Full Length Research Paper

Characterization of UTI associated Protease from *Serratia marcescens* Strain TW1 and its Immunogenic properties

Tariq A.L[1] and Reyaz A.L[2]

[1]Department of Biotechnology, KSR Autonomous College of Arts and Science, Tiruchengode-637215
[2]Department of Biotechnology, Periyar University, Salem-636011

The extracellular UTI associated protease produced from *Serratia marcescens* TW1. The protease was purified to homogeneity from the production medium by ammonium sulphateation, acetone precipitation with 80% saturation and DEAE cellulose column chromatography. The molecular weight of protease was found approximately 50 KDa weight by the sodium dodecyl sulfate polyacrylamide gel electrophoresis. The isoelectric point was close to 6.5. The maximal activity towards case in was found at 40^0C and its pH activity was maximum at 8.0. The protease was strongly inactivated by $HgCl_2$ metal ion and reactivated by $FeSO_4$ thus indicated as metalloprotease. The protease was inhibited by Na_2EDTA. The antibodies rose against protease of *Serratia marcescens* strain TW1 when tested immunologically by showing line of precipitation against the antiserum thus indicated that protease is immunologically identical.

Keywords: UTI associated protease, *Serratia marcescens* TW1, Extracellular protease, Immunogen, line of precipitin, therapeutic application.

INTRODUCTION

Serratia marcescens is a gram-negative bacteria belonging to the genus *Serratia* and family enterobacteriaceae (Grimont and Grimont 2006). Have the ability to produce protease were separated by thin layer chromatography (Matsuyama et al., 1986). The Proteolytic enzymes from microorganisms may be located within the cell or excreted into the media (Kohlman et al., 1991). The mechanism in each case is the ability of an enzyme to cleave or cut protein target into two or more pieces usually at a very specific cleavage sites (Bhatti et al., 2002; Carbonell et al., 2003). In hospital patients are frequently infected with *Serratia marcescens* a well known nosocomial species of *Serratia*. It is an oppurtunitic pathogen causing infection in immuo-compromised patients. The ability of *Serratia* cells to attach human buccal epithelial cells (Ismail and Som, 1982) and to human urinary bladder surfaces (Yamamoto et al., 1985). *Serratia* poses a serious public health threat by showing resistance against beta lactam ring antibiotics (Choi et al., 2007). The incidence increase with advancing age and males are at high risk in which 92% of isolates were *Serratia marcescens* (Laupland et al., 2008). *Serratia marcescens* has increasingly been recognized as a cause of infection in adult pediatric and neonatal intensive care units (Teertstra et al., 2006). It is found that insulin and sedative solutions used for the patients were contaminated with *Serratia marcescens* was proof to be a source of outbreak (Alfizah et al., 2004).

MATERIALS AND METHODS

Bacterial strains

Urine samples were collected from the urinary tract infected woman admitted at general Govt. Hospital,

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*Corresponding Author E-mail: tariqtasin@gmail.com

Namakkal District in Tamil Nadu. The organism was isolated (Matsumato et al., 1984) by using Caprylate-thallious agar medium. The urine samples were serially diluted and spread over the medium then incubated at 37^0C for 24 hours. The purification of the isolated potential proteolytic organism was done by following a method of Rifaat et al., (2007). The strain TW1 showed the highest proteolytic activity and identified by biochemical tests and 16S rDNA genomic sequence.

Enzyme production

The protease was produced by following a method of Salamone and Wodzinski (1997) using the enzyme production medium Tryptone-yeast extract glucose broth containing: Tryptone 5g/l, Yeast extract 2.5g/l, Glucose 1g/l and pH 7.2. For the study of protease production 250ml of medium was poured into 1000ml of Erlenmeyer flask capacity, and were sterilized at 121^0C for 15 minutes. After cooling 0.5ml of stationary phase culture of strain TW1 was inoculated and incubated on shaker at 28^0C for 48 hours.

Enzyme purification

The protease was purified by following method of Matsumato et al., (1984) from culture broth by centrifugation at 10000rpm for 20minutes at 4^0C. The supernatant was collected and filtered through membrane filter having porosity of 0.022µm at 4^0C then subjected to 80% ammonium sulphate and actone saturation. The pellet was obtained by centrifugation at 10000rpm for 20 minutes at 4^0C and resuspended in 20mM Tris HCl pH 8.0 then dialyzed against 500ml of 5mM Tris-HCl pH 8.0 containing 1mM $MgCl_2$ over night at 4^0C with stirring conditions. The dialyzate was centrifuged at 8000rpm for 20 minutes at 4^0C and supernatant were subjected to DEAE-cellulose anion exchange column chromatography equilibrated with 10mM Tris-HCl buffer pH 8.3. The 15ml of dialysate eluted with 10mM Tris-HCl buffer pH 8.3 at the flow rate of 20ml/h. A linear gradient consisting of 50ml of 10mM Tris-HCl buffer pH 8.3 and 50ml of the same buffer with 0.3M NaCl. The 5ml of fractions elute were collected and absorbance measured at 280nm and enzyme activity was determined.

Determination of protein content and assay of proteolytic activity

The protein concentration of strain TW1 was determined by the method of Lowry et al., (1951) by taking bovine serum albumin as standard. The proteolytic activity was determined by following a method of Kunitz (1947) using casein as substrate and absorbance for the supernatant was determined at 280nm. One unit of proteolytic was defined as the amount of enzyme that produced an increase of absorbance at 280nm of 0.1 under the conditions of the assay.

Molecular weight determination by SDS-PAGE

The molecular weight of the protein in strain TW1 was determined by the method of Laemmli et al., (1970) staining the protein with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 hours and destained with 10% methanol, 25% acetic acid solution for 12 hours.

Isoelectric focusing of protein

The isoelecric focusing of purified protease of strain TW1 was determined using mini-gel system (Robertson et al., 1987). The gel was placed in staining solution for 30minutes and destained for one hour. The bands were observed in white light transilluminator.

Effect of temperature on protease activity

0.2ml protease sample of strain TW1 was added to the substrate mixture containing 1.5ml of 1.0% (w/v) casein in 100mM Tris-HCl in 1mM $MgCl_2$ at pH 8.0 and incubated at 30^0C, 35^0C, 40^0C, 45^0C, 50^0C, 55^0C, 60^0C for one hour. After the incubation, the proteolytic activity was determined by the protease assay, an optical density was measured at 280nm.

Effect of pH on protease activity

0.2ml protease sample of strain TW1 was added to the substrate mixture containing 1.5ml of 1% (w/v) casein, and 0.1mM $MgCl_2$ in various buffers. Such as Glycine-HCl buffer having pH 2.0, 2.5, 3.0, 3.5, Acetate buffer having pH 4.0, 4.5, 5.0, 5.5, phosphate buffer having pH 6.0, 6.5, 7.0, Tris- HCl buffer having pH 7.5, 8.0, 8.5, 9.0 and carbonate bicarbonate buffer having pH 9.5, 10.0, 10.5, 11.0 and incubated at 37^0C for 60 minutes. After incubation the proteolytic activity was determined by the protease assay.

Effect of metal ions on protease activity

0.2ml protease sample of strain TW1 was added to the 1.5ml of 0.1 M Tris-HCl pH 7.5 and to the same buffer supplemented with 100µl of 8.3mM of metal ions viz $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$

Table 1. Biochemical identification of UTI associated bacterial strain *Serratia marcescens* TW1

S.No	Biochemical test	Result
1	Indole	N
2	Methyl Red	N
3	Voges Proskauer	P
4	Citrate	P
5	Triple sugar iron	A/K G[-ive]
6	Hydrogen sulphide production	P
7	Glucose	F
8	Sucrose	F
9	Sorbitol	F
10	Fructose	F
11	Xylose	NF
12	Rhaminose	NF
13	Lactose	N
14	Arabinose	NF

Where N-negative, P-positive, A/K – Acid/Alkaline, G[-ve] –gas negative, F- fermentation,
NF- no fermentation

and mixtures were incubated at temperature of 25^0C for 30mintues and proteolytic activity was determined by protease assay. In addition the purified protease sample preparation (200µl/ml) was incubated for 30 minutes at 25^0C in 0.1 M acetate buffer having p^H 5.0 supplemented with (100µl/ml) Na_2EDTA and protease activity was determined by protease assay.

Effect of inhibitors on protease activity

0.2ml protease sample of strain TW1 preparation was added into 1.5ml of 0.1M tris-hydrochloride buffer having pH 7.5 and to the same buffer supplemented with 100µl of various inhibitors 20mM Na_2EDTA, 8.3mM iodioacetic acid, 8.3mM dithiothreitol, 8.3mM leupeptin, 1% of 2 β-mercaptoethanol, 1% of tween-20 and 3% of ethanol and mixtures were incubated at 25^0C for 30 minutes and proteolytic activity was determined by protease assay.

Assessment of Therapeutic Role of Protease

0.2 ml of the protease samples of strain TW1 was emulsified with 0.8 ml of incomplete Freund's adjuvant (Hum and Chantler, (1980) by vortaxing thoroughly. The emulsion was injected subcutaneously into Rabbit (NCP/IAEC /KSR/04 /2009) by using 21-gauge needle. Similarly three booster doses were given at intervals of 10 days following the initial injection. After the eight days of last booster dose the rabbit was bled at the marginal vein near in ear and 15ml of blood was collected and were allowed to clot for 3 to 4 hours at room temperature. The serum was separated from the clot by centrifugation

at 5000rpm for 15 minutes. The straw yellow colored serum was stored at 4^oC in the presence of 0.1% sodium oxide as antibacterial agent. The antiserum was then tested by immunodiffusion for the formation of immunoprecipitate.

Immunodiffusion by Ouchterlony method

The immunodiffusion analysis for the antiserum of protease strain TW1 was followed by method of Ouchterlony (1968) in a double diffusion technique both the antigen and antibody are allowed to diffuse into the gel and meet to each other. The antigen and antibody react to produce the antigen-antibody complex. At equilibrium the complex produced immobile and form a thin band of precipitin. The precipitin is visualized either directly or after protein staining for interpretation.

RESULT

Bacterial soil isolate

After 48 hours of incubation it was found to be 1.25x103 CFU/ml in the *Serratia* medium ATCC from the urine sample. The total 205 strains were isolated from the urine samples of UTI infected women and the potential proteolytic strain TW1 was found gram negative rod shaped bacterium by biochemical tests are presented in Table 1. The 16s rDNA gene sequences confirmed that it belongs to *Serratia marcescens* therefore this bacterium was named as *Serratia marcescens* TW1 under Gene Bank ACC. No. GU046545.

Table 2. Purification of Protease from *S. marcescens* TW1 in the supernatants of tryptone yeast extract glucose medium.

S. No	Purification Stage	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Activity (U/mg)	Specific activity (U/mg)	Total activity (U)	Purification (-fold)	Recovery (%)
1	Cell free culture supernatant	2500	0.8	2000	799	998	1997500	1	100
2	Ammonium sulfate fraction	200	3.9	780	6985	1791	1397000	1.8	90
3	Acetone fraction	50	5.7	285	23708	4159	1185400	4.1	80
4	Dialysate	100	0.5	50	3590	7180	359000	7.1	70
5	DEAE cellulose Fraction	50	0.3	15	2532	8440	126600	8.5	50
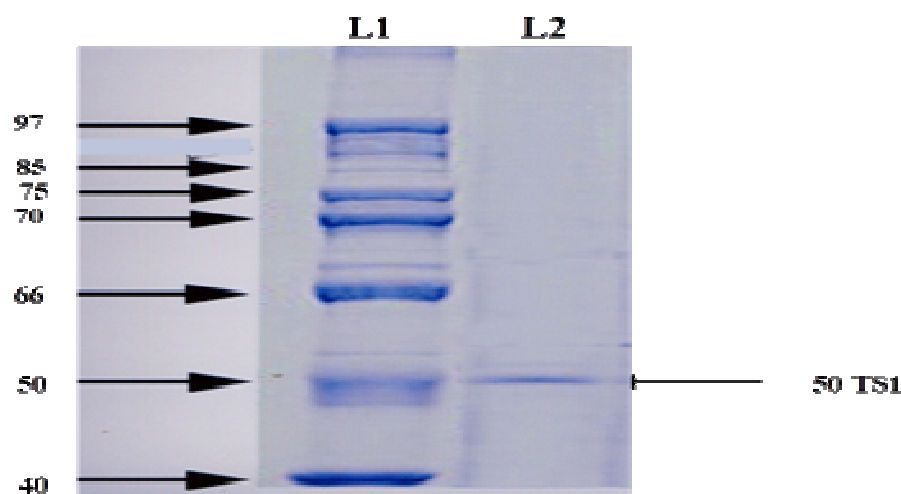
Figure 1. Determination of molecular weight of proteases by sodium dodecyl sulphate agarose gel electrophoresis.
L1—Molecular marker mass standards: phosphorylase b (97 kDa), tyrosine (85kDa), acid phosphate (75kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (50 kDa) and aldolase (40 kDa)
L2—Protease sample of *S. marcescens* TW1 (50KDa)

Enzyme purification

The purification process showed that 80% ammonium sulphate saturation had precipitated the protease in the solution by salt out mechanism and recovered with 80% acetone saturation. After the dialysis the precipitated protease of *Serratia marcescens* strain TW1 got purified by the DEAE Cellulose anion exchanged chromatography with 10mM Tris HCl buffer pH 8.3 (Table 2).

Determination of protein content and proteolytic activity of protease enzyme

The proteolytic activity was 83.84 IU/ml in casein as substrate. The molecular weight of protease *Serratia marcescens* strain TW1 was found approximately 56 KDa protein band when observed under white transilluminator (Figure 1) and isoelectric point was 6.5 in an ampholyte buffer having pH ranges from 2.0 to 11.0 (Figure 2).

Effect of temperature on protease activity

The temperature optimum for the protease of *Serratia marcescens* TW1 was $40^{0}C$ in 100mM Tris HCl buffer as shown in Figure 3. The activity declined rapidly above $40^{0}C$ and was negligible above $55^{0}C$. The enzyme retained its 82% activity at $40^{0}C$ when temperature increased the enzyme activity decreases rapidly and lost at $60^{0}C$.

Effect of pH on protease activity

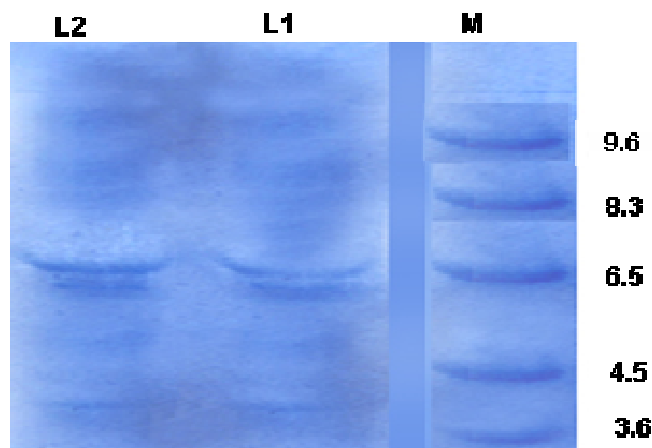The pH optimum of protease *Serratia marcescens* strain

Figure 2 . Isoelectric focusing electrophoretogram, pH 2.0 to 11.0 stained with coomassie blue.
M—Isoelectric focusing standards: amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.5), carbonic anhydrase II (pI 6.5), lentil lectin (pI 8.3) and ribonuclease A (pI 9.6)
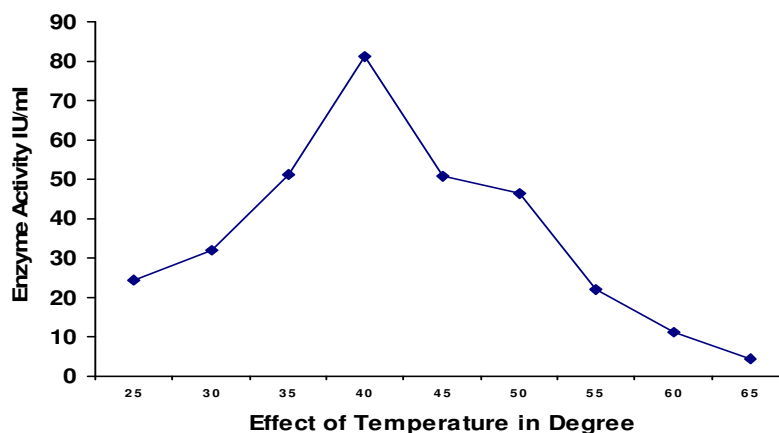**L1 and L2**—*Serratia marcescens* TW1 showing pI 6.5

Figure 3. Effect of Temperature on protease activity was examined in 100mM Tris-HCl buffer having pH 8.0 at 25^0C, 30^0C, 35^0C, 40^0C, 45^0C, 50^0C, 55^0C, 60^0C and 65^0C for 30 minutes. The *Serratia marcescens* strain TW1 showed maximum activity at optimum 40^0C .

TW1 was 8.5 with a sharp decrease in activity above pH 9.0. The protease had half maximal activity near pH 7.5 and exhibited a little activity below pH. The protease retained its maximum activity from pH 6.5 to 9.0 (Figure 4).

Effect of metal ions on protease activity

The metal ions have altered the protease activity of *Serratia marcescens* strain TW1. The $HgCl_2$ and Na_2EDTA have inactivated the protease at both pH 8.5 and pH 6.5. The protease have retained maximum activity in $FeSO_4$, $MgSO_4$, $ZnCl_2$ and minimum activity in $MnCl_2$, $CaCl_2$, $CuSO_4$ and lost its activity in $HgCl_2$ and

Na_2EDTA (Table 3).

Effect of inhibitors on protease activity

The protease of *Serratia marcescens* strain TW1 showed the resistance against the all inhibitors but lost its activity in 20mM EDTA (Table 4).

Antiserum production

Antibodies rose against protease of *Serratia marcescens* strain TW1 got confirmed by immunodiffusion precipitation; the protease of *Serratia marcescens* strain
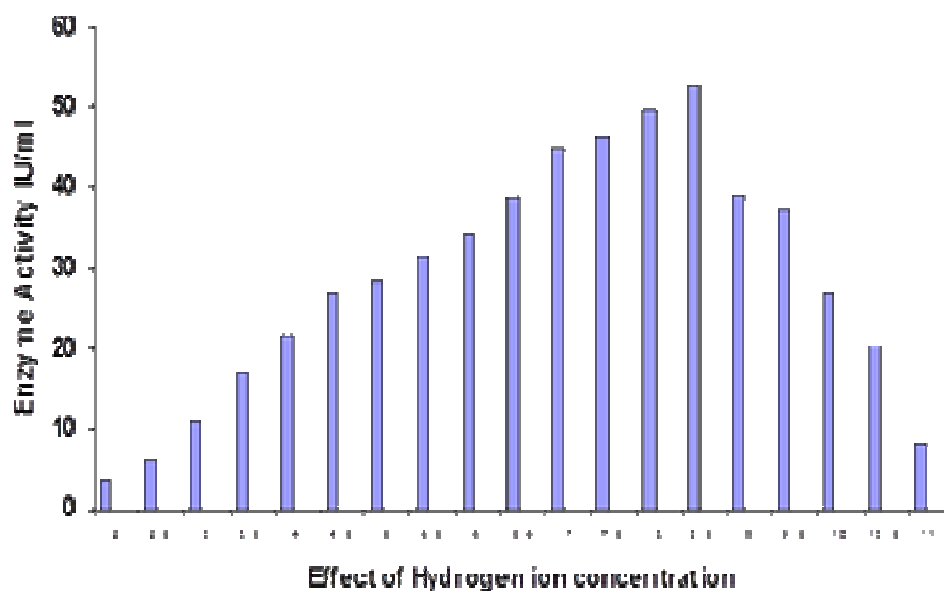
Figure 4. Effect of pH on protease activity was examined in various buffers such as Glycine-HCl buffer having pH 2.0 to 3.5, Acetate buffer having pH 4.0 to 5.5, Phosphate buffer having pH 6.0 to7.0, Tris-HCl buffer having pH 7.5 to 9.0 and Carbonate-Bicarbonate buffer having pH 9.5 to 11.0. at 30^0C for 30 minutes. The pH optimum of *Serratia marcescens* strain TW1 was at pH 8.5 Tris-HCl buffer.

Table 3. Effect of metal ions on protease activity was examined in 8.3mM of $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$ in 0.1M Tris-HCl buffer having pH 7.5 at 25^0C for 30 minutes. The metal ions $HgCl_2$ and 20mM Na_2EDTA have inactivated the protease of *Serratia marcescens* TW1.

S. No	Metal Ion	Residual Protease Activity (%)
1	Native protease	100
2	$FeSO_4$	75
3	$MnCl_2$	26
4	$CaCl_2$	19
5	$CoSO_4$	14
6	$ZnCl_2$	60
7	$MgSO_4$	70
8	$HgCl_2$	00
9	Na_2EDTA	00

Table 4. Effect of inhibitors on protease activity was examined in 20mM EDTA, 8.3mM Iodioacetic acid, 8.3mM Dithiothreitol, 8.3mM Leupeptin, 1% of 2- β Mercaptoethanol, 1% of Tween 20 and 3% of ethanol in 0.1M Tris-HCl having pH 7.5 at 25^0C for 30 minutes. The protease of *Seratia marcescens* strain TW1 was inactivated completely by 20mM EDTA.

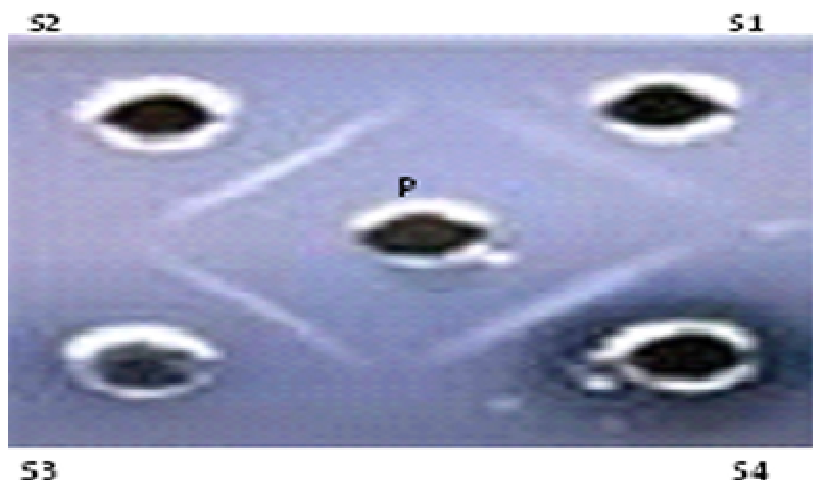| S.No	Inhibitors	Residual protease activity (%)
1	Native protease	100
2	Iodioacetic acid	78
3	2- Mercaptoethanol	80
4	Tween 20	88
5	3% Ethanol	85
6	Dithiothreitol	70
7	Leupeptin	84
8	20mM EDTA	00

Figure 5. Ouchterlony double immunodiffusion: The 1.2% (wt/vol) agarose gel was prepared in 0.05M borate buffer having pH 8.5. The distance form the peripheral wells to the centre well is approximately 5.5mm and each cut of well is 4mm in diameter, **P**—Centre well contained 20µl of antiserum, **S1**—Cultural broth supernatant of 20µl *Serratia marcescens* strain TW1, **S2**—Ammonium sulphate fraction 20µl *Serratia marcescens* strain TW1, **S3**—Dialysate protease 20µl *Serratia marcescens* strain TW1, **S4**—DEAE cellulose purified of 20µl *Serratia marcescens* strain TW1

TW1 showed line of precipitatin against the antiserum (Figure 5).Thus indicated that protease is immunologically identical.

DISCUSSION

The *Serratia marcescens* strain TWI secretes large extracellular enzyme protease in the surrounding medium (Yanagida et al., 1988). The production was stopped at early stationary phase at that time maximum protease was produced (Henriette et al., 1993). The 80% ammonium sulphate saturation leads the precipitation of the protease at 4^0C and fractional precipitation with acetone (Salamone and Wodzinski, 1997). The excess salt was removed from protease by means of a dialysis (Morita et al., 1997). The dialyzate of *Serratia marcescens* strain TW1 purified by ion exchange chromatography relied on the attraction between oppositely charged particles. The net charge exhibited by these compounds was depends on their p^{ka} and pH of the solution. The proteolytic activity was determined by using casein as substrate (Kunitz, 1947) in Tris HCl buffer pH 8.0 and showed the protease activity of 83.84 IU/ml. The purified exocellular protease turned out to be one polypeptide chain with a molecular weight of 56 KDa averages of the values obtained by SDS-PAGE (Laemmli, 1970). The isoelectric point of protease *Serratia marcescens* strain TW1 was 6.5 (Robertson et al., 1987). As proteins are differing in the composition each and every protein has its own characteristic p^I value.

The optimal temperature of protease *Serratia marcescens* strain TW1was 40^0C in Tris HCl buffer containing $MgCl_2$ having pH 8.0. The protease activity lost when temperature was increased to 60^0C there was negligible activity. The pH characteristics of proteases *Serratia marcescens* strain TW1 showed high enzyme activity between 6.5 to 9.0 and maximum at 8.5 in Tris HCl buffer (Lyerly and Kreger, 1979). The metals ions $HgCl_2$ and Na_2EDTA completely inactivated the protease activity (Matsumoto et al., 1984) and reactivated by Mg^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} are essential for the enzyme activity so named as metalloprotease (Aiyappa and Haris 1976). The 20mM EDTA inhibited the enzyme activity completely while as other inhibiters did not showed much impact on enzyme activity. The proteolytic enzymes offer a powerful treatment for pain and inflammation with widespread use in arthritis, fibrocystic breast disease, chronic bronchitis, sinusitis, atherosclerosis, wound debridement and carpal tunnel syndrome (Kee *et al.*, 1989; Coulthrust *et al.,* 2006; Lee *et al.,* 2006). These produce pharmacological effects by absorption through the intestines into blood stream (Miyata, 1980). But oral bioavailability of these peptide drugs is generally very low, owing to acidic conditions of the stomach and poor permeability across intestinal mucosa (Rawat *et al.,* 2006). The proteolytic enzymes and protease inhibitors have shown invitro capacity to depress or inhibit some fundamental activity of bacteria (Grenier, 1992). Sarratiopeptidase has been widely used in therapy as an anti-inflammatory drug because of its ability to enhance the penetration of antibiotics in infected cells (Coria-

Jimenez *et.,* 2004). *Serratia* peptidase was effective for eradicating infection caused by biofilm forming bacteria (Mecikoglu *et al.,* 2006).

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