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Application studies of the halotolerant protease from a newly isolated *Virgibacillus dokdonensis* VIT P14

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Protease extracted from halotolerant bacterium *Virgibacillus dokdonensis* was tested for possible industrial applications. This enzyme was able to dissolve blood clot and coagulated egg within 30 min. The enzyme exhibited substantial keratinolytic activity. It was compatible with all the tested commercial detergents like Rin, Surfexcel, Henko, Tide, Ariel and Technobright and was found to be effective in the removal of blood strains from cotton fabric in the presence of these detergents. The enzyme was compatible with the organic solvents like xylene, toluene, hexane and ethanol and the maximum activity was observed in the presence of ethanol. The enzyme was tested for antimicrobial activity against gram positive and gram negative bacteria and it was found that it possesses good inhibition capability against *Escherichia coli, Streptococcus eqiuns, Staphylococcus aureus* and *Salmonella enterica.* The present report indicates that this halotolerant protease has a wide range of properties and the conditions could be optimized to suit any particular industrial application.

Keywords: *Virgibacillus dokdeninsis,* Halotolerant protease, antimicrobial, organic solvent, detergent

Introduction

Protease belongs to an important group of industrial enzymes (Anwar and Saleemuddin, 1998) which is being studied in detail. It has a variety of industrial applications such as in detergent industries, peptide synthesis, leather treatment, food, pharmaceutical, medicine and dairy industries (Samal et al., 1990; Shimogaki et al., 1991; Gupta et al., 2002). The largest area of protease application is in detergents industries for removing the protein stains from cloths (Banerjee et al. 1999). Keratin is a natural protein with high stability (Bradbury 1973). It is difficult to degrade the polypeptide which is closely packed and highly stabilized by disulphide, hydrogen and hydrophobic interactions. These bonds confer high mechanical stability which resists the degradation of this protein by the useful proteases (Parry and North 1998; Kreplak et al. 2004). The bacterial proteases are the important group of secondary metabolites that are widely exploited (Ferrari et al., 1993). Also, use of these enzymes in peptide synthesis has numerous advantages than chemical process (Sergeeva et al., 1997). However, the enzyme should be stable in the

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presence of solvents. Investigation of solvent stable proteases is a major area of research (Geok et al., 2003). This paper report the possible applications of protease, extracted from the halotolerant bacterium *Virgibacillus dokdonensis* VITP14 isolated from Kumta coast.

Materials and Methods

Microorganism and Phylogenetic analysis

Virgibacillus dokdonensis producing halotolerant protease was isolated from Kumta coast (Pooja and Jayaraman, 2009). The authenticity of the strain was verified by morphological, biochemical and genetic characterization and the details of which has been reported in our earliest study (Devi et al 2012).

Media and culture conditions

Enzyme production was carried in Zobell marine broth. From the overnight culture, 5% inoculum was added to Zobell broth and the broth was incubated for 48 hrs at 37 ˚C (100 rpm). After incubation, the culture was withdrawn and centrifuged at 10,000 rpm for 10 min at 4˚C. Then the centrifuged culture supernatant was collected and used for the possible application analysis.

Assay of protease activity

The enzyme activity was assayed by the modified method of Kunitz using casein as a substrate. A 500 µl casein substrate (2% w/v in 10mM Tris–HCl buffer pH 8.0) was mixed with enzyme and incubated at 37˚C for 10 min. After incubation, the reaction was terminated by adding 2.5 ml of 10% (w/v) trichloroacetic acid (TCA), kept at room temperature for 30 min and then centrifuged at 10,000 rpm for 8min. The absorbance of the clear supernatant was measured against a blank at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1µmol of tyrosine in 1min under standard assay conditions (Hadj-Ali *et al*., 2007).

Applications

Digestion of natural proteins

The enzyme (10 U) was incubated with blood clot and coagulated egg white (separately) in 20 mM Tris-HCl (pH 8.0) at 37^{o}C. Conditions of the substrates were monitored at different time interval of incubation (Najafi *et al*., 2005).

Removing blood stain

A clean piece of cloth was soaked in blood and allowed to dry. Subsequently, the cloth was treated in 2% formaldehyde. After 30 min, the cloth was washed with water to remove the excess formaldehyde. Then the cloth was cut into 3 equal sizes and incubated with the (a) preheated detergent, (b) 10 U enzyme and (c) preheated detergent with enzyme at 37^{o}C for different time incubation. After specified incubation time, each piece was rinsed with water for 2 min and then dried. Treatment of the stained cloth with detergent alone served as the control (Sana et al., 2006).

Keratinolytic activity

To check the presence of keratinolytic activity of the enzyme, 0.5gm of hair was measured suspended in 10mM Tris-HCl buffer (pH – 8.0). Hair containing tube was added with enzyme (10 U) and incubated for 1hr (Yamauchi and Okazaki., 2007). A control tube was also maintained with hair and buffer. After incubation, both the tubes were centrifuged and the supernatant was analyzed for keratinolytic activity by monitoring the changes in the elution pattern in a reverse phase (C_{18}) HPLC column.

Antibacterial activity

Antibacterial activity of the protease was assayed by well diffusion method using Cappucino and Sherman method (2004). Three gram positive and three gram negative bacteria, namely *Bacillus subtilis, Staphylococcus aureus, Streptococcus eqiuns, Escherichia coli, Salmonella enterica,* and

Pseudomonas aeruginosa were used in the study. These organisms were grown individually in nutrient broth for 24 hr at 37°C. Muller Hinton agar was prepared and poured into the sterile petriplates. The overnight grown cultures were spread on the plates and allowed to dry. 6 mm bore was made on the set agar plates and the protease was added to the well. The plates were incubated at 37°C for 24 hr. After incubation, the plates were examined for zone of clearance around the individual wells. The diameter of the zone of clearance was measured and expressed as arbitrary units per ml (AU/ml).

$$AU/ml = \frac{\text{Diameter of the zone of clearance (mm) X 1000}}{\text{Volume taken in the well (µl)}}$$

Effect of solvent

To test the stability of partially purified protease on different solvents, 1 ml of different organic solvents (xylene, toluene, hexane and ethanol) and 10U of enzyme solution in 100 mM Tris-HCl buffer (pH 8.0) were mixed and incubated for 1 hr. Residual activity of the enzyme was assayed by the method described above (Sana *et al*., 2006).

Detergent Compatibilty

The stability of the protease in the presence of laundry detergents was examined by incubating the enzyme (250 U/ml) for 1 hr at 37°C and the residual activities were determined at every 10 hr interval (10, 20, 30, 40, 50 and 60 min). The enzyme activity of a control sample (without detergent), incubated under the similar conditions, was taken as 100%. The commercial detergents used were Rin, Surf excel, Tide, Ariel, Technobright and Henko. The detergents were diluted in water to give a final concentration of 7 mg/ml to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by pre-heating treatment (Haddar et al., 2009).

Results and Discussion

Digestion of natural proteins

The blood clot and the coagulated egg white was incubated with protease and observed for solubilization in different time periods. After 30 min of incubation, the insoluble forms of them were converted to soluble form (Fig 1 & 2). The ability of protease to digest different natural substrates including blood clot and coagulated egg white suggest the usefulness of this enzyme for different applications such as waste treatment, in removing the blood proteins from the medical instruments (Kumar et al. 1998) and other related applications.

Fig – 1: Blood clot treated with enzyme (Test and control)

Fig-2: Coagulated egg white treated with enzyme (Test and control)

Blood stain removal from fabrics

In removing the blood stain from the cloth, the protease enzyme in the presence of commercial detergents showed moderate effect (Fig 3). Thus, its effect with the presence of solvents and detergents could be exploited for such applications. Similarly, Oberoi et al. (2001) reported the increase in wash performance of the enzyme with detergents. Anwar and Saleemuddin, (1997) stated the usefulness of protease in removing the blood stains from cotton cloth both in the presence and absence of detergents. This enzyme can be believed to degrade hemoglobin from the blood and also effect in removing recalcitrant blood stains (Najafi et al., 2005).

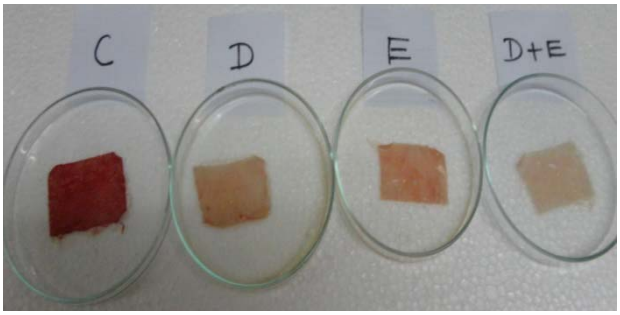


Fig – 3: Blood stain removal from fabrics. C – Control D – Detergent E – Enzyme D +E – Detergent + Enzyme

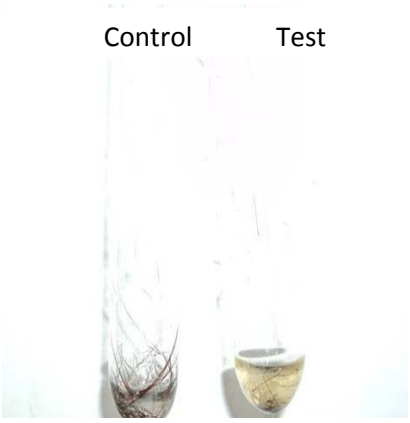
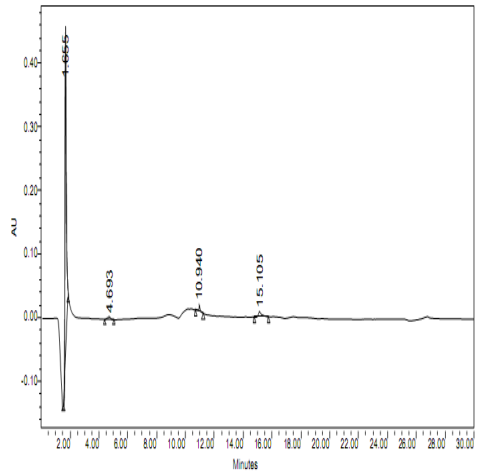


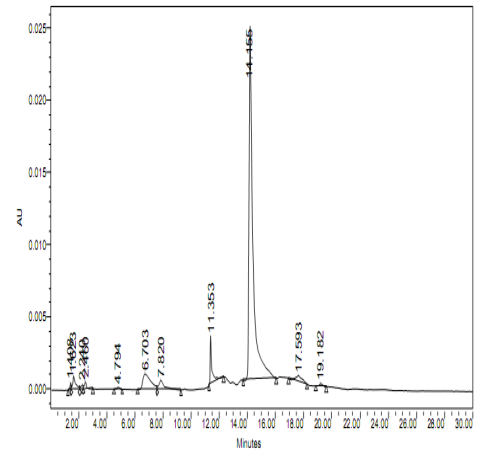
Fig – 4: Hair treated with enzyme (Test and control)

Keratinolytic activity

The keratinolytic activity was checked by treating the hair suspension with enzyme. There was a color change in buffer with enzyme treated hair than the buffer alone (Fig 4), clearly indicating a chemical change in the hair suspension treated with the protease enzyme. The absorbance value showed increase with increase in time which indicates the degradation of hair taking place. The hair treated buffer with enzyme and without enzyme was analyzed using reverse phase HPLC. Treatment with enzyme showed more peaks compared to without enzyme (Fig 5), indicating the presence of hydrosylates.



(a)



(b)

Fig – 5: HPLC Analysis of Buffer treated with hair (a) and buffer with enzyme + hair (b)

This suggested the degradation of keratin protein and the release of aminoacids (or short peptides). Keratin is an insoluble protein which is difficult to degrade by common proteases. Keratinases is the endo-proteases which has broad spectrum of activity (Gradisar et al. 2000, 2005; Brandelli 2005), commonly hydrolyze both soluble proteins as well as insoluble proteins such as keratin (Lin et al. 1992; Suh and Lee 2001; Farag and Hassan 2004). Very few enzyme show effective hydrolyzing capacity of keratin than the soluble proteins (Muhsin and Aubaid 2000). The reason behind this is more number of disulphide bonds in keratin than the water soluble globular proteins (Xie et al. 2010). Keratin degradation is not only by the production of keratinases but also the release of thiol groups leads to the reduction of disulphide bonds results in efficient keratin degradation (Mabrouk 2008).

Antibacterial activity

The halotolerant protease isolated from kumta coast was tested for its ability to inhibit the growth of *Bacillus subtilis, Staphylococcus aureus, Streptococcus eqiuns, Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa*. Among the tested bacterial strains, the enzyme showed maximum inhibition against *Escherichia coli* followed by *Streptococcus eqiuns, Staphylococcus aureus, Salmonella enterica.* The zones of inhibition of the protease are presented in Table 1. This shows that the protease enzyme has antibacterial activities and may cause some morphological changes (Moriarty, 1998). Several microorganisms which produce proteolytic enzymes have been reported and are commercially exploited for their application (Rebecca et al., 1991). These clearly indicated that the halotolerant protease reported in the present study could have the potential for the formulation of anti-bacterials and similar such applications.

Table 1: Antibacterial activity of the protease against 3 G +ve and 3 G –ve bacteria.

Micro organism	**MTCC Num**	**Inhibition zone (mm)**
Bacillus subtilis	9878	8
Staphylocous aureus	3160	13
Streptococcus eqiuns	3523	14
Escherichia coli	1698	14
Samonella enterica	9844	12
Pseudomonas aeruginosa	4673	9

Table 2: Effect of solvent on enzyme activity

Solvent	**Relative activity (%)**
Control	100
Xylene	54
Toluene	62
Hexane	67
Ethanol	83

Effect of solvent

The residual activity of the enzyme was measured after incubating the enzyme for 1 hr with the specified organic solvent. Activity in control without enzyme was taken as 100%. Maximum activity was seen in ethanol (83%) and the least activity was observed in the presence of xylene (54%). Thus the enzyme showed moderate stability with the tested solvents Table 2. This result is in correlation with a study (Sana et al., 2006) that showed maximum activity with ethanol and in contrast to a literature (Simon et al., 2004) stating decrease in activity with ethanol.

Detergent Compatibilty

The halotolerant protease is stable with all the tested commercial detergents at

concentration of 7 mg/ml. It exhibited better stability with Ariel followed by Henko, Rin, Surfexcel, Tide and Technobright (Fig 6). This result is in correlation with a study (Sellami-Kamoun et al., 2008) that reported the stability of protease enzyme with a wide range of commercial solid detergents at similar concentration.

Thus the present study aimed at exploiting the application of a halotolerant protease revealed the compatibility and stability under various chemical conditions and therefore has a great potential in many industrial formulations.

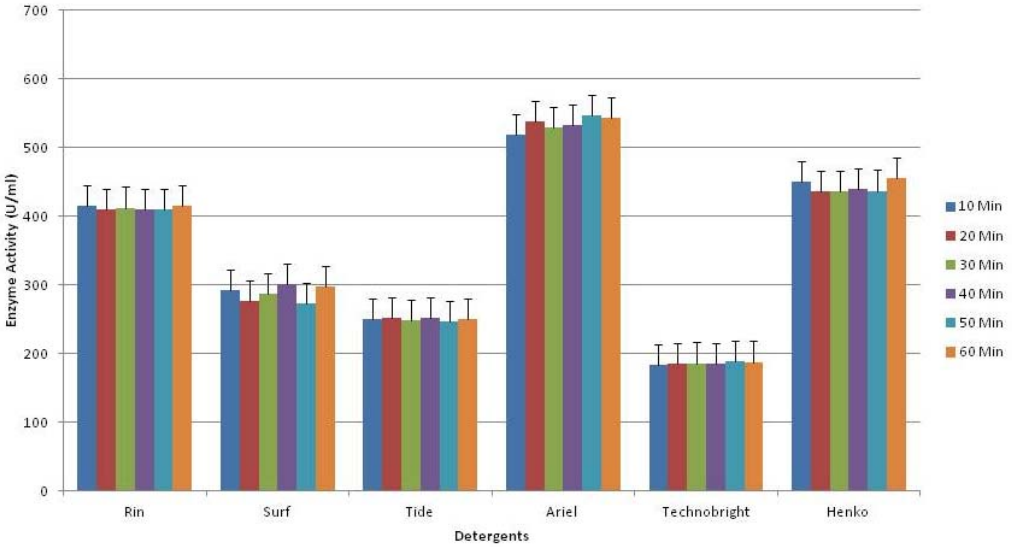


Fig – 6: Detergent compatibility of enzyme at different time interval (10, 20, 30, 40, 50 and 60 min)

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