Separation and properties of the haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*

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Summary. Desalted ammonium-sulphate (0–65%) precipitates from the cell-free supernates of 16–24-h cultures of *Listeria monocytogenes* Boldy and *L. ivanovii* (previously *L. monocytogenes*) Type 5 were eluted through Sephadex G-200. The enzyme activities gave rise to two main peaks. The first peak (approximate mol. wt of protein 150 000) contained only phosphatase activity (assayed by hydrolysis of 4-nitrophenylphosphate at pH 5.0 and 7.0). The second peak (approximate mol. wts of proteins 40 000–60 000) contained the haemolysin activity and the following hydrolytic activities (assay substrates are given in parentheses): phospholipase C (phosphatidyl choline and 4-nitrophenyl-phosphoryl-choline); phosphodiesterase (*bis*-4-nitrophenyl-phosphate); acid phosphatase (4-nitrophenylphosphate); and esterases and lipases (4-nitrophenyl acetate, naphthyl-acetate and -oleate, triacetin and triolein). DEAE-Sephadex chromatography of appropriate fractions from the Sephadex G-200 purification step separated the first peak into two phosphatases and resolved the second peak into its constituent activities. Polyacrylamide gel electrophoresis showed that the individual fractions from the DEAE-Sephadex step consisted of mixtures of protein. The effects of pH and potential activators and inhibitors on the active proteins purified by DEAE-Sephadex chromatography were examined.

Introduction

Two laboratories have published methods for purification of the extracellular lipolytic and haemolytic activities of *Listeria monocytogenes* by gel chromatography which gave conflicting results (Jenkins and Watson, 1971; Siddique *et al.*, 1974). Jenkins and Watson (1971) reported that Sephadex G-75 gel filtration of *L. monocytogenes* strain NCTC 7973 exoproteins gave a single peak of haemolytic and lipolytic activity. Siddique *et al.* (1974) demonstrated with *L. monocytogenes* strain 9–125 that a single peak of haemolytic activity and two of lipolytic activity could be resolved by chromatography on Sephadex G-200 and that the haemolytic activity could further be separated into two components by ion exchange chromatography. The mol. wt of the haemolysin was estimated by Jenkins and Watson (1971) to be at least 171 000 and that of the lipolytic “antigen” to be 52 000. However,

these figures were determined by gel filtration through Sephadex G-75, which has a maximum exclusion limit of 80 000 for peptides and globular proteins. Siddique *et al.* (1974) claimed that the mol. wt of the haemolysins were <10 000 as determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), but the isolated proteins were eluted soon after the void volume in Sephadex G-200 gel chromatography, which suggests that the proteins involved were large molecules. No estimation of the mol. wt of the lipolytic activity was given by Siddique *et al.* (1974). In a personal communication of Smyth and Duncan (1978), the mol. wt of the haemolysin was reassessed and estimated by Watson to be *c.* 100 000 but no confirmation of any value for the mol. wts of the haemolysin or lipolytic activity has been given.

In this study chromatography was used to separate the haemolytic and lipolytic activities of *L. monocytogenes* Boldy and *L. ivanovii* Type 5, which was previously classified as *L. monocytogenes* serovar 5, and to compare the elution of these proteins with the additional hydrolytic activities that we have recently reported (Barclay *et al.*, 1989).

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Materials and methods

Organisms and media

L. monocytogenes Boldy and *L. ivanovii* Type 5 were grown in proteose peptone broth as described by Barclay *et al.* (1989).

Chemical assays

These were done as described before (Barclay *et al.*, 1989).

Enzyme assays

Haemolysin was assayed by serial dilution in tubes as before (Barclay *et al.*, 1989), but fractions from column chromatography were screened by incubating 50 μ l of the sample with 50 μ l of erythrocytes 3% v/v in Cook microtitration wells at 37°C for 1 h in a humid chamber. Haemolytic titres of the positive fractions were then determined by serial dilution in tubes. Comparison of the tube and microtitration methods indicated that the microtitration method gave slightly lower estimates of haemolytic titres than those obtained by the tube dilution method. Thiol-activated haemolytic activity was assayed by including 20 mM cysteine-HCl, pH 7.2, in the buffered saline.

Esterase/lipase activity was detected in column fractions by adding 50 μ l of naphthyl-acetate or naphthyl-oleate (10 mg/ml) to 50 μ l of chromatographic fraction in precipitin tubes, incubating for 16 h at 37°C, and assaying as before (Barclay *et al.*, 1989). All other methods were as before (Barclay *et al.*, 1989).

Phospholipase was assayed by the hydrolysis of phosphatidylcholine to produce acid releasable Pi as described previously (Barclay *et al.*, 1989) with 0.5 ml of each column fraction. The hydrolysis of sphingomyelin and of phosphatidylethanolamine was determined by the same method as that for phosphatidylcholine. Phospholipase, which had been partially purified by gel- and ion-exchange chromatography was used as 1.5 mg in 0.5 ml. The effects of potential inhibitors and activators were assayed by inclusion of the following in the reaction mixture: 40 mM sodium fluoride, 10 mM dithiothreitol, 20 mM iodoacetate, anti-exoprotein antiserum, normal serum, or one of the following divalent cations—Ca²⁺, Mg²⁺, Zn²⁺, Fe²⁺ (as tenfold dilutions from 1 M to 10⁻⁹ M).

Acid phosphatase, neutral phosphatase and phosphodiesterase were determined by hydrolysis of 4-nitrophenol phosphate or bis-4-nitrophenol phosphate (Barclay *et al.*, 1989) with 0.2 ml of the chromatography fraction. The following potential activators and inhibitors of phosphatase and phosphodiesterase activities were tested: 40 mM sodium fluoride, 10 mM sodium tartrate, 0.6 mM mercaptoethanol, K₂HPO₄ (tenfold dilutions from 1 M to 10⁻⁷ M), and Ca²⁺, Mg²⁺ or Zn²⁺ (as tenfold dilutions from 1 M to 10⁻⁹ M).

Neutral phosphatase and phosphodiesterase were assayed

by a method similar to that for acid phosphatase except that buffers of appropriate pH were used, and the substrate for phosphodiesterase was bis-4-nitrophenol phosphate (see Barclay *et al.*, 1989).

Catalase was assayed by adding 2 ml of H₂O₂ 5% v/v to 1 ml of each chromatography fraction and examining the mixture visually for the release of oxygen.

NADase activity was monitored as before (Barclay *et al.*, 1989) with 1.0 ml of each column fraction plus 1.0 ml of 0.1 M KH₂PO₄.

Inhibitors and activators of haemolysis

The haemolytic titre was determined as before (Barclay *et al.*, 1989) but the haemolysin or the erythrocytes were pre-incubated at either 0°C or 37°C for 30 min with one of the following potential inhibitors before the two were mixed together: normal rabbit serum, anti-exoprotein antiserum, anti-exoprotein IgG, cholesterol 25 μ g, 20 mM iodoacetamide, 10 mM L-histidine, the divalent cations Zn²⁺, Ca²⁺, Mg²⁺, Mn²⁺, Fe²⁺ or Fe³⁺ (all at 0.01, 0.1 or 1.0 M), or 10 mM L-histidine + 0.01 M Zn²⁺. The solutions were incubated at 37°C for 30 min and the amount of substrate hydrolysed was determined as above.

Antiserum and IgG

Anti-exoprotein antiserum was prepared by immunising young adult New Zealand White rabbits with intravenous injections of 0.5 ml of strain Type 5 exoprotein 50 mg/ml + 0.5 ml of Freund's complete adjuvant. Normal (control) serum was collected before injection. Two further injections of 0.5 ml of protein + 0.5 ml of adjuvant were made after 7 and 14 days and the rabbits were bled after a further 7 days. IgG was prepared by saturation of the antisera with 50% saturated ammonium sulphate, desalting and then isolation of the immunoglobulin by elution through a DEAE-cellulose column with 50 mM phosphate buffer, pH 7.5, at 4°C.

Polyacrylamide gel electrophoresis (PAGE)

Disk-gel electrophoresis was by a modification of the method of Davis (1964). Polymerisation of the separating gel (1.2 ml), which consisted of acrylamide 11.7% w/v and 'Bis' 0.153% was catalysed with ammonium persulphate 0.07% w/v. The stacking gel (0.18 ml) was photopolymerised with riboflavin 0.0005% w/v and contained acrylamide 4% w/v and 'Bis' 1% w/v. Samples 50 μ l containing 70–100 μ g of protein were applied and the gels were electrophoresed at 2 mA per tube at 4°C. Proteins were detected with Coomassie Blue.

Gel chromatography

Exoproteins were isolated by 0–65% ammonium-sulphate saturation of the culture supernate from cells grown without shaking for 16–20 h at 37°C. The protein precipitate was desalted by gel filtration on Sephadex G-25 and 200-mg samples of the proteins were separated

chromatographically through Sephadex G-200 and eluted with 5 mM Tris-HCl buffer, pH 7.0, at 4°C with a flow rate of 15–18 ml/h. Fractions (3 ml) were assayed for protein, haemolysin and the following hydrolytic activities (the assay substrates are given in parentheses): phospholipase (phosphatidyl choline); acid phosphatase (4-nitrophenol phosphate); phosphodiesterase (*bis*-4-nitrophenol phosphate); esterase (4-nitrophenol acetate); catalase (H_2O_2); and nicotinamideadenine-dinucleotide (NAD).

Results

After gel filtration two main peaks of activity were observed (fig. 1). The first peak (mol. wt

$150\,000 \pm 5000$) contained only phosphatase activity (assayed at pH 5.0 and 7.0). The second peak (mol. wt $50\,000 \pm 1000$) contained haemolysin, as well as the following hydrolytic activities: phospholipase, acid phosphatase, phosphodiesterase and esterase. Neither catalase nor NADase was detected, probably because of the initially low concentrations of these enzymes in the extract (Barclay *et al.*, 1989). Low recovery rates and specific activities were obtained for the haemolytic activity (table I), possibly as a result of inactivation or loss of essential co-factors during chromatography. Apart from one of the esterases, the specific activities of the enzymes were increased.

Fig. 1. Sephadex G-200 gel filtration of 300 mg of desalted strain Type 5 exprotein: **a** — protein, --- acid phosphatase, -.-.- diesterase; **b** — protein, --- phospholipase, -.-.- esterase, CHU, ○—○ thiol-CHU.

Table I. Recovery of exoproteins from Sephadex G-200 chromatography

Enzyme/haemolysin	Crude precipitate		Proteins eluted from Sephadex G-200					
	Total activity applied (nmol/assay)	Specific activity (nmol/mg of protein/min)	Fraction number(s)	Total activity recovered (nmol/assay)	Specific activity (nmol/mg of protein/min)	Recovery (%)	Total recovery (%)	Relative purification
Phospholipase*	765 000	2550	31–37	411 000	14 500	53.7	53.7	5.7
Acid phosphatase	13 647	84.2	17–22	5734	436	36.6	74.0	5.2
			30–40	5844	130	37.4		1.5
Esterase	1008	6.22	34–39	197	7.84	19	43.0	1.3
			55–65	157	5.54	24		0.9
Phosphodiesterase	14 730	72.4	28–42	14 872	2.44	100	100	3.4
	(CHU)	(CHU/mg)		(CHU)	(CHU/mg)			
Thiol-independent haemolysin	3840	12.8	35–37	24	1.94	0.625	0.625	0.15
Thiol-activated haemolysin	7680	25.6	31–40	132	3.21	1.72	1.72	0.13

* Assayed by acid releasable Pi method.

† Assayed by hydrolysis of 4-nitrophenolacetate.

Ion exchange chromatography

Proteins with mol. wts 40 000–60 000 from two similar G-200 chromatographic runs were pooled and eluted through a column of DEAE-Sephadex A50 (1.5 × 30 cm) with a linear gradient of 0–1 M NaCl in 5 mM Tris-HCl buffer, pH 7.0. Fractions (3 ml) were assayed for protein, haemolysin, phospholipase, acid phosphatase, phospholipase, and phosphodiesterase activities (fig. 2). The haemolysin was resolved into two components, the second of which was active only in the presence of a thiol-reducing agent (cysteine hydrochloride). Acid phosphatase was eluted with the first of these haemolytic peaks, and phosphodiesterase with the second. Phospholipase was eluted later, with about 0.6 M NaCl, and was associated with a small amount of phosphodiesterase activity. Esterase was not detected, probably because of the low yield from Sephadex G-200 gel chromatography. Recoveries of the activities (in relation to the activities in the crude protein precipitate) were generally low (table II), although the specific phospholipase (70 µg) isolated by ion exchange chromatography was not pure and contained at least six proteins when examined by PAGE (data not shown).

Gel filtration of the phosphatases

The phosphatases from strain Type 5 exoprotein (300 mg) were separated by Sephadex G-200 chromatography into two acid phosphatase compo-

nents, with approximately equal activities, and two co-eluting neutral phosphatases (fig. 3). The phosphatase of mol. wt 150 000 was then eluted through the DEAE-Sephadex A50 column described above. About 30% of the acid and neutral phosphatase activities passed through the column without binding (fig. 4), but most of the neutral phosphatase remained bound to the DEAE-Sephadex. This bound phosphatase was not released with low (pH 5.0) or high (pH 10) pH buffers of high ionic strength (1 M NaCl).

The free eluting phosphatase contained at least five proteins; the second, which was predominantly acid phosphatase, had two slowly migrating bands and the third, which was mostly neutral phosphatase, also showed two bands (PAGE; data not shown), the slowest migrating of which coincided with the faster migrating band from the predominantly acid phosphatase peak.

Biochemical properties of the haemolysins

Cholesterol, zinc and anti-haemolytic antiserum had been shown to inhibit streptolysin O (Avigad and Bernheimer, 1978; Smyth and Duncan, 1978), and other thiol-activated haemolysins related to listeriolysin. We examined the effects on haemolysis of these, and also of divalent cations other than zinc, as well as sodium fluoride and iodoacetamide—a thiol group inhibitor reported to have no effect on thiol-activated listerial haemolytic activity

Fig. 2. DEAE Sephadex chromatography of the $50\,000 \pm 1000$ -M_r peak from Sephadex G-200 chromatography (fig. 1): **a** — protein, --- thiol-CHU, ···· CHU; **b** — protein, --- diesterase, ···· phospholipase.

Fig. 3. Fractionation of acid and neutral phosphatase activities from strain Type 5 by Sephadex G-200 gel chromatography: — protein, ···· neutral phosphatase, --- acid phosphatase.

(Jenkins and Watson, 1971). None of the compounds stimulated haemolysis but cholesterol, zinc, normal serum (which contains cholesterol), anti-exoprotein antiserum and IgG all inhibited both thiol-activated and thiol-dependent haemolytic activity (table III). Inhibition by zinc was reversed with L-histidine in a manner similar to perfringolysin O rather than like streptolysin O where zinc inhibits an early step in haemolysis (Avigad and Bernheimer, 1978). As with other thiol-activated cytolytins, haemolysis was not induced by the haemolysin at 0°C.

Two experiments were done in a preliminary

attempt to determine the nature of the inhibition by zinc, cholesterol and IgG (the sera were not considered because of the interfering presence of cholesterol). No cholesterol or cholesterol esters were detected in the IgG preparation with a serum cholesterol diagnostic kit (Sigma). In one experiment, either the haemolysin or the erythrocytes were incubated with the inhibitors at 0°C for 30 min before the two were mixed together. In the other experiment the haemolysin and erythrocytes were mixed for 30 min at 0°C before the inhibitor was added. After 30 min the haemolysins from both experiments were assayed for haemolytic activity

Table II. Recovery of proteins from DEAE-Sephadex chromatography

Enzyme/haemolysin	Crude precipitate		Proteins eluted from DEAE-Sephadex column			Recovery (%)	Relative purification
	Total activity applied (nmol/assay)	Specific activity (nmol/mg of protein/min)	Fraction number(s)	Total activity recovered (nmol/assay)	Specific activity (nmol/mg of protein/min)		
Phospholipase*	1.53 × 10 ⁶	2550	36–45	9.7 × 10 ⁵	2.21 × 10 ⁵	63.4	86.7
Acid phosphatase	27 294	84.2	12–17	1300	593.6	4.76	7.0
Phosphodiesterase	29 460	72.4	17–30	1034	354.1	3.51	4.9
Esterase†	2016	6.22	—	0	0	0	0
	(CHU)	(CHU/mg)		(CHU)	(CHU/mg)		
Thiol-independent haemolysin	7680	12.8	11–14	12	12.1	0.16	0.9
Thiol-activated haemolysin	15 360	25.6	11–18 19–26	106 48	41.7 34.5	0.69 0.31	1% 1.3

* Assayed by acid-releasable Pi.

† Assayed by hydrolysis of 4-nitrophenolacetate.

at 37°C as before. Cholesterol, IgG and zinc each inhibited haemolysis when they were added to the haemolysin or erythrocytes before the two were mixed together. When the inhibitor was added after the two were mixed, haemolysis was not affected by cholesterol, was only partially inhibited by IgG and was completely inhibited by zinc.

Properties of phospholipase C

In systems other than those used for *L. monocytogenes*, Ca^{2+} had been reported to either stimulate phospholipase C activity or to have no effect. Therefore, calcium was initially included in the phospholipase C assays, but was later found to have

no effect on the reaction (fig. 5), although Zn^{2+} , Mg^{2+} and Mn^{2+} inhibited phospholipase activity. Phospholipase C activity was inhibited only slightly by anti-exoprotein antiserum or by 20 mM iodoacetate and neither 40 mM NaF nor 10 mM dithiothreitol had any effect. Phospholipase C had optimal activity at two pH values. Thus, phosphatidyl choline was preferentially hydrolysed at either pH 6.0 or pH 8.5 in 10 mM Tris-HCl buffer at approximately equal rates. The partially purified phospholipase C also hydrolysed phosphatidyl ethanolamine and sphingomyelin at rates equivalent to 79% and 35% respectively of that when phosphatidyl choline was the substrate.

Properties of the phosphatases

Acid phosphatase activities were inhibited by K_2HPO_4 (fig. 6) and NaF (table IV). High mol. wt acid and neutral phosphatase activities were unaffected by Zn^{2+} , Ca^{2+} and Mg^{2+} ions. However low mol. wt acid phosphatase was activated by low concentrations of these cations (fig. 7). Phosphodiesterase activity was trebled by the inclusion of 0.6 mM mercaptoethanol at pH 6 (table IV). This enzyme was inhibited by tartrate, but unaffected by NaF or divalent cations (Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+}) (see table IV).

Discussion

The estimated mol. wt of the haemolysin of c. 50 000 is consistent with those reported for

Table III. Inhibitors of haemolysis

Inhibitor	Haemolytic titres (CHU) for 10 mg of exoprotein	
	Thiol-independent	Thiol-activated
Saline	64	512
Cholesterol (25 µg)	0	0
Iodoacetamide (20 mM)	64	512
L-Histidine (10 mM)	64	512
L-Histidine + Zn^{2+}	64	512
Normal serum	16	128
Antiserum	2	16
IgG	0	0
Zn^{2+} (0.01, 0.1, 1M)	0	0

Fig. 4. Fractionation by DEAE Sephadex ion exchange chromatography of the $150\,000 \pm 5000$ -M_r peak of phosphatase activity from sephadex G-200 chromatography (fig. 3): — protein, --- acid phosphatase, ··· neutral phosphatase.

Fig. 5. Phosphatidylcholine (100 mM) hydrolysis by strain Type 5 exoprotein (25 mg/ml) at 37°C after 16 h in the presence of chlorides of Ca²⁺ (○), Mn²⁺ (●), Zn²⁺ (□) and Mg²⁺ (■), as measured by release of Pi (total μmol/assay).

related thiol-activated haemolysins (Smyth and Duncan, 1978). Interestingly, most of the other extracellular hydrolytic activities (i.e., esterase/lipase, phosphodiesterase, phospholipase and phosphatase) from strain Type 5 cultures had a similar mol. wt. The exceptions were an acid and a neutral phosphatase with mol. wts *c.* 150 000. These estimations need to be confirmed by other methods

Fig. 6. Effect of K₂HPO₄ on acid phosphatase (○), neutral phosphatase (●) and phosphodiesterase (□) activities of strain Type 5 exoprotein (1 mg/0.5 ml) after incubation at 37°C for 30 min, as measured by release of 4-nitrophenol (total μmol/assay).

Fig. 7. Effect of divalent cations (added as chlorides) on partially purified acid phosphatase (0.5 mg of fraction 36 from Sephadex G-200 chromatography) after incubation for 30 min at 37°C, as assayed by release of 4-nitrophenol (total μmol/assay).

such as gel electrophoresis and ultracentrifugation because gel filtration can give false estimates (Andrews, 1985). Similar elution patterns to those for strain Type 5 were found for *L. monocytogenes* Boldy (data not shown). The separation of listerial

Table IV. Inhibitors of acid and neutral phosphatases and phosphodiesterase

Enzyme	4-Nitrophenol released (nmol) with			
	No addition	Tartrate (10 mM)	NaF (40 mM)	Mercaptoethanol (0.6 mM)
Acid phosphatase	1950	1907	270	1997
Neutral phosphatase	937	1017	278	792
Phosphodiesterase	249	142	284	1007

haemolytic activity into two components by DEAE-Sephadex chromatography was consistent with similar reports (Siddique *et al.*, 1974) from studies with strain 9-125. In the present study, the two haemolytic activities were antigenically identical as observed by immunodiffusion (data not shown), and were inhibited by cholesterol, zinc, normal sera and antisera directed against the exoprotein.

Phospholipase and phosphodiesterase activities remained as a single protein fraction after chromatography with Sephadex G-200 and DEAE-Sephadex but there was no evidence that these were the same proteins. A partially purified phospholipase preparation had a greater specificity than the unpurified preparation for phosphatidyl ethanolamine than for sphingomyelin. Thus phospholipase activity seems to be composed of more than one protein.

None of the activities in the chromatography fractions showed single bands of protein when 70–100 μ g of each protein was examined by PAGE. The purification methods used in the present study were similar to those used by Siddique *et al.* (1974) who used PAGE to show that their haemolysin had been purified to a single protein. However, Siddique *et al.* used only 4.5 μ g of protein in their PAGE analysis which may well have been too low to detect the contaminating proteins. If this was the case, then care must be taken in the interpretation of the amino acid analysis of the haemolysin as reported by Siddique *et al.* (1974).

The inhibition of the haemolysin by "normal" serum would seem to question the role of the haemolysin in pathogenesis, but it is possible that a low haemolysin concentration, possibly in conjunction with the other exoproteins, is enough to cause the loss of small molecules and essential ions such as K^+ and could partly explain the increased blood potassium levels and death by cardiotoxicity following the administration of haemolysin as a crude exoprotein preparation (Siddique and Cooper, 1969; Sword and Kingdon, 1971; Williams and Siddique, 1972; Takeda *et al.*, 1978), and it

would not seem unreasonable to suggest that other exoprotein activity could enhance cardiotoxicity.

The mechanism of action of the haemolysin is not known, but haemolysis appears to be divided into two steps. The first step is binding of the haemolysin to the cell. Binding, which could occur at 0°C, was blocked by the extracellular addition of cholesterol, which probably acted as a competitive inhibitor for the cholesterol in the erythrocyte cell membrane, whilst the cytolytic action of the second step could be prevented with zinc but was negated by the addition of L-histidine. IgG partially blocked both steps. This phenomenon is also characteristic of other thiol-activated haemolysins including pneumolysin (Neill and Fleming, 1927), streptolysin O (Herbert and Todd, 1941) and Θ toxin (Van Heyningen, 1941).

It is generally believed that thiol-activation occurs by reduction of protein-S-S-groups to -SH groups. This is a property of many enzymes (e.g., succinate dehydrogenase, papain and urease), which are, in addition, strongly inhibited by iodoacetamide. However, in agreement with Jenkins *et al.* (1964) we found that the haemolysin was not markedly affected by iodoacetamide. It is possible that this insensitivity might be because the iodoacetamide cannot gain access to the disulphide bonds because of steric factors. Alternatively, it has been found that, in haem metabolism, certain heavy metals and a series of transition elements (e.g., cobalt, nickel and platinum) bind several functional groups including sulphhydryl and carbonyl groups (Maines and Kappas, 1977), but only the ionic forms of the metals appear capable of altering haem metabolism, which suggests that it was binding of the metals with such functional groups that perturbed metabolism. Cysteine and glutathione blocked the toxic actions of the metals by competing for them. Thus it might be that a similar situation occurs in the thiol-activation of this haemolysin.

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