The Chittagong Univ. J. B. Sci.,Vol. 7(1 &2):109-119, 2012.

CHARACTERIZATION OF KERATINASES FROM *BACILLUS* SPP.

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ABSTRACT

The present study deals with the characterization of keratinases from *Bacillus brevis* and *Bacillus cereus* isolated from the poultry wastes. During the enzyme-substrate reaction, the crude keratinases of the two bacteria showed their maximum activity at 35 and 50^0C and pH 10 and pH 8 with optimum incubation period of 80 and 70 minutes, respectively. The keratinases showed their activity towards casein, keratin, BSA and gelatine. It was found that keratinases belongs to metalloprotease and serine protease. Ca^{2+}, Mg^{2+} and Triton X-100 increased the activity of both the keratinases, while Cu^{2+} and Hg^{2+} decreased the activity. β-Mercaptoethanol and Dimethyl sulfoxide increased the crude keratinase activity of *B. brevis* and *B. cereus* respectively, while β-Mercaptoethanol had negative effect on keratinase of *B. cereus.*

Key words: Keratinases, β-Mercaptoethanol, *Bacillus* spp, BSA.

INTRODUCTION

Considerable amount of feathers are produced as waste products of commercial poultry-processing areas in our country. Feathers are composed primarily of keratin. Keratin also occurs in hair, horn, nails and cornified tissue. It is an insoluble protein and is not degraded by normal proteases such as trypsin, pepsin and papain, as it contains molecular conformation of their structural amino acids, which is tightly packed in the α-helices (hairs) and β-sheets (feather) in the presence of cystine disulfide bonds, hydrogen bonds and hydrophobic interactions (Parry and North 1998). The fibers of keratins on both forms are intensively twisted forming micro and macro fibrils that ensure their stability against proteolysis (Krelpak *et al*. 2004). For this, feathers have limited use as dietary components in animal feedstuffs, although they contain large amounts of potentially useful protein and amino acids. Conventional physical and chemical treatments, for increasing digestibility of feather keratin, require consumption of large amounts of energy and also destroy certain amino acids, thus yielding products of poor digestibility and variable nutrient quality. Alternatively, this

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keratin waste can be efficiently degraded by a few microorganisms due to the secretion of keratinolytic peptidases (keratinases) into the culture medium (Onifade *et al*. 1998). Keratinolytic activity has been reported in *Bacillus* (Williams *et al*. 1990, Lin *et al*. 1999, Manczinger *et al*. 2003, Suntornsuk and Suntornsuk 2003, Zerdani *et al*. 2004, Suntornsuk *et al*. 2005), *Thermoanaerobacter* (Riessen and Antranikian 2001), *Chryseobacterium* (Riffel *et al*. 2003), *Flavobacterium* (Riffel and Brandelli 2003, Nam *et al*. 2002) and *Vibrio* (Sangali and Brandelli 2000). Keratinolytic enzymes are largely used in the removal of keratin containing feathers from the environment through nonpolluting process and also in industry for various biotechnological and agricultural applications. After treatment with keratinase, feather can be used as feeders, fertilizers and insoluble polymers (Yamauchi 1996). Feather- hydrolysate can be used as food additives. In leather and pharmaceutical products, the enzyme can be used as de-hairing agent (Thanikaivel *et al*. 2004).

In the present investigation, the preparation of crude enzymes from *Bacillus brevis* and *B. cereus* from Chittagong region to characterize the indigenous keratinase enzymes.

MATERIALS AND METHODS

Microorganisms and maintenances

The bacteria *Bacillus brevis* and *B. cereus* were isolated from feather-decomposed soil collected from a poultry farm yard and dumping site of poultry waste areas in Chittagong, Bangladesh. Stock cultures of the organism were maintained at -20°C in nutrient broth containing 10% glycerol.

Media used for enzyme production

Feather Meal Medium-1 (NaCl, 0.5 g l^{-1}; K_2HPO_4, 0.3 g l^{-1}; KH_2PO_4, 0.4 g l^{-1} and Feather, 10 g l^{-1}; pH 7.5) and Horikoshi Medium (Soluble starch, 5 g l^{-1}; Peptone, 5 g l^{-1}; Glucose, 5 g l^{-1}; K_2HPO_4, 1 g l^{-1}; $MgSO_4$ $7H_2O$, 0.2 g l^{-1}; Na_2CO_3, 1 g l^{-1}; Yeast extract, 5 g l^{-1} and Feathers, 10 g l^{-1}; pH 7.5) were used for enzyme production (Sarita and Neeraj 2010).

Crude enzyme production

The bacterial cultures were centrifuged at 8000 rpm, at 4˚C for 20 minutes. The supernatant was collected and filtered by Whatman No. 540 (Ash less) and was used as crude enzyme.

Keratinase assay

Enzyme assay was done following modified method (Meyers and Ahearn 1977). Three ml of culture filtrates, 3 ml of citrate phosphate buffer and 3 ml of 1% casein was taken in a 25 ml test tube. Then the test tube was placed in a water bath at 37^0C for 1 hour. After reaction, 5 ml of 20% TCA was added with the solution for stopping the reaction. After one hour, the solution was filtered by Whatman No. 540 (Ash less). From the filtrate, 1 ml was taken into a test tube and 2 ml of 20% Na_2CO_3 was added to it. To this mixture 1 ml of Folin Ciocalteu Reagent was added and mixed well. After 30 minutes, 6 ml distilled water was added to it and absorbance of the solution was measured at 650 nm in a spectrophotometer and calculated the amounts of amino acids released from a standard curve plotted from known concentration of bovine serum albumin. The enzyme activity was expressed in Unit. One unit of enzyme was defined as the amount of enzyme that releases 1 gm of tyrosine/ml of crude extract/hour.

Characterization of crude enzyme

Effect of incubation period

To ascertain the effect of incubation period on keratinase activity crude enzyme-substrate mixtures were incubated for different incubation time i. e. 40, 50, 60, 70, 80 and 90 minutes and its effect on enzyme activity were studied.

Effect of temperature

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures ranged from 20 to 60°C.

Effect of pH

The effect of pH on keratinase activity was determined by incubating the reaction mixture at pH value ranging from 6.0 to 11.0 using citrate phosphate buffer and phosphate buffer solutions.

Effect of substrate concentration

To determine the effect of substrate concentration, keratinase activity was measured at different substrate concentrations i. e. 0.5, 1.0, 1.5, 2.0 and 2.5%.

Substrate specificity

Four different types of natural proteins such as casein, keratin, BSA and gelatin were used to observe the substrate specificity and enzyme activity was measured separately for each substrate.

Effects of different chemical reagents and metal ions

To investigate the effects of different solvents, detergents, reducing agents, protease inhibitors and metal ions on keratinase activity, the crude enzyme solution was pre-incubated in phosphate buffer solutions (pH 8.0) for 1 h at 40^{o}C with different reagents at concentration of 0.1% (wt/vol or vol/vol) or 1mM. Keratinase activity was determined as a percentage of residual activity relative to control. The chemicals and metal ions used were: Triton X-100, Dimethyl sulfoxide (DMSO), β-Mercaptoethanol, 1,2-epoxy-3-(p-nitrophenoxy) propane, phenylmethylsulphonyl fluoride (PMSF), ethylendiaminetetraacetate (EDTA), and Ca^{2+}, Mg^{2+},Cu^{2+}, Hg^{2+}.

RESULTS AND DISCUSSION

Characterization of keratinase

In the table 1, the crude enzyme of *Bacillus brevis* showed highest keratinase activity after 80 minutes incubation period, whereas *B. cereus* showed highest keratinase activity after 70 min. The enzymatic hydrolysis of keratin increased linearly with incubation in reaction mixture up to 45 min of reaction time and then gradually increased up to 60 min (Thanaa *et al*. 2011).

The crude enzyme of *B. brevis* was active in the temperature range of 25 to 60°C with maximum activity (160.89 U/ml) at 35°C, although, a small peak at 50°C was also observed, suggesting probable presence of two proteases. The isolates *B. cereus* showed maximum crude enzyme activity at 50°C (Table 2). The high keratinase activity at 50°C of incubation temperature is in agreement with Thys and Brandelli (2006). In the table 3, *B. brevis* and *B. cereus* showed their maximum keratinase activity at pH10 and 8 respectively. Most keratinases were found to be active in neutral to alkali conditions. The optimum activity of keratinase of *Microbacterium* kr10 was pH 7.0, *B. pumilus* FH9 of pH 8.0 (Thys *et al*. 2004), *Fervidobacterium islandicum* AW-1 of pH 9.0 (Nam *et al.* 2002), and a few of extreme alkalophilic optima at pH 12~13 (Takami *et al*. 1992).

The maximum enzyme activity of *Bacillus cereus* was found with 1.0% substrate concentration, while *B. brevis* showed maximum keratinase activity with 2.0% substrate concentration (Table 4). The table 5 showed that the highest enzyme activity of 188.90 U/ml with casein was recorded by the enzyme of *Bacillus brevis*. The enzyme of *B. cereus* gave the highest enzyme activity of 166.62 U/ml with gelatin as substrate. The keratinase prefers gelatin and keratin type of protein which usually contains a substantial number of disulfide bonds

(Rosita *et al.* 2007). In addition, the purified keratinase enzymes showed higher activities with soluble proteinaceous substrates such as casein than with insoluble proteinaceous substrates (Fuhong *et al.* 2010).

The table 6 summarizes the effects of metal ions and other chemical substances on the activity of keratinase. Keratinase from the isolate *B. brevis* may belong to the metalloprotease, as it was completely inhibited by EDTA. On the other hand, keratinase from the isolates *B. cereus* may belong to serine protease, as it was completely inhibited by PMSF and partially inhibited by EDTA. Cu^{2+} and Hg^{2+} ions had inhibitory effect on enzyme activity of both the isolates. In contrast, both Ca^{2+} and Mg^{2+} ions as well as triton X-100 had positive effect on crude enzyme activity of *B. brevis* and *B. cereus*. DMSO had no effect on keratinase activity of the isolates *B. brevis*, whereas β-Mercaptoethanol inhibited the keratinase activity of *B. cereus* by 29% comparison.

So, it can be concluded that in addition to optimizing environmental conditions, pre-treatment of chicken feather by reducing agents, or detergents, or activation of the enzyme by adding metal salts are required for improvement of their degradation. The bacteria and their keratinases can be used to improve nutritional values of animal feed containing keratin and to reduce poultry processing waste in Chittagong region and also might be used in other biotechnological processes such as leather industry, keratin waste treatment, and cosmetic industry.

TABLE 1: EFFECTS OF INCUBATION PERIOD ON KERATINASES ACTIVITY OF *BACILLUS* SPP.

Incubation period (minutes)	Keratinases activity (U/ml)	
	Bacillus brevis	*Bacillus cereus*
40	71.63	59.77
50	79.79	66.82
60	148.33	107.43
70	150.77	112.17
80	155.09	101.70
90	144.45	92.54

TABLE 2: EFFECTS OF TEMPERATURE ON KERATINASES ACTIVITY OF *BACILLUS* SPP.

Temperature (˚C)	Keratinase activity (U/ml)	
	Bacillus brevis	*Bacillus cereus*
25	46.75	52.87
30	113.65	86.08
35	160.89	102.46
40	141.76	110.45
45	132.09	120.86
50	158.77	139.20
55	64.87	108
60	51.09	42.57

TABLE 3: EFFECTS OF pH ON KERATINASES ACTIVITY OF *BACILLUS* SPP.

pH	Keratinase activity (U/ml)	
	Bacillus brevis	*Bacillus cereus*
6	78.54	82.24
7	167.00	140.93
8	171.57	151.31
9	178.16	143.20
10	185.38	91.78
11	110.33	64.23

TABLE 4: EFFECTS OF SUBSTRATE CONCENTRATION ON KERATINASES ACTIVITY OF *BACILLUS* SPP.

Substrate conc. (%)	Keratinase activity (U/ml)	
	Bacillus brevis	*Bacillus cereus*
0.5	158.82	132.55
1.0	184.67	152.70
1.5	187.41	143.28
2.0	191.96	93.61
2.5	161.24	87.27

TABLE 5: EFFECTS OF SUBSTRATE SPECIFICITY ON KERATINASE ACTIVITY OF *BACILLUS* SPP.

Substrate	Keratinase activity (U/ml)	
	Bacillus brevis	*Bacillus cereus*
BSA	18.87	14.18
Casein	188.90	151.95
Keratin	176.55	158.56
Gelatin	86.65	166.62*

TABLE 6: EFFECTS OF DIFFERENT CHEMICALS AND METAL IONS ON KERATINASES ACTIVITY.

Types of Substance	Substance	Concen-tration (%)	Residual activity (%)	
			Bacillus brevis	*Bacillus cereus*
Control		0.1	100	100
Detergents	Triton X-100	0.1	116	139
Organic solvents	Dimethyl sulfoxide (DMSO)	0.1	100	111
Reducing agents	β-Mercaptoethanol	0.1	114	71
Inhibitors	Phenylemethylsulfonyl fluoride (PMSF)	0.1	115	0
	Ethylenediaminetetra acetic acid (EDTA)	0.1	0	90
	1,2-epoxy-3-(p-nitrophenoxy) propane	0.1	105	108
Metal ions	Ca^{2+}	1 mM	106	105
	Mg^{2+}	1 mM	112	117
	Cu^{2+}	1 mM	90	94
	Hg^{2+}	1 mM	50	83

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Manuscript received on 08.08.2012; Accepted on 01.01.2013
The Chittagong University Journal of Biological Sciences, Vol. 7 (1 & 2). Page No.