resulting when 1 ml of HA at 70 µg/ml was incubated with 1 ml of enzyme sample solution and incubated at 37° for 10 min and reduction in turbidity was read by measuring the absorbance at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that reduced the absorbance by 0.1 at 600 nm (A_{600}) in 30 min at 37°, pH 7.0 under assay conditions similar to that caused by one unit of an international standard. Some of the metal ions[10] and few synthetic compounds like Imidazoles, benzimidazoles, N-acetylated, S-alkylated, N-alkylated benzimidazole derivatives, 2-mercaptobenzimidazole derivatives, 2- oxazoles, 1,3-diacetylbenzimidazoles-2-thione and its derivatives[11] were reported to be potent inhibitors[12] of hyaluronidase. An attempt has been made to evaluate the inhibitory effect of some synthesized compounds with 2- aryl acenaphthimidazoles and their N-Mannich bases on hyase.

To investigate the influence of initial pH (4.0-9.0), temperature ranging from 20° to 55°, incubation period for 96 h, cell growth, inoculum age and inoculum level at 0.1 to 12 % and the samples were withdrawn at regular interval of 12 h, assayed for biomass (OD A_{675nm}) and enzymatic activity.

Different metallic ions (Sr^{2+}, Mo^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Fe^{3+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Ag^{+}, W^{2+}, Ca^{2+} (10 mM)) in salt forms and chelators(N, N, N', N'-Tetraacetyl ethylene diamine and Ethylene diamine tetraacetate (10 mM)) were added to the production medium aseptically and assayed for enzyme activity.

The inhibitory effect of some 2- aryl acenaphthimidazoles and their N-mannich bases compounds[13] (**1a-e** and **2a-e**) were tested at 0.001 M against the enzyme hyaluronidase by colorimetric (Morgan-Elson) assay[14].

The results of incubation period and temperature on the fermentation cycle by *S. mitis* and *S. equi* at pH 7.0 was noticed at 48 h at 37°C, exhibiting highest enzyme activity (151 and 143 U/mL) and biomass (0.87 and 0.68). The biomass and optimum enzyme production was detected at *p*H 6.0 (1.61, 181 U/mL) and 5.5 (1.44, 165 U/mL) from *S. mitis* and *S. equi* respectively. The highest enzyme activity (179 and 168 U/mL) and cell mass (1.64 and 1.47) was found in case of *S. mitis* and *S. equi* respectively

Among metallic ions used Mo^{2+} (135 and 119 %) and Ca^{2+} (117 and 88 %) stimulated enzyme production by *S. mitis* and *S. equi* SED 9 respectively where as other metallic salts decreased the enzyme activity by both the strains. The metals like Fe^{3+}, Zn^{2+} Mn^{2+}, Ag^{+}, Ni^{2+}, Sr^{2+} and Cu^{2+} acted as strong inhibitors of the enzyme hyase produced by both the strains. Metals like Co^{2+}(94 and 33 %), Hg^{2+}(56 and 22 %), Cd^{2+}(46 and 11 %), Pb^{2+}(48 and 18 %) and W^{2+} (69 and 75 %) decreased the percentage activity of hyase by *S. mitis* and *S. equi* SED 9 respectively.

From the synthesized compounds of both the series N-Mannich bases of 2-aryl acenaphthimidazoles (**2a-e**) exhibited promising inhibitory activity in comparison to derivatives of 2-aryl acenaphthimidazoles (**1a-e**). The compounds 2b exhibited the highest percentage of activity (71 and 89 %) followed by 1a (49 and 81 %), 2c (64 and 79 %), 1b (63 and 77 %) and the lowest activity was found to be 1e (13 %) and 2e (17 %) against hyase produced by *S. mitis* and *S. equi* SED 9 respectively.

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