Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*

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The cytotoxicity of the two different enterotoxin complexes of *Bacillus cereus* was compared after isolation from three different strains. Protein components of non-haemolytic enterotoxin (NHE) of 39 kDa, 45 kDa and 105 kDa were isolated from all of the three strains, whilst proteins B, L₁ and L₂ of haemolysin BL (HBL) were isolated from supernatants of two strains (F837-76 and 1230-88). These proteins were not detected in strain 0075-95. Inhibition of protein synthesis in Vero cells was used as a measure of cytotoxicity. The HBL complex from strain F837-76 was highly toxic. This strain also produced the NHE complex. However, when purified, at least two of the components of NHE had to be present in higher amounts than those of the components of HBL to cause the same degree of toxicity. Both complexes purified from strain 1230-88 were cytotoxic. The amount required to cause the same degree of cytotoxicity was approximately equal for the components of the two complexes, except that higher amounts of the 105 kDa protein of NHE had to be present than for the other components. None of the purified complexes from strain 1230-88 was toxic in amounts comparable to those of the HBL complex of strain F837-76 and NHE of strain 0075-95. These results indicate that when measuring cytotoxic enterotoxins from *B. cereus* at least two different complexes and six different proteins have to be taken into consideration.

Keywords: *Bacillus cereus*, enterotoxin, haemolysin

INTRODUCTION

Bacillus cereus is responsible for diarrhoeal and emetic types of food poisoning, which are caused by different toxins (for reviews see Granum, 1997; Kramer & Gilbert, 1989). The diarrhoeal type of food poisoning is caused by enterotoxin(s) produced during vegetative growth of the bacteria in the small intestine (Granum, 1994).

Different enterotoxin complexes of *B. cereus* have been described. Haemolysin BL (HBL), containing the protein components B, L₁ and L₂, has been well characterized. The B component was cloned and sequenced by Heinrichs *et al.* (1993) and the entire HBL operon characterized recently by Ryan *et al.* (1997). This complex has haemolytic, dermonecrotic and vascular permeability

activities, and causes fluid accumulation in ligated rabbit ileal loops (Beecher & Wong, 1994a, 1997; Beecher *et al.*, 1995). HBL has therefore been suggested to be a primary virulence factor in *B. cereus* diarrhoea (Beecher *et al.*, 1995). The components of HBL have been isolated only from strain F837-76, although multicomponent enterotoxins from other strains of *B. cereus*, containing similar proteins, have been reported by others (Thompson *et al.*, 1984; Bitsaev & Ezepechuk, 1987). In HBL and the related complexes all three components were required for maximal activity. Another, non-haemolytic enterotoxin (NHE) complex, consisting of proteins of molecular mass 39, 45 and 105 kDa, which together are cytotoxic in low amounts, has been isolated from a strain which caused a large food poisoning outbreak (Lund & Granum, 1996). Some similarities between L₁ and the 39 kDa protein in size and amino acid sequence at the N-terminus have been shown, and it has been suggested that they might be homologous proteins in the two different strains (Lund & Granum, 1996). Although some components of possible enterotoxin complexes are

Abbreviations: HA, hydroxylapatite; HBL, haemolysin BL (haemolytic enterotoxin); B, binding protein of HBL; L₁ and L₂, lytic proteins of HBL; NHE, non-haemolytic enterotoxin; ReQ, Resource Q.

known, the understanding of the molecular nature of *B. cereus* virulence is limited.

Two commercial immunoassays for identifying enterotoxigenic strains of *B. cereus* are currently available. One of the assays detects the L₂ component of HBL, whilst the other detects the 45 kDa protein of the NHE complex (Beecher & Wong, 1994b; Lund & Granum, 1996).

HBL and NHE have each been isolated from only one strain of *B. cereus* (F837-76 and 0075-95, respectively) and in different laboratories. The activity of the two complexes has never been compared with respect to toxicity. We have therefore isolated and compared the cytotoxicity of the two enterotoxin complexes from three different *B. cereus* strains.

METHODS

Strains, culture medium and culture conditions. Strains F837-76 (Turnbull *et al.*, 1979), 1230-88 (Granum *et al.*, 1993) and 0075-95 (Lund & Granum, 1996) were used for enterotoxin production. Strain F837-76, an isolate from a surgical wound infection, was obtained from J. M. Kramer, Public Health Laboratory Services, London. The other strains were responsible for outbreaks of diarrhoeal syndrome food poisoning in Norway in 1988 and 1995, respectively. The culture medium used for production of enterotoxins was a modification of the CGY medium (Beecher & Wong, 1994a), consisting of 2% casein hydrolysate (Merck), 0.4% glucose, 0.6% yeast extract, 0.2% (NH₄)₂SO₄, 1.8% K₂HPO₄, 0.2% KH₂PO₄, 0.1% sodium citrate and 0.2% MgSO₄. The cells were grown at 32 °C for 5 h and EDTA (1 mM) was added at the time of harvesting. Extracellular proteins were separated from the cells by centrifugation (10000 g at 4 °C for 20 min).

Purification of proteins. The purification schemes were similar to those previously described in detail for purification of HBL (Beecher & Wong, 1994a) and NHE (Lund & Granum, 1996). DEAE-Sephacel (Pharmacia) was, however, used instead of Whatman DE-52. Proteins from 3 litres culture supernatant were precipitated with 70% saturated (NH₄)₂SO₄, resuspended in 40 ml water and dialysed overnight at 4 °C against 1 litre 25 mM Bistris/HCl, containing 1 mM EDTA (pH 6.0), with two buffer changes. DEAE-Sephacel (Pharmacia) was packed into a column (diameter 1.6 cm, height 10 cm) and equilibrated with 25 mM Bistris/HCl (pH 6.0). Proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M over 200 ml. Chromatography was carried out as described by Lund & Granum (1996) and selected fractions from chromatography were pooled and applied directly to a column of Bio-Gel HT hydroxylapatite (HA) (Bio-Rad). Selected fractions from HA were dialysed overnight at 4 °C against 20 mM triethanolamine (Sigma) containing 1 mM EDTA, with two buffer changes. The pH of the buffer was 7.5 for fractions containing the 105 kDa protein, 8.1 for fractions containing the 39 kDa protein and 7.8 for fractions containing the other proteins. Dialysed fractions were finally applied to a Resource Q (ReQ) column (1 ml) (Pharmacia), equilibrated with 20 mM triethanolamine at the same pH as the dialysis buffer. Proteins were eluted in 20 mM triethanolamine with a linear gradient of NaCl from 0 to 0.5 M over 40 ml. Protein concentrations were estimated by measuring absorbance at 260 nm, 280 nm and 320 nm.

Enzymic cleavage. The 105 kDa proteins of the three strains (0.2–0.3 mg ml⁻¹) were treated with trypsin (Sigma) (50:1,

w/w) for 1 h at 37 °C at pH 7.8. The 45 kDa protein of strain 0075-95 (38 µg ml⁻¹) was treated with the same enzyme (50:1, w/w) for 30 min at 37 °C at pH 7.8.

Protein sequencing. Purified proteins were sequenced from the N-terminus by Edman degradation using an Applied Biosystems 477A automatic sequence analyser with an on-line 120A phenylthiohydantoin amino acid analyser.

Electrophoresis. SDS-PAGE was carried out using a Bio-Rad Mini-Protein II Dual Slab Cell. The gels (10% acrylamide) were stained using the Bio-Rad Silver Stain Plus kit, and the molecular mass of the proteins were estimated using Bio-Rad SDS-PAGE low-molecular-mass standards.

Vero cell assay. Toxicity was determined using the inhibition of protein synthesis in Vero cells as an assay, according to Sandvig & Olsnes (1982). The toxin concentrations were chosen in ranges which allowed an estimate of the concentrations giving 50% inhibition. The experiments were repeated twice and mean values are given. In some experiments the Vero cells were incubated with one of the toxin components for 15 min. The cells were then washed in the growth medium and new medium containing the two other components was added. The subsequent procedure was identical to the procedure when all three components were added at the same time.

Kit for detection of enterotoxin. The *Bacillus* Diarrhoea Enterotoxin (BDE) Visual Immunoassay kit from Tecra was used according to the instruction manual.

RESULTS

Proteins from supernatants from strains F837-76, 1230-88 and 0075-95 of *B. cereus* were precipitated with ammonium sulphate. SDS-PAGE with silver staining of the precipitates revealed at least 20 extracellular protein bands from each strain, but each strain showed a different pattern (Fig. 1). Chromatography on DEAE-Sephacel at pH 6.0 was used as the first step to purify the

Fig. 1. SDS-PAGE of ammonium-sulphate-precipitated proteins from three different strains of *B. cereus*. Lanes: 1, strain 1230-88; 2, strain F837-76; 3, strain 0075-95; 4, low-molecular-mass markers. Positions and molecular masses in kDa of markers are indicated on the right.

Fig. 2. SDS-PAGE of selected fractions resulting from separation of ammonium-sulphate-precipitated proteins from strain F837-76 by DEAE-Sephacel. Lanes 1–10 contain fractions 2–11 from the DEAE-Sephacel column, respectively. Positions and molecular masses in kDa of markers are indicated on the left.

components of HBL and NHE, and gave results similar to those previously obtained with Whatman DE (Beecher & Wong, 1994a; Lund & Granum, 1996). SDS-PAGE of some of the fractions resulting from strain F837-76 is shown in Fig. 2. Fraction 4 was used for further purification for the putative L_2 protein, seen as a band slightly above 45 kDa in lane 3. Fractions 5 and 6 were combined for further fractionation of L_1 and the 45 kDa protein (putative 45 kDa protein is seen as a doublet just below L_2 , and above L_1 at approximately 37 kDa in lane 4). Fraction 8 was used for purification of the B protein (putative B is seen as a band at approximately 37 kDa in lane 7). Fraction 10 was used for purification of the 105 kDa protein (seen as a doublet). The protein of 39 kDa from NHE did not bind to the column at pH 6.0. The elution patterns from the two other strains were similar to that of strain F837-76.

DEAE-Sephacel fractions from the three different strains, corresponding to lane 9 in Fig. 2 were further purified directly on ReQ at pH 7.5. The proteins giving the double band at 105 kDa were eluted between 195 mM and 220 mM NaCl. SDS-PAGE (Fig. 3a, lanes 2–4) revealed that the molecular mass of these proteins from the three strains was nearly identical. The protein in the upper band from strain 0075-95 has previously been referred to as the 105 kDa protein of NHE (Lund & Granum, 1996). The proteins in the two bands were only partly separated on the ReQ column, and were not separated by chromatography on HA. Tryptic peptides from a fraction enriched for the upper band and a fraction containing mainly the lower band gave nearly identical patterns on SDS-PAGE (not shown). We therefore suggest that the protein in the lower band is a nicked form of the 105 kDa protein.

DEAE-Sephacel fractions from strain F837-76 and strain 1230-88 corresponding to lane 3 in Fig. 2 were further fractionated by HA. Proteins with similar molecular mass to L_2 (Beecher & Wong, 1994a) were eluted between 150 mM and 190 mM sodium phosphate. These proteins were finally purified using ReQ at pH 7.8, and were eluted at 90–100 mM NaCl. Purity was evaluated by SDS-PAGE (Fig. 3a, lanes 5 and 6). The identity of L_2 from strain 1230-88 was further established by N-terminal amino acid sequence analysis (Fig. 4). Strain 0075-95 did not express a protein with chromatographic or immunological properties similar to L_2 .

Fractions from DEAE-Sephacel which gave patterns similar to those in lanes 4 and 5 in Fig. 2 were pooled and chromatographed on HA. The putative 45 kDa proteins were eluted at 190–215 mM sodium phosphate. Proteins with the same molecular mass as L_1 (Beecher & Wong, 1994a) from strains F837-76 and 1230-88 were eluted from HA at 120–140 mM sodium phosphate.

The 45 kDa-like proteins were finally purified by ReQ at pH 7.8, and were eluted at approximately 120 mM (strain 1230), 150 mM (strain 837) and 180 mM NaCl (strain 0075-95). The purified proteins were analysed by SDS-PAGE (Fig. 3a, lanes 7–10). Strains F837-76 and 0075-95 gave doublet proteins. The doublet proteins from strain 0075-95 were separated from each other when narrow fractions were selected at each purification step (lanes 9 and 10). Digestion of the upper protein with trypsin yielded a fragment with mobility identical to the smaller one (lane 9), indicating a nicked form (not shown). The upper form is identical to the protein previously used in cytotoxicity tests (Lund & Granum, 1996). The doublet proteins from strain F837-76 are

Fig. 3. SDS-PAGE of purified proteins from three different strains of *B. cereus*. (a) Lanes: 1, Low-molecular-mass markers; 2, 105 kDa protein of strain 1230-88; 3, 105 kDa protein of strain F837-76; 4, 105 kDa protein of strain 0075-95; 5, L_2 of strain 1230-88; 6, L_2 of strain F837-76; 7, 45 kDa protein of strain 1230-88; 8, 45 kDa protein of strain F837-76; 9, 10, lower and upper forms, respectively, of 45 kDa protein of strain 0075-95. (b) Lanes: 1, Low-molecular-mass markers; 2, B of strain F837-76; 3, B of strain 1230-88; 4, L_1 of strain F837-76; 5, L_1 of strain 1230-88; 6, 39 kDa protein of strain F837-76; 7, 39 kDa protein of strain 1230-88; 8, 39 kDa protein of strain 0075-95 (slightly degraded form); 9, 39 kDa protein of strain 0075-95 (intact form). Positions and molecular masses in kDa of markers are indicated on the left.

L_2 , 1230-88	ETQQEGMDISSS
L_2 , 837-76*	ETQQENMDISSS
39 kDa, 837-76	AESTVKQAPVHVAKAYNDYE
39 kDa, 0075-95†	AESTVKQAPVXAVAKAYNDY
Nicked 39 kDa, 0075-95†	VAKAYNDYEEYSLGPEGLKDAMEXTGSNALVMD
L_1 , 837-76*	QETTAQE QKVGNY ALGPEGLK KAL AETGSH ILVMD

Fig. 4. Amino acid sequence of the N-termini of putative L_2 from strains 1230-88 and F837-76, the 39 kDa protein from strains F837-76 and 0075-95 (including a putative nicked form), and L_1 from strain F837-76. The sequence of L_1 is aligned to visualize similarity with the nicked 39 kDa protein and the residues in bold indicate identity between them. *, Deduced sequence from Ryan *et al.* (1997); †, from Lund & Granum (1996); X, uncertain residue.

probably identical to proteins described by Beecher & Wong (1994b). The N-terminal sequence of the lower form has been shown to be identical to the corresponding part of the 45 kDa protein from strain 0075-95, and according to Beecher & Wong (1994b) both proteins in the doublet react strongly in the Tecra test.

The 45 kDa-like protein from strain 1230-88 (lane 7) was further identified by the immunological Tecra assay. It was detected at 3 ng ml^{-1} , a value corresponding to the those reported previously for the 45 kDa protein from strains F837-76 and 0075-95 (Beecher & Wong, 1994b; Lund & Granum, 1996).

Table 1. Approximate concentrations in ng of components of HBL and NHE necessary for 50% inhibition of protein synthesis in Vero cells

Strains	NHE			HBL		
	39 kDa	45 kDa	105 kDa	B	L ₁	L ₂
Strain F837-76	ND	ND	ND	30	25	25
Strain 1230-88	30	70	500	90	75	70
Strain 0075-95*	30	20	20	—	—	—

ND, Not determined, but proteins were functional (see text).

—, Not present.

* From Lund & Granum (1996).

Fractions from HA chromatography containing L₁-like proteins were pooled and applied to ReQ. The L₁-like proteins were eluted at approximately 110 mM NaCl at pH 7.8. SDS-PAGE (Fig. 3b, lanes 4 and 5) revealed that the molecular mass of the L₁-like protein from strain 1230-88 was close to that of L₁ from strain F837-76. Cytotoxicity tests (see below) showed that the L₁-like protein from strain 1230-88 was toxic in combination with L₂ and B. This protein is therefore probably a homologue of L₁ from strain F837-76. No protein with chromatographic properties similar to L₁ was detected from strain 0075-95. Proteins from this strain were also used in cytotoxicity tests, together with B and L₂ from strain F837-76, without cytotoxic effect.

Fractions similar to DEAE-Sephacel fraction 8 (Fig. 2) were used for further purification of B-like proteins by HA. Proteins with molecular mass similar to the B protein were eluted between 180 mM and 210 mM sodium phosphate. These proteins were further purified on ReQ at pH 7.8 and eluted at 140–160 mM NaCl. SDS-PAGE showed that the B proteins of strains F837-76 and 1230-88 were pure and had similar molecular mass (Fig. 3b, lanes 2 and 3). Amino acid sequence analysis of the first 15 N-terminal amino acids of the B component from strain 1230-88 were identical to those deduced for the B component from strain F837-76 (Heinrichs *et al.*, 1993). No B-like component was detected from supernatants from strain 0075-95.

The void fraction from chromatography on DEAE-Sephacel was used to purify proteins similar to the 39 kDa protein of strain 0075-95 (Lund & Granum, 1996). These fractions were applied to a HA column and proteins of approximately 39 kDa were eluted at 105–130 mM sodium phosphate. Chromatography on ReQ at pH 8.1 was the final purification step (eluted at 50 mM NaCl). The resulting proteins are shown in Fig. 3b (lanes 6–9). From strain F837-76 a band appeared (lane 6) that corresponded to the intact 39 kDa protein from strain 0075-95, whilst from strain 1230-88 a band appeared (lane 7) corresponding to the nicked form of the same protein. The sequence of the first 21 N-terminal amino acids of the 39 kDa protein from strain F837-76 was determined and compared to the sequence

of the 39 kDa protein from strain 0075-95 (Fig. 4). Except for valine substituting for alanine in position 12, the sequence was identical to that of the 39 kDa protein from strain 0075-95.

Toxicity tests on Vero cells showed that B, L₂ and L₁ components (in combination) from strains F837-76 and 1230-88 were cytotoxic at low concentrations. Approximately 25–30 ng of each component from strain F837-76 was sufficient for 50% inhibition of protein synthesis in Vero cells, whilst the corresponding values from strain 1230-88 were approximately 70–90 ng (Table 1). Combinations of two of the components, or single components, were not cytotoxic at comparable concentrations. L₁ from strain 1230-88 used in this test was slightly contaminated with a smaller protein (Fig. 3b, lane 5). This protein was purified, and shown to be non-toxic in combination with B and L₂.

We have previously shown that NHE isolated from strain 0075-95 gave 50% inhibition of protein synthesis in Vero cells using approximately 20–30 ng of each component (Lund & Granum, 1996). The values for the corresponding proteins from strain 1230-88 were approximately 30 ng (39 kDa protein), 70 ng (45 kDa protein) and 500 ng (105 kDa protein) (Table 1). The 105 kDa protein from strain 1230-88 was a mixture of the intact protein and the putative degraded form (Fig. 3a, lane 2). The degraded form bound slightly better than the intact form to ReQ and could therefore be obtained in a nearly pure form. For 48% inhibition of protein synthesis, 700 ng of the degraded protein was needed, indicating that it contributed less to the cytotoxicity of NHE than the intact protein. In combination with the proteins of 45 kDa and 105 kDa (from strain 1230-88), the 39 kDa protein of strain 0075-95 could substitute for the 39 kDa protein of strain 1230-88, further indicating the similarity or identity of these proteins. Different combinations of only two components, in much higher amounts (2–3 µg) than used in the experiments above, gave less than 6% inhibition of protein synthesis in Vero cells.

Cytotoxicity tests with pure proteins of NHE from strain F837-76 in relatively high amounts (194 ng 39 kDa protein, 242 ng 45 kDa protein and 648 ng 105 kDa

Table 2. Toxicity to Vero cells caused by addition of the different components of NHE at different times

Vero cells were initially incubated with one of the toxin components for 15 min. Non-bound component was then removed by washing in growth medium and the other two components were added in new medium.

Initial protein component	Additional protein components added after 15 min	Inhibition of protein synthesis (%)
39 kDa (1.5 µg)	45 kDa (200 ng) + 105 kDa (1 µg)	6
45 kDa (1 µg)	39 kDa (160 ng) + 105 kDa (1 µg)	15
105 kDa (3 µg)	39 kDa (160 ng) + 45 kDa (200 ng)	92
105 kDa (2 µg)	39 kDa (160 ng) + 45 kDa (200 ng)	71
105 kDa (1 µg)	39 kDa (160 ng) + 45 kDa (200 ng)	43

protein) gave only 32% of inhibition of protein synthesis, and no value for 50% inhibition was estimated. However, the 39 kDa protein (97 ng) and 45 kDa protein (105 ng) from this strain together with the 105 kDa protein (345 ng) from strain 0075-95 gave inhibition of 97%. Furthermore, a combination of the 39 kDa protein (97 ng) and the 45 kDa protein (105 ng) with partly purified 105 kDa component (DEAE-Sephacel fraction 10, Fig. 2) (approximately 400 ng), all from strain F837-76, resulted in 90% inhibition. The other proteins of DEAE-Sephacel fraction 10 were tested together with the 39 kDa and 45 kDa proteins and gave no inhibition of protein synthesis.

Cytotoxicity of NHE was also tested with only one of the components added to the Vero cells initially. After 15 min, non-bound component was removed by washing and the other components were added. When the 39, 45 and 105 kDa proteins from strain 1230-88 were tested, a high degree of toxicity was achieved only when the 105 kDa protein was added before washing (Table 2). Combinations of only two components, or the 105 kDa protein alone, at the highest concentrations used, yielded less than 6% inhibition. The same result was found using components from strain 0075-95.

We also examined if the components of the two enterotoxin complexes could co-operate. In one experiment, using the components from strain 1230-88, B and L₂ were added separately and together to the components of NHE in amounts similar to that of the 45 kDa protein (about 100 ng). When the B component was added the toxicity to Vero cells increased slightly from 52% to 58% inhibition. Protein synthesis was inhibited at 66% with addition of both B and L₂. When either the 45 kDa or the 105 kDa protein was omitted, the inhibition was reduced to 15% or less. Co-operation between the components of the two different complexes therefore seems to be limited.

DISCUSSION

This work has shown that *B. cereus* strains may produce both the HBL and the NHE complexes. The two complexes were cytotoxic at comparable amounts when

isolated from the strains from which they were originally characterized (HBL from strain F837-76 and NHE from strain 0075-95). When comparing cytotoxicity based on amounts of protein used it must also be taken into consideration that five of the components have a similar size, whereas the sixth (the 105 kDa protein) has a molecular mass about 2.5-fold higher. Both complexes are probably important *B. cereus* enterotoxins, as judged by analysis of three different strains. For some strains NHE is probably the only functional complex, as in strain 0075-95 where the HBL complex is not present. We have also observed, using Western blots and the same polyclonal antibodies as described by Lund & Granum (1996), that several strains of *B. cereus* gave bands corresponding to the 39 kDa, 45 kDa and 105 kDa proteins, whilst a band corresponding to L₂ was lacking.

It has been suggested that L₁ and the 39 kDa protein could be homologous proteins in the two different strains due to similarities in amino acid sequence and molecular mass (Lund & Granum, 1996). The gene encoding L₁ from strain F837-76 has now been cloned and sequenced (Ryan *et al.*, 1997). Comparing this sequence with the amino acid sequence of the 39 kDa protein from strain 0075-95 revealed a stretch of 23 amino acids with 70% identity (Fig. 4). These two proteins may therefore have similar functions. Strain F837-76, however, expressed another protein, designated the 39 kDa, with an N-terminal sequence almost identical to that of the 39 kDa protein of strain 0075-95 (Fig. 4). The 39 kDa protein of strain F837-76 had toxic properties (was functional together with the other two components of NHE) and chromatographic properties similar to the 39 kDa protein of strain 0075-95, and is therefore probably a true homologue of this protein. We therefore now conclude that the two toxin complexes consist of six different proteins.

The degree of cytotoxicity of the NHE complex appears to vary from strain to strain. This variation is probably first and foremost due to differences in the 105 kDa protein. Indeed, small structural differences between the strains could be detected for this protein. Although the tryptic peptide pattern from the three strains was similar, different peptides could be detected, and long

SDS-PAGE gels revealed that the mobility of the 105 kDa protein from strain 0075-95 was slightly faster than the corresponding proteins from the other two strains (not shown). Variation in the toxicity of the 45 kDa protein between strains was also observed.

Nicked forms of different bacterial toxins, produced by bacterial proteases, have been described (Gordon & Leppla, 1994). The nicking may activate some toxins while others are inactivated. We have detected degraded forms of all the proteins of NHE. The nicked form of the 105 kDa proteins from strain 1230-88 was less toxic than the intact form. The 105 kDa protein from strain F837-76 was probably reduced in activity during the last purification step. Conformational changes due to loss of co-operative components may have caused the inactivation. Taking into consideration the loss of activity of some of the components of NHE during purification, it could be that the intact NHE is much more toxic than the mixture of purified components shows.

In some toxicity tests with NHE, one component was added to the cells at time zero. Unbound protein was washed away before addition of the remaining two components. In these experiments high toxicity was obtained only when the 105 kDa protein was added first, before washing. This protein is therefore most probably the binding protein and the other two components bind to the 105 kDa protein, in a similar manner to the anthrax toxin of *Bacillus anthracis* (Leppla, 1991). Another possibility is that the two other components bind to structures on the target cells which have to be modified by the binding of the 105 kDa protein. It has previously been suggested that the B protein is the binding component of the HBL complex (Beecher & Wong, 1994a). The same authors have, however, recently claimed that the interactions between the HBL components and the target cells are more complicated than first suggested (Beecher & Wong, 1997).

Polyclonal antibodies raised against partly purified enterotoxin from *B. cereus* have previously been used to identify toxic strains (Beecher *et al.*, 1995; Lund & Granum, 1996; Thompson *et al.*, 1984). The 105 kDa protein, and probably L₂, the 45 kDa protein and the upper form of the 39 kDa protein, may be identified by such antibodies. The mobilities of B, L₁ and the lower form of the 39 kDa protein are, however, too close to allow identification in Western blots with such polyclonal antibodies alone.

The multicomponent diarrhoeal enterotoxin described by Thompson *et al.* (1984) has previously been compared to HBL and it was claimed from immunological analysis that the two complexes were identical (Beecher *et al.*, 1995). On Western blots (Beecher *et al.*, 1995) it appears, however, that the antiserum developed by Thompson *et al.* (1984), in addition to B, L₁ and L₂, also recognized proteins corresponding to the 45 kDa and 105 kDa protein of the NHE complex. Thus, it cannot be excluded that the enterotoxin described by Thompson *et al.* (1984) was a mixture of HBL and NHE.

Neither of the two available commercial immunoassays will completely quantify the toxicity of the enterotoxins from *B. cereus*. The assay measuring L₂ (Oxoid) will not detect strains producing only the NHE complex, whilst the other kit, detecting mainly the 45 kDa protein (Tecra), will not detect strains producing only the HBL complex. If one or both of the commercial kits reacts positively with proteins from *B. cereus* supernatants it is likely that the strain is enterotoxin-positive. If the supernatants are also shown to be cytotoxic (on Vero or Caco-2 cells), they can be regarded as enterotoxin-positive. Because variations in cytotoxicity of NHE seem to be mostly due to differences in the 105 kDa component, it is not possible to even estimate activity by using an immunological kit which detects the 45 kDa component. Indeed, we have shown large differences in cytotoxicity between NHE complexes containing the same amount of the 45 kDa protein.

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