Jour. Microbiol. December 1995 p. 289-294 Vol. 33, No. 4

Physiological Characterization of Kinetics and Action Mechanism of Vibrio Hemolysin

Youngchool Choe and Gajin Jeong*

Department of Microbiology, Research Center for Molecular Microbiology
Seoul National University Seoul 151-742, Korea

(Received November 8, 1995/Accepted November 21, 1995)

The action mechanism of hemolysin rendering virulency of *Vibrio anguillarum* has not been clarified as yet, even though there were several possible factors explained. We have studied hemolytic kinetics perfomed by hemolysin from *V. anguillarum* strain V7 as well as binding of hemolysin to RBC membrane. Maximal rate of hemolysis and duration of lag phase were directly and inversly correlated to the concentration of hemolysin used. Hemolysin molecules are known to bind consumptively to RBC membrane. The hemolysis could be prevented by dextran, an osmotic pressure protectant with proper diameter, while other protectants with smaller diameter could not. In conclusion, hemolysin should bind irreversibly to RBC membrane exert hemolysis distorting osmotic pressure. The binding could be hindered by spatial structure of the RBC surfacem which might be caused by sialic acid.

Key words: hemolysin, *Vibrio anguillarum*, kinetics, action mechanism

Vibriosis is caused by *Vibrio anguillarum,* a Gram negative halophilic bacterium. Intensive studies on the pathogenicity of vibriosis suggested various factors and products such as siderophore (11), outer membrane protein (1, 2), and extra cellular toxin (14, 15, 18). Iron deficiency in vibriosis (10) was believed to be one of the most possible explanations turning on some unknown metabolic pathways under an unfavourable condition. Therefore, it is essential to analyse the action mechanisms of hemolysin to understand how the host could lose irons during vibriosis. Nevertheless, the mechanism of how this pathogenic microbe develops vibriosis is not clear as yet.

We have previously reported that vibriosis could be rendered by an outer membrane protein of *V. anguillarum* (14) and by hemolysin (8). In this study, we have evaluated the kinetics of hemolysis as well as the binding property and osmotic disturbing characteristics of hemolysin from *V. anguillarum V7.*

Materials and Methods

Strains

Vibrio anguillarum strain V-7 (NCMB 6, serotype J01) was kindly provided by Professor Kimura of Hokkaido University, Japan in 1987 and maintained in our laboratory thereafter. To keep the virulency, *in vivo* passages were carried out with a group of gold fish *Cyprinus caprio* L. weighing 4~5 g each. They were injected intraperitoneally with V-7 cultured in tryptic soy broth supplemented with 1.5% NaCl at 22~23℃. Media for bacterial culture were purchased from Difco (Detroit, MI), unless otherwise mentioned. Through *in vivo* passages, virulent bacteria were recovered from the peritoneal cavity and spleen of dead fish on tryptic soy agar (TSA) which was supplemented with 1% NaCl when incubated at 22℃ for 24~48 hrs. They were always identified by O/129 test and culture in TCBS medium (8).

Preparation of RBCs

Heparinized normal peripheral blood from healthy volunteers with blood type O was diluted in an equal volume of Hanks' Balanced Salt Solution (HBSS) and separated from leukocytes using Lymphocyte Separation Medium (LSM ρ= 1.077, Flow Laboratory, North Ryde, N.S.W., Australia). Recovered RBCs from the sediment after centrifugation at 1,000×g for 20 min were washed twice in Alserver's solution to make the final concentration

* To whom correspondence should be addressed.

40% and kept at 4℃ until use. RBC preparation was washed twice again before use in 10 mM phosphate buffered saline (PBS) solution and finally adjusted to 1% solution (v/v, 1.1×10^8 cells/ml) or 1.5%.

Hemolytic activity

Virulent bacterium was cultured in TSB and human RBC suspension was added to estimate the different hemolytic activities (7). Bacterial cultures were adjusted to $A_{600}=0.5$ before evaluation and added to equal volume of 1.5% human RBC suspension. Reaction was carried out at 25℃ for 20 min. Lysed RBCs were quantified by measuring absorbency of supernatant at 540 nm (Shimadzu Model UV-265).

Hemolytic activity in the culture supernatant was measured as previously described (17) with some modification. In brief, each of hemolysin in two fold serial dilution was added to the equal volume of 1% RBC suspension. With gentle stirring for 30 min, hemolytic activity was evaluated in an hour by measuring absorbency at 540 nm. The maximum dilution to lyse 50% RBCs was converted to 50% hemolysis unit (HU_{50}).

Lysis of RBCs at room temperature was continuously detected by spectrophotometer (Shimadzu UV-265) according to Rennie *et al.* (16). The initial optical density at 650 nm of RBCs in PBS was adjusted to 0.7, which was equivalent to 0.25% v/v. Hemolysin preparation in various concentrations (8, 16, 32, 64, 128, and 256 HU_{50}/ml) was mixed in equal volume and changes in A_{650} was detected every 20 seconds (light path=1 cm). Hemoglobin from RBCs did not affect the optical density at 650 nm. The rate of hemolysis in every 20 seconds was converted into the decrease of absorbancy ($-dA_{650}/20$ sec). Lag phase was defined as the range where the rate of hemolysis was less than 0.02.

Effect of neuraminidase treatment on RBCs for hemolytic activity

Neuraminidase (type VIII from *Clostridium perfrigens*, Sigma) has been treated to RBCs in order to see if hemolysis could be affected by hemolysin. Normal and neuraminidased RBCs were added with hemolysin at the final concentration of 0.25, 0.5, and 1 HU_{50}. Hemolytic activity was measured as described above.

Effect of chelating reagnets on Hemolysin

The final concentration of 10 mM solution of sodium citrate (Oriental Chemical Inc., Seoul, Korea) and ethylene-diaminetetraacetic acid (EDTA; Sigma Chemical Co., St. Louis, MO) in PBS was reacted with 1HU_{50}/ml. Chemicals were purchased from Sigma unless otherwise mentioned.

Attribution of hemolytic activity to phospholipase C (NPPC Assay)

The presence of phosphlipase C was detected according to the modified method of Berka *et al.* (3) by measuring the absorbancy at 405 nm of the supernatant after the addition of one part of p-nitrophenylphosphorylcholine (NPPC) to three parts of the bacterial culture in TSB. *Psuedomonas aeruginosa* ATCC 25619 was used as the positive control after the hemolytic activity was confirmed on blood agar plates.

Effects of osmotic protectants

Final concentration 30 mM of Sucrose (molecular diameter 0.9 nm), Raffinose (Janssen Chimica, molecular diameter 1.2~1.4 nm), and dextran (m.w. 8,800) was reacted with 1 HU_{50}/ml according to the method of Bhakdi *et al.* (4).

Binding of hemolysin onto the target cell membrane

We followed the method of Berka *et al.* (3) to reveal whether binding of hemolysin molecule to the target cell membrane is essential for hemolysis. Human RBCs at 1% was mixed with an equal volume of hemolysin to get final concentration of 1 HU_{50}/ml. RBCs in one mixture were coated with dextran (20 mM) to be osmosis-protected at room temperature. After an hour, supernatant was removed to measure the optical density at 540 nm (primary hemolysis), and cells were resuspended to the previous volume in PBS. A_{540} was observed again after 30 min (hemolysis after washing). To the supernatant removed, new RBCs were added and kept at room temperature for an hour, then it was checked again if RBCs were further lysed (secondary hemolysis).

Results and Discussion

Kinetic study on hemolysin activity

Activity of hemolysin upon addition into RBC suspension was detected by continuously measuring observance at 600 nm. Hemolysis occured through initial lag phase, phase of accelerated hemolysis, and phase of declining rate as was previously reported (16). The result was shown both by hemolytic activity and by rate of hemolysis (Fig. 1). Analysing the kinetics of hemolysin, it was revealed that when concentration of hemolysin was high enough there was short lag phase and strong hemolysis (Fig. 2).

It has been suggested for *E. coli* that hemolysin binds to RBC like an ionophore making channels through which ion transport becomes available, espeicially Ca^{++} activating ATPase. After ATPase activation, unique stru-

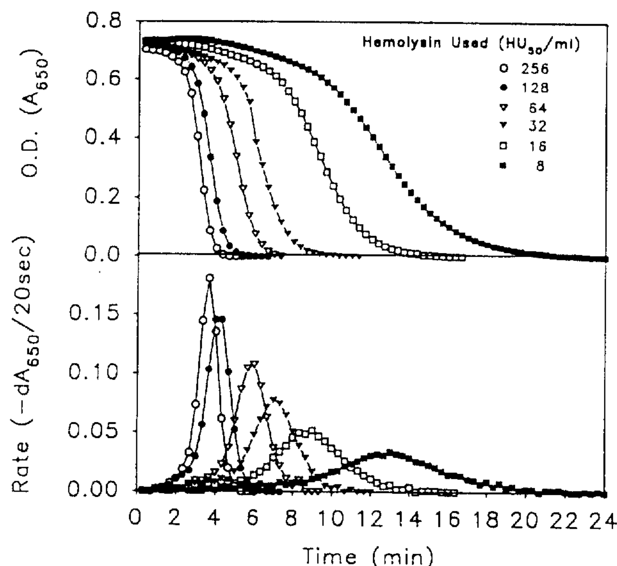

Fig. 1. Hemolysin kinetics from *Vibrio anguillarum* V7. The reduction of A_{650} was continuously estimated at 20 sec intervals induced with hemolysin at the given concentrations (upper pannel). The hemolysis rate ($-dA_{650}$/20 sec) for each reaction with different concentration of hemolysin was calculated from the kinetic study.

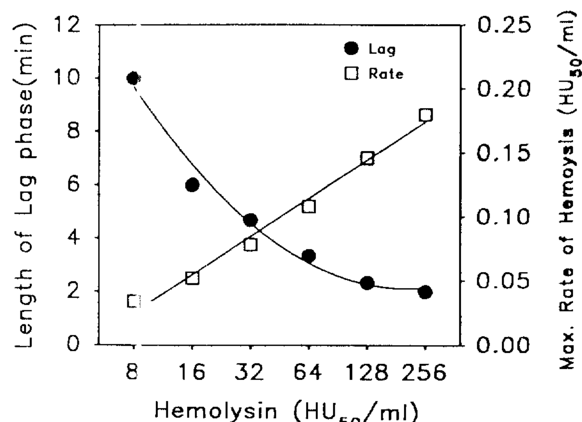

Fig. 2. The length of lag phase and maximum rate of hemolysis were in directly and inversely proportional to the concentration of hemolysin, respectively.

Table 1. Effect of chelating agents on hemolytic activity from Vibrio hemolysin

	Concentration (mM)	Hemolysis (A_{540})	Relative activity (%)
Control	None	0.675± 0.024[a]	100.0
EDTA	10	0.824± 0.010	122.1
Na-Citrate	10	0.762± 0.011	112.9

[a] mean± standard error of the mean (S.E.M.).

ctural transformation (17) induced by changes of spectrin-actin cytoskeletion or lipase activity was expected but not clearly proved yet.

In addition, rapid Ca^{++} influx and K^{+} outflux was also suggested as one of the possible explanation for hemolysis (4). Rennie *et al.* (16) proposed that hemolytic reaction in *E. coli* occurs irreversibly and non-enzymatically as well from the finding that duration of lag phase is inversely proportional to logarithm of hemolysin amount. Inasmuch as duration of lag phase should be inversely proportional to arithmetic of hemolysin amount, if hemolysis was processed in enzymatic reaction.

The process of hemolysis by Vibrio hemolysin from strain V7 seems to have similar properties, but it can not be clear until ruling out the possible presence of other active molecules such as proteolytic enzymes by purification of hemolysin.

Effects of chelators on hemolytic activity

To understand the nature of action mechanism for Vibrio hemolysin, EDTA or sodium citrate, as chelating agent, was added to observe if hemolysis could be affected. Both reagents could not inhibit hemolytic activity at 10 mM concentration. For hemolysin from *E. coli*, Ca^{++} is absolutely required for activity (16, 19).

It is not clear how it exerts hemolysis, but it is known that Ca^{++} is required for production and activity of hemolysin making the molecule firmly bound to RBCs (5). Short and Kurtz (17) also showed that hemolysis was abolished upon the addition of chelating agent such as EDTA or sodium citrate, while others found EDTA and EGTA did not affect hemolysis at all (4, 7).

From this study, hemolysis was rather stimulated when 10 mM EDTA or sodium citrate was added, implying the possibility that cation may not be essentially required for Vibrio hemolysin (Table 1).

Protease Inhibitors on hemolysin activity

EDTA, as metalloprotease inhibitor, and PMSF and leupeptin, as serine protease and thiol protease at the same time, were added to RBC reaction mixture at the concentration of 10 mM, 1 mM, and 1 μM, finding hemolytic activity was not affected. Many pathogenic microorganisms were reported to produce extracellular protease as virulency factor(s). Metalloprotease had been found stimulating toxic activity from *V. cholerae*, meanwhile proteolytic enzyme, sensitive to protease inhibitors, was detected in *V. mimicus* (6). In addition, collagenase from *V. vulnificus* (18), proteolytic enzyme fatal to mice or gold fish (12), and metalloprotease with m.w. of 40 kd (9) were also intensively studied as the important virulency factor(s).

These proteolytic enzymes play an important role in destruction of mucous layer and tissue and in activation of exotoxins such as cholerae and diphtheria toxin. St-

Table 2. The phospholipase C activity of *V. anguillarum* V7.

Temperature	Bacteria	Time (Hr)		
		1	4	16
25℃	None[a]	0.022[d]	0.019	0.030
	P. Aeruginosa[b]	0.190	0.404	1.093
	V. anguillarum[c]	0.050	0.113	0.581
37℃	None	0.016	0.015	0.016
	P. aeruginosa	0.283	0.591	1.056
	V. anguillarum	0.074	0.174	0.727

[a] PBS only was used as the control.
[b] *Pseudomonas aeruginosa* ATCC 25619.
[c] A_{600} of the bacterial suspension was adjusted to 0.5.
[d] Enzymatic activity was estimated by A_{405}.

rains V7 and V104 of *V. anguillarum* also have proteolytic activity (14). Therefore it is important to estimate if the proteolytic activity is directly related to hemolysis in vibriosis. In conclusion, the hemolytic activity was not affected by protease inhibitors (data not shown).

Activity of phospholipase C

Pseudomonas aeruginosa infecting pulmonary organs has been intensively studied for its phospholipase C (PLC) activity, which degrades phosphatidylcholine into phosphorylcholine and diacylglycerol acting as a heat-labile hemolytic toxin (3). PLC activity of *V. anguillarum* was evaluated with *Ps. aeruginosa* as a control (Table 2). Even though *V. anguillarum* did not show strong hemolytic activity on blood agar plate when compared with *Ps. aeruginosa,* it evidently had PLC activity in NPPC assay.

Hemolysin from culture supernatant of *V. anguillarum* reacted very slow, and it is not clear as yet if the hemolytic activity was rendered by PLC. Small hemolytic zone on blood agar plate developed by *V. anguillarum* colonies might have been explained that hemolysis was performed non-enzymatically as was discussed before, and it was thought that hemolysin was consumed up after binding to RBCs.

Hemolytic activity change with neuraminidase treatment on RBCs

Since it was suspected that hemolysin was binding to the surface of RBCs, it was estimated if neuraminidase digestion of sialic acid could facilitate binding of hemolysin onto RBCs. RBCs with neuraminidase treatment was relatively more fragile to hemolysin (Fig. 3). Microbial toxins modifies host cell metabolism or destroy cells after they bind to receptor molecules on target cells (11). It is also well known that if toxic molecules binds to receptors for hormone or cytokine molecules disturbing normal cellular metabolism. Host specificity shown by pathogenic microorganisms can be explained in part as ligand blinding to receptor molecules generally expressed on the surface of given tissues only. When surface ganglioside GM of *V. cholerae* was removed from the cell membrane, cholera toxin could not affect cellular metabolism at all.

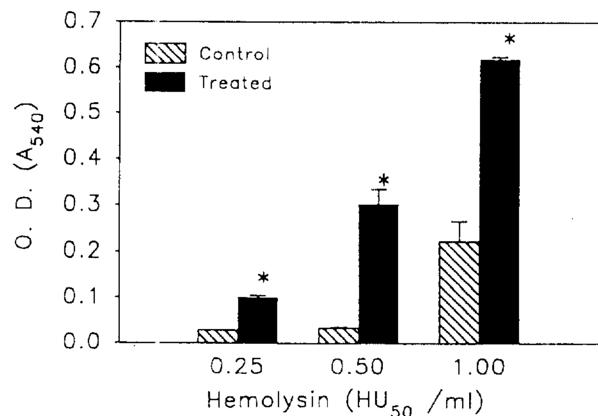

Fig. 3. Facilitation of hemolysin binding to RBCs by neuraminidase treatment. Hemolysin could easily bind to the surface of RBCs when surface sialic acid was removed by neuraminidase, implying that hemolysin is binding to RBC membrane with spatial hindrance.

Table 3. The hemolysis prevented by osmotic protectants.

Protectant	Size (nm)	Concentration (mM)	Inhibition (%)[a]
Sucrose	0.9	30[b]	20.0
Raffinose	1.2~1.4	30	22.4
Dextran	>3.0	30[c]	99.6

[a] compared with none-treated control. Hemolysin was used at $1HU_{50}$/ml.
[b] no comlete hemolysis at higher concentration.
[c] complete hemolysis detected even at 20 mM concentration.

Furthermore, sialic acid or glycoprotein containing sialic acid residue is not used as the receptor for hemolysin molecules. Neuraminidase removed sialic acid making it easy for hemolysin to bind onto certain receptor molecules or to penetrate into plasma membranes.

Osmotic pressure protectant effects on hemolysis

On the assumption that hemolysis could be exerted via disturbance of osmotic pressure between in and outside of plasma membrane by binding of hemolysin onto RBCs, osmotic pressure protectant was evaluated to prevent hemolysis. As summarized in Table 3, only dextran could protect RBCs from hemolysis at final concentration of 30 mM. Sucrose and raffinose, at the same concentration, did not affect hemolysis. Among three, dextran was the largest molecule with molecular diameter larger than 3 nm. When *E. coli* hemolysin, 107 kd, bound to RBC

Table 4. Consumptive binding of hemolysin to RBC membrane

Activity (A_{540})	Hemolysin only ($1HU_{50}$/ml)	Dextran protected (20 mM)
Primary hemolysis[a]	0.558	−0.002
After washing in PBS[b]	0.008	0.498
Secondary hemolysisc	0.008	0.001

[a] activity was measured 1 hr after initiation of reaction.
[b] hemolysis detected after washing only.
[c] remaining hemolytic activity from supernatant of primary hemolysis.

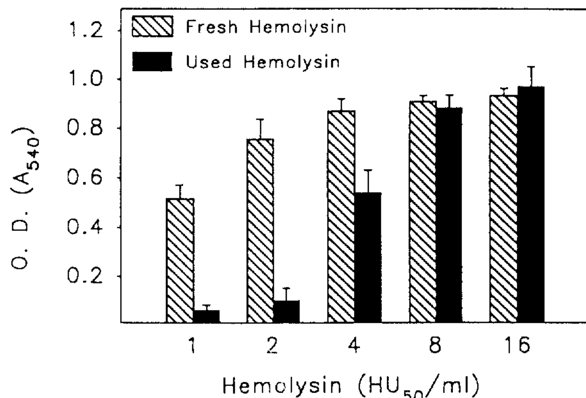

Fig. 4. Consumptive binding of hemolysin to RBCs. Primary (fresh) hemolysis used up bound hemolysin, and remaining activity in the supernatant was evaluated as secondary (used) hemolysis. As was discussed in the text, hemolysin is used up once bound to RBCs.

surface, it reacted like an integrated protein and remained as an 80 kd protein after trypsin treatment (4). As was observed in Vibrio hemolysin, hemolysis by *E. coli* could be also protected only with dextran. Furthermore, outflux of K^+, and influx of Ca^{++}, [^{14}C]mannitol, and [^{3}H]sucrose was detected, but no [^{3}H]dextran transport was recognized. This suggests that hemolysin develop pores 3 nm in diameter on the surface of RBCs.

Binding of hemolysin onto plasma membrane of RBCs

No hemolysis was observed with RBCs protected with dextran even 1 hr after hemolysin was reacted. These RBCs, then, were quickly washed twice in PBS and kept still at R.T. for another hour. Clear hemolysis was detected as if they were challenged with the same amount of hemolysin in the absence of protectant (Table 4). This is in the same context with the results of Bhakdi *et al.* (4), implying that hemolysin could bind to cellular membrane even in the presence of dextran, and hemolysin, in turn, could exert its activity when dextran was completely removed.

In addition, supernatant from reaction mixture either protected or non-protected with dextran was transferred to new RBC suspension to estimate remaining hemolytic activity after the first reaction. Both supernatant did not show much activity in the second reaction, and it confirmed again that hemolysin is irreversibly binding to cellular membrane of RBC (Fig. 4).

Acknowledgement

This study was supported in part by SNU Dae Woo Research Fund 91-06-2059 and by the research grant from Ministry of Education, Korea, BSRI-94-4414.

References

1. **Actis, L.A., W. Fish, J.H. Crosa, K. Kellerman, S.R. Ellenberger, F.M. Hauser, and J. Sanders-Loehr,** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). *J. Bacteriol.* **167**, 57-65.
2. **Actis, L.A., M.E. Tolmasky, D.H. Farrel, and J.H. Crosa,** 1988. Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid mediated iron-transport system. *J. Biol. Chem.* **263**, 2853-2860.
3. **Berka, R.M., G.L. Gray, and M.L. Vasil,** 1981. Study of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa. Infect. Immun.* **31**, 1071-1074.
4. **Bhakdi, S., N. Mackman, J.M. Micaud, and I.B. Holland,** 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *J. Bacteriol.* **152**, 239-245.
5. **Boehm, D.F., R.A. Welch, and I.S. Snyder,** 1990. Calcium is required for binding of *Escherichia coli* hemolysin (Hly A) to erythrocytes membranes. *Infect. Immun.* **58**, 1955-1958.
6. **Brubaker, R.P.,** 1985. Mechanism of bacterial virulence. *Ann. Rev. Microbiol.* **39**, 21-50.
7. **Cavalieri, S.J. and I.S. Snyder,** 1982. Cytotoxic activity of partially purified *Escherichia coli* alpha-hemolysin. *J. Med. Microbiol.* **15**, 11-21.
8. **Choe, Y. and G. Jeong,** 1994. Virulence evaluation of *Vibrio anguillarum* in accordance with hemolytic activity on Human Red Blood Cells. *Kor. Jour. Microbiol.* **32**, 539-544.
9. **Crosa, J.H. and L.L. Hodges,** 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum. Infect. Immun.* **31**, 223-237.
10. **Crosa, J.H., M.H. Schiewe, and S. Falcow,** 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum. Infect. Immun.* **18**, 509-513.
11. **Eidels, K., R.L. Proia, and D.A. Hart,** 1983. Membrane receptors for bacterial toxins. *Microbial. Rev.* **47**, 596-620.
12. **Kanemori, Y., K. Muroga, and T. Nakai,** 1987. The role of extracellular products of *Vibrio anguillarum. J. Fish Dis.* **6**, 461-471.

13. **Kodama, H., M. Mustafa, T. Mikami, and H. Izawa,** 1985. Partial purification of extracellular substance of *Vibrio anguillarum* toxigenic for rainbow trout and mouse. *Fish Pathol.* **20**, 173-179.
14. **Lee, J.S., G. Jeong, and Y.C. Hah,** 1994. Characterization of the nature of virulency caused by *Vibrio anguillarum*: Identification of an outer membrane protein rendering virulency. *Kor. Jour. Microbiol.* **32**, 531-538.
15. **Munn, C.B.,** 1980. Production and properties of a hemolytic toxin by *Vibrio anguillarum. In* Fish disease, Third COPRAQ session. Ed. Ahne, W., p. 69-74, Springer-Verlag, Berlin.
16. **Rennie, R.P., J.H. Freer, and J.P. Arbuthnott,** 1974. The kinetics of erythrocyte lysis by *Escherichia coli* hemolysin. *J. Med. Microbiol.* **7**, 189-195.
17. **Short, E.C. Jr. and H.J. Kurtz,** 1971. Properties of the hemolytic activity of *Escherichia coli. J. Bacteriol.* **9**, 763-767.
18. **Smith, I.W.,** 1961. A disease of finnock due to *Vibrio anguillarum. J. Gen. Microbiol.* **24**, 247-252.
19. **Snyder, I.S. and N.A. Koch,** 1966. Production and characterization of hemolysis of *Escherichia coli. J. Bacteriol.* **9**, 763-767.