Purification and characterization of a lecithin-dependent haemolysin from *Escherichia coli* transformed by a *Vibrio parahaemolyticus* gene

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(Received 29 April 1991; revised 16 July 1991; accepted 15 August 1991)

Lecithin-dependent haemolysin (LDH) of *Vibrio parahaemolyticus* was purified from *Escherichia coli* C600 transformed with a plasmid (pHL591) ligated with a 1.5 kb DNA fragment of *V. parahaemolyticus*. The final preparation comprised two LDH proteins with different molecular masses which were immunologically cross-reactive and had the same enzymic activity. The LDH was a phospholipase hydrolysing both fatty acid esters of phospholipid, i.e. it hydrolysed phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) and then LPC to glycerophosphorylcholine (GPC). From this point of view, LDH should be classified as a phospholipase B. Phospholipase B, however, does not usually show haemolytic activity, because the intermediate (LPC), which is the actual haemolytic agent, is immediately hydrolysed to the final product (GPC). On the other hand, LPC formed by LDH action was comparatively stable, because the rates of the two reactions catalysed by LDH, PC to LPC and LPC to GPC, are almost the same. This is the reason that LDH shows haemolytic activity. Therefore, LDH of *V. parahaemolyticus* is an atypical phospholipase to be designated as phospholipase A₂/lysophospholipase.

Introduction

Vibrio parahaemolyticus is a slightly halophilic bacterium and a major causative agent of food poisoning in Japan. Although several kinds of haemolysins have been reported as potential pathogenic factors, the actual cause of the diarrhoea has not been clarified. Among these haemolysins, thermostable direct haemolysin (TDH) is the best-documented toxin, and is well known as the causative agent of Kanagawa phenomenon, a haemolysis on a blood-agar medium caused by pathogenic strains of the vibrio (Sakazaki *et al.*, 1968; Miyamoto *et al.*, 1969; Miwatani & Takeda, 1976). TDH-related haemolysins produced by KP-negative strains of *V. parahaemolyticus* (Honda *et al.*, 1988, 1989) or vibrios other than *V. parahaemolyticus* (Yoh *et al.*, 1986a, b) have also been reported. Delta-VPH is a haemolysin produced by a

strain of *Escherichia coli* transformed by a plasmid containing a DNA fragment of *V. parahaemolyticus*, but the production of delta-VPH by the donor vibrio has not been demonstrated (Taniguchi *et al.*, 1990).

Yanagase *et al.* (1970) reported a haemolytic factor of *V. parahaemolyticus* which was activated by the addition of lecithin (phosphatidylcholine) and was denoted as lecithin-dependent haemolysin (LDH). We purified LDH from a culture supernatant of *V. parahaemolyticus*, but the yield of the final preparation was too small to allow its characterization. In the course of cloning the TDH gene of *V. parahaemolyticus*, Taniguchi *et al.* (1985, 1986) obtained a transformant [*E. coli* C600(pHL591)] producing LDH, which was designated as thermolabile haemolysin in their paper. The transformant has a 1.5 kb *Hind*III–*Hinc*II DNA fragment of *V. parahaemolyticus* and produces LDH using the endogenous vibrio promoter. We found that LDH produced by the transformant is localized in the periplasmic space of the cell and is easily released by osmotic shock treatment. Thus, we could obtain an LDH fraction with high specific activity. This paper shows the existence of two immunologically cross-reactive LDH molecules and the atypical property of LDH as a phospholipase (PLase).

Abbreviations: GPC, glycerophosphorylcholine; LDH, lecithin-dependent haemolysin; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PLase, phospholipase.

The amino acid sequence data reported in this paper have been submitted to PIR and have been assigned the accession number A39996 [LDH(S)] and B39996 [LDH(L)].

Methods

Purification of LDH. LDH was purified from a periplasmic fraction of the transformant *E. coli* C600(pHL591) (Taniguchi *et al.*, 1986). The seed inoculum was prepared by cultivating the cells in 5 ml heart infusion (HI) broth (Difco) at 37 °C overnight without shaking, and inoculated into 500 ml HI broth. Cultivation was carried out at 37 °C for 16 h with shaking (80 cycles min⁻¹). The cells were harvested and washed with saline by centrifugation at 7000 *g* for 40 min. A periplasmic fraction was obtained by an osmotic shock method described by Neu & Heppel (1965). Briefly, the washed cell pellet obtained from 3 l of the culture was suspended in 100 ml cold water. The suspension was centrifuged at 7000 *g* for 40 min and the supernatant was collected as the periplasmic fraction. Ammonium sulphate was added to 65% saturation, and the precipitate was collected by centrifugation. The precipitate was resuspended in 3 ml 20 mM-histidine/HCl buffer (pH 5.5), dialysed against 1 litre of the buffer and applied to a Pharmacia LKB Fast Protein Liquid Chromatography system equipped with a Mono Q HR 5/5 anion exchange column. The column was washed with 16 ml 20 mM-histidine/HCl buffer and the fractions were eluted with 14 ml 0.9 M-NaCl/20 mM-histidine/HCl buffer. Lecithin-dependent haemolytic activity of each fraction was assayed as described below and fractions with haemolytic activity were pooled. The LDH preparation thus obtained from the transformant is designated as Tr-LDH, while LDH produced by *V. parahaemolyticus* is designated as Vp-LDH.

To obtain a preparation of Vp-LDH, a clinical strain, *V. parahaemolyticus* MY 80-11 (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), was cultivated in HI dialysate medium [200 g HI medium powder dissolved in 1.6 l water, and then dialysed against 6.4 l 1.5% (w/v) NaCl solution] at 37 °C for 40 h. The LDH preparation obtained by ammonium sulphate fractionation (65% saturation) of the culture supernatant was suspended in 3 ml 20 mM-histidine/HCl buffer and dialysed against the buffer overnight. The dialysed preparation was centrifuged at 100 000 *g* for 120 min and the supernatant was used as a partially purified Vp-LDH.

Protein assay. The amount of protein was determined by the Lowry method with bovine serum albumin as the standard.

Determination of haemolytic activity. A 0.1 ml aliquot of sample solution containing 0.9% NaCl was mixed with 0.3 ml 1.3% (v/v) sheep erythrocyte suspension in 0.9% NaCl/10 mM-Tris/HCl buffer (pH 7.5) containing 10 µg phosphatidylcholine (PC) (egg-yolk lecithin, type XIII-E, Sigma) and the mixture was incubated at 37 °C. After 1 h incubation, 2.5 ml 0.9% NaCl solution was added and the mixture was centrifuged at 1000 *g* for 15 min. Haemolysis was determined by measuring the *A*₅₄₀ of the supernatant and the haemolytic activity, in haemolytic units (HU), was expressed as the reciprocal of the dilution of the haemolysin solution which showed 50% haemolysis.

Determination of phospholipase and lysophospholipase activity. Hydrolysis of PC to lysophosphatidylcholine (LPC) or LPC to glycerophosphorylcholine (GPC) was determined by measuring the decrease in the amount of fatty acid ester residues of the phospholipids by the hydroxamate method (Nishida & Tamiya, 1986). Sample solution (40 µl) was mixed with 160 µl Tris/HCl buffer containing 0.125% Triton X-100, 0.025 M-CaCl₂, 0.875 mM-EDTA and 2.5 mM-PC, and incubated at 37 °C for 30 min. Ethanol (400 µl) and 1 M-hydroxylamine/1.75 M-NaOH solution (200 µl) were added and the mixture was incubated for 30 min at room temperature. Finally 120 µl 3.3 M-HCl and 100 µl FeCl₃ solution (50 g FeCl₃·6H₂O dissolved in 10 ml 3.6 M-HCl, and then diluted nine times with ethanol) were added and *A*₅₂₀ was measured.

Electrophoresis. PAGE was carried out by the method of Davis & Ornstein (1986) with 12% (w/v) acrylamide gel. SDS-PAGE was carried out by the method of Laemmli (1970) with 12.5% (w/v) acrylamide gel. The gels were stained with 0.25% Coomassie brilliant blue.

Determination of N-terminal amino acid sequence. Protein bands from PAGE were transferred to siliconized glass fibre sheet by electroblotting (Eckerson *et al.*, 1988) and the N-terminal amino acid sequence was determined with a Pulse-liquid Sequencer (model 477A, Applied Biosystems).

TLC of phospholipids. Phospholipid spots on a silica gel 60 plate were developed with chloroform/methanol/water (65:35:5, by vol.) and detected by spraying with 50% (v/v) H₂SO₄ and drying at 200 °C for 20 min.

GLC of fatty acids. Fatty acids were converted to their methyl ester derivatives (Carreau & Dubacq, 1978) and analysed with a gas chromatograph equipped with a flame-ionization detector (GC-6AM, Shimadzu, Japan). Gas chromatographic conditions were as follows: column, 15% DEGS on Unipore HP (80–100 mesh), 1.5 m × 3 mm i.d.; column temperature, 200 °C; N₂ flow rate, 50 ml min⁻¹.

Preparation of antibody against LDH. Two millilitres of a mixed emulsion of equal volumes of purified Tr-LDH solution (1 mg ml⁻¹) and Freund's complete adjuvant was injected into the foot pads of a guinea pig. At 2 and 4 weeks after the injection, booster injections of 1 ml of the emulsion were performed, and antiserum was obtained by bleeding the animal at 5 weeks after the first injection. The antiserum was fractionated with ammonium sulphate (40% saturation) and the precipitate was collected as an IgG fraction. Control serum was prepared with the same method by injecting an emulsion of 0.9% NaCl/10 mM-Tris/HCl buffer and adjuvant.

Reagents. PC (egg-yolk lecithin type XIII-E), LPC (egg-yolk lecithin type I, 1-palmitoyl-2-oleyl-PC, 1-oleyl-2-palmitoyl-PC, PLase A₂ (*Naja naja* venom) and PLase B (*Vibrio* sp.) were purchased from Sigma. Silica gel 60 was purchased from Merck.

Results

Tr-LDH was purified from the periplasmic fraction of the transformant by ammonium sulphate fractionation and Mono-Q column chromatography (Table 1). The recovery of lecithin-dependent haemolytic activity was 14.8% of that of the periplasmic fraction. The specific activity was 21 times that of the starting material. Conventional PAGE of the final preparation showed two protein bands (Fig. 1*a*). When the acrylamide gel column was embedded in lecithin/blood-agar gel [1% (w/v) agar, 4% (v/v) sheep erythrocytes, 3% (w/v) NaCl, 10% (w/v) lecithin, 50 mM-Tris/HCl buffer, pH 7.5] and incubated at 37 °C overnight, a stretched haemolytic zone with a constricted part in the middle was observed around the protein bands. The constricted part was thought to be formed by overlapping of the two haemolytic zones around the two protein bands. The diameters of the haemolytic zones were measured (Fig. 1*b*), especially at the constricted and swollen parts. When the gel column was embedded in agar gel (1% agar in 50 mM-Tris/HCl buffer, pH 7.5) and antiserum against LDH was applied in a trough parallel to the gel

Table 1. Purification of Tr-LDH

Purification step	Total volume (ml)	Total protein (mg)	Total activity* (HU)	Specific activity* (HU)	Recovery (%)
Periplasmic fraction	100	144.5	315000	2180	100
Preparation obtained by ammonium sulphate fractionation	3.3	17.2	178200	10360	56.6
Preparation obtained by Mono-Q column chromatography	2.0	1.0	46500	46500	14.8

* Measured as lecithin-dependent haemolytic activity.

Fig. 1. PAGE of LDH. A Tr-LDH preparation (50 µg protein) obtained by Mono-Q column chromatography was applied to a polyacrylamide gel column. Electrophoresis was carried out by the method of Davis & Ornstein (1968). One gel was stained with Coomassie brilliant blue (a). Another gel was subjected to the haemolysin assay with blood-agar as described in Methods (b).

column, precipitin arcs were formed against both bands and the arcs fused with each other. These results suggest that the two proteins in the final preparation are both LDH. It is thought that the transformant produces two LDH proteins which are immunologically cross-reactive. SDS-PAGE showed that the molecular masses of the two Tr-LDH molecules were 43 and 45 kDa. The two proteins, small- and large-sized Tr-LDH, were designated as Tr-LDH(S) and Tr-LDH(L), respectively.

The N-terminal amino acid sequences of the two LDHs were determined. As shown in Fig. 2, both sequences were in agreement with the sequence suggested by DNA sequence analysis (Taniguchi *et al.*, 1986). Tr-LDH(S) seems to be a protein which has 15 N-terminal amino acids deleted from the Tr-LDH(L) molecule. If so, the molecular masses of these proteins can be calculated as 41 453 and 42 794 kDa, respectively. These values are roughly in agreement with the results from SDS-PAGE.

The final preparation of Tr-LDH that we obtained was a mixture of two proteins, but the above results suggest that both proteins are products of the same gene and have the same haemolytic activity. Therefore, this fraction was used for the study of properties of LDH in subsequent experiments.

Such different-sized LDH molecules were also observed in Vp-LDH. When the partially purified Vp-LDH fraction was applied to conventional PAGE and the gel column was embedded in PC/blood-agar gel, the two overlapped haemolytic zones were observed in the same place as those of Tr-LDH (Fig. 3). In the gel diffusion test, Tr-LDH and Vp-LDH formed a fused precipitin line against anti-Tr-LDH antibody (data not shown).

It is well known that PLase A of snake or bee venom shows indirect haemolytic activity by hydrolysing phospholipids to lysophospholipids, which, due to their surface activity, dissociate erythrocyte membranes. Haemolysis by LDH is caused after the addition of phospholipid, suggesting that LDH has PLase A activity. Thus, the activity of LDH was compared with that of two reference enzymes: PLase A₂ of snake venom

(A) GCT TCT GCA GTT GCC GAA GAG CCA ACC TTA TCA CCA GAA ATG GTT TCA GCG TCT GAA
(B) Ala Ser Ala Val Ala Glu Glu Pro Thr Leu Ser Pro Glu Met Val Ser Ala Ser Glu
(C) Thr Glu Glu Pro Thr Leu Ser Pro Glu Met Val Ser Ala
(D) Ser Glu

(A) GTG ATC AGC ACG CAA GAA AAC CAA ACC TAT ACC TAT GTT CGC TGT TGG TAT CGC
(B) Val Ile Ser Thr Gln Glu Asn Gln Thr Tyr Thr Tyr Val Arg Cys Trp Tyr Arg
(C)
(D) Val Ile Ser Thr Gln Glu Asn ? Thr Tyr Thr ? Val

Fig. 2. N-terminal amino acid sequences of Tr-LDHs. Lines: A, DNA nucleotide sequence (Taniguchi *et al.*, 1986); B, amino acid sequence suggested by the DNA sequence; C and D, amino acid sequences of LDH(L) and LDH(S), respectively, determined in this paper.

Fig. 3. PAGE of LDHs obtained from *V. parahaemolyticus* (●, 500 µg partially purified protein) and transformed *E. coli* (○, 50 µg purified protein). The electrophoresis was carried out by the method of Davis & Ornstein (1968) and the gel was subjected to the haemolysin assay with blood-agar as described in Methods.

Fig. 4. Haemolytic activity of LDH and reference enzymes. Tr-LDH (a), PLase A₂ of *Naja naja* venom (b) and PLase B of a *Vibrio* sp. (c) were subjected to the assay as described in Methods.

and PLase B of a *Vibrio* species. Fig. 4 shows the haemolytic activities of Tr-LDH and the reference enzymes in the test-tube method. Tr-LDH caused haemolysis at low, but not high, concentrations. On the

Fig. 5. Hydrolysis of PC (○) and LPC (●) by Tr-LDH. LDH and the substrates were incubated at 37 °C for 30 min and the hydrolysis was assayed by the hydroxamate method as described in Methods.

Table 2. *Hydrolytic activity of LDH and reference enzymes against phosphatidylcholine and lysophosphatidylcholine*

ED₅₀ values (50% effective dose: µg enzyme necessary to hydrolyse 50% of 400 nmol substrate in the assay system) are given; they were determined by the hydroxamate method as described in Methods.

Substrate	Tr-LDH	PLase B	PLase A ₂
Phosphatidylcholine	13	332	19
Lysophosphatidylcholine	12	5	> 1000

other hand, haemolysis by PLase A₂ was dose dependent, and did not disappear at high concentration. PLase B did not show any haemolytic activity at the concentrations used, although it has enough enzymic activity against phospholipid, as shown below. Fig. 5 shows enzymic activity of Tr-LDH against PC and LPC, which was determined by measuring the decrease in the amount of fatty acid ester residues of the phospholipids. Tr-LDH showed almost the same degree of hydrolytic activity to both PC (diglyceride) and LPC (monoglyceride), suggesting that LDH hydrolyses both fatty acid esters of phospholipid at roughly the same rate. The ED₅₀ values (50% effective dose: µg enzyme necessary to hydrolyse 50% of 400 nmol substrate in the assay system) for PC and LPC were almost the same, as shown in Table 2. In contrast, PLase B was more effective on LPC, and PLase A₂ was effective only on PC (Table 2). These results suggest that LDH is an enzyme which hydrolyses both fatty acid esters of phospholipid but is different from PLase B in the rate of action against the two fatty acid ester residues.

Fig. 6 shows the results of the phospholipid-agar gel assay of LDH and reference enzymes. Agar gel plates (1% agar in 50 mM-Tris/HCl buffer, pH 7.5) containing

Fig. 6. Enzymic activity of Tr-LDH and reference enzymes in agar gels containing phospholipids. Purified PLase B (350 µg) (1), PLase A₂ (380 µg) (2) and Tr-LDH (100 µg) (3) were placed into wells of agar gels containing phosphatidylcholine (a) or lysophosphatidylcholine (b) and the gels were incubated at 37 °C as described in Methods.

0.1% PC (PC-agar) or 0.1% LPC (LPC-agar) were prepared, the PLases were applied in the wells and the agar plates were incubated at 37 °C overnight. In PC-agar (an opaque gel because of the insolubility of PC), PLase A₂ formed a clear zone around the well, whereas Tr-LDH and PLase B formed a concentric circle, an outer clear ring and an inner turbid zone, the clear ring of PLase B being very thin compared with the inner turbid zone. Gradients of the enzyme concentration formed

around the wells. These results suggest, therefore, that PC was partially hydrolysed to soluble LPC in the outer ring and LPC was further hydrolysed to GPC and insoluble free fatty acids in the inner ring. In LPC-agar (a translucent gel because of the solubility of LPC), on the other hand, PLase B and Tr-LDH formed a turbid zone because of insoluble fatty acid, whereas PLase A₂ had no effect. Although the same amount of enzyme was applied to both gels, PLase B formed a larger zone in the LPC-gel, whereas zones formed by Tr-LDH were almost the same in both gels. The extent of the clear or turbid circles in PC-agar corresponds to the progress of the PLase action, i.e. formation of the reaction products: LPC and a free fatty acid in the clear zone, and GPC and two free acids in the turbid zone. To confirm this, chloroform extracts of the circles were applied to a TLC plate. In the extract of the clear zone on PC-agar formed by LDH action, LPC and free fatty acid were detected. In the turbid zone, however, no LPC remained (data not shown). The phospholipid-agar assay thus demonstrated the different reactions of these PLases.

To determine which fatty acid ester of PC was hydrolysed first, PCs with two different fatty acid residues were used for the phospholipid-gel assay. Tr-LDH was applied to the agar gel containing such PCs, and the fatty acid liberated in the outer clear zone, in which only one ester was hydrolysed (as shown above), was determined by gas chromatography. When 2-oleyl-1-palmitoyl-PC was used, only oleic acid was detected, whereas palmitic acid was detected from the 2-palmitoyl-1-oleyl-PC-agar, suggesting that LDH hydrolysed the ester in the 2-position in the first step (data not shown).

The LDH preparation used in this experiment is a mixture of two immunologically cross-reactive protein molecules. As shown above, the LDH preparation hydrolyses both fatty acid esters of the phospholipid. Therefore, one might suspect that the two proteins have different functions, one being a PLase A hydrolysing diglyceride to monoglyceride and the other, a lyso-PLase hydrolysing the monoglyceride. If so, only one of the two proteins should form a clear zone in PC-agar gel. When the gel of conventional PAGE was embedded in PC-agar gel, however, the double circled zone (outer clear and inner turbid) was formed around both protein bands, suggesting the identity of the activity of the two protein bands.

Discussion

LDH of *V. parahaemolyticus* was purified from the periplasmic fraction of *E. coli* C600 transformed by a recombinant plasmid, pHL591, containing a DNA fragment of the chromosome of *V. parahaemolyticus*.

Purification was accomplished in only two steps, ammonium sulphate fractionation and FPLC with a Mono-Q column. This was easier than purification from culture supernatant of *V. parahaemolyticus*, in which more steps are necessary and the yield is very low (unpublished observation). The LDH (Tr-LDH) obtained from the transformant seems to be identical with LDH produced by *V. parahaemolyticus* (Vp-LDH), because these LDHs showed the same mobility on PAGE and were cross-reactive in the immuno-gel diffusion test.

The preparation obtained was a mixture of two LDH molecules, LDH(L) and LDH(S). Application of the two proteins separated by conventional PAGE to blood- or phospholipid-agar gel revealed that both molecules have the same haemolytic and enzymic activities. The size of the cloned DNA fragment with the LDH gene is 1.5 kb, which is able to encode an approximately 50 kDa protein. Moreover, the fragment contains a promoter region. The sizes of LDH(L) and LDH(S) estimated by SDS-PAGE are 45 and 43 kDa, respectively. Therefore, it is thought that the two LDH molecules are products of the same structural gene, and are separated in the processing step. Two sites for signal peptidase may exist in the sequence. This was supported by analysis of the N-terminal amino acid sequence of LDHs. The sequences of the regions analysed roughly agreed with the sequence estimated from the DNA sequence (Taniguchi *et al.*, 1986). The sequences suggest that LDH(S) is a protein in which 15 amino acids of the N-terminal of LDH(L) are deleted. Such processing seems to take place not only in the transformant but also in the donor, *V. parahaemolyticus*, because the same haemolytic zones were observed around Tr-LDH and Vp-LDH migrated by PAGE.

The LDH of *V. parahaemolyticus* has been thought to be a PLase A (Yanagase *et al.*, 1970). The LDH we obtained, however, hydrolysed not only PC to LPC but also LPC to GPC. PLase A is defined as an enzyme hydrolysing one of two fatty acid esters of phospholipid, and PLase B as an enzyme hydrolysing both esters. According to this definition, the LDH that we obtained should be classified as PLase B. However, the activity against LPC (mono-glyceride) of the typical PLase Bs obtained from various sources is much higher than that against PC (diglyceride). Our result in the PC- or LPC-agar assays with a commercially available PLase B preparation also showed this property by PLase B. Therefore LPC formed by PLase B action seems to be immediately hydrolysed to GPC. PLase B has no haemolytic activity because the final product, GPC, is no longer a detergent. On the other hand, LPC formed by PLase A action is a detergent which disturbs erythrocyte membranes, leading to haemolysis. Although LDH hydrolyses LPC, the reaction rate is much lower than

that of PLase B. Therefore, the intermediate, LPC, is relatively stable and present in sufficient quantity to cause haemolysis, although addition of too much LDH decreases the haemolytic activity because of the rapid hydrolysis of LPC. Thus LDH of *V. parahaemolyticus* is an atypical PLase, an exceptional PLase B, or a new type of enzyme which should be designated as PLase A₂/lysophospholipase, which has been demonstrated in guinea pigs (Gassama-Diagne *et al.*, 1989). Such an enzyme was also reported in rats (Pind & Kuksis, 1989), and Nishijima & Nojima (1974) reported that PLase A₁ of *Mycobacterium phlei* has some lysophospholipase activity.

Misaki & Matsumoto (1978) reported purification of lysophospholipase of *V. parahaemolyticus*. However, this enzyme is a hexamer which has a molecular mass of 89 kDa and cannot hydrolyse PC. Therefore, the LDH reported here is different from lysophospholipase.

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

- CARREAU, J. P. & DUBACQ, J. P. (1978). Adaptation of a macroscale to the microscale for fatty acid methyl transesterification of biological lipid extracts. *Journal of Chromatography* **151**, 384-390.
- DAVIS, B. J. & ORNSTEIN, L. (1968). Disc electrophoresis, acrylamide gel columns. In *Methods in Immunology and Immunochemistry*, vol. II, pp. 38-47. Edited by C. A. Williams & M. W. Chase. New York & London: Academic Press.
- ECKERSKORN, C., MEWES, W., GORETZKI, H. & LOTTSPEICK, F. (1988). A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. *European Journal of Biochemistry* **176**, 509-519.
- GASSAMA-DIAGNE, A., FAUVEL, J. & CHAP, H. (1989). Purification of a new, calcium-independent, high molecular weight phospholipase A₂/lysophospholipase (phospholipase B) from guinea pig intestinal brush-border membrane. *Journal of Biological Chemistry* **264**, 9470-9475.
- HONDA, T., NI, Y. & MIWATANI, T. (1988). Purification and characterization of a haemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct haemolysin. *Infection and Immunity* **56**, 961-965.
- HONDA, T., NI, Y. & MIWATANI, T. (1989). Purification of TDH-related haemolysin produced by a Kanagawa phenomenon-negative clinical isolate of *Vibrio parahaemolyticus* O6:K46. *FEMS Microbiology Letters* **57**, 241-246.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- MISAKI, H. & MATSUMOTO, M. (1978). Purification of lysophospholipase of *Vibrio parahaemolyticus* and its properties. *Journal of Biochemistry* **83**, 1395-1405.
- MIYAMOTO, Y., KATO, T., OBARA, Y., AKIYAMA, S., TAKIZAWA, K. & YAMAI, S. (1969). *In vitro* haemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *Journal of Bacteriology* **100**, 1147-1149.
- MIWATANI, T. & TAKEDA, Y. (1976). Discovery of *Vibrio parahaemolyticus*. In *Vibrio parahaemolyticus: Causative Bacterium of Food Poisoning*, pp. 1-5. Tokyo: Saikon Publishing Co.

- NEU, H. C. & HEPPEL, A. L. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *Journal of Biological Chemistry* **240**, 3685–3692.
- NISHIDA, S. & TAMIYA, N. (1986). Phospholipase A₂ of snake and bee venom (in Japanese). *Protein, Nucleic Acid and Enzyme* **31**, 158–165.
- NISHIJIMA, M. & NOJIMA, S. (1974). Membrane-bounded phospholipase A₁ of *Mycobacterium phlei* (in Japanese). *Protein, Nucleic Acid and Enzyme* **12**, 277–285.
- PIND, S. & KUKSIS, A. (1989). Association of the intestinal brushborder membrane phospholipase A₂ and lysophospholipase activities (phospholipase B) with a stalked membrane protein. *Lipids* **24**, 357–362.
- SAKAZAKI, R., TAMURA, K., KATO, T., OBARA, Y. & YAMAI, S. (1968). Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. III. Enteropathogenicity. *Japanese Journal of Medical Science and Biology* **21**, 325–331.
- TANIGUCHI, H., OHTA, H., OGAWA, M. & MIZUGUCHI, Y. (1985). Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct haemolysin and thermolabile haemolysin genes. *Journal of Bacteriology* **162**, 510–515.
- TANIGUCHI, H., HIRANO, H., KUBOMURA, S., HIGASHI, K. & MIZUGUCHI, Y. (1986). Comparison of the nucleotide sequences of the genes for the thermostable direct haemolysin and the thermolabile haemolysin from *Vibrio parahaemolyticus*. *Microbial Pathogenesis* **1**, 425–432.
- TANIGUCHI, H., KUBOMURA, S., HIRANO, H., MIZUE, K., OGAWA, M. & MIZUGUCHI, Y. (1990). Cloning and characterization of a gene encoding a new thermostable haemolysin from *Vibrio parahaemolyticus*. *FEMS Microbiology Letters* **67**, 339–346.
- YANAGASE, Y., INOUE, K., OZAKI, M., OCHI, T., AMANO, T. & CHAZONO, M. (1970). Hemolysins and related enzymes of *Vibrio parahaemolyticus* I. Identification and partial purification of enzymes. *Biken Journal* **13**, 77–92.
- YOH, M., HONDA, T. & MIWATANI, T. (1986*a*). Purification and partial characterization of a non-O1 *Vibrio cholerae* haemolysin that cross-reacts with thermostable direct haemolysin of *Vibrio parahaemolyticus*. *Infection and Immunity* **2**, 319–322.
- YOH, M., HONDA, T. & MIWATANI, T. (1986*b*). Purification and partial characterization of a *Vibrio hollisae* haemolysin that relates to the thermostable direct haemolysin of *Vibrio parahaemolyticus*. *Canadian Journal of Microbiology* **32**, 632–636.