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SCREENING AND PARTIAL CHARACTERIZATION OF HEMOLYSINS FROM *BACILLUS* SP.: STRAIN S128 & S144 ARE HEMOLYSIN B (HBL) PRODUCERS

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Abstract

The potential of 130 different strains of the genus *Bacillus* for hemolysin production and their effects against erythrocytes from different sources was examined.

Complete hemolytic units (CHU) of three randomly selected strains of *B. cereus* group *viz.* S128, S140(c) and S144 were determined at different temperatures and pH. The highest CHU in yeast extract-tryptone (YT) broth was found at pH 5 & 7 for S140(c); pH 7 and 9 for S144 and S128, respectively. Hemolysins from the strains S128 and S140(c) remained stable at 50°C for 30 min, while hemolysin from strain S144 completely lost its activity at this temperature. However, no hemolytic activity was noted at 60°C in any of the test strains. Strains S128 and S144 showed discontinuous pattern of hemolysis which is an indication of hemolysin BL (HBL) producers.

Introduction

Some members of Group I *Bacillus* are associated with certain clinical conditions (Brooks *et al*., 1998). Among these members, *Bacillus subtili*s and *Bacillus cereus* are important as they cause food-spoilage and food-poisoning and thereby pose threat to food industry and public health (Rozovitz *et al*., 1998). Although *B. cereus* is ubiquitous soil-borne saprophyte but can produce a variety of virulence factors such as hemolysins.

The hemolysins of *B. cereus* group are of several types including: (i) cereolysin (hemolysin I) - a thiol activated heat-sensitive hemolysin of streptolysin O family. Its activity is lost, *In vitro*, on pre-incubation with cholesterol and papain. It is also inactivated reversibly in the presence of oxygen (Cowell *et al.,* 1976); (ii) cereolysin AB - a cytolytic complex of phosphotidalcholine hydrolase and sphingomyelinase which act together to cause hemolysis (Gilmore *et al*., 1989, Beecher & Macmillan, 1990); (iii) Hemolysin II - a β-barrel pore-forming toxin of the staphylococcal α-hemolysin family, its activity is generally unaffected by cholesterol or anti-streptolysin O antibody (Coolbaugh & Williams, 1978; Sinev *et al*., 1993); (iv) Hemolysin III- a hemolysin exhibiting temperature-dependent binding and pore formation followed by temperature independent erythrocyte lysis (Baida & Kuzmin, 1996); (v) Hemolysin BL (HBL) - a complex comprising of three components; B, L_1 and L_2. The B component binds to the target cell permitting L components to bind to increase permeability resulting in hemolysis (Beecher & Macmillan, 1991). As its activity is not affected in the presence of cholesterol so it seems similar to hemolysin II. HBL not only acts as enterotoxin but is also associated with non-gastrointestinal diseases like endophthalmitis (Beecher *et al*., 1995a, Beecher *et al*., 1995b) and (vi) cytotoxin K- a cytotoxic necrotic and hemolytic protein of 34 kDa similar to *S. aureus* α-hemolysin (Lund *et al*., 2000). Most of these hemolysins are produced by the members of *Bacillus cereus* group especially by *Bacillus thuringiensis* as it is closely related to *B. cereus* (Budarina *et al*., 1994).

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In the present study, an attempt was made to evaluate the hemolytic potential of 130 indigenously isolated strains of genus *Bacillus* against chicken, human, rabbit and sheep blood along with partial characterization of the hemolysins from three selected strains (S128, S140(c) and S144), all belonging to *B. cereus* group.

Materials and Methods

Bacterial strains: A total of 130 strains of *Bacillus* isolated from the soil samples and insect-guts, 40 years ago from the different parts of Pakistan were used. Isolates were identified on the basis of their cultural and biochemical characteristics (Clauss & Berkley, 1986). A vast majority (99/130) of strains were of *Bacillus cereus* group, identified on the basis of their growth characteristics on the chromogenic *Bacillus cereus* agar (CM1036; Oxoid). The strains were maintained on nutrient agar, pH 7 (Oxoid) slants and stored as 15% (v/v) glycerol stock at -20°C.

Screening for hemolytic activity: Overnight cultures were spot inoculated on plates containing nutrient agar supplemented with 6% citrated chicken, human, rabbit or sheep blood (pH 7). Hemolytic zone around each spot was noted after 24 hours of incubation at 37°C.

HBL producing strains were identified on the basis of discontinuous hemolysis (DC) pattern on blood-agar plates containing 6% (3-4 week old or partially-lysed) citrated sheep-blood. One set of plates was incubated for 24 hr. at 18°C and another at 28°C. The plates were observed for discontinuous zones of hemolysis after 5 hr. and continued to observe periodically until 24 hr.

Agar-well diffusion assay was performed, as a confirmatory test for HBL production, on blood-agar plates supplemented with 0.15M NaCl, 2% donkey serum and 2.5% defibrinated and washed erythrocytes of chicken, human, rabbit or sheep, separately. Each well was filled with 100 µl of crude hemolysin and the plates were incubated initially at 37°C for 8 hours followed by an overnight incubation at 4°C (Beecher & Wong, 1994).

Hemolysin production: Three strains of *B. cereus* group S128, S140(c) and S144, were grown at 28° and 37°C in YT broth (Yeast extract: 10, Tryptone: 16 and NaCl: 5g/l) having pH 5, 7 and 9. Cultures were propagated after adjusting an initial OD_{600} to ~0.01 in a shaking incubator (120 cycles/min) for 7 days. Aliquots were withdrawn after every 24 hr and cell-free culture supernatant/crude hemolysin preparations were obtained by centrifugation at ____ 5500g for 15 minutes followed by membrane-filter sterilization through 0.2 µm filters (Millipore, USA). Hemolytic activity was measured as complete hemolytic unit (CHU) according to Girard *et al*., (1963). Gel-diffusion assay was performed for hemolysin BL (HBL) activity according to Beecher & Wong (1994).

Characterization of Hemolysin

Determination of thermal stability: Crude hemolysin preparations were exposed to 50°, 60° and 100°C for 30 min., followed by the determination of CHU.

Determination of pH stability: The pH of cell-free culture supernatant was adjusted to 3, 5, 7, 9 & 11. After an incubation for 2 hr at 37°C the pH of the supernatant was neutralized and the residual hemolytic activity was measured.

Determination of enzyme sensitivity: Crude hemolysin preparations were treated with filter-sterilized solutions of lipase, α chymotrypsin (MP Biochemicals Inc. France), trypsin (Sigma-Aldrich Inc. Germany), papain (Arco-organics, USA) and lysozyme

(Pierce, USA) to obtain a final concentration of 5 mg/ml, incubated at 37°C for an hour followed by hemolytic activity assay.

Effect of lipids and other chemicals on hemolysin: Sensitivity of hemolysins to cholesterol (200mg/ml and 100mg/ml), lecithin (100mg/ml), dithioerythritol (100mg/ml), EDTA (10mM) and organic solvents including acetone, chloroform, ethanol and toluene (5% v/v and 10% v/v) was studied by exposure of hemolysin to these agents and incubation at 37°C for an hour followed by the determination of residual CHU.

SDS PAGE analysis: Ammonium sulphate fractionated (80% saturation) undialysed crude hemolysin preparations were separated on 12% Tris-glycine SDS-PAGE gel with slight modification. For hemolysin activation, samples were diluted with loading buffer containing DTT (as reducing agent) and incubated for 20 min at 37°C. In order to detect the hemolytic activity *in situ*, gel was divided into two halves after electrophoresis. The first half was fixed with 25% 2-propanol and 10% glacial acetic acid for an hr, washed several times with sterile distilled water and overlaid with Tris buffered-agar containing 0.15M NaCl and 1% washed suspension of human erythrocytes. After incubation at 37°C, in a humid chamber, for 6 hours the gel was placed in a refrigerator overnight and observed for zone of hemolysis. The other half of the gel was silver stained to ascertain the position of bands involved in hemolysis (Dunn, 1992).

Results

Detection of hemolysin on solid agar: The patterns of hemolysis as revealed by the *Bacillus* strains are summerised in Table 1a and 1b. The continuous β-hemolytic zones were observed on nutrient agar supplemented with fresh 6% (v/v) either chicken, human, rabbit or sheep blood while discontinuous hemolytic zones were observed only when the nutrient agar was supplemented with either 3-4 weeks old partially-lysed citrated sheep-blood or supplemented with 0.15M NaCl and 2% donkey serum together with 6% (v/v) fresh blood (Fig. 1).

The characteristic discontinuous hemolytic pattern for HBL was exhibited by fifty (38%) strains. More pronounced and distinct discontinuous patterns were noted when plates were incubated at 18°C compared to 28°C. It was also noted that at 28°C less distinct pattern of discontinuous hemolysis merges with the continuous hemolytic zone within 10 hours, however, the incubation time varied with strains (Beecher & Wong, 1994).

Furthermore, the β-hemolytic strain S140(c) that causes partial hemolysis (α) on partially lysed sheep-blood agar at 18°C and 28°C while its characteristic continuous β-hemolytic pattern was only noted when it was grown at 37°C. This indicates the temperature-dependent production of hemolysin by S140(c).

Gel-diffusion assay: Gel diffusion assays revealed the clear, continuous zones of hemolysis appeared against all types of blood after 15 min. with a higher rate of hemolysis in the case of S128 compared to S144 (both are HBL producers). In addition to this zone, a transient zone of discontinuous hemolysis appeared after 2 hr around the wells containing S128 hemolysin against chicken, human and sheep blood (Fig. 2); this pattern was, however, more pronounced in gels containing 2.5% (v/v) human blood. whereas S144 hemolsin produced discontinuous hemolysis after 8 hr of incubation against human and sheep blood while in agar well-diffusion assay on YT agar it only became evident against chicken erythrocytes. Moreover, the crude preparations of S140(c) hemolysin caused partial hemolysis of sheep and human; and complete hemolysis of chicken and rabbit erythrocytes after 18 hours of incubation.

Table 1(a). Summary of hemolytic patterns of strains of *Bacillus* sp. against the blood from different sources.

Type of hemolysis	Chicken		Human		Rabbit		Sheep		Partially lysed blood	
	C	NC	C	NC	C	NC	C	NC	C	NC
β	92	11	93	15	86	15	90	16	80 (50*)	12
α	0	7	2	1	2	4		2	14	4
γ	7	13	4	15	11	12	4	13	5	15

*HBL producers
C: No. of Bacilli of *B. cereus* group
NC: No. of Bacilli other than *B. cereus* group

Table 1(b). Variability in hemolytic patterns of few selected *Bacillus* strains against the blood from different sources.

Group	Organisms	Chicken	Human	Rabbit	Sheep
Non- *B. cereus*	S 4	β	γ	α	β
	S 12L	α	γ	γ	γ
	S 19	β	β	α	β
	S 21	β	γ	β	β
	S 11S (b)	γ	γ	β	β
	S 53	β	β	γ	β
	S 60 (a)	α	α	γ	γ
	S 60 (b)	α	β	β	γ
	S 61	α	γ	α	β
	S 62 (b)	α	γ	γ	γ
	S 64 (c)	β	β	β	γ
	S 64 (d)	γ	β	γ	α
	S 65	γ	β	γ	α
	S66	β	γ	β	γ
	S 72 (b)	γ	γ	β	γ
	S 103	γ	γ	β	β
	S 108 (b)	γ	γ	β	γ
	S 109	γ	γ	α	γ
	S 130 (b)	γ	β	γ	β
	S 140 (a)	γ	β	γ	β
	S 140(b)	β	β	α	β
B. cereus	S 6	β	α	α	β
	S 11S (a)	β	β	β	α
	S 26 (a)	β	α	γ	β
	S 55 (b)	β	β	β	α
	S 56	β	β	β	α
	S 72 (a)	β	β	γ	β
	S 102	β	β	γ	γ
	S 108 (a)	β	β	γ	β
	S 119 (a)	β	β	γ	β
	S 119 (b)	γ	γ	β	α
	S 127	β	β	γ	β
	S 129	γ	β	γ	α
	S 133	γ	β	γ	β
	S 134	β	γ	β	β
	S 137	γ	β	γ	γ

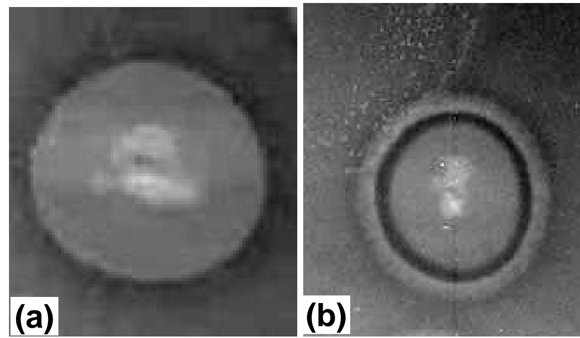

Fig. 1. Hemolytic pattern on solid agar. (a) Strain S 140(c) exhibiting continuous hemolysis. (b) Discontinuous hemolysis pattern by strain S144.

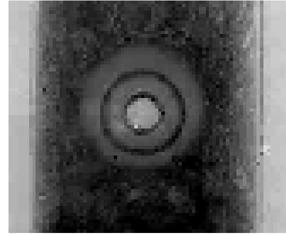

Fig. 2. Discontinuous hemolytic pattern of S128 hemolysin by gel-diffusion on slide against chicken blood.

Complete hemolytic unit (CHU) production kinetics in growth medium: The results indicate that the CHU production was influenced with incubation temperature and initial pH of the medium (Table 2). The data further show that the hemolysin production by S140(c) was maximum at pH 5 & 7 at 37°C, while for strains S144 and S128 it was pH 7 and 9, respectively. Hemolysin production was best at 37°C in all cases. The strain S144 has nonetheless exhibited an exception of producing higher amounts of hemolysin (as determined by CHU) against sheep blood at 28°C and pH 9 after 2 days of incubation. Furthermore, chicken erythrocytes were found more sensitive to hemolysins of S128 and S144 than of S140(c). The hemolysin of S144 additionally exhibited activity against sheep RBC, which was comparable to the chicken RBC's

Characterization of hemolysin: The sensitivity of hemolysins to enzymes, lipids, chemicals, pH and heat was measured as CHU, as summarized in Table 3. The result reveal that the α-chymotrypsin sensitive hemolysins of S128 and S144 were unstable at pH 3 and 11.

Although S128 and S144 hemolysin seemed similar but it was noted that there was a loss of hemolytic activity when crude preparations of S144 was exposed to acetone and chloroform. The insensitivity of hemolysins of S128 and S144 to cholesterol and ability to show characteristic discontinuous hemolytic pattern suggests that they are possibly hemolysin BL, as reported earlier (Beecher & Wong, 1994).

Hemolysin of S140(c) was sensitive to cholesterol and papain which indicates that it is probably hemolysin I or cereolysin (Cowell *et al.,* 1976). This hemolysin was also sensitive to acetone, pH 3 and high temperature. Moreover, the S140(c) hemolysin showed partial sensitivity at pH 11.

Table 3. Effect of plant extracts on mean diameter of fungal colonies (cm) on agar medium at 25^{0}C.

Treatment	Conc (%)	*Alternaria alternata*	*A. flavus*	*A. niger*	*Curvularia lunata*	*D. tetramera*	*Rhizopus* sp.	*Penicillium* sp.
Datura stramonium L. (Jimson weed)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	3.050 B	3.050 B	2.550 B	3.050 B	2.300 A-C	2.000 B	3.375 A
	0.01	2.800 C	2.450 CD	1.72C E	2.475 CD	2.075 B-D	1.525 B-D	2.525 B
	0.015	2.000 EF	1.950 EF	1.400 EF	2.050 DE	1.650 D-F	1.350 B-E	1.950 BC
Allium sativum L. (Garlic)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.650 C	2.650 C	2.700 B	2.725 BC	2.400 AB	1.950 BC	2.000 BC
	0.01	1.800 FG	2.075 D-F	1.550 DEF	2.250 C-E	1.900 B-E	1.200 C-F	1.800 C
	0.015	1.650 H	1.700 F	1.250 EF	1.875 D-F	1.400 E-G	0.6750 E-G	1.525 C
Zingiber officinale Roscoe (Ginger)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.375 D	1.950 EF	2.200 BC	2.250 C-E	1.975 B-E	1.750 BC	1.575 C
	0.01	1.800 FG	1.275 G	1.500 EF	1.750 EF	1.675 C-F	0.8500 D-G	1.350 C
	0.015	1.150 H	1.025 G	1.050 F	0.9500 G	1.100 FG	0.5750 FG	0.700 D
Azadirachta indica (L.) A. Juss. (Neem)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.050 E	2.225 DE	2.025 CD	2.125 C-E	1.375 E-G	1.900 BC	1.425 C
	0.01	0.8750 I	1.775 F	1.400 EF	1.675 EF	0.8250 GH	0.525 F-G	0.5250 D
	0.015	0.5500 J	1.125 G	1.150 F	1.375 FG	0.4500 H	0.3750 G	0.4250 D
LSD value		**0.457**	**0.729**	**0.754**	**0.52**	**0.67**	**0.48**	**0.43**

$p<0.05$ values within the same column show the same letters are not significantly different from each other

Table 3. Physicochemical Characterization of hemolysins.

Treatments	Residual activity (CHU)*		
	S128	**S140(c)**	**S144**
Untreated	8	32	32
Enzymes			
α Chymotrypsin	0	8	0
Trypsin	4	4	4
Lipase	8	16	4
Papain	8	0	8
Lysozyme	8	32	32
Lipids			
Cholesterol	8	0	32
Lecithin	8	16	32
Chemicals			
EDTA	8	16	32
Dithioerythretol	8	32	16
Acetone	4	0	0
Chloroform	4	8	0
Ethanol	8	8	16
Toulene	8	8	32
pH			
3	0	0	0
5	4	8	8
7	8	32	32
9	8	16	8
Heat			
50°C/30 min	8	8	0
60°C/30 min	0	0	0
100°C/30 min	0	0	0

* Organisms were propagated in YTB at pH 7.

SDS PAGE analysis: SDS-PAGE analysis shows that the hemolysins of S128 and S144 are possibly HBL, as by definition HBL is a tri-partite hemolysin and all the three components work synergistically. While the hemolysin from the S140(c) was not HBL and hence produced clear zone of hemolysis on SDS-PAGE gel overlaid with agar containing blood (see material & method; Fig. 3). The size of S140(c) hemolysin was ~45,000 kD when compared with low-range molecular markers (Bio Rad).

Discussion

Most members of the genus *Bacillus* produce hemolysins but the members of *Bacillus cereus* group, particularly *B. cereus*, *B. thuringiensis* and *B. mycoides*, simultaneously produce several types of hemolysins, and contribute to the pathogenesis of these, otherwise saprophytic soil-borne bacteria. Reports suggest similarity between hemolysins of different species of *B. cereus* group, hence the type of hemolysin produced cannot be considered as an important criteria for their identification up to species level (Beecher & Wong, 1994).

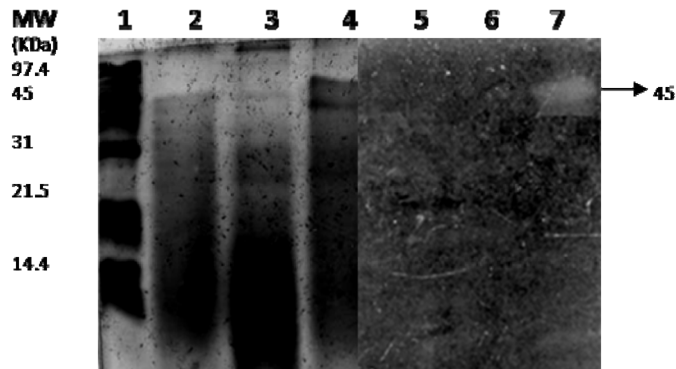

Fig. 3. SDS-PAGE of crude hemolysins. Lanes 1: low range protein marker; 2: S128; 3: S144; 4: 140(c). The SDS-Page gel was overlaid with Tris buffered-agar containing 0.15M NaCl and 1% washed suspension of human erythrocytes. Lanes 5, 6 & 7 loaded with crude hemolysin preparation of S128, S144 & S140(c), respectively. Hemolysis was produced by ~ 45 kDa protein (arrow) by the strain S140(c), lane 7.

The most extensively studied hemolysins are cereolysin (Hemolysin I) and hemolysin BL (HBL). The cereolysin are oxygen labile, requires cholesterol or lipid-rafts rich in cholesterol for its binding to the target cell (Watson *et al*., 1972; Tweten, 2005). The binding follows the pore formation thereby permitting the hemolysin to enter the erythrocyte and cause lysis. In the past few decades, much emphasis was given to HBL because not only it acts as an enterotoxin (Beecher & Macmillan, 1991, Beecher *et al*., 1995a) but associated with non-gastrointestinal disease like endophthalmitis (Beecher *et al*., 1995b). The HBL is similar to hemolysin II as it does not require cholesterol as membrane receptor for binding to erythrocytes, and has a binding component B which helps it to bind to the target cell.

In the present study, various isolates of *Bacillus* sp., were tested against a variety of bloods including chicken, human, rabbit and sheep. Most of the hemolytic strains 76.15% (99 strains) belonged to *B. cereus* group. Around 27.6% of the strains (36/136) exhibited variable hemolytic patterns. This variation in hemolytic pattern may be attributed to the variable concentrations of cholesterol found in blood from different sources or may possibly be due to the differences in overall structure and composition of erythrocytes. For instance, chicken erythrocytes are nucleated, flattened and have high glutathione concentration which helps to resist oxidative stress (Gradinski-Verbanak *et al.,* 2002).

The HBL production is unique to *B. cereus* group and the present study on 3-4 weeks old citrated sheep-blood agar revealed a discontinuous pattern of hemolysis which was similar to an earlier study which suggests that blood agar prepared from expired human blood or partially lysed blood and containing 0.15M NaCl and 2% calf-serum results in discontinuous hemolytic pattern (Beecher & Wong, 1994).

Both the HBL producers (S128 and S144) were lecithinase and protease positive (data not shown) indicating that they are possibly the members of *B. cereus* group. The lecithinase production by these strains can be correlated with the production of phosholipase C which also causes hemolysis of erythrocytes. In the case of strain 140(c), it is evident that the CHU was reduced to half upon pre-incubation with lecithin indicating that the hemolytic activity might be due to Phospholipase C/ lecithinase.

The phenomenon of sudden drop in CHU after 48 hrs of growth might be due to the proteolytic cleavage of hemolysin which is in line with other studies on *Staphylococcus aureus*, *B. anthracis* and *Pseudomonas aeruginosa* (Elliot, 1945; Harris-Smith *et al*., 1958; Liu & Hsieh, 1969). Besides, the variable values of CHU against chicken, human and sheep blood at different temperature and pH indicate the dependence of hemolysin production to temperature and pH which follows the pattern of hemolysin II production (Andreeva *et al*., 2007).

The present study demonstrates that most members of *B. cereus* group produce a variety of hemolysins which are active against erythrocytes of different sources.

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