Iranian Biomedical Journal 6 (2 & 3): 55-61 (April & July 2002)

The Extractability of Inner-Membrane Proteins from *Salmonella typhimurium* Intact Cells, Spheroplasts and Inner-Membrane Fragments by Non-Denaturing Detergents

Dariush Minai-Tehrani*, Ezzatollah Keyhani and Zahra Sobhani-Damavandifar

Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384, Tehran, Iran

ABSTRACT

The effect of Triton X-100, Na cholate and Tween 80 on the solubilization of integral membrane proteins in intact cells, spheroplasts and inner-membrane fragments of *Salmonella typhimurium* was studied. The detergents were used in various concentrations (1.6 to 64 mM) and cytochromes *b* and *d* were used as marker to monitor the solubilization of membrane-bound proteins. Results showed that no inner-membrane protein solubilization was detected after the treatment of intact cells with detergents. The effect of Na cholate and Tween 80 on spheroplasts and inner-membrane fragments was also negligible in comparison to Triton X-100. Triton X-100 solubilized cytochromes from inner-membrane fragments more efficiently than from spheroplasts. The ratio of total protein solubilization to solubilize cytochromes showed that in spheroplasts this ratio was maximum at 1.6 mM Triton X-100 while it was maximum at 16-32 mM Triton X-100 in inner-membrane fragments. This difference between spheroplasts and inner-membrane fragments may be due to the orientation of the inner-membrane in spheroplasts (right side out) and inner-membrane fragments (in-side out as well as right side out), and to the presence of peripheral proteins attached to cytoplasmic membrane in spheroplasts. *Iran. Biomed. J. 6 (2 & 3): 55-61, 2002*

Keywords: Detergents, Cytochromes, *Salmonella typhimurium*

INTRODUCTION

The solubilization of the membrane proteins is one of the most important steps in protein purification in both eukaryotic and prokaryotic cells and still represents a major challenge in membrane proteins investigations [1, 2]. The envelope of prokaryotic cells such as Gram-negative bacteria has a complex structure which consists of an outer membrane, a cell wall, and an inner membrane (called plasma membrane). Each of these layers has a distinct morphology and structure and contains proteins that play essential roles for the cell. In order to investigate the structure and function of these proteins, it is often required to extract them from the membranes or to solubilize them.

Detergents have been widely used to solubilize membrane-bound proteins [3, 4]. Non-ionic detergents, in particular, are useful to solubilize proteins without affecting their structure and function [5, 6], but their action is sometimes limited. It was shown that Triton X-100, a non-ionic detergent, could solubilize proteins from the plasma membrane of *Escherichia coli* but not those from its outer membrane [7-11]. Furthermore, the procedure used in one biological system may not be applicable to the others. When we applied this procedure to solubilize respiratory pigments from *Salmonella typhimurium*, the results were not satisfactory.

The respiratory chain of *S. typhimurium* is similar to that of *E. coli* [12, 13]. It has been shown that the cytochrome *bd* complex is the predominant terminal oxidase of *S. typhimurium* cells in the stationary phase, when the level of oxygen has fallen down in the culture medium [14]. The cytochrome *bd* complex contains two subunits: the subunit I, consisting of cytochrome b_{558} and the subunit II, containing cytochromes b_{595} and *d* [15, 16]. The heme group in cytochrome *d* (chlorin) is different from that of *b* type cytochromes [17, 18]. These cytochromes are all integral membrane proteins. It has been suggested that subunit I and II contain nine and eight transmembrane helices, respectively [19].

In this paper, we report the solubilization of inner-membrane proteins of intact *S. typhimurium*,

*Corresponding Author; Tel. (98-21) 611 3373; Fax: (98-21) 640 4680; E-mail: minae@ibb.ut.ac.ir

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

spheroplasts and inner-membrane fragments with various detergents.

MATERIALS AND METHODS

Bacterial strain. *S. typhimurium* strain 3507 was obtained from Dr. Roseman's laboratory at Johns Hopkins University, USA. Cells were cultured at 37°C for 48 h in a medium containing peptone (7 g), yeast extract (5 g) and NaCl (3 g) per liter of water, pH 7, in an incubator shaker (150 rpm). This culture was then in the stationary phase.

Spheroplasts and inner-membrane fragments preparation. Spheroplasts and inner-membrane fragments were prepared according to the references 20 and 21, respectively with the following modifications. Spheroplasts were lysed in 0.05 M Tris buffer pH 9. The suspension was homogenized in a blender and centrifuged at 10,000 ×g for 15 minutes to separate unlysed spheroplasts. The supernatant, consisting of inner-membrane fragments, was centrifuged at 40,000 ×g for 30 minutes. The pellet, which consisted of plasma membrane fragments, was washed with 0.1 M phosphate buffer pH 7, and centrifuged again at 40,000 ×g for 30 minutes. Then, the pellet (inner-membrane fragments) was frozen at -25° C until use.

Samples treatment and cytochrome release. The samples, including intact cells, spheroplasts and inner-membrane fragments were treated with either Na cholate, Tween 80 or Triton X-100, at various concentrations (1.6, 8, 16, 32 and 64 mM) for 45 minutes. The treated samples were centrifuged at 50,000 ×g for 60 minutes [7]. The supernatant, which contained the released proteins, was analyzed for the presence of cytochromes *b* and *d* and for total proteins.

The amount of released cytochromes was measured spectrophotometrically. Difference absorption spectra (dithionite-reduced-minus-air-oxidized) of the samples were recorded with an Aminco DW2 spectrophotometer adapted for bacterial suspension [22, 23]. The amounts of cytochromes *b* and *d* were calculated by measuring the peak at 560 nm (ε = 10.8 $mM.cm^{-1}$) and 630 nm (ε = 19 $mM.cm^{-1}$), respectively [24]. For each condition studied, two samples were taken for spectrophotometric studies after detergent treatment. One sample was taken before centrifugation as control, and another sample (soluble fraction), was taken from the supernatant after centrifugation for the extent of protein release.

Total protein determination. The total amount of proteins was determined by the Biuret method. A standard curve was prepared each time, using different concentrations (1-6 mg/ml) of ovalbumin protein.

RESULTS

The reduced-minus-oxidized absorption spectrum of the respiratory chain in *S. typhimurium* is shown in Figure 1. The peak at 630 nm is due to cytochrome *d*, a special terminal oxidase that has a chlorin heme as prosthetic group and the peak at 560 nm is due to *b* type cytochrome. Identical absorption spectra were obtained with intact cells, spheroplasts and inner-membrane fragments. To detect the effect of the various detergents studied on membrane-bound protein solubilization, the release of cytochromes *b* and *d* from the inner-membrane was monitored spectrophotometrically.

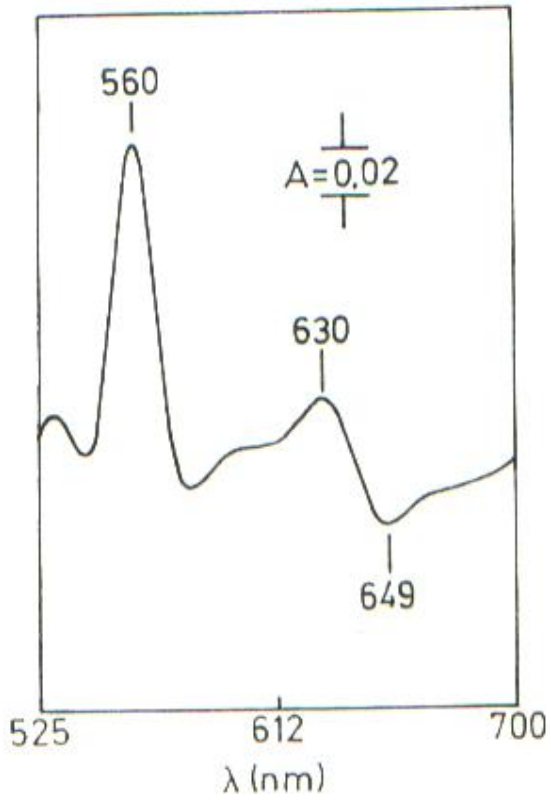


Fig. 1. Reduced-minus-oxidized difference absorption spectrum of *S. typhimurium* cell suspension. The 560 nm peak is due to *b* type cytochrome and the 630 nm peak is due to cytochrome *d*.

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

Effect of detergents on intact cells. Protein determination indicated that no more than 3.5% of the total proteins was solubilized after the treatment of intact cells with the highest concentration of Triton X-100 (64 mM) (Fig. 2). Similarly, only 4% of the total proteins were solubilized after treatment with 64 mM sodium cholate (Fig. 2). No protein release was detectable after treatment of intact cells with up to 64 mM Tween 80.

At low concentrations (1.6 to 16 mM), only Triton X-100 was able to release proteins (Fig. 2). No cytochrome solubilization was detectable after treatment of the intact cells with any of the three detergents. The results showed that intact cells were resistant to the effects of these detergents and little or no lysis was observed during treatment by detergents. The viscosity of the working solution would increase after lysing the cells, due to the release of genetic materials.

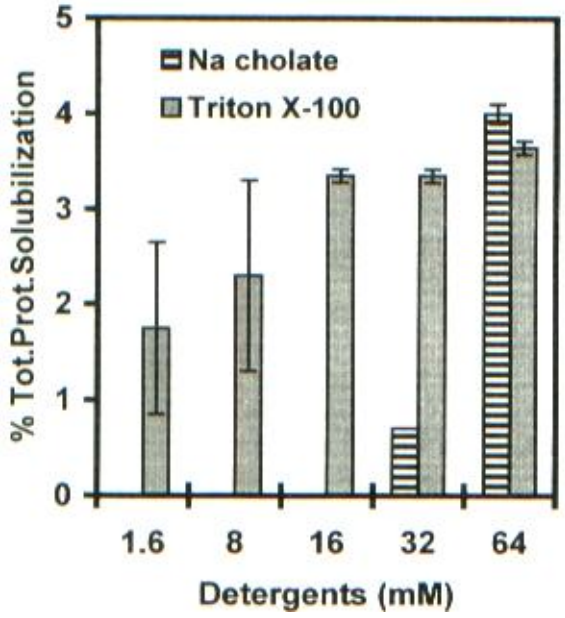


Fig. 2. The effect of Na cholate and Triton X-100 on protein solubilization in intact cells.

Effect of detergents on spheroplasts. Figure 3 shows the effect of the detergents on spheroplasts. Triton X-100 solubilized total proteins with higher efficiency than Na cholate and Tween 80; however, Na cholate was more efficient than Tween 80 (Fig. 3A).

By increasing the detergent concentration, the solubilization of all proteins as well as cytochromes was increased. No *b* type cytochromes solubilization was detectable with Tween 80. Na cholate at high concentration could release a small amount of *b* type cytochromes, however, Triton X-100 solubilized *b* type cytochromes either in low or high concentrations (Fig. 3B), suggesting that it could penetrate to inner membrane of spheroplast better than the two other detergents. No cytochrome *d* was released after the treatment of spheroplasts with Na cholate and Tween 80, while Triton X-100 either in low or high concentrations solubilized cytochrome *d* (Fig. 3C). Figure 4 shows the ratio of solubilized cytochrome *b* to solubilized total proteins for each detergent concentration. Results show that 1.6 mM Triton X-100 could solubilize cytochrome *b* from the inner membrane. The amount of cytochrome *b* solubilized by either Na cholate or Tween 80 was negligible. The control value referred to the ratio of cytochrome *b* to total proteins in the spheroplasts suspension before treatment.

Effect of detergents on inner-membrane fragments. Protein release from inner-membrane fragments was considerable in comparison to spheroplasts and intact cells. At 64 mM, Triton X-100 solubilized 78% of total proteins while Tween 80 and Na cholate could solubilize about 25% of total proteins of the membrane (Fig. 5A). Both cytochrome *b* and *d* were well solubilized by the effect of Triton X-100, but only a few amounts of cytochromes released after the effect of high concentrations of the two other detergents (Fig. 5B and C). These results showed that Triton X-100 is a potent detergent for solubilization of inner-membrane fragments in *S. typhimurium.* The comparison of the ratio of cytochrome *b* and *d* to solubilized total proteins from inner-membrane fragments showed that this ratio decreased at 1.6 mM in all detergents in comparison to the control (Fig. 6A and B). This ratio became higher than control when Triton X-100 concentrations was more than 1.6 mM. In that situation, the peak for cytochromes *b* and *d* were 16 mM and 32 mM, respectively (Fig. 6, A and B), and in two other detergents it remained lower than control.

There was an inverse effect at concentration of 1.6 mM for releasing of both cytochrome *b* and *d* between spheroplasts and inner-membrane fragments (Fig. 6, A and B). In spheroplasts, the ratio of cytochromes *b* and *d* to solubilize total proteins was maximum at 1.6 mM and for inner-membrane fragments it was minimum at 1.6 mM Triton X-100. As a result, in spheroplasts, 1.6 mM Triton X-100 was more selective for the release of cytochromes.

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

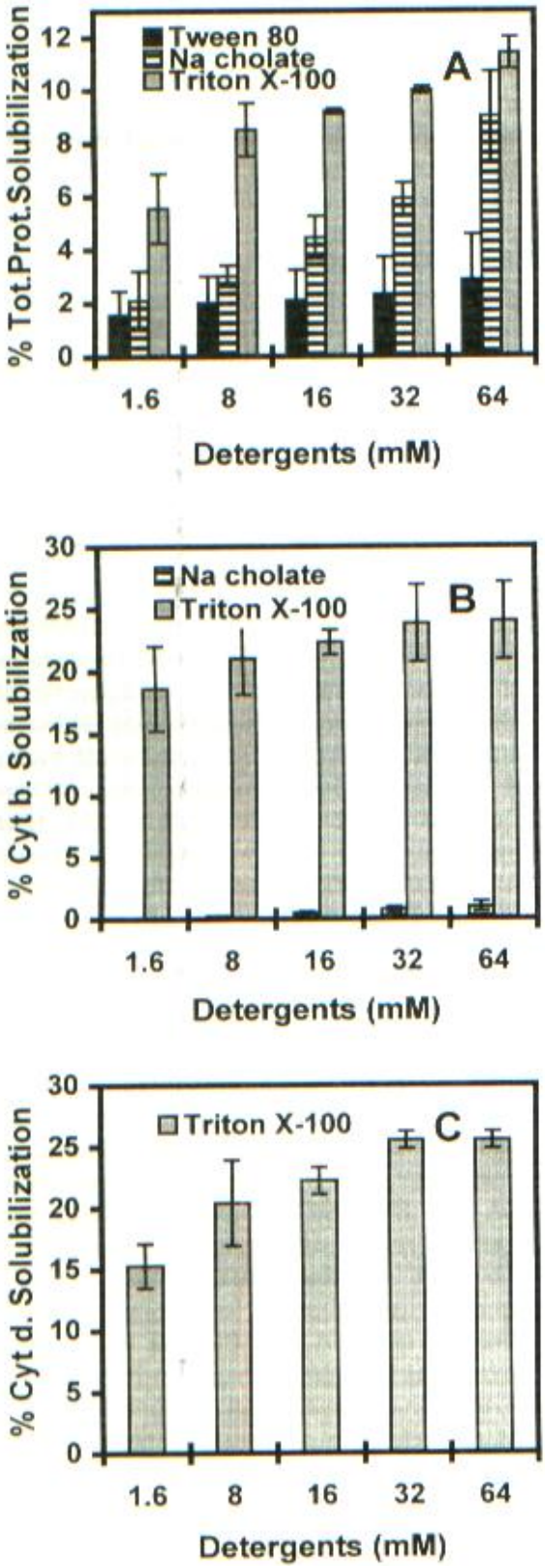


Fig. 3. The effect of Tween 80, Na cholate and Triton X-100 on spheroplasts. **(A)** Effect on total protein solubilization; **(B)** Effect on solubilization of *b* type cytochrome; **(C)** Effect on solubilization of cytochrome *d*.

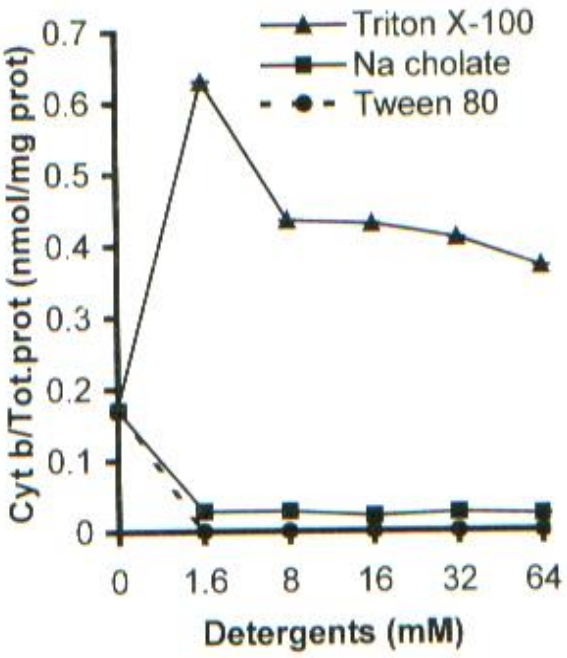


Fig. 4. The ratio of solubilized cytochrome *b* to solubilized total proteins in spheroplasts after treatment with Triton X-100, Na cholate or Tween 80.

DISCUSSION

Because each detergent is suitable for a given experimental condition, the solubilization of membrane by detergents is still a subject of extensive investigation [25, 26].

The failure to release cytochromes (as a determinant of integral inner-membrane proteins) from intact cells after treatment with various detergents suggested that the outer membrane of *S. typhimurium* was resistant to the effect of these detergents. Other reports also showed that in the presence of Mg^{++} the solubility of outer membrane proteins was low in Gram-negative bacteria such as *E. coli* [7, 9]. Furthermore, the amount of lysed cells was negligible, a result which also emphasized the barrier formed by the outer membrane and the cell wall against the entrance of detergent micelles to contact inner-membrane. After removal of the outer membrane and most parts of the cell wall in the process of spheroplast preparation, the effect of detergents on cytochrome solubilization was detectable. Tween 80 and Na cholate could not well solubilize cytochromes, but the total proteins

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

solubilization showed that these detergents may solubilize the peripheral proteins with little effect on integral proteins such as cytochromes. Triton X-100 solubilized cytochromes from spheroplasts better than the two other detergents, suggesting that Triton X-100 solubilized integral proteins as well as peripheral proteins. Triton X-100, at 1.6 mM caused high ratio of cytochromes to solubilized total proteins, suggesting that most of the solubilized proteins were integral membrane proteins, such as cytochromes. Increasing Triton X-100 concentrations decreased this ratio probably because of the solubilization of peripheral proteins.

With inner-membrane fragments, the detergents solubilized the proteins with higher efficiency than with intact cells and spheroplasts. However, Na cholate and Tween 80 were less efficient than Triton X-100, which solubilized about 90% of both cytochromes *b* and *d*. As reported previously, the lower the CMC, the higher the solubilization was occurred [27]. Triton X-100 was able to well penetrate the inner-membrane, substitute inner-membrane phospholipids and dissolve integral proteins with high efficiency. Similar results were also reported for purple bacteria [10, 11] and *E. coli* [7].

Only in 1.6 mM Triton X-100, the ratio of cytochromes to total proteins was very low, suggesting that at this concentration the ability of Triton X-100 to dissolve integral proteins was low. This was in contrast with spheroplasts where at 1.6 mM Triton X-100 this ratio was high. The interaction of Triton X-100 with spheroplasts membrane and inner-membrane fragments may be different. Spheroplasts are still viable cells and have maintained their cytoplasmic membrane orientation (right side out); However, inner-membrane fragments form vesicles and membrane could be oriented in reverse (inside out). Besides, in the process of inner-membrane preparation most of the peripheral proteins, which could help to stabilize the membrane structure, dissociate from the cytoplasmic membrane. These factors could influence the accessibility of the cytoplasmic membrane to detergents in spheroplasts and membrane fragments.

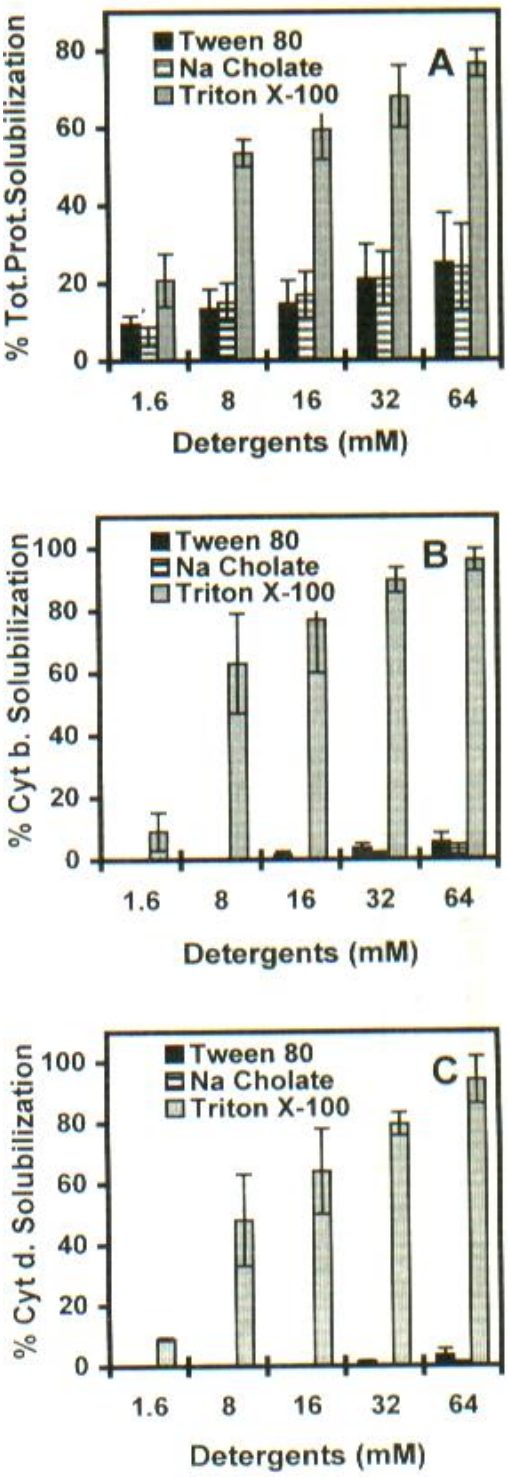


Fig. 5. Effect of the detergents on inner-membrane fragments. **(A)** Effect on total protein solubilization; **(B)** Effect on solubilization of *b* type cytochrome; **(C)** Effect on solubilization of cytochrome *d*.

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

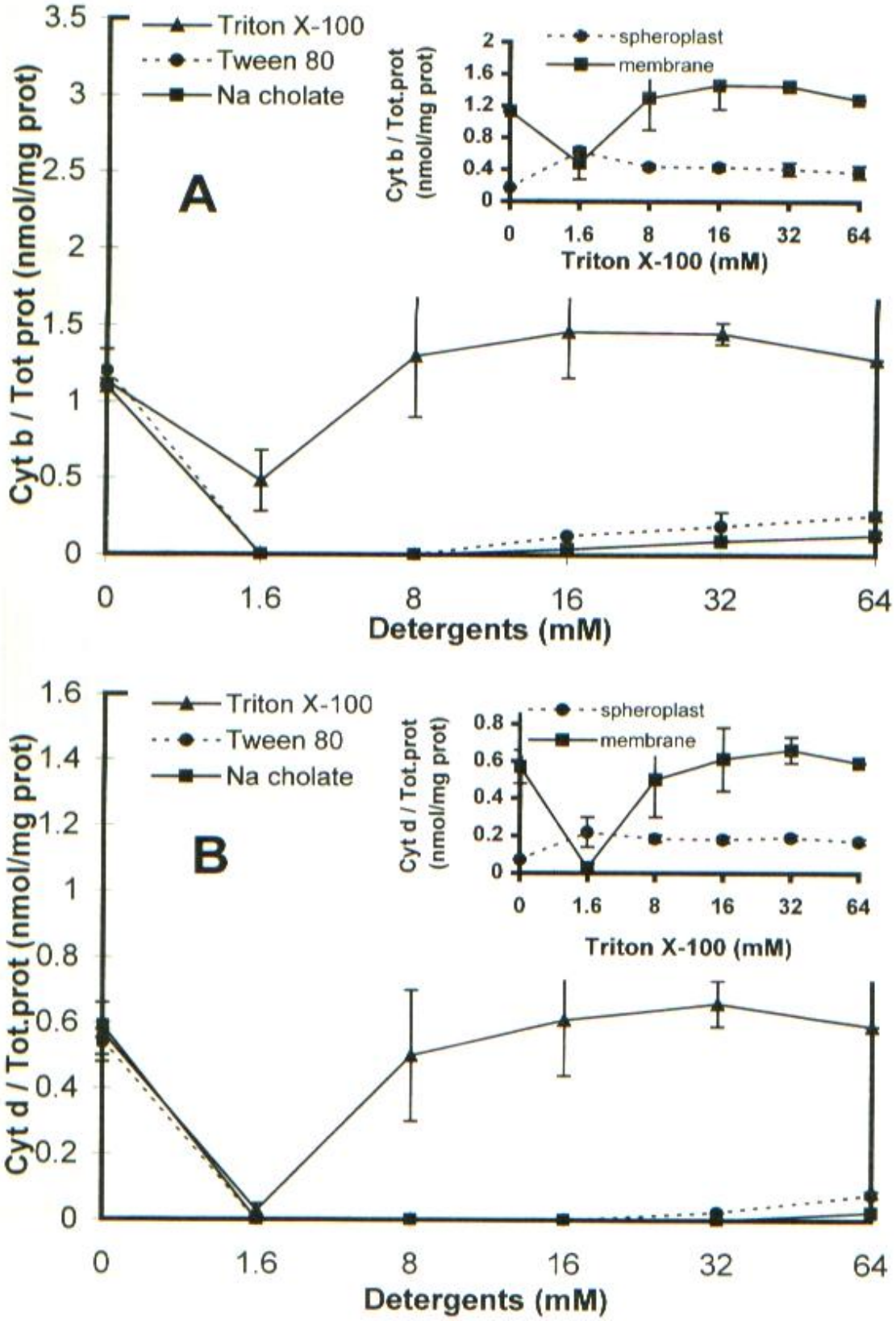


Fig. 6. (A) The ratio of solubilized cytochrome *b* to solubilized total proteins after treatment with Triton X-100, Na cholate or Tween 80; **Insert (A)** The ratio of solubilized cytochrome *b* to total proteins in spheroplast and inner-membrane fragments after treatment with Triton X-100; **(B)** The ratio of solubilized cytochrome *d* to solubilized total proteins after treatment with Triton X-100, Na cholate or Tween 80; **Insert (B)** The ratio of solubilized cytochrome *d* to solubilized total proteins in spheroplasts and inner-membrane fragments after treatment with Triton X-100.

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]

REFERENCES

1. Le Miare, M., Champeil, P. and Moller, J.V. (2000) Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim. Biophys. Acta 1508: 86-111*.
2. London, E. and Brown, D. A. (2000) Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholestrol membrane domains (rafts). *Biochim. Biophys. Acta 1508: 182-195.*
3. Helenius, A. and Simons, K. (1975) Solubilization of membrane by detergents. *Biochim. Biophys. Acta 415: 29-79.*
4. Helenius, A., McCaslin, D. R., Fries, E. and Tanford, C. (1979) Properties of detergents. *Meth. Enzymol. 56: 734-744*.
5. Tzagoloff, A. (1971) Extraction and purification of lipoprotein complexes from membranes. *Meth. Enzymol. 22: 219-230.*
6. Thompson, M.F. and Bachelard, H.S. (1970) Cerebral cortex hexokinase. Comparison of properties of solubilized mitochondrial and cytoplasmic activities. *Biochem. J. 118: 25-34.*
7. Schnaitman, C.A. (1971) Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J. Bacteriol. 108: 545-552.*
8. Filip, C., Fletcher, G., Wulff, J.L. and Earhart, C.F. (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent Sodium-Lauryl Sarcosinate. *J. Bacteriol. 115: 717-722.*
9. Schnaitman, C.A. (1971) Effect of Ethylenediaminetetraacetic acid, Triton X-100, and Lysozyme on the morphology and chemical composition of the isolated cell walls of *Escherichia coli*. *J. Bacteriol. 108: 553-563.*
10. Gonzalez-Manas, J.M., Virto, M.D., Gurtubay, J.G. and Goni, F.M. (1990) The interaction of Triton X-100 with purple membranes. *Eur. J. Biochem. 188: 673-678.*
11. Viguera, A.R., Gonzales-Manas, J.M., Taneva, S. and Goni, F.M. (1994) Early and delayed stages in the solubilization of purple membrane by polyoxyethylenic surfactant. *Biochim. Biophys. Acta 1196: 76-80.*
12. Shipp, W.S. (1972) Cytochromes of *Escherichia coli*. *Arch. Bichem. Biophys. 150: 459-472.*
13. Ingledew, W.J. and Poole, R.K. (1984) The respiratory chain of *Escherichia coli*. *Microbiol. Rev. 48: 222-271.*
14. Pudek, M.R. and Bragg, P. D. (1974) Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. *Arch. Biochem. Biophys. 164: 682-693*.
15. Juneman, S. (1997) Cytochrome *bd* terminal oxidase. *Biochim. Biophys. Acta 1321: 107-127.*
16. Borisov, V.B. (1996) Cytochrome *bd*: Structure and properties. A review. *Biochemistry (Moscow) 61: 565-574.*
17. Barrette, J. (1954) The prosthetic group of cytochrome a_2. *Biochem. J. 64: 626-639.*
18. Timkovich, R. Cork, M.S. and Gennis, R.B. (1985) Proposed structure of heme *d*, a prosthetic group of bacterial terminal oxidases. *J. Amer. Chem. Soc. 107: 6069-6075*.
19. Osborne, J.P. and Gennis, R.B. (1999) Sequence analysis of cytochrome *bd* oxidase suggests a revised topology for subunit I. *Biochim. Biophys. Acta 1410: 32-50.*
20. Kaback, H.R. (1971) Bacterial membrane. *Meth. Enzymol. 22: 99-106.*
21. Keyhani, E. and Minai-Tehrani, D. (1998) Kinetics of KCN binding to cytochrome d in *Salmonella typhimurium*. *Biotermokinetics in the Post Genomic Era.* (Larsson, C. and Gustfsson, L. eds.), Chalmers Reproservice, Goteborg, pp. 236-239.
22. Keyhani, J. and Keyhani, E. (1975) Cytochrome *c* oxidase biosynthesis and assembly in *Candida utilis* yeast cells. Subunit structure and molecular weight determination. *Arch. Biochem. Biophys. 167: 588-595.*
23. Keyhani, E. and Minai-Tehrani, D. (2001) The binding of cyanide to cytochrome *d* in intact cells, spheroplasts, membrane fragments and solubilized enzyme from *Salmonella typhimurium*. *Biochim. Biophys. Acta 1506: 1-11.*
24. Jones, C.W. and Poole, R.K. (1985) The analysis of cytochromes. *Meth. Microbiol. 18: 285-328.*
25. Knol, J., Sylloma, K., and Poolman, B. (1998) Detergent-mediated reconstitution of membrane proteins. *Biochemistry 37 (46): 16410-16415.*
26. Kragh-Hansen, U., Le Maire, M., and Moller, J.V., (1998) The mechanism of detergent solubilization of liposomes and protein-containing membranes. *Biophys. J. 75 (6): 2932-2946.*
27. Neugebauer, J.M. (1990) Detergents: an overview. *Meth. Enzymol. 182: 239-282.*

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2002.6.2.2.5]