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# Comparison of four methods for extracting periplasmic proteins

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**Summary**

Overall, chloroform (CHL) extracts contained higher quantities of periplasmic proteins from three of seven bacteria than either cold osmotic shock (COS) or slow- and rapid-freeze/thaw (SF/T, RF/T) methods. CHL extracts contained equally higher levels for the six periplasmic enzymes tested. Considerably more protein was extracted by all four methods from two *Pseudomonas* spp. than from two *Acinetobacter* spp., and the RF/T method extracted considerably more from *Acinetobacter* than the other methods.

**Key words:** Periplasmic protein

## Introduction

The classical method for extracting periplasmic proteins from gram-negative bacteria is the cold osmotic shock (COS) method of Neu and Heppel [1]. Although widely used, this method is laborious and large volumes of culture broth are required. A simpler and more rapid method is the chloroform (CHL) extraction procedure of Ames et al. [2]. With this method, smaller volumes of culture fluids can be used, and it is quantitative. More recently, the selective release of periplasmic proteins from *Aquaspirillum magnetotacticum* by freezing and thawing in buffer was reported by Paoletti et al. [3]. This simple, slow-freeze/thaw method produced SDS-PAGE for the organism noted that was nearly identical to COS and CHL.

For the preparation of periplasmic proteins, a relatively small range of bacterial genera have been reported, with *Escherichia coli* being by far the most common. Because of our interest in periplasmic proteins from a wide variety and number of bacter-

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ia, we were interested in a method that does not require large volumes of culture broth for analysis, that produced results similar or identical to COS, and, in general, that lends itself to the routine examination of large numbers of cultures. To this end, we compared 4 methods for the extraction of periplasmic proteins from 7 species representing 5 genera of bacteria.

## Materials and Methods

Cultures were obtained from fresh and spoiled ground beef [4] from the American Type Culture Collection (ATCC) and from the clinical laboratories of W. J. Brown and J. Eisses of Hutzel and Henry Ford Hospitals, respectively, in Detroit, MI. The cultures were chosen primarily because of their importance in refrigerated fresh foods with the exception of *E. coli*, which as previously noted is widely used for periplasmic extractions.

For periplasmic extractions, cultures were grown in the low-phosphate medium of Neu and Heppel [1] at pH 6.5 in 170 ml quantities at 30 °C for 18 h. COS was carried out by the method of Neu and Heppel; CHL extraction by the method of Ames et al. [2]; and the slow-freeze/thaw (SF/T) extraction by the method of Paoletti et al. [3]. The latter was overnight freezing at -20 °C followed by a room temperature thaw. Rapid-freeze/thaw (RF/T) was carried out by washing cells twice in about 40 parts (v/v) of cold washing buffer (Tris-HCl), pH 7.3) and resuspended in the residual buffer. The suspension was transferred to lyophilizer vials with another volume (2 ml) of washing buffer to rinse the centrifuge tubes. The vials were frozen in an acetone-dry ice mixture for 10 min followed by a room temperature thaw. The mixture was centrifuged at $15000 \times g$ for 15 min and the supernatant was collected.

For cell-free extracts, cells were washed 3 times in 40 parts (v/v) cold washing buffer and suspended in 20 ml of the same buffer. They were disrupted by sonication using a Model W 350 unit (Heat Systems Ultrasonics) for approximately 30 min. The suspension was centrifuged at 20000 rpm $\times g$ for 30 min and the supernatant was collected. The same procedure (sonication) was performed on cells immediately after a CHL extraction.

To analyze the extracts, approximately equal quantities were subjected to SDS-PAGE and stained with Coomassie blue. Protein content was determined using the Bio-Rad assay (Bio-Rad Labs., Richmond, CA), and the presence/absence of certain periplasmic and cytoplasmic enzymes was ascertained by the API ZYM assay (Analytab Products, Plainview, NY). The viability of spheroplasts was determined by the surface plating of extracted cells onto tryptic soy agar (Difco Labs., Detroit, MI), following serial dilutions in saline.

## Results and Discussion

The relative quantities of periplasmic proteins obtained by the 4 methods from 7 organisms are presented in Table 1. CHL extracts contained the highest quantity of protein from 4 of the 7 species; RF/T was highest for 2; and the other culture yielded the highest quantity by COS. Overall, CHL extracted more protein than the other 3 methods. It is clear from the table that the 4 methods varied in their capacity to extract

TABLE 1

RELATIVE QUANTITY OF PERIPLASMIC PROTEINS EXTRACTED FROM 7 BACTERIA BY 4 EXTRACTION METHODS

Values are o.d. at 595 nm using the Bio-Rad assay

| Organisms, source | CHL | COS | RF/T | SF/T |
|---|---|---|---|---|
| *Pseudomonas fragi*, ATCC 27363 | 1.358* | 0.674 | 1.152 | 1.069 |
| *Hafnia alvei*, meats | 0.433 | 0.760* | 0.393 | 0.364 |
| *Pseudomonas cepacia*, meats | 0.943* | 0.143 | 0.167 | 0.374 |
| *Escherichia coli*, clinical | 0.493* | 0.265 | 0.248 | 0.115 |
| *Yersinia enterocolitica*, meats | 0.383* | 0.359 | 0.158 | 0.137 |
| *Acinetobacter calcoaceticus*, ATCC 23220 | 0.143 | 0.065 | 0.223* | 0.151 |
| *Acinetobacter lwoffii*, ATCC 15309 | 0.071 | 0.093 | 0.103* | 0.064 |

* Highest value of the 4 methods. CHL, chloroform; COS, cold osmotic shock; RF/T, rapid freeze/thaw; SF/T, slow freeze/thaw.

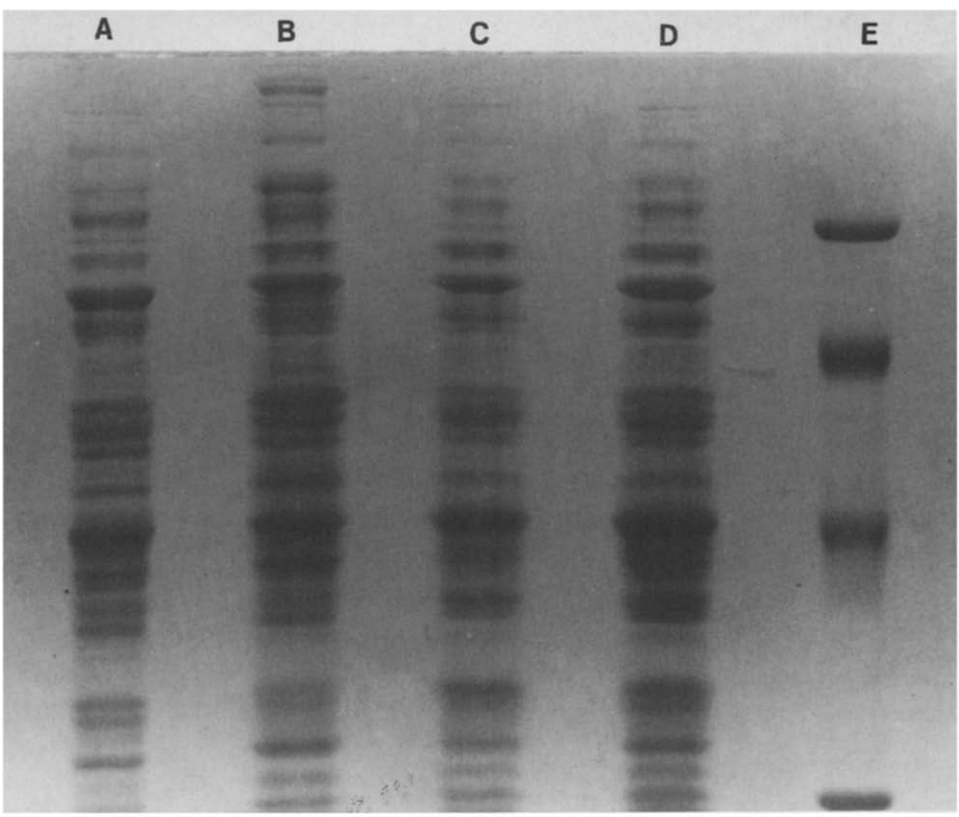


Fig. 1. SDS-PAGE of periplasmic proteins released from cells of *Pseudomonas fragi* (ATCC 27363) using 4 extraction methods. A = chloroform; B = cold osmotic shock; C = slow freeze/thaw; D = rapid freeze/thaw; and E = molecular weight markers (from top to bottom: 94000; 67000; 43000; and 20000).

proteins from different organisms. From this and data not presented, the pseudomonads yielded the highest quantities of periplasmic proteins of all organisms tested. The similarity of extracts by the 4 methods for *Pseudomonas fragi* can be seen from Fig. 1. *Acinetobacter* spp. consistently yielded lower quantities of periplasmic

TABLE 2

PRESENCE AND RELATIVE QUANTITIES OF 6 ENZYMES IN PERIPLASMIC EXTRACTS OBTAINED BY 4 EXTRACTION METHODS

Assay was by the API ZYM System, with 0 = no activity and 5 = maximum

| Organism | Extraction methods | Alkaline phosphatase | Esterase | Esterase lipase | Acid phosphatase | Phospho-hydrolase | α-Glucosidase |
|---|---|---|---|---|---|---|---|
| *Pseudomonas fragi* | CHL | 5 | 4 | 5 | 5 | 5 | 5 |
| | COS | 5 | 4 | 5 | 5 | 5 | 5 |
| | RF/T | 3 | 4 | 5 | 5 | 5 | 5 |
| | SF/T | 5 | 4 | 5 | 5 | 5 | 5 |
| | CFE[a] | 5 | 4 | 5 | 5 | 5 | 5 |
| | SS[b] | <1 | 0 | <1 | <1 | <1 | 0 |
| *Hafnia alvei* | CHL | 5 | 2 | 2 | 5 | 5 | 5 |
| | COS | 4 | 2 | 2 | 4 | 5 | 3 |
| | RF/T | 3 | 1 | 2 | 4 | 5 | 1 |
| | SF/T | 3 | 1 | 2 | 5 | 5 | 1 |
| *Pseudomonas cepacia* | CHL | 5 | 2 | 3 | 5 | 5 | 5 |
| | COS | 5 | 1 | 2 | 4 | 5 | 5 |
| | RF/T | 5 | 1 | 1 | 4 | 4 | 4 |
| | SF/T | 5 | 2 | 2 | 5 | 5 | 4 |
| *Escherichia coli* | CHL | 5 | 2 | 4 | 5 | 5 | 5 |
| | COS | 2 | 1 | 3 | 5 | 5 | 0 |
| | RF/T | 2 | 1 | 2 | 3 | 3 | 1 |
| | SF/T | 2 | 1 | 2 | 4 | 4 | 0 |
| *Yersinia enterocolitica* | CHL | 5 | 2 | 3 | 5 | 5 | 5 |
| | COS | 5 | 1 | 2 | 4 | 5 | 5 |
| | RF/T | 5 | 1 | 2 | 5 | 5 | 5 |
| | SF/T | 4 | 2 | 2 | 5 | 5 | 4 |
| *Acinetobacter calcoaceticus* | CHL | 2 | 3 | 5 | 2 | 2 | 0 |
| | COS | 1 | 2 | 4 | 2 | 2 | 0 |
| | RF/T | 2 | 2 | 2 | 2 | 2 | 0 |
| | SF/T | 1 | 2 | 2 | 2 | 2 | 0 |
| *Acinetobacter lwoffii* | CHL | 2 | 2 | 4 | 2 | 1 | 0 |
| | COS | 1 | 1 | 3 | 1 | 1 | 0 |
| | RF/T | 2 | 1 | 2 | 1 | 1 | 0 |
| | SF/T | 2 | 2 | 4 | 1 | 2 | 0 |
| | CFE[a] | 5 | 4 | 4 | 2 | 1 | 0 |
| | SS[b] | 5 | 3 | 4 | 2 | 1 | 0 |

[a] Cell free extracts of sonicated whole cell supernatants.

[b] CHL extracts of sonicated spheroplasts after CHL extraction of whole cells. CHL, chloroform; COS, cold osmotic shock; RF/T, rapid freeze/thaw; SF/T, slow freeze/thaw.

proteins than the other 4 genera noted in Table 1, with RF/T extracts containing more than those by the other methods. Turbidity measurements performed using a Klett-Summerson colorimeter indicated that approximately equal numbers of cells were used for the extractions. Therefore, the differences in protein release were not due to differences in numbers of cells.

It appears that the lower quantity of proteins in *Acinetobacter* extracts was due to a greater difficulty in extracting periplasmic proteins from this genus by these methods, and evidence for this can be seen from Table 2 for *Pseudomonas fragi* and *Acinetobacter lwoffii.* When spheroplasts of CHL-extracted cells of the former organism were disrupted by sonication, the cell-free extracts of sonicated spheroplasts (SS) contained no detectable esterase or $\alpha$-glucosidase and only traces of acid and alkaline phosphatase, esterase lipase, and phosphohydrolase. On the other hand, the SS of *A. lwoffii* contained levels of the 6 enzymes noted that were comparable to those from cell-free extracts (CFE) of whole cells following disruption by sonication, and we presume that the other organisms tested are intermediate to *P. fragi* and *A. lwoffii.* Spheroplasts of cells extracted by each method were viable following serial dilutions and platings.

In general, the cultures that yielded the highest total quantity of periplasmic proteins also yielded the highest quantities for the 6 periplasmic enzymes tested (Table 2). The 4 extraction methods were equally effective in extracting maximum quantities from *P. fragi* and *P. cepacia,* and equally lower quantities of these enzymes from the 2 *Acinetobacter* spp. Overall, CHL extracts contained higher quantities of the 6 enzymes than the other methods.

In summary, the CHL extraction method was better than the other methods for the species tested although RF/T was more effective for *A. calcoaceticus* and *A. lwoffii*; and COS extracted the most protein from *Hafnia alvei.* The findings indicate that for pseudomonads CHL is preferred, while for *Acinetobacter* and related organisms (such as *Branhamella*) the RF/T method may be preferred.

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