Characterization of staphylococcal γ -lysin

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γ -Lysin was purified from *Staphylococcus aureus* strains Smith 5R and PG23 (a toxic shock syndrome isolate) by a combination of heparin-agarose and hydroxylapatite chromatography. Both strains produced two haemolytic components, designated γ_1 and γ_2 . Though each component was weakly haemolytic they acted synergistically to potentiate haemolysis on rabbit, sheep and human blood. Rabbit and sheep erythrocytes were more sensitive to lysis by γ -lysin than human erythrocytes. The molecular mass of γ_1 was 32 kDa and its pI value was 9.4. γ_2 had a molecular mass of 36 kDa and a pI value of 9.3. While both trypsin and papain acted synergistically with γ_2 to induce increased haemolysis, no such synergism was seen with γ_1 . Also, protease inhibitors acted to inhibit synergism between γ_1 and γ_2 . These findings suggest that γ_1 could be a protease.

Introduction

γ -Lysin is the least well documented staphylococcal haemolysin. It is inhibited by agar (Jackson, 1963) and therefore its presence is usually missed when strains are screened for haemolysis on blood plates. For this reason the importance of γ -lysin as a possible virulence determinant in the pathogenesis of staphylococcal disease has been largely overlooked. Attention has focussed on the principal haemolysins (i.e. α , β and δ), and the existence of a fourth haemolysin has been largely ignored.

The relatively small amount of work published indicates considerable controversy about the physicochemical characteristics of γ -lysin and a distinct lack of information on its mode of action. Different workers have used different methods to purify γ -lysin from strain Smith 5R, a well known γ -lysin producer first described in 1938 by Smith & Price. Plommet and his group in France (Plommet & Bouillanne, 1966) purified two components, designated γ_1 and γ_2 , by chromatography on hydroxylapatite, which complemented one another to give haemolytic activity. Both components were very basic proteins and had similar molecular masses (γ_1 , 29 kDa; γ_2 , 26 kDa). This finding was confirmed by Taylor & Bernheimer (1974). However, Mollby &

Wadstrom (1971), using isoelectric focussing in the pH range 9–11, isolated only one basic protein with a molecular mass of 31 kDa. It is possible that they failed to separate the two components since resolution by isoelectric focussing in this high pH range is poor. Fackrell & Wiseman (1976a), using a combination of ultrafiltration, gel-filtration and ammonium sulphate fractionation, isolated only one protein but this had markedly different physicochemical characteristics to the toxin isolated by other workers with a molecular mass of 45 kDa and a pI value of 6.0 (Fackrell & Wiseman, 1976b). This protein also differed from the toxin isolated by other workers in that it had a very high specific activity against human erythrocytes. It is likely that this protein was some other haemolytic protein produced by *S. aureus*. Birkbeck & Niven (1988) isolated a single component haemolysin with a molecular mass of 33 kDa and a pI value of 9.0 from strain Smith 5R by heparin-agarose affinity chromatography followed by chromatography on hydroxylapatite. A second component, which was not haemolytic on its own, was also isolated which potentiated the haemolytic titre of γ -lysin on both human and sheep erythrocytes.

The γ -lysin determinant of strain Smith 5R has been cloned in phage λ and plasmid vectors in *Escherichia coli* (Cooney *et al.*, 1988). Two genes (*hlgA* and *hlgB*) were mapped and their polypeptide products identified by immunoblotting and in the minicell system as proteins of 32 and 36 kDa. These two proteins acted synergistically to give haemolysis on blood agarose plates. Nothing is

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known about how these two components interact to enhance haemolysis.

Clearly there is a need to characterize γ -lysin isolated from other strains besides Smith 5R before firm conclusions about its physicochemical characteristics can be made and studies on its mode of action can be carried out. It was decided therefore to purify γ -lysin from both Smith 5R and from a strain isolated from a patient suffering from toxic shock syndrome (TSS). TSS isolates of *Staphylococcus aureus*, which had previously been reported to be weak producers of haemolysins (Barbour, 1981; Chow *et al.*, 1983), are now known to produce γ -lysin when agarose is substituted for agar in blood plates or when the strains are grown in the presence of 20% (v/v) CO₂ (Carlson, 1986; Clyne *et al.*, 1988). In this investigation a comparison of the properties of γ_1 and γ_2 -isolated from the two strains was made and the results of a preliminary study on how the two components act synergistically is presented.

Methods

Bacterial strains. *S. aureus* Smith 5R is a well known γ -lysin producer first described by Smith & Price (1938). *S. aureus* PG23 is a TSST-1+ strain isolated from a patient suffering from TSS.

Production of crude culture supernate. Smith 5R was grown using the dialysis membrane technique (Fackrell & Wiseman, 1976a) with Trypticase Soy Broth (TSB) (Difco), plus 1% (w/v) agarose. Each plate was then inoculated with 5 ml of an overnight TSB culture of Smith 5R. Plates were incubated at 37 °C for 24 h in an atmosphere of 20% (v/v) CO₂/80% (v/v) O₂. Bacteria and extracellular products were harvested by rinsing the surface of each plate with 20 ml 10 mM-potassium phosphate buffer (pH 6.8) and removing growth with a glass spreader. Bacteria were removed by centrifugation at 10000 r.p.m. for 20 min.

The dialysis membrane technique with added CO₂ regularly yielded small volumes (100 ml) containing 5120 HU ml⁻¹ with strain Smith 5R and 128 HU ml⁻¹ with strain PG23. For purification, however, crude toxin was prepared from strain PG23 grown in Todd-Hewitt broth without added CO₂ as described previously (Clyne *et al.*, 1988) though yields of haemolytic activity were lower. Five hundred millilitres of culture supernate was used as the starting material.

Chromatography on heparin-agarose. Crude culture supernate was stirred overnight at 4 °C with 5 ml heparin-agarose (Sigma) and the supernate recovered by allowing the gel to settle for 1 h. The heparin-agarose was packed into a 1 cm diameter column and washed with 0.01 M-potassium phosphate buffer (pH 6.8; 50 ml), and a gradient of 0.1–1.5 M-NaCl in 0.01 M-potassium phosphate buffer was applied. Fractions of 2 ml were collected and monitored for haemolytic activity. Protein was detected by measuring A₂₈₀.

Chromatography on hydroxylapatite. Hydroxylapatite was prepared according to the method of Tiselius *et al.* (1956). The hydroxylapatite thus obtained was stored in 0.001 M-potassium phosphate buffer (pH 6.8). Enough hydroxylapatite was poured into a column to give 5 ml packed volume and the column was washed with 50 ml 0.01 M-potassium phosphate buffer. Selected fractions from heparin-agarose chromatography were pooled and applied to the hydroxylapatite column which was then washed with 25 ml 0.01 M-potassium phosphate buffer (pH 6.8); elution was achieved with a 0.1–1.0 M continuous

gradient of potassium phosphate buffer (50 ml total). Fractions of 2 ml were collected and monitored for haemolytic activity. Protein was detected by measuring A₂₈₀. Chromatography was done at 4 °C.

Measurement of haemolytic activity. Haemolytic activity in the fractions from the heparin-agarose and hydroxylapatite columns was detected on blood-agarose plates. Molten 1% (w/v) agarose in PBS (0.067 M phosphate, 0.077 M-NaCl, pH 7.2; 10 ml) containing 5% (v/v) washed erythrocytes was poured into a Petri dish and allowed to solidify. Small wells, 4 mm in diameter, were punched in the agarose about 2 cm apart; fractions (5 μ l) were allowed to diffuse at 37 °C for 16 h, after which time zones of haemolysis occurring around the wells were noted. Each haemolytic fraction was then titrated against rabbit, sheep and human erythrocytes in microdilution trays as previously described (Clyne *et al.*, 1988).

Both of the above methods were also used to demonstrate synergism between γ_1 and γ_2 . When using blood plates, γ_1 and γ_2 were placed in neighbouring wells and examined after incubation for zones of haemolysis occurring between the wells.

SDS-PAGE. Fractions from each of the chromatography steps were analysed on SDS-polyacrylamide gels and stained with either Coomassie blue or silver nitrate according to the method of Arezzo & Rose (1987). The molecular mass of the proteins were determined by the method of Weber & Osborn (1969). Proteins used as standards were lysozyme (14.3 kDa), β -lactoglobulin (18.4 kDa), trypsinogen (24 kDa), egg albumin (45 kDa) and bovine serum albumin (66 kDa) (Sigma).

Western immunoblotting. Haemolytic fractions from hydroxylapatite chromatography were checked for contamination with α , β or δ -lysin by Western immunoblotting (Sarvas & Nurminen, 1985) using specific antisera. Sera were used at a 1:100 dilution and the antigen-antibody complexes were detected with a protein A-peroxidase conjugate (Sigma). Blots were developed with 4-chloro-1-naphthol as the substrate.

Analytical isoelectric focussing. The isoelectric points (pI) of γ_1 and γ_2 were determined using polyacrylamide isoelectric focussing gels, (1 mm thick) with a pH gradient of 3.5–9.5 (LKB). The gels were run and stained as described previously (Clyne *et al.*, 1988).

N-terminal sequencing. The first twenty amino acids at the N-terminal of γ_1 were determined by Dr C. Bailey of the Department of Biochemistry, Trinity College, Dublin, Republic of Ireland using an Applied Biosystems 477A protein sequencer.

Antiserum against γ -toxin. Rabbits were injected intradermally at multiple sites on the back. For the first immunization the toxin was diluted in sterile saline and mixed with an equal volume of Freund's complete adjuvant (Sigma) until a stable emulsion was achieved. For the three subsequent booster injections, which were given at fortnightly intervals, Freund's incomplete adjuvant (Sigma) was used. For γ_1 the total amount of toxin injected was 30 μ g for the initial immunization and 40 μ g for the subsequent booster doses. For γ_2 100 μ g of protein was used for all immunizations. Test bleeds were taken from the rabbits at regular intervals; when antiserum reacted in Western blots at a 1:100 dilution, rabbits were bled out. The blood was allowed to clot overnight and the serum removed. Immunoglobulin was then precipitated with ammonium sulphate and purified according to the method of Harboe & Ingild (1973).

For γ_1 , the antiserum raised in rabbits was monospecific but due to impurities in the γ_2 fraction this antiserum also reacted with a band of 45 kDa present in the culture supernatant of different strains of *S. aureus*.

Proteases and protease inhibitor. The effect of the following proteases and protease inhibitors on synergism between γ_1 and γ_2 was investigated. Proteases used were trypsin (BDH), papain (BDH), chymotrypsin (Sigma) and *S. aureus* V8 protease (Sigma). Each enzyme

Table 1. Purification of γ -lysin

Step in purification	Haemolytic activity (HU ml ⁻¹)*	Protein (mg ml ⁻¹)	Specific activity (HU mg ⁻¹)	Total activity (HU)	Recovery (%)
Culture supernate					
Smith 5R†	5120	ND	ND	358400	100
PG 23‡	128	1	128	64000	100
Heparin-agarose chromatography					
Smith 5R	6250	ND	ND	250000	69.7
PG 23	2048	7.5	273.0	40960	64
Hydroxylapatite chromatography					
Smith 5R γ_1	320	0.67	477.6	3200	0.89
γ_2	160	0.27	592.6	1920	0.53
$\gamma_1 + \gamma_2$	2560	0.47	5446.8	56320	15.70
PG 23 γ_1	32	0.16	200.0	256	0.40
γ_2	64	0.12	533.3	512	0.80
$\gamma_1 + \gamma_2$	512	0.14	3657.14	8192	12.80

ND, Not done

* Titrated against rabbit erythrocytes.

† γ -Lysin produced by dialysis membrane technique.‡ γ -Lysin produced by shake culture method.

was dissolved in PBS (pH 7.2) prior to use at a concentration of 1 mg ml⁻¹. Trypsin and papain were used at concentrations ranging from 10 to 0.75 mg ml⁻¹.

The protease inhibitors benzamidine (Sigma), *N* α -p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma), aprotinin (Sigma), trypsin inhibitor (BDH analar), EDTA (Sigma) and PMSF were tested for inhibitory activity. Each inhibitor, except for PMSF, was dissolved in PBS (pH 7.2) and used at a concentration of 10 mM. PMSF was first dissolved in dimethyl formamide (BDH) at a concentration of 400 mM and then diluted to 2 mM in PBS (pH 7.2). The effect of cysteine at a concentration of 1 mg ml⁻¹ was also tested.

Determination of the susceptibility of γ_2 to proteolytic digestion by γ_1 , trypsin and papain. Trypsin (25 μ g) and papain (25 μ g) were incubated separately with γ_2 (1 μ g) for 45 min at 37 °C; γ_1 (2 μ g) and γ_2 (1 μ g) were incubated together at 37 °C for times ranging from 10 to 120 min. In addition γ_2 (1 μ g) was incubated with increasing concentrations of γ_1 ranging from 0.25 to 3.0 μ g for 45 min. The resulting digests were then run on SDS-polyacrylamide gels and stained with silver nitrate (Arezzo & Rose, 1987). Gels were screened for immunoreactive products of digestion by Western immunoblotting, as described above, using γ_1 and γ_2 antiserum.

Binding of γ_1 and γ_2 to erythrocyte membranes. γ_1 and γ_2 were each diluted so that neither component was haemolytic on its own and they were each incubated at 37 °C with freshly washed erythrocytes. After washing three times in 0.85% NaCl erythrocyte ghosts were prepared according to the method of Freer *et al.* (1973). Erythrocytes were lysed by the slow dropwise addition of the packed cells to 0.01 M-potassium phosphate buffer (pH 7.4) which was stirred slowly throughout this step. The resulting lysate was centrifuged at 13000 r.p.m. for 15 min and the membrane pellet was then resuspended and washed a further twice with Tris/HCl buffer (pH 7.4). Detection of γ_1 and γ_2 bound to the erythrocyte membranes was determined by Western immunoblotting using either γ_1 or γ_2 antiserum. The Western blotting procedure was carried out as described above.

Results

Purification of γ -lysin

Though the material obtained by heparin-agarose chromatography was not homogeneous, the step served to concentrate γ -lysin and many of the lower molecular mass impurities were removed.

Chromatography on hydroxylapatite separated two haemolytic proteins from both strain Smith 5R and strain PG23, designated γ_1 and γ_2 . The haemolytic titres, protein concentrations, specific activities and recovery yields of the material at the different stages throughout the purification procedure are given in Table 1.

Characterization of γ -lysin components

Preparations of γ_1 and γ_2 from both strains were found to be identical and to have molecular masses of 32 and 36 kDa, respectively, by SDS-PAGE. Whilst γ_1 was homogeneous on SDS-polyacrylamide gels when stained with either Coomassie blue or silver nitrate, γ_2 preparations contained minor contaminating protein bands (Fig. 1). Neither γ_1 nor γ_2 was contaminated with α -, β - or δ -toxin when tested by Western immunoblotting. The isoelectric points of γ_1 and γ_2 were 9.4 and 9.3, respectively, as determined by analytical isoelectric focussing.

Antibodies against γ_1 were raised easily in rabbits. However, it proved difficult to raise antibodies against γ_2

Fig. 1 SDS-PAGE of purified preparations of γ_1 and γ_2 . The gel was run and stained with Coomassie blue as described in Methods. Lanes: 1, bovine serum albumin (66 kDa); 2, egg albumin (45 kDa); 3, trypsinogen (24 kDa); 4, β -lactoglobulin (18.4 kDa); 5, lysozyme (14.3 kDa); 6, γ_2 (1.5 μ g) eluted from hydroxylapatite; 7, a non-haemolytic fraction eluted from hydroxylapatite; 8, γ_1 (1.5 μ g) eluted from hydroxylapatite. When up to 10 μ g of either γ_1 or γ_2 was loaded onto a gel, no additional bands were visible even when stained with silver nitrate.

and the antiserum raised, when tested by Western immunoblotting, also reacted with a band with a molecular mass of 45 kDa present in the culture supernatant of some strains of *S. aureus*. Western immunoblotting confirmed that γ_1 , isolated from strains Smith 5R and PG23 was serologically identical (Fig. 2). There was no immunological cross-reactivity between either γ_1 or γ_2 .

Isoleucine was the most common amino acid found within the sequence of the first twenty N-terminal amino acid residues of γ_1 : Glu-Asn-Lys-Ile-Glu-Asp-Ile-Gly-Glu-Gly-Ala-Glu-Ile-Ile-Lys-Arg-Thr-Glu-Asp-Ile. Due to impurities in the fractions containing γ_2 it was not possible to obtain an N-terminal amino acid sequence for this component.

Fig. 2. Western blot of γ_1 from strains Smith 5R and PG23 using specific antiserum raised against γ_1 from strain Smith 5R. Lanes: 1, γ_1 from Smith 5R; 2, γ_1 from PG23; 3, Smith 5R culture supernate concentrated tenfold; 4, PG23 culture concentrated tenfold. An excess of protein was loaded on to lane 3 and the higher molecular mass band is thought to be due to either non-specific staining caused by insufficient blocking or the presence of a cross-reacting protein.

Haemolytic activity of γ_1 and γ_2 .

Rabbit and sheep erythrocytes were more sensitive than human erythrocytes. γ_1 and γ_2 acted together to potentiate haemolysis on rabbit, sheep and human erythrocytes. This was demonstrated on blood-agarose plates (Fig. 3). However, when γ_1 and γ_2 were first allowed to diffuse from adjacent wells in a plain agarose plate incubated for 8 h at 37 °C which was then overlaid with rabbit blood-agarose, no haemolysis occurred. Synergism between γ_1 and γ_2 could also be demonstrated in microtitre plate assays (Table 2).

The haemolytic activity of γ_1 and γ_2 was inhibited by agar. Synergism tests showed that γ_1 was resistant to treatment at 70 °C for 10 min whereas γ_2 was inactivated after 10 min at 55 °C.

When γ_1 and γ_2 were mixed and incubated together at 37 °C prior to the addition of the erythrocytes the degree of synergism between the two components, as determined by microtitre plate assays, decreased markedly

Fig. 3. γ -Lysin synergism and the effect of proteases on a rabbit blood-agarose plate. Wells contain: A–C, γ_1 (2 μ g); D, papain (25 μ g); E, trypsin (25 μ g); F, γ_1 (2 μ g); G–I, γ_2 (1 μ g).

Table 2. Synergistic haemolysis between γ_1 and γ_2 on rabbit, sheep and human erythrocytes determined by microtitre plate haemolysin assays when the erythrocytes were added immediately after and 45 min after the components were mixed

γ_1 and γ_2 were premixed for 45 min at 37 °C prior to the addition of the erythrocytes. The concentration of γ_1 was twice that of γ_2 . This was the concentration which gave optimal haemolysis.

	Haemolytic activity (HU ml ⁻¹)					
	Rabbit		Sheep		Human	
	0 min	45 min	0 min	45 min	0 min	45 min
γ_1	8	4	8	4	0	0
γ_2	16	8	16	8	0	0
$\gamma_1 + \gamma_2$	128	8	128	8	8	2

with time. A decrease in synergism was noted after 10 min and the synergism between the two components continued to decrease with time up to 45 min (Table 2) after which time further incubation did not result in any further decrease in titre.

The proteases trypsin and papain each acted synergistically with γ_2 to potentiate haemolysis on rabbit erythrocytes (Fig. 3) but not on sheep or human erythrocytes (Table 3). Indeed, when tested on sheep and human erythrocytes trypsin and papain appeared to

Fig. 4. Proteolytic digestion of γ_2 by trypsin and papain. Samples 1, 2, 5, 6, and 7 were incubated at 37 °C for 45 min. Digestion was terminated by boiling for 2 min. The samples were then run on a 15% (w/v) SDS-polyacrylamide gel which was stained with silver nitrate. Lanes: 1, γ_1 (2 μ g); 2, γ_2 (1 μ g); 3, trypsin (25 μ g); 4, papain (25 μ g); 5, γ_1 (2 μ g) + γ_2 (1 μ g); 6, γ_2 (1 μ g) + trypsin (25 μ g); 7, γ_2 (1 μ g) + papain (25 μ g).

Table 3. Haemolytic activity of γ -lysin components in the presence of proteases on rabbit, sheep and human erythrocytes as determined by microtitre plate haemolysin assays

Haemolysin titres were determined as described in Methods. Lysin components and enzymes were not preincubated prior to the addition of the erythrocytes.

	Haemolytic activity (U ml ⁻¹)		
	Rabbit	Sheep	Human
γ_1 (2 μ g)	2	2	0
γ_2 (1 μ g)	2	4	0
Papain (25 μ g)	0	0	0
Trypsin (25 μ g)	0	0	0
γ_1 (2 μ g) + γ_2 (1 μ g)	64	32	8
γ_2 (1 μ g) + papain (25 μ g)	32	4	0
γ_2 (1 μ g) + trypsin (25 μ g)	64	4	0

Fig. 5. Western immunoblot of erythrocyte membranes incubated with one component of γ -lysin prior to incubation with the other component. Freshly washed erythrocytes were incubated with one component of γ -lysin at 37 °C for 30 min to allow the component to bind. Erythrocytes were then washed and the second component added. After incubation, erythrocyte membranes were prepared as described in Methods. The binding of the second component to the membranes was determined by Western immunoblotting. (a) Western immunoblot probed with γ_1 antiserum. Lanes: 1, concentrated culture supernate from strain PG23; 2, γ_1 ; 3, γ_1 bound to rabbit erythrocyte membranes containing bound γ_2 ; 4, γ_1 bound to sheep erythrocyte membranes containing bound γ_2 ; 5, γ_1 bound to human erythrocyte membranes containing bound γ_2 . (b) Western immunoblot probed with γ_2 antiserum. Lanes: 1, γ_2 ; 2, γ_2 bound to rabbit erythrocyte membranes containing bound γ_1 ; 3, γ_2 bound to sheep erythrocyte membranes containing bound γ_1 ; 4, human erythrocyte membranes containing bound γ_1 that were incubated with γ_2 .

inhibit the synergism between γ_1 and γ_2 . This was most noticeable with papain on human blood. Neither chymotrypsin nor *S. aureus* V8 protease acted synergistically with γ_2 .

None of the proteases tested acted synergistically with γ_1 . Incubation of either γ_1 or γ_2 with a protease inhibitor decreased the synergism between the two components. In order to abolish the synergism completely both components had to be treated with a protease inhibitor prior to use. Cysteine had no effect on the synergism between the two components.

Susceptibility of γ_2 to proteolytic degradation by γ_1 , trypsin and papain

γ_2 (1 μ g) was completely digested by both trypsin (25 μ g) and papain (25 μ g) after incubation for 45 min as shown by SDS-PAGE (Fig. 4). There was no apparent digestion

of γ_2 (1 μ g) by γ_1 (2 μ g) or *vice versa* when the two components were incubated together. Likewise, no immunoreactive fragments could be detected by Western immunoblotting using either γ_1 or γ_2 antiserum. The final concentrations of γ_1 and of proteases used in these experiments were the same as those used to potentiate haemolysis with γ_2 on rabbit erythrocytes. However, it must be noted that the concentration of γ_1 used was approximately tenfold less than that of trypsin or papain.

Binding of γ_1 and γ_2 to erythrocyte membranes

Both γ_1 and γ_2 bind to rabbit, sheep and human erythrocyte membranes. Prior binding of γ_1 to human erythrocyte membranes prevented subsequent binding of γ_2 . This effect of γ_1 applied only to human erythrocyte membranes; the effect was not seen for either rabbit or sheep erythrocyte membranes (Fig. 5). On the other

hand, prior binding of γ_2 to membranes did not affect subsequent binding of γ_1 to rabbit, sheep or human erythrocyte membranes.

Discussion

Chromatography on hydroxylapatite allows separation of the two components of γ -lysin. In the past only workers who used hydroxylapatite in the purification procedure reported the existence of two separate components (Guyonnet *et al.*, 1968; Guyonnet & Plommet, 1970; Bezard & Plommet, 1973; Taylor & Bernheimer, 1974). In view of the very similar pI values of γ_1 and γ_2 , 9.4 and 9.3, respectively, it is not surprising that Mollby & Wadstrom (1971) were unable to separate the two components by isoelectric focussing. The molecular masses of γ_1 and γ_2 estimated by SDS-PAGE were found to be 32 and 36 kDa, respectively. These differ from values reported by Taylor & Bernheimer (1974), who found the molecular masses of γ_1 and γ_2 to be 26 and 29 kDa, respectively, by gel filtration on a G-50 Sephadex column. Molecular cloning and genetic analysis of the determinant for γ -lysin also showed that γ -lysin requires the co-operative action of two polypeptides with molecular masses of 32 and 36 kDa (Cooney *et al.*, 1988).

Both components of γ -lysin have similar molecular masses and pI values. They differ, however, in their heat stability and in their immunogenicity. Staphylococcal γ -lysin has been reported previously to be heat-sensitive with haemolytic activity being completely destroyed after heating to 55 °C for 10 min (Jackson, 1963). In this study only γ_2 was found to be destroyed by heating to 55 °C. The antigenicity of γ -lysin has been well established (Guyonnet & Plommet, 1970; Taylor & Plommet, 1973). Taylor & Plommet (1973) used the fact that γ -lysin is antigenic in man to show that estimation of anti- γ -lysin titres in serum can be useful in the diagnosis and treatment of staphylococcal osteomyelitis. The poor immunogenic response to γ_2 in the rabbit and the extent of its immunogenicity in other species requires further study.

Clearly γ_1 and γ_2 act together to potentiate haemolysis on rabbit, sheep and human erythrocytes. Exactly how they act together, however, is unknown. It seems possible that proteolytic activation may be involved since the synergism between the two components is inhibited by a number of protease inhibitors. The fact that either trypsin or papain can be substituted for γ_1 to give synergistic haemolysis with γ_2 on rabbit blood supports the view that γ_1 is a protease.

If γ_1 is proteolytic it might nick γ_2 to yield a product that is haemolytic. The observation that haemolytic

activity decreases when γ_1 and γ_2 are in contact with each other for more than a few minutes prior to the addition of erythrocytes could be due to degradation of γ_2 by γ_1 to such an extent that haemolytic activity is destroyed.

The optimum concentrations of trypsin and papain in synergism experiments with γ_2 was 1 mg ml⁻¹; this contrasts with the much lower concentration required for optimum activation of γ_2 by γ_1 (100 µg ml⁻¹). Zones of synergism demonstrated on blood plates between γ_2 and γ_1 , or between γ_2 and trypsin or papain always occurred at the mid-point between the two wells indicating haemolysis only occurs where the two components meet in optimum proportions. *S. aureus* V8 protease did not act synergistically with γ_2 to give potentiated haemolysis. Collectively these results can be interpreted by proposing that if γ_1 is a protease it acts specifically on γ_2 and that γ_2 is not degraded by other staphylococcal proteases.

Synergism with trypsin and papain was observed only on rabbit blood; on sheep or human blood the enzymes acted to inhibit the synergism between γ_1 and γ_2 . As trypsin and papain at a concentration of 1 mg ml⁻¹ are both capable of digesting γ_2 completely it is necessary to provide an explanation for the synergy between γ_2 and proteases on rabbit erythrocytes. It is possible that rabbit erythrocytes contain a protease inhibitor which prevents trypsin and papain from completely digesting γ_2 or that under the conditions of a diffusion experiment in the presence of rabbit erythrocytes, the haemolytic component found after interaction between γ_2 and protease binds irreversibly to the erythrocytes thus rendering it inaccessible to further degradation by a protease.

The haemolytic effect observed with rabbit erythrocytes in the presence of γ_2 and protease is unlikely to be due to a direct effect of trypsin or papain on the erythrocytes. There are no reports in the literature of the release of a factor from erythrocytes that potentiates haemolysis by other bacterial haemolysins. Also, the results with protease inhibitors suggest that direct interaction between γ_1 and γ_2 generates enhanced haemolytic activity. Both γ_1 and γ_2 had to be treated with a protease inhibitor in order to inhibit haemolysis completely which suggests that γ_2 could also have some proteolytic activity.

Trypsin belongs to the serine class of enzymes and catalyses the hydrolysis of only those peptide bonds in a polypeptide chain whose carbonyl function is donated by either a lysine or an arginine residue. Papain on the other hand belongs to the cysteine class of enzymes and a covalent thioester bond is formed between an acyl group of the substrate and the sulphhydryl group of a specific cysteine residue in the active site of the enzyme. There is a report that cysteine inhibits γ -toxin (Jackson, 1963) but this observation has not been confirmed by either us or other workers (Taylor & Bernheimer, 1974).

Degradation of γ_2 by γ_1 could not be shown by silver nitrate staining of SDS-polyacrylamide gels and no immunoreactive fragments could be detected by Western immunoblotting. This does not necessarily disprove the protease hypothesis. It is quite possible that γ_1 nicks a short peptide fragment from γ_2 consisting of only a few amino acid residues; such a nicked peptide might be very small and could easily run off the gel. Also, such a change would make no appreciable difference to the molecular mass of γ_2 as determined by SDS-PAGE. A way of unequivocally proving that γ_1 nicks a few amino acid residues from γ_2 would be to determine the N- and the C-terminal sequence of γ_2 before and after treatment with γ_1 . This would also locate the precise point where γ_1 nicks γ_2 .

The γ_1 and γ_2 components were each able to bind to rabbit, sheep and human erythrocyte membranes when the toxin was incubated with whole cells prior to the preparation of the membranes. The presence of bound γ_2 on the membranes did not inhibit binding of γ_1 but γ_1 did inhibit binding of γ_2 to human erythrocyte membranes. This could explain why human erythrocytes are less sensitive to haemolysis by γ -lysin than are sheep or rabbit erythrocytes. If an excess of γ_1 was present, it would bind to the membranes and block the action of the haemolytic derivative of γ_2 .

This is the first study which reports the purification of γ -lysin from a strain of *S. aureus* other than Smith 5R. Previous work has shown that γ -lysin is commonly produced by strains of *S. aureus* associated with TSS (Clyne *et al.*, 1988). Both the genetic analysis of the γ -lysin determinant in strain Smith 5R (Cooney *et al.*, 1988) and this study show that γ -lysin is a two component haemolysin. Little is known about the mode of action of γ -lysin but it is reasonable to suggest, as a result of this study, that γ_1 is a protease or a protease-like molecule that acts specifically on γ_2 to give enhanced haemolysis.

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