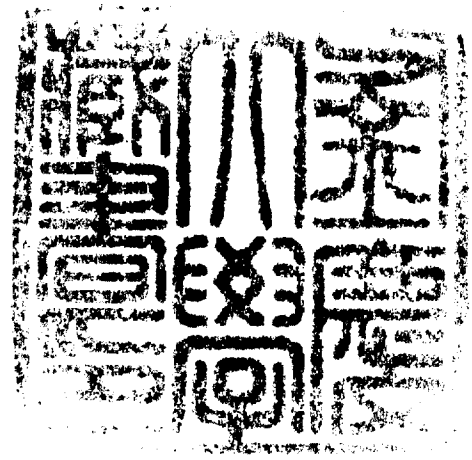
Characterization of hemolysin produced by NAG vibrio PFM5 isolated from seawater

해수에서 분리한 NAG vibrio PFM5가 생산하는 용혈독소에 관한 연구

Advisor : Dong-Suck Chang

by

Ki-Hyun Kim



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A Dissertation

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Characterization of hemolysin produced by NAG vibrio PFM5 isolated from seawater

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Abstract

NAG virbio has been reported to cause gastroenteritis, and secretes a pore-forming hemolysin, which causes hemolysis of erythrocytes. The author isolated several Non O1 agglutination *Vibrio cholerae* from sea water. Among the isolated strains, one strain which has the strongest hemolytic activity against sheep erythrocytes was chosen. The chosen strain named NAG vibrio PFM5 was submitted in this study. It was confirmed that the chosen strain has cholerae toxin gene by PCR with VCT1 and VCT2 as CT gene detection kit. NAG vibrio PFM5 was cultured

in various media. Specific activity of ammonium sulfate precipitate collected from the culture supernatant was stronger in modified marine broth than in any other media such as brain heart infusion, heart infusion and marine broth 2216. The crude hemolysin from modified marine broth was partially purified by using phenyl sepharose high performance column 16/10 with FPLC. The specific activity of partially purified hemolysin was 1133HU. The hemolytic activity of hemolysin to the erythrocytes was dependent on the temperature. There was no hemolysis of erythrocytes at 4℃ for 180 min. While it appeared after transfer to 20, 37, 50℃. The optimum temperature for hemolysis was at 37℃. The relative hemolytic activities compare with that of optimum temperature(100%) were 75.9% at 50℃ and 54.4% at 2 0℃, respectively.

The changes of hemolytic activity was investigated under various cations such as Na^+, K^+, Li^+, Mg^{2+}, Ca^{2+} and Fe^{2+}. The concentrations of the used cations were 2mM. Hemolytic activity was almost completely inhibited by the reaction at 37℃ for 1 hr in the presence of Fe^{2+}, and also inhibited by 28% and 43% in the presence of Mg^{2+} and Ca^{2+}, respectively.

Adding EGTA without the cations had no effect on the hemolysis against sheep erythrocytes. While the protective action of Fe^{2+} sheep erythrocytes was decreased by adding 1mM EGTA.

EGTA was added to reaction buffer at the time maintaining protective action of Fe^{2+} to erythrocytes. There were little effect on the hemolysis unlike simultaneous addition of Fe^{2+} and EGTA.

It can be suggested that hemolysin and Fe^{2+} bind to erythrocytes competitively. It can be also suggested that Fe^{2+} may inhibit not the pore-forming stage, but the binding of hemolysin and erythrocytes. The mechanism of Fe^{2+} to hemolysin produced by NAG vibrio PFM5 seems to be different from that of the other cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}. These results were identical to the microscopic observation. The sheep erythrocytes without Fe^{2+} in the reaction buffer were rugged features, but they were normal features with Fe^{2+} in spite of after incubation for 60 min.

Indroduction

Vibrio cholerae non-O1 has been named as nonagglutinable *Vibrio cholrae* or NAG vibrio, since they do not agglutinate with O1 *V. cholerae* antiserum. *V. cholerae* non-O1 has also been reported to cause not only gastroenteritis, though typically less severe than that caused by *V. cholerae* O1 but also extraintestinal infections, septicemia, meningitis. It is biochemically and genetically indistinguishable from *V. cholerae* O1 except serological difference.

Vibrio cholerae non-O1 can be found in seawater, estaurine water and also in foods. It produces thermostable direct hemolysin (NAG-rTDH) similar with that of *Vibrio parahaemolyticus*. TDH has a variety of biological activities, including hemolytic activity, cytotoxicity on red blood cell and intestine cell(Honda et al., 1985; Craig et al., 1981; Balakrish et al., 1992).

As a result of rabbit ligated intestinal loop test, TDH caused fluid accumulation and is considered to be the major virulent factor (Gyobu et al., 1988, 1992). Hemolysis by TDH has been thought to have some serial processes. Recent studies suggest that TDH bind to the erythrocye surface, and then form pores on the membrane. These pores increase the cation permeability of the

cell membrane and finally cause colloidal osmotic lysis of erythrocytes (Shinoda et al., 1985; Tang et al., 1994; Zitzer et al., 1995; Miyake et al., 1989).

There are many reports on *V. cholera*e non-O1 in foreign countries but few in Korea. Besides there are few research for TDH produced by *V. cholerae* non-O1 compared with *V. parahaemolyticus*.

The author isolated several strains of NAG virbio from seawater and identified hemolytic activity on sheep blood agar medium. The strongest strain in hemolytic activity was selected among those strains, and then named as NAG vibrio PFM5. The biochemical and genetical characteristics were confirmed with API kit system and polymerase chain reaction (PCR). The examined items were to find better medium for hemolysin pruduced by NAG vibrio PFM5, purification of hemolysin with FPLC, effects of cations, EGTA and temperature on the hemolytic activity and so on.

Materials and Methods

Ⅰ. Materials

1. strain

The strain submitted in this study was isolated from seawater and named as NAG vibrio PFM5. *Vibrio cholerae* non-O1 ATCC 25872 was used as reference strain.

2. Media and kit for rapid test

Media for production of hemolysin was brain heart infusion (BHI), heart infusion (HI), marine broth 2216 added 3% glycerol (MB), marine broth 2216 added 3% glycerol and 1% tryptose (modified marine broth, MMB). All media were products of Difco laboratories(USA). API kit system (BioMerieux, France) was used for identification of NAG vibrio PFM5. Reagents for hemolytic activity measurement and protein assay were products of Sigma Co.(USA).

3. Equipments and facilities

Thermocycler (Bio-Rad, Japan) and VCT1 & VCT2 (Takara, Japan) were used for detection of CT gene. FPLC

and phenyl sepharose column 16/10(pharmacia LKB, Sweden) was used for purification of hemolysin.

Ⅱ. Methods

1. Selection of strain

NAG vibrios were isolated from seawater and its biochemical characteristics was identified by classical method and API kit system. This strain was cultured on tryptic soy agar plate containing 5% sheep blood for 24hrs at 37℃. The strongest strain in hemolytic activity was selected among those strains, and then named as NAG vibrio PFM5.

2. Detection of CT gene

Genetic characteristics was confirmed by operating gene thermocycler. PCR was done with primers VCT1 & VCT2 for detection of CT gene from NAG vibrio PFM5 isolated from sea water. Amplification products were analyzed by electrophoresis on a 1% agarose gel.

3. Effects of culture media on the hemolysin production

NAG virbio PFM5 was cultured in various media such as BHI, HI, MB, MMB. The crude hemolysin was obtained as described

below and then hemolytic activity was compared.

4. Preparation of hemolysin

1) Crude hemolysin

The hemolysin was purified by the modified method of Oh et al. (1993). NAG vibrio PFM5 was inoculated in marine broth 2216 and precultured at 26℃ for 8hrs. After preculture, 5ml of culture fluid reinoculated in 400㎖ of modified marine broth and shanking-cultured at 26℃, 150rpm for 18hrs. Culture supernatant including hemolysin was precipitated with 50% saturated ammonium sulfate at 4℃ for 6 hrs. The precipitated protein was washed and collected with 50% ammonium sulfate solution. After centrigugation at 6,000 rpm for 20 min, the pellet was dissolved in 10mM Tris-HCl buffer (pH 7.0). The supernatant after centrifugation at 12,000 rpm for 30 min was used as crude hemolysin(Fig. 1).

2) Purification of hemolysin

Partially purified hemolysin was obtained from the crude hemolysin by using phenyl sepharose high performance FPLC column 16/10 (Pharmacia LKB, Sweden).

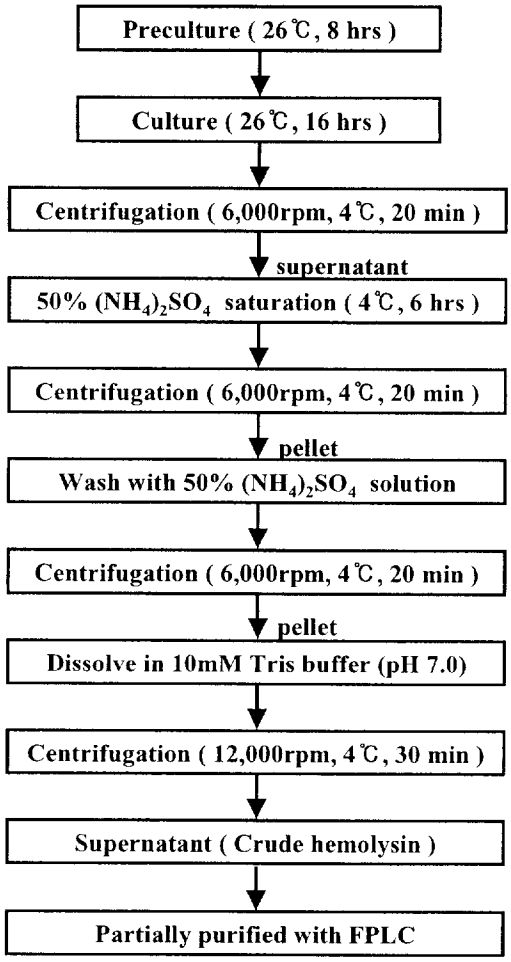


Fig. 1. Purification scheme of the hemolysin produced by NAG vibrio PFM5.

Column was equilibrated with 100mM Tris-HCl buffer at pH 7.0. After injection of crude hemoysin (20㎖), materials not associated with column was eluted with the same buffer. Then, associated hemolysin was eluted with 20mM Glycine buffer (pH 10.0). Flow rate was 0.5㎖/min and fraction collector included 4.5㎖ partially purified hemolysin.

5. Measurement of hemolytic activity

1) Sheep erythrocytes suspension

Sheep erythrocytes were used for hemolytic activity assay, which was carried out by the method of Oh et al. (1993). Sheep blood was washed with 0.85% NaCl buffer. After washing, the solution was centrifuged at 700 rpm for 30 min and supernatant was removed. Sheep erythrocytes obtained from repetitious treatment were suspended in 0.85% NaCl buffer at 1% concentration.

2) Hemolytic activity

Hemolytic activity assay solution is 10mM Tris-HCl buffer containing 0.85% NaCl, 0.01% bovine serum albumin and 0.04% sodium azide. The erythrocytes suspension (1% v/v) was added to reaction buffer containing hemolysin at same volume (1:1).

After centrifugation at 3,000 rpm for 5 min, the optical density of treated sample supernatant was measured at 540nm with a DR/4000U spectrophotometer (HACH, Japan). As negative controls, reaction buffer contained no hemolysin and as positive control, reaction buffer was substituted for same volume of distilled water. Hemolysis was calculated as a percentage of controls.

6. Protein assay

Protein assay was carried out by the modified method of Lowry et al. (1951). Protein assay solution is the mixture of 4% Na_2CO_3-0.2N NaOH, 2% $CuSO_4$ and 4% sodium potassium tartarate (100:1:1). Properly diluted sample solution was brought to 1㎖ with distilled water and reacted with same volume of protein assay solution for 10 min. After adding 100㎕ Folin phenol reagent, reaction solution was kept at room temperature for 30 min and the optical density was measured at 500nm with a DR/4000U spectrophotometer (HACH, Japan). Protein assay standard curve was presented by using 1㎎/㎖ bovine serum albumin (BSA, Sigma) as standard protein.

7. Effects of environmental factors on the hemolytic activity

1) Temperature

Sheep erythrocytes were incubated with the hemolysin (5HU/㎖) at 4℃ for 90 min. After 30 min incubation at 4℃, the suspension was transferred and incubated at 20℃, 37℃, 50℃ for 60 min. Hemolysis was calculated as a percentage of controls.

2) Cations

Various cations such as K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}, Fe^{2+} were added to hemolytic activity assay solution (final contration 2mM/㎖). All cations were chloride salts. Sample solution was incubated at 37℃ for 30 min. As positive control, no cation was added and relative hemolysis was calculated as a percentage of positive control.

Effect of Fe^{2+} on the hemolysis was examined changing concentration of hemolysin or reaction time. Reaction buffer was incubated at 37℃ for 30 min at various hemolytic unit (HU) or reaction buffer was incubated at 37℃ through 180 min. Hemolysis was calculated as a percentage of the positive control, respectively.

3) EGTA

Hemolysis of sheep erythrocytes was compared in absence or presence of EGTA (final concentration 2mM/㎖). Effects of EGTA on hemolysis were also examined in presence of Fe^{2+}.

8. Morphological changes of sheep erythrocytes by various treatments

Morphological change of sheep erythrocytes was examined in the absence or presence of Na^{+}, Fe^{2+} after 0, 30, 60 min, respectively. Morphological change was examined at a magnification of ×400 with bright field light microscopy (Olympus BX50, Japan).

Results & discussion

1. Characteristics of isolated strain

1) Selection of the strain

NAG vibrios were isolated from seawater and these biochemical characteristics were identified by classical method and API kit system. The strains producing beta-hemolysin on the sheep blood agar plate induced the lysis of red blood cells and finally it showed clear zone surrounding the colonies. The strongest strain in hemolytic activity was selected among those strains, and then named as NAG vibrio PFM5 (Fig. 2). Biochemical characteristics of NAG vibrio PFM5 was identical with *V. cholerae* non-O1 ATCC 25872 as the reference strain (Table 1).

2) Detection of CT gene

PCR was done with primers VCT1 & VCT2 (Takara kit) for detection of CT gene from NAG vibrio PFM5. As the result of PCR, CT gene (307bp) was detected from NAG vibrio PFM5 like *Vibrio cholerae* O1 and *Vibrio cholerae* O139 (Fig. 3). Biochemical and genetical characteristics of NAG vibrio PFM5 were shown in Table 1.

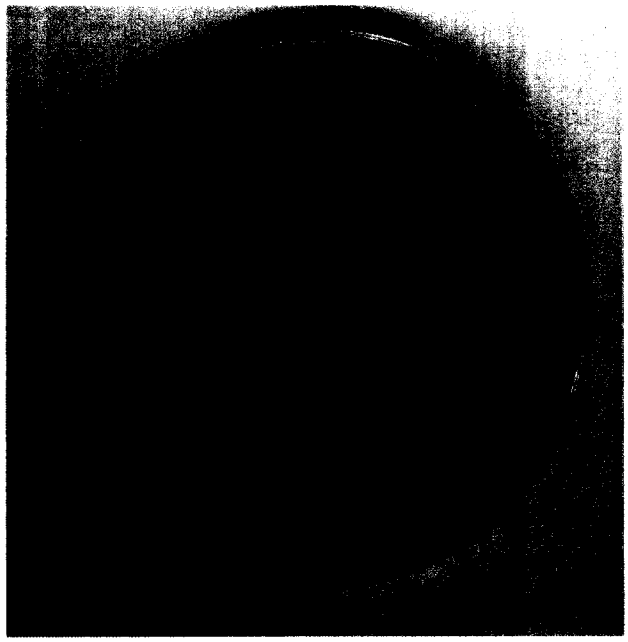


Fig. 2. Production of hemolysin on sheep blood agar plate.

Hemolysin were applied to the paper disk on tryptic soy agar plate containing 5% sheep blood and incubated overnight at 37℃.

Table 1. Biochemical and genetical characteristics of NAG vibrio PFM5 included in this study

Biochemical test	ONPG	+(+)	Biochemical test	Acid from	
	Arginine Dihydrolase	-(-)		Mannitol	+(+)
	Lysine decarboxyase	+(+)		Inositol	-(-)
	Ornithine decarboxylase	+(+)		Sorbitol	-(-)
	Citrate	-(-)		Rhamnose	-(-)
	H2S	-(-)		Sucrose	+(+)
	Urease	-(-)		Melibilse	-(-)
	Tryptophan deaminase	-(-)		Amygdalin	-(-)
	Indole	+(+)		Arabinose	-(-)
	Voges-Proskauer	+(+)		Galactose	+(+)
	Gelatin	+(+)	Sheep blood agar hemolysis		+
	Oxidase	+(+)	PCR	*CT* gene	+

· ( ) : reference strain (*Vibrio cholerae* non O1 ATCC 25872)

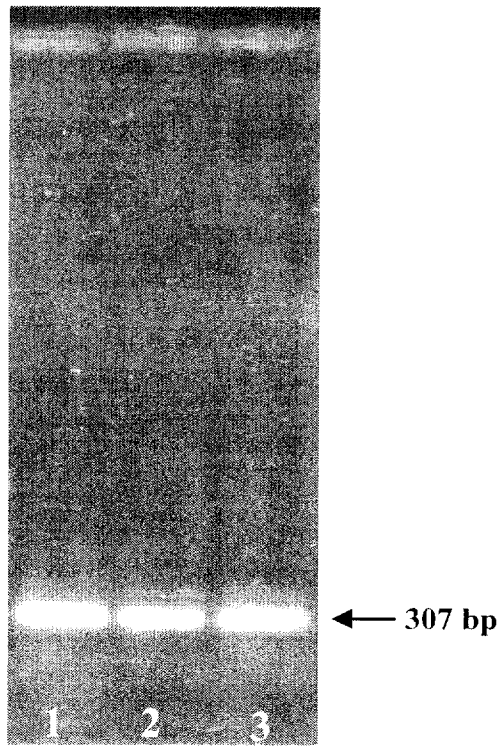


Fig. 3. The detection of CT gene from NAG vibrio PFM5 isolated from seawater.

PCR was done with primers VCT1 & VCT2 (Takara kit). Amplification products were analyzed by electrophoresis on a 1% agarose gel.

Lane 1 : NAG vibrio PFM5 isolated from seawater

Lane 2 : *Vibrio cholerae* O1

Lane 3 : *Vibrio cholerae* O139

Vibrio cholerae non-O1, non-O139 strains have been considered negligible significance, since they have been associated with illness only in a low percentage of patients hopitalized with secretory diarrhea. Furthermore, the majority of these strains isolated from the environment do not produce cholera toxin and lack the virulence gene cassette for cholera toxin. (Koch et al., 1993; Sharma et al., 1998; Singh et al., 2001). Recent studies reported that the non-choera-toxin-producing strains of *V. cholerae*, whether of clinical or environmental origin, possess the ability to produce a new secretogenic toxin that is entirely different from the toxin produced by toxigenic *V. cholerae* O1, O139 strains, and the susceptibility to CTXΦ, leading to the origination of potential new epidemic clones. (Rivera et al., 1995; Faruque et al., 1998; Jiang et al., 2000; Singh et al., 2001). However, the strain submitted in this study not only produced hemoysin but also had cholera toxin gene. It means that NAG vibrio PFM5 is very inportant bacteria which has serious potential hazard to the human health. Therefore, the author sincerely suggest that to study on NAG *vibrio* is one of the urgent problems not only for bacteriological field but also sanitary measurement for fisheries foods in Korea.

2. Effects of culture media on the hemolysin production

NAG vibrio PFM5 was cultivated in various media such as BHI, HI, MB, and MMB. There was little effect of protease on the production of hemolysin. Therefore, inactivator such like EGTA was not supplemented.

In case of MMB, 1% tryptose was added to get higher hemolytic activity of NAG virbio PFM5. It was known that an agar medium prepared with tryptose without tissue infusion is an excellent blood agar base and the growth of organisms on tryptose blood agar base is luxuriant and the zones of hemolysis produced are distinct and clear (Difco manual 11th Ed., USA). From these report, tryptose was applied to production of hemolysin from NAG vibrio PFM5. The crude hemolysin was obtained from these medium and then hemolytic activity was compared (Table 2). Total hemolytic activity was the highest in HI (747 HU/㎖). Heart infusion broth is widely applied to purification of hemolysin produced by *V. mimicus*, *V. parahaemolyticus*, and *V. cholerae*. However, the crude hemolysin from MMB showed the highest specific activity; MMB (108 HU/㎎) > MB (81 HU/㎎) > HI (38 HU/㎎) > BHI (7.5 HU/㎎).

Marine broth containing high concentration of salt, vitamins and minerals is more effective than the other tissue infusion

media for producing hemolysin (Bae, 2001). Addding tryptose to marine broth as nitrogen source enhanced the specific activity. Therefore hemolysin production for this study, MMB was applied to examine characteristics of hemolysin produced by NAG vibrio PFM5.

Table 2. Effects of cultivation media on the hemolysin production by NAG vibrio PFM5

Medium	Protein (㎎/100㎖)	Hemolytic activity (HU/㎖)	Specific activity (HU/㎎)
BHI	14.1	352	7.48
HI	5.8	747	64
MB	0.34	81	81
MMB	1.6	560	108

* 1 HU(hemolytic unit) is defined as the amount of hemolysin that cause 50% hemolysis

3. Hemolysin purification

NAG virbio PFM5 was incubated in MMB and precipitated with 50% saturated ammonium sulfate. And then, the crude hemolysin from MMB was partial purified by using phenyl sepharose colum with FPLC.

The amounts of protein and the hemolytic activity from culture supernatant, ammonium sulfate precipitate and partially purified hemolysin are shown in Table 3. Volume of culture supernatant was 1,600㎖ and the amount of protein was 12,960㎎. From the culture supernatant, 5.3㎎ of partial purified hemolysin was obtained, and the purification fold was 472 times. The specific activity was 1133 HU/㎎ and the yield of activity was 20%.

4. Effects of environmental factors on the hemolytic activity

1) Temperature

Sheep erythrocytes were incubated with the hemolysin (5HU/㎖) at 4℃ for 90 min. There was no hemolysis at 4℃ for 90 min. After 30 min incubation at 4℃, the suspension was transferred and incubated at 20℃, 37℃, 50℃ for 60 min, respectively. However, the hemolysis

Table 3. Speicific activity and purification fold of hemolysin produced by NAG virbio PFM5 cultivated in MMB broth

Purification step	Total			Hemolytic activity (HU/㎖)	Specific activity (HU/㎎)	Purification fold	Yield of activity (%)
	Volume (㎖)	Protein (㎎)	Activity (HU)				
Culture supernatant	1,600	12,960	30,400	19	2.4	1	100
Ammonium sulfate	30	156	16,800	560	108	45	55
Purified with FPLC	4.5	5.3	6,005	1,334	1,133	472	20

appeared after transfer to 20, 37, 50℃.

It is known that hemolysin produced by *V. cholerae* non-O1, *V. vulnificus* binds to erythrocytes temperature-independently at first stage, and forms pore at surface of erythrocytes temperature-dependently (Oh, 1993; Kim, 1997). The hemolysin obtained from NAG vibrio PFM5 was shown same pattern as reported others. The sheep erythrocytes were hemolyzed readily at 37℃ but were less sensitive to lysis at 50℃, 20℃. The optimum temperature for hemolysis was 37℃ (Fig. 4).

2) Cations

Various cations such as K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}, Fe^{2+} were added to hemolytic activity assay solution at final concentration 2mM/㎖ and 5mM/㎖. Hemolytic activity was almost completely inhibited by the reaction at 37℃ for 1hr in the presence of Fe^{2+}.

There were little inhibitory or promotive effects in the other cations at 2mM of cations as following results (Table 4). However, the hemolytic activity was inhibited by addding cations such as Mg^{2+}, Ca^{2+} at 5mM.

There have been various reports about the effects of cations on the hemolytic activity represented by many kinds of bacteria. The hemolytic reactions by various hemolysin have been documented to be inhibited by divalent cations. The hemolysins of

V. mimicus, V. vulnificus, V. metschinikovii were reported to be inhibited by divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} (Miyoshi et al, 1997;

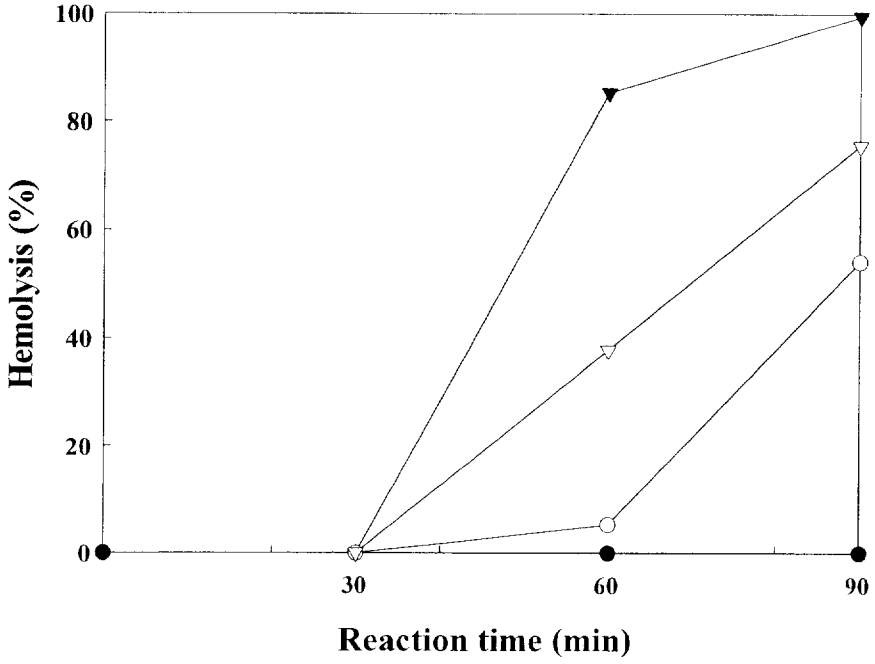


Fig. 4. Effects of temperature on the hemolytic activity.

Hemolysin mixture was stored at 4℃(●). After 30 min storage at 4℃, the mixture transferred and incubated at 20℃(○), 37℃(▼), 50℃(▽), respectively. Hemolytic activity was measured as in Table 2.

Table 4. Effects of cations on hemolytic action

Cation	Relative hemolytic activity (%)*	
	2mM	5mM
None	100	100
K^+	98	104
Na^+	97	110
Li^+	94	98
Mg^{2+}	94	72
Ca^{2+}	92	57
Fe^{2+}	0.5	<0.1

* Mixture of hemolysin (5HU) and sheep erythrocytes (1%) was incubated at 37℃ for each time in the absence or presence of various cations. The cations was added to reaction buffer at 2mM and 5mM. After centrifugation, relative hemolytic activity was measured.

Shinoda et al., 1985; Miyake et al., 1989). The result from this study was identical with the report of them. There was different report that the hemolytic activity of NAG vibrio is promoted by the addition of Ca^{2+}, Mg^{2+}, Mn^{2+} but inhibited by Zn^{2+} (Kim, 1997).

In this study, the author investigated the effect of cations on hemolytic activity. It was shown that the addition of various cations such as K^{+}, Na^{+}, Li^{+}, Mg^{2+}, Ca^{2+}, Fe^{2+} at low concentration (2mM), the inhibitory effect of Fe^{2+} was very strong. These results were interesting and following experiments were done to understand the mechanism with which Fe^{2+} functions as protectant from hemolysin to erythrocytes.

Effect of Fe^{2+} on the hemolysis was examined changing concentration of hemolysin. As the concentration of hemolysin increase, hemolysis of erythrocytes increased in the absence of Fe^{2+}. However, hemolysis of erythrocytes was inhibited by about 99% at 7HU in presence of Fe^{2+} (Fig. 5).

Effect of Fe^{2+} on the hemolysis was examined as reaction time (Fig. 6). Hemolytic activity assay solution was incubated at 37℃ through 180 min. Hemolysis was calculated at each time.

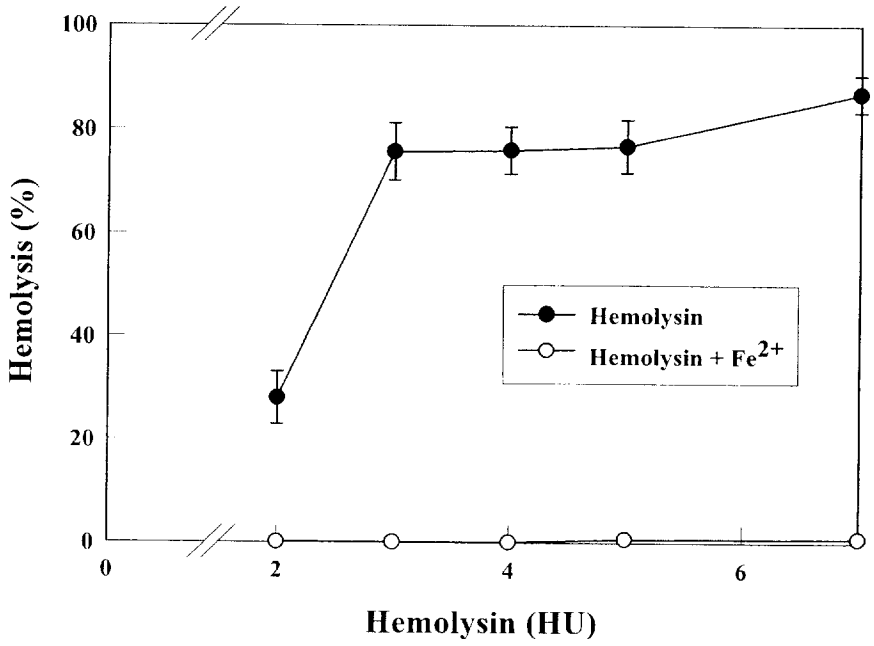


Fig. 5. Inhibition pattern of hemolytic activity by addding Fe^{2+} according to concentration of hemolysin.

Hemolytic activity was measured as in Table 2. The sample were incubated for 30 min. Concentration of Fe^{2+} was 2mM.

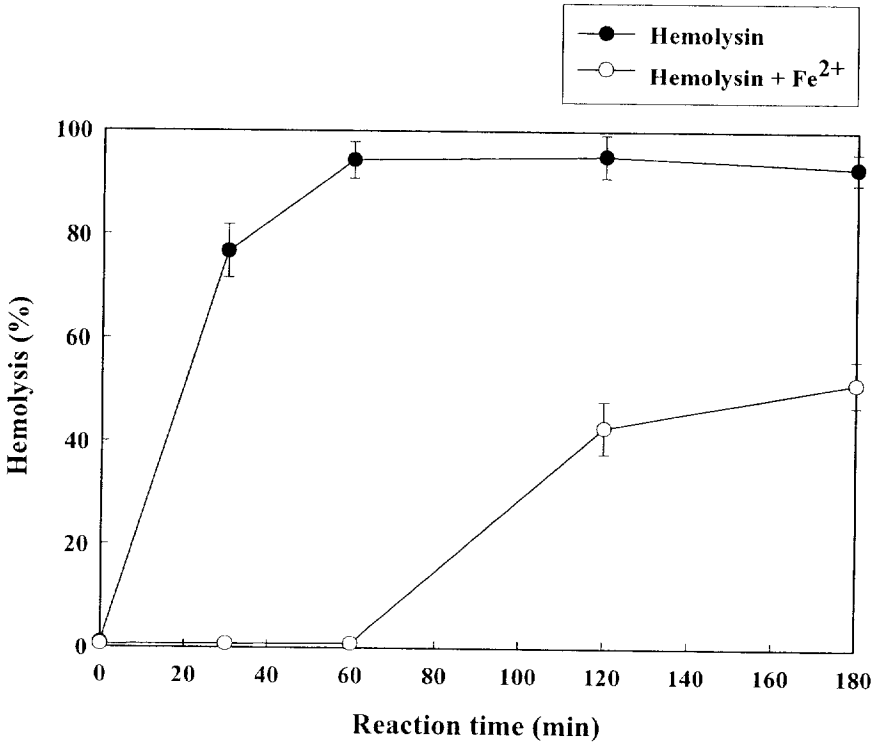


Fig. 6. Inhibition pattern of hemolytic activity by adding Fe^{2+} according to reaction time.

Concentration of Fe^{2+} was 2mM in reaction buffer containing hemolysin (5HU). Hemolytic activity was measured as in Table 2.

Hemolysis of erythrocytes increased until 180 min in the absence of Fe^{2+}. Hemolysis of erythrocytes was inhibited by about 99% (3, 5HU), 97% (7HU) until 60 min at 37℃ in the presence of Fe^{2+}. However, the hemolysis was increased after 60 min and the hemolysis was 3.7%(3HU), 51.6% (5HU), 67.9% (7HU) at 180 min, respectively. It was shown that the inhibitory effects of Fe^{2+} to erythrocytes decrease as concentration of hemolysin increase and reaction time is longer.

Fe^{2+} was added to hemolytic activity buffer after lysis of erythrocyes. Hemolysis of erythrocytes was 76.4% (none), below 1% (in the presence of Fe^{2+}) after 60 min at 37℃. However, adding Fe^{2+} after 10 min, the hemolysis was 59.2%. Inhibitory effect of Fe^{2+} was decreased rapidly (Fig. 7).

3) EGTA

It has been reported that hemolysin cause colloidal osmotic lysis of erythrocytes and divalent cations affect on the hemolysis. EGTA was examined how to effect on the hemolysis. Hemolysis of sheep erythrocytes was compared in absence or presence of EGTA (final concentration 2mM/㎖). EGTA did not inhibit hemolysin-induced hemolysis of erythrocytes (Fig. 8). This result was identical with the report of Naim et. al (2001).

Effects of EGTA on hemolysis were also examined in the

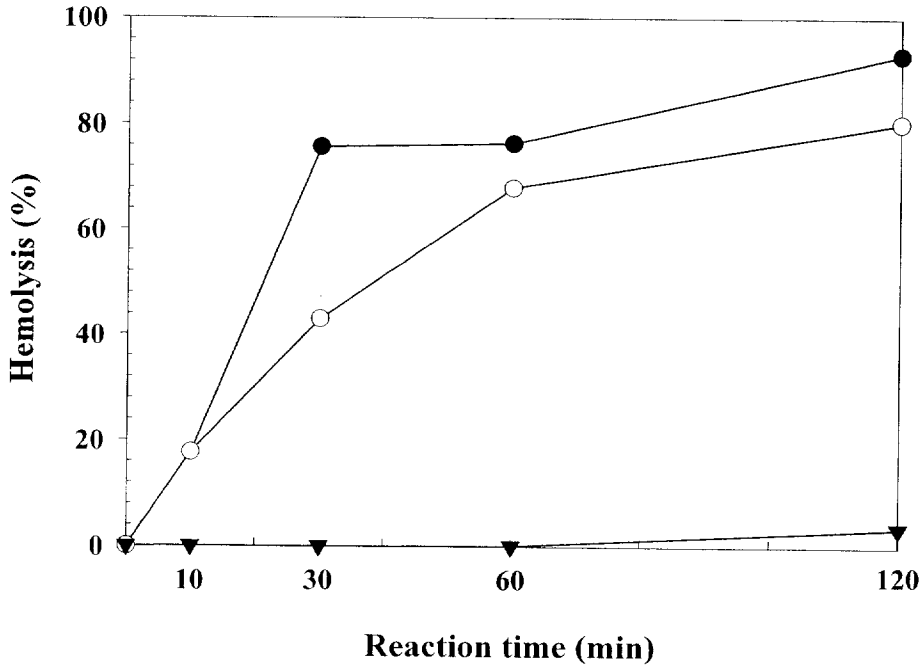


Fig. 7. Effects of adding Fe^{2+} on the hemolytic activity after hemolysis.

● : Control sample (hemolysin + 1% sheep erythrocytes)

▼ : 2mM of Fe^{2+} was added to control sample

○ : 2mM of Fe^{2+} was added to control sample incubated for 10 min at 37℃.

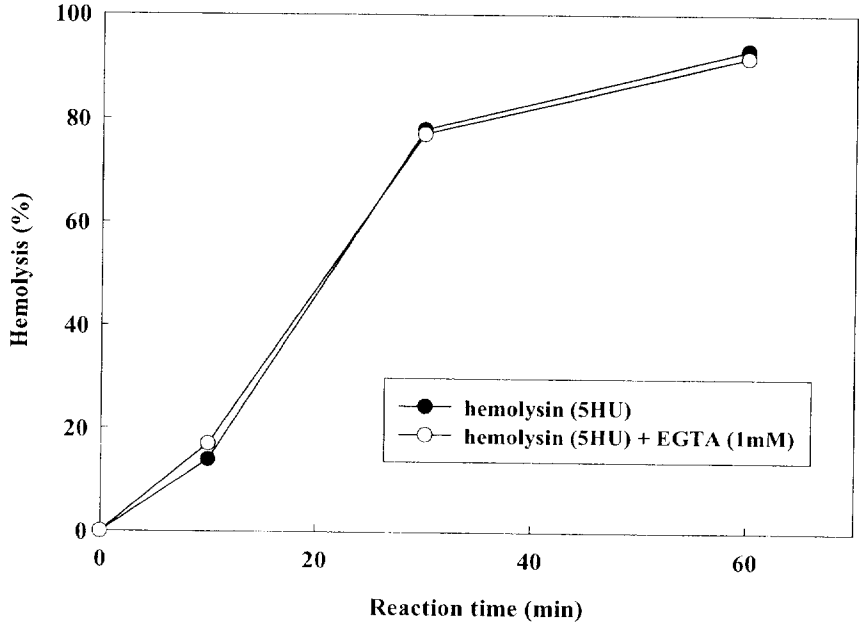


Fig. 8. Effect of EGTA on hemolysis of sheep erythrocytes.

presence of Fe^{2+}. The hemolysis was below 1% (3, 5HU), 41.5% (7HU) in the presence of Fe^{2+}. However, EGTA decreased the inhibitory effects of Fe^{2+} and the hemolysis was 17.8% (3HU), 43.3% (5HU), 66.2% (7HU), respectively (Fig. 9).

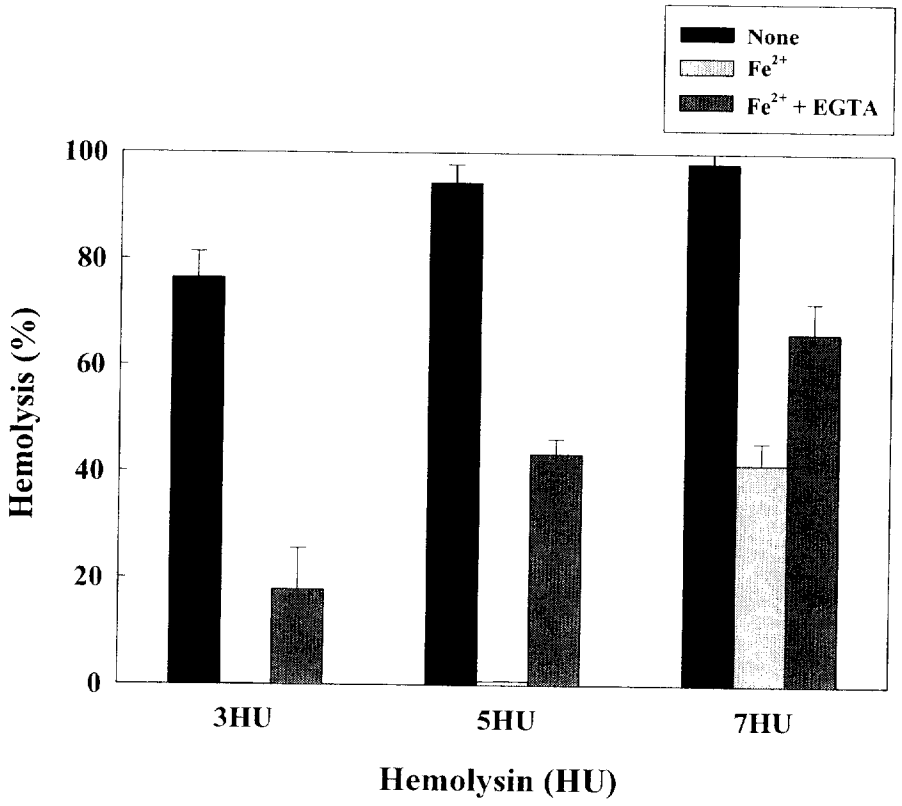


Fig. 9. Inhibition pattern of hemolytic activity by adding Fe^{2+} and EGTA according to the concentration of hemolysin.

Hemolytic activity was measured as in Table 2 (Conc. of Fe^{2+} : 2mM, Conc. of EGTA : 1mM).

It can be suggested that Fe^{2+} can rather protect erythrocytes and EGTA may act as a metal chelates.

EGTA was added to hemolytic activity buffer after 30, 60 min maintaining protective action of Fe^{2+}. There were little effect on the hemolysis unlike simultaneous addition of Fe^{2+} and EGTA (Fig. 10). These results seem to be related to the binding of hemolysin to erythrocytes or Fe^{2+}.

The mechanism by which the divalent cations inhibit the hemolytic reaction is pooly understood. Shinoda et al. (1985) suggested that the divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} do not inhibit the step in which hemolysin produced by *V. vulnificus* is bound to erythrocytes but inhibit the membrane destruction step. It was also reported that the cations inhibited the hemolysin action even when they were added after the hemolysin had already bound to the erythrocytes. Park et al. (1994) reported that a transmembrane pore formed by *V. vulnificus* hemolysin was impermeable to Ca^{2+}, so that this cation possibly functions as an osmotic protectant which protects from the increase in the intracellular osmotic pressure through blockage of influx of extracellular water via the pore.

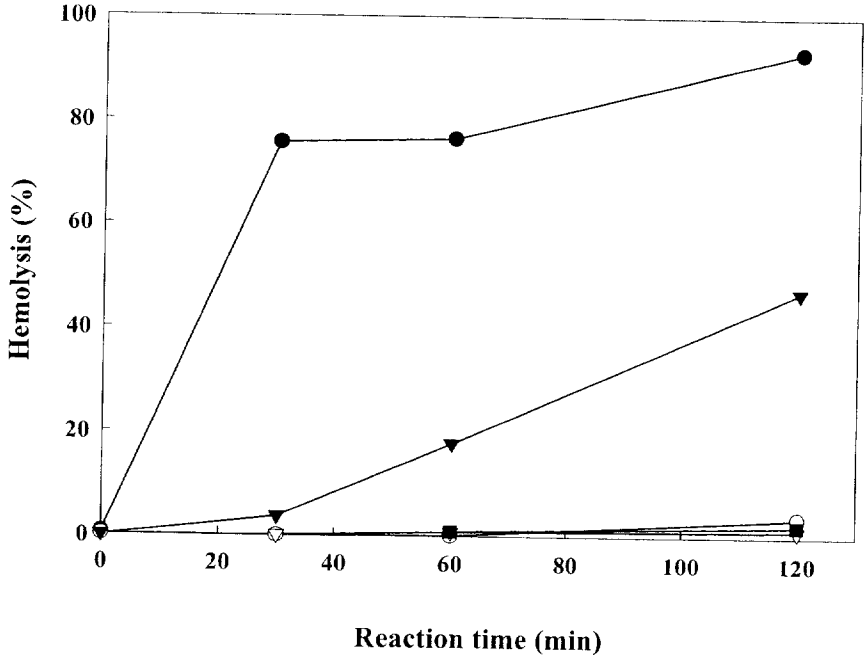


Fig. 10. Effects of adding EGTA on the hemolytic activity after reaction hemolysin and Fe^{2+}.

● : Control sample (hemolysin + 1% sheep erythrocytes)

▼ : Sample mixed with hemolysin, 1% sheep erythrocytes, 2mM Fe^{2+} and 1mM EGTA

○ : Sample mixed with hemolysin, 1% sheep erythrocytes and 2mM Fe^{2+}

▽ : 1mM EGTA was added to sample mixed with Fe^{2+}, hemolysin, 1% sheep erythrocytes after 30 min

■ : 1mM EGTA was added to sample mixed with Fe^{2+}, hemolysin, 1% sheep erythrocytes after 60 min.

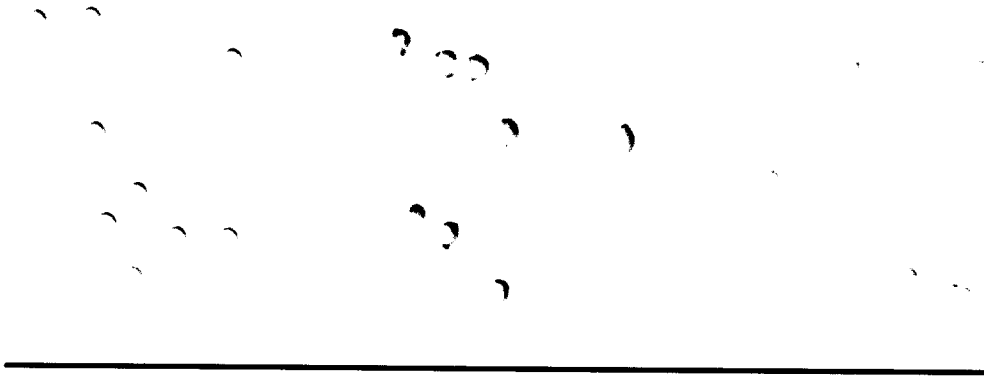
In case of Fe^{2+}, hemolytic activity was inhibited effectively compared with the other cations at low concentration of cations (2mM). It was confirmed that the addition of Fe^{2+} after hemolysis is less effective on the inhibitory function than simultaneous addition. And the addition of EGTA as metal chelates had no effect until the period Fe^{2+} is maintaining its hemolysis-inhibition. Furthermore, the author observed decrease of O.D value when the hemolysin and Fe^{2+} were reacted at high concentraion of Fe^{2+} without sheep erythrocytes (data not shown).

From these results, it can be suggested that hemolysin and Fe^{2+} bind to erythrocytes competitively. It can be also suggested that Fe^{2+} may inhibit not the pore-forming stage, but the binding of hemolysin and erythrocytes. In presence of Fe^{2+}, hemolysin did not destroy erythrocytes and may use Fe^{2+} as substrates. The mechanism of Fe^{2+} to hemolysin produced by NAG vibrio PFM5 seems to be different from that of Ca^{2+} to VVH reported by Park et al. (1994).

5. Morphological changes of sheep erythrocytes by various treatment

Morphological change of sheep erythrocytes was examined in the absence or presence of Na^{+}, Fe^{2+} after 0, 30, 60 min, respectively. Morphological change was examined at a

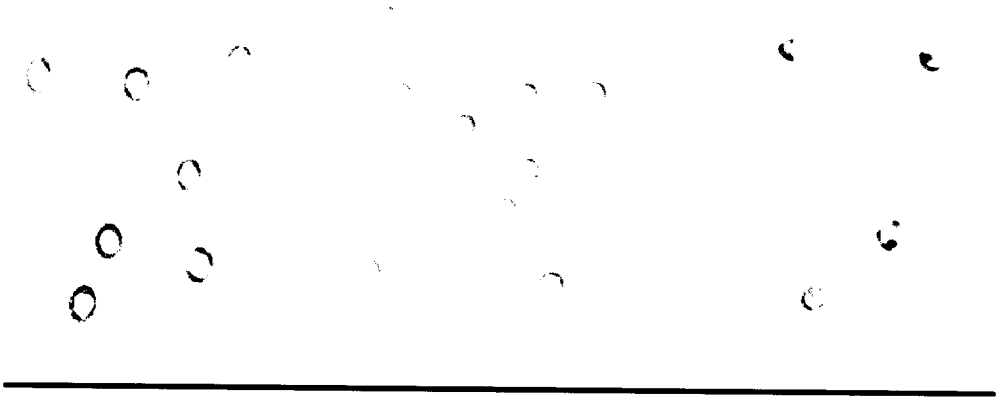
magnification of ×400 with bright field light microscopy (Olympus BX50, Japan). Namely, sheep erythrocytes in the presence of ferric cation showed normal figures after treatment by hemolysin but disrupted in the other cation (Fig. 11, 12, 13).



control 30min 60min

Reaction time

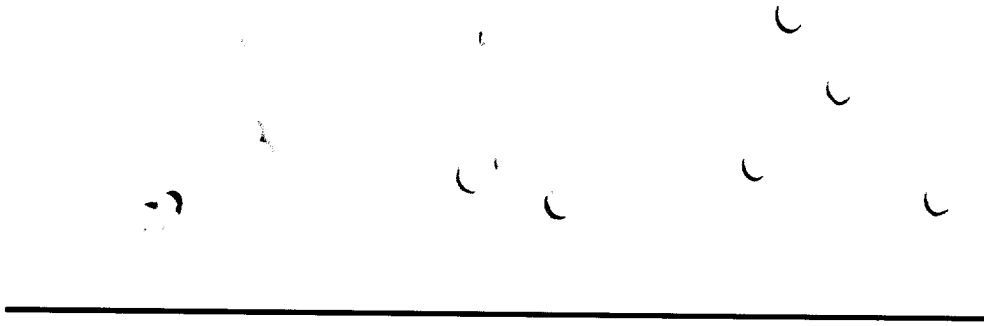
Fig. 11. Morphological changes of erythrocytes treated with hemolysin.



0 time 30 time 60 time

Reaction time

Fig. 12. Morphological changes of erythrocytes treated with hemolysin and Na^+.



0 time 30 time 60 time

Reaction time

Fig. 13. Morphological changes of erythrocytes treated with hemolysin and Fe^{2+}.

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