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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.

Empigen BB: A useful detergent for solubilization and biochemical analysis of keratins

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Intermediate filament (IF) proteins make up some of the most insoluble proteins known, and within the IF protein family, keratins are the least soluble. We compared the efficiency of nonionic, cationic, mixed nonionic and anionic, and zwitterionic detergents in solubilizing keratins from insect cells that express recombinant human keratins and from human colonic cell lines and normal keratinocytes. The cationic detergent cetyltrimethylammonium bromide was similar to the zwitterionic detergent Empigen BB in its ability to efficiently solubilize keratins, but the latter detergent was superior in that it maintained antibody reactivity and allowed for immunoprecipitation of the keratins. Although Nonidet-P40 partially solubilizes keratins, Empigen BB solubilizes a significant amount of keratins not solubilized by Nonidet-P40. In the case of vimentin, differences in solubilization efficiency among the detergents was not as dramatic as with keratins. Our results show that Empigen BB solubilizes a significant amount of epidermal and glandular keratins while preserving antigenicity. This detergent should prove useful for carrying out biochemical and molecular studies on these proteins and may be similarly beneficial for other IF proteins. © 1995 Academic Press, Inc.

Intermediate filaments (IF) make up one of the three major cytoskeletal protein groups (1-4). One characteristic feature of IF, in contrast to microfilament and microtubule proteins, is their relative insolubility in aqueous buffers (5). Of the cytoplasmic IF (e.g. keratins, vimentin, desmin, and neurofilaments), keratins are the least soluble. To date, twenty different epithelial keratin proteins (K1-K20) have been identified and catalogued (6,7). Keratins are found in epithelial tissues as

obligate heteropolymers, and more than one keratin pair may be found in a given tissue. One advantage of keratin insolubility is the ability to obtain crude but near-quantitative recovery of keratins. This is achieved by brief solubilization of cells in nonionic detergent, followed by extraction of the nonsolubilized material with high salt, thereby generating an insoluble keratin pellet (8). However, disadvantages of keratin insolubility include the inability to carry out immunobiochemical studies, and the general difficulties encountered in working with insoluble material.

The importance of being able to immunologically isolate IF proteins, and keratins in particular, has several practical applications. For example, co-immunoprecipitation of detergent-solubilized proteins is a widely used simple technique that allows for the identification of protein-protein associations. Co-immunoprecipitation of two or more proteins implies physical associations, in contrast with co-purification of a mixture of insoluble proteins which make it difficult to distinguish association due to insolubility from true association. Another advantage of being able to immunopurify detergent-solubilized keratins is the ability to carry out biochemical studies, such as the characterization of K8/18 glycosylation and phosphorylation (e.g. 9-11). Recently, the cationic detergent cetyltrimethylammonium bromide (CTAB) was shown to be effective in the preferential solubilization of vimentin from BHK-21 cells, while leaving approximately two-thirds of actin in the CTAB-insoluble fraction (12). However, the ability of vimentin to be immunoprecipitated after CTAB solubilization was not tested.

In this study, we used the zwitterionic detergent Empigen BB (Emp) and showed that it can efficiently but not quantitatively solubilize keratins and vimentin. Its ability to solubilize keratins in an immunologically intact form included epidermal keratins (K5, 6, 14, and 16) and simple epithelial keratins (K8 and 18). In contrast, although CTAB was similarly efficient in its ability to solubilize these keratins, it did not preserve their antigenicity to allow for immunoprecipitation. Our results show that Emp is a highly effective detergent for solubilizing significant amounts of keratins, and that its utility is further enhanced by its ability to allow for antibody binding and immunoprecipitation in solution.

Materials and Methods

Cells and Reagents: Tissue culture cell lines used in this study were HT29 (human colon), Molt 4 (human T cell) (American Type Culture Collection, Rockville, MD), and Sf9 (insect) (Pharmingen, San Diego, CA). The cells were grown in the appropriate serum-supplemented medium, as recommended by the supplier. Human epidermal keratinocytes were kindly provided by Dr. Eugene Bauer. They were isolated from neonatal foreskin and cultured essentially as described (13), then used at passages three or four. Sf9 cells were infected with a recombinant baculovirus-K8/18 construct for 4 days as described (11), followed by solubilization of the cells. The detergents used were: Empigen BB and cetyltrimethylammonium bromide (CalBiochem, La Jolla, CA); Nonidet P40 (NP40)

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and sodium deoxycholate (Sigma); and sodium dodecyl sulfate (SDS) (BioRad, Hercules, CA). Anti-K8/18 monoclonal antibodies (MAb) used were L2A1 (10), CK5 and 8.13 (Sigma), and CR48 (14). Other antibodies used were anti-vimentin MAB V9 (Sigma), and rabbit anti-K16 (15). An enhanced chemiluminescence (ECL) kit (Amersham) was used for visualization of immunoblotted proteins.

Detergent Solubilization and High Salt Extraction: Cells were harvested by scraping, washed twice with phosphate-buffered saline (PBS) pH 7.4, then solubilized for 45 minutes at 4 °C in one of the following detergents: 1% NP40, 2% Emp, 1% CTAB, or 1% deoxycholate/1% NP40/0.1% SDS (DNS). The concentration of 2% Emp was optimal, from a range of 0.2-3% that was tested, based on the amount of K8/18 that can be immunoprecipitated (not shown). All detergents were prepared in PBS containing 5 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride, 10 μ M pepstatin, 10 μ M leupeptin, and 25 μ g/ml aprotinin. The insoluble material was pelleted (16,000g; 5 min) and discarded, and the supernatant cell lysate was used for immunoprecipitation and immunoblotting as described below.

High salt extraction (HSE) was done by solubilizing harvested cells in 1% Triton X-100 in PBS/5 mM EDTA (2 min, 4 °C), then pelleting the insoluble keratin (16,000g; 10 min; 4 °C). The pellet was homogenized using a Dounce in HSE buffer (10 mM Tris-HCl pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% Triton X-100), incubated at 4 °C for 30 min, then centrifuged (16,000g; 20 min; 4 °C). The resulting pellet was washed once with PBS to remove excess salt, and re-centrifuged (16,000g; 10 min; 4 °C).

Immunoprecipitation: Detergent lysates (100-200 μ l) were mixed with 3 μ l of antibody and incubated for 30 min at 4 °C. Twenty μ l of goat anti-mouse IgG Sepharose (for L2A1, CK5, 8.13, and V9 MAb), goat anti-mouse IgM Sepharose (for CR 48), or protein A Sepharose (for rabbit anti-K16 antibody) were then added to precipitate the keratin-antibody complexes (30 min, 4 °C). Alternatively, agarose-coupled L2A1 (20 μ l) was used to directly immunoprecipitate K8/18. Immunoprecipitates were washed 3 times in the detergent used for solubilization, followed by SDS-PAGE analysis using 10% acrylamide gels (16).

Sequential immunoprecipitation was carried out in the following manner: equal numbers of HT29 cells (3x10⁶ cells) were solubilized in triplicate in 1% NP 40 or 2% Emp for 1 hour (4 °C), then centrifuged (16,000g; 5 minutes) to pellet insoluble material. The solubilized material (250 μ l) from one of the triplicates was used to immunoprecipitate K8/18 by adding 20 μ l of L2A1-agarose (which contains a saturating amount of antibody, not shown). The pellets from the remaining two triplicates were again solubilized in 2% Emp or 1% NP 40 (1 hour, 4 °C). After centrifugation, the supernatants were used to immunoprecipitate K8/18.

Western Blotting: Equal fractions of each detergent lysate (usually 10 μ l of 300 μ l of detergent lysate prepared from 3x10⁶ cells) were separated by SDS-PAGE, then transferred to nitrocellulose membranes (4 °C, 40 volts, 12-16 hours). Membranes were blocked in MT buffer (5% nonfat dried milk, 0.2% Tween 20 in PBS) (2 h, 22 °C), then incubated in primary antibody (1:1000 dilution) in MT buffer for 1 h (22 °C). After 3 washes in MT buffer (10 min each), membranes were incubated (1 hour, 22 °C) in a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit IgG (for anti-K16) or peroxidase-conjugated goat anti-mouse IgG (for other mouse antibodies). Membranes were subsequently washed 3x with MT buffer (10 min/wash), then 2x with 0.2% Tween 20 in PBS. Proteins were visualized using the ECL kit as recommended by the manufacturer.

Results

Comparison of several detergents for their ability to solubilize K8/18: We tested the ability of several detergents to solubilize K8/18 by Western blotting of the solubilized material using anti-K8/18 MAb L2A1. We also tested whether the solubilized material is immunologically preserved for immunoprecipitation. As shown in Fig. 1, CTAB and Emp were superior to NP40 and DNS in solubilizing K8/18 from human colonic HT29 cells (panel A) and from insect Sf9 cells that were infected with a recombinant baculovirus-K8/18 construct (panel B). However, when immunoprecipitation was carried out using lysates from cells solubilized with the different detergents, Emp was superior in that it not only solubilized significant amounts of keratin, but also maintained antigenicity and antibody reactivity to allow for immunoprecipitation. Although DNS solubilized slightly more K8/18 from Sf9 cells than NP40 as determined by immunoblotting (Fig. 1B), NP40 was a milder detergent than DNS in terms of allowing for more K8/18 to be immunoprecipitated. The efficiency of solubilization by Emp and immunoprecipitation of K8/18 from HT29 using the L2A1 MAb was approximately 40% of the total keratin pool (not shown), assuming that the total keratin pool is that obtained after high salt extraction as described (9).

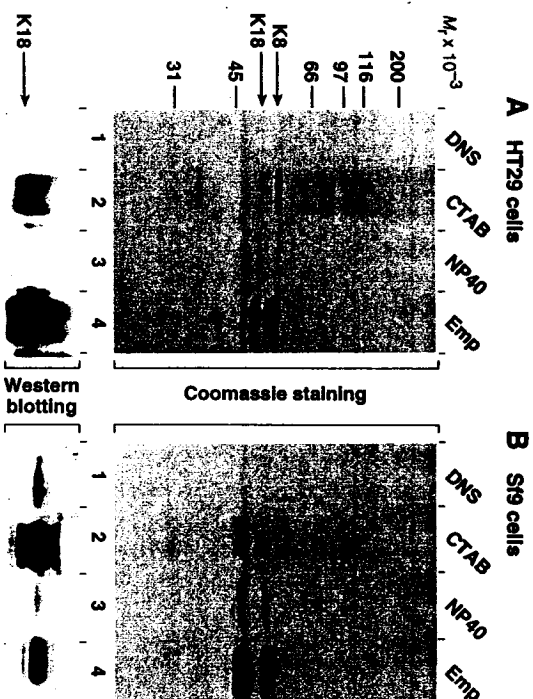


Fig. 1. Solubilization of K8/18 from HT29 and Sf9 cells using different detergents: Equal number of cells were solubilized with the indicated detergents for 45 minutes as described in Materials and Methods. After pelleting non-solubilized material, equivalent fractions (by volume) of the supernatants were used to immunoprecipitate K8/18 with MAb L2A1 followed by SDS-PAGE and Coomassie staining. Alternatively, equivalent fractions from the supernatant were directly analyzed by Western blotting using MAb L2A1.

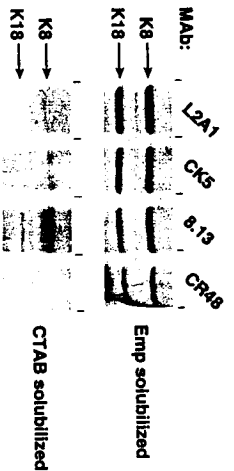


Fig. 2. Immunoprecipitation of K8/18 from CTAB or Empigen solubilized HT29 cells using a panel of anti-K8/18 MAB: HT29 cells were solubilized in 2% Emp or 1% CTAB as described in Materials and Methods. The solubilized material was used for immunoprecipitation with the indicated anti-K8/18 MAB which recognize different K8/18 epitopes.

We asked if the inefficient immunoprecipitation of K8/18 after CTAB solubilization was related to inactivation of the L2A1 antibody epitope and if other MAB that recognize different epitopes of K8/18 could have a better K8/18 immunoprecipitation efficiency. As shown in Fig. 2, CTAB solubilization prevented a panel of anti-K8/18 MAB from being able to efficiently immunoprecipitate the antigen. The increased level of immunoprecipitated K8/18 after Emp solubilization was not due to a detergent effect on antibody binding since immunoprecipitation of K8/18 from a cytosolic aqueous cellular fraction that was adjusted to 2% Emp or 1% NP40 afforded identical amounts of K8/18 (not shown).

Empigen efficiently solubilizes significant quantities of K8/18 not solubilized by the nonionic detergent NP40: The improved efficiency of solubilization of K8/18 by Emp versus NP40 suggested that Emp should be able to solubilize a significant amount of keratin not solubilized by NP40. To test this, we resolubilized the material that could not be solubilized by the first detergent (NP40 or Emp) with Emp or NP40, and analyzed the initially solubilized and re-solubilized material by immunoprecipitation using a saturating amount of antibody. As shown in Fig. 3, Emp again solubilized K8/18 better than NP40 (compare lane 2 with lane 1, Fig. 3). Resolubilizing of the nonsolubilized material with the same detergent used in the first solubilization afforded a small amount of K8/18 relative to the first solubilization (lanes 3 and 4, Fig. 3), indicating that the first solubilization was nearly complete. However, resolubilization of the NP40 nonsolubilized material with Emp followed by immunoprecipitation, resulted in a significant amount of K8/18 (lane 5, Fig. 3). A small amount of keratin was noted after resolubilization of non-Emp-solubilized material with NP40 (lane 6, Fig. 3), which is likely related to some keratin trapping in the pellet after spinning of the first solubilized material.

Partial solubilization of epidermal keratins by Empigen: Epidermal keratins are notoriously insoluble proteins that require high concentrations of urea for solubilization (e.g. 17). However, simple epithelial keratins are more soluble than epidermal keratins and require lower urea concentrations for dissociation (18).

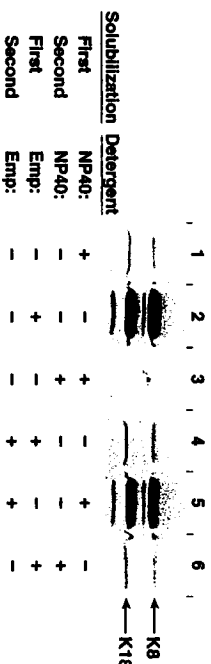


Fig. 3. Empigen is able to solubilize significant quantities of K8/18 not solubilized by NP40: Equivalent number of HT29 cells were solubilized with 1% NP40 or 2% Emp in triplicates for 60 min. After centrifugation, the supernatant from one of the triplicates was used to immunoprecipitate K8/18 (lanes 1,2). The pellets from the remaining two triplicate samples were then re-solubilized (second solubilization) in the same detergent to ensure complete first solubilization (lanes 3,4), or re-solubilized in the detergent that was not used for the first solubilization (lanes 5,6). After the second solubilization, samples were centrifuged to remove non-solubilized material followed by immunoprecipitation of K8/18, SDS-PAGE, then Coomassie staining.

Analysis of the keratin fraction obtained from cultured keratinocytes after high salt extraction showed the keratin pairs K5/14 and K6/16 (Fig. 4A). The band labeled for simplification as K16 in Fig. 4A, in fact contains a significant amount of K17 that is typically not resolved using one-dimensional SDS-PAGE conditions. We tested

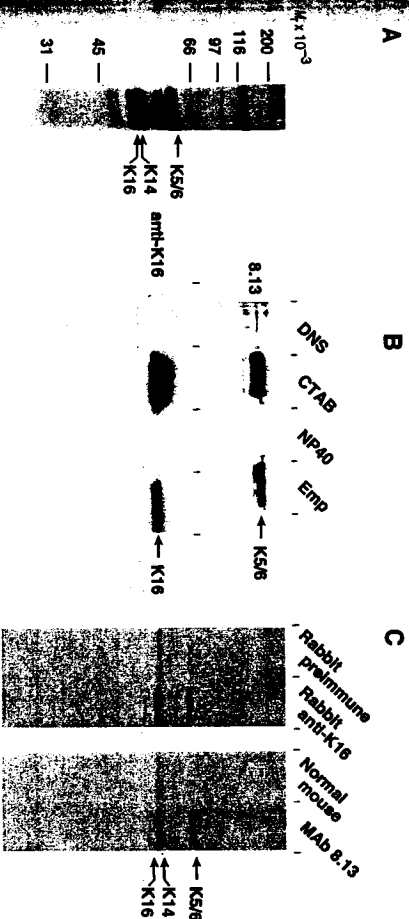


Fig. 4. Biochemical analysis of keratins in keratinocytes: *Panel A:* Keratins from normal human keratinocytes were purified by high salt extraction as described in Materials and Methods followed by analysis using SDS-PAGE. Note that K17 (not shown) and K16 generally co-migrate under standard one-dimensional SDS-PAGE. *Panel B:* Equal numbers of keratinocytes were solubilized in the indicated detergent followed by analysis of equivalent volume of solubilized material by Western blotting using MAb 8.13 or rabbit polyclonal anti-K16 antibody. *Panel C:* Human keratinocytes were labeled with ^{35}S -met for 12 h, solubilized with 2% Emp, followed by immunoprecipitation of the keratins using polyclonal or monoclonal anti-keratin antibodies and the corresponding control non-immune ascites or serum.

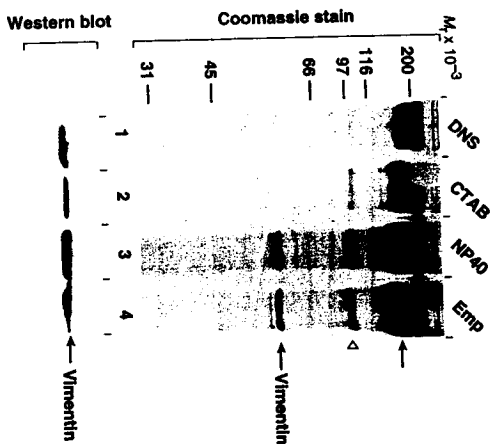


Fig. 5. Solubilization of vimentin from Molt-4 cells using a panel of detergents: Equal numbers of Molt-4 cells were solubilized with the indicated detergent for 45 min. Mab V9 was used to immunoprecipitate vimentin from equal fractions of the detergent lysates. In addition, equal fractions of the detergent lysates were used to analyze the solubilized vimentin content by immunoblotting with Mab V9.

several detergents for their ability to solubilize epidermal keratins by immunoblotting with an anti-K16 or anti-K5/6 antibody after solubilization (Fig. 4B). Immunoprecipitation of the Emp-solubilized material (after labeling of the keratinocytes with ^{35}S -methionine) resulted in clearly detectable K5/14 and K6/16. The amount of protein that was immunoprecipitated was barely detectable by Coomassie staining (not shown), and therefore was significantly less than what was obtained from K8/18-containing cells (e.g. Fig. 1A). Of note, when NP40 or CTAB-solubilized lysates were used for immunoprecipitation, no detectable epidermal keratin was seen despite a 40-fold increase in the exposure time (not shown).

Solubilization of vimentin: We also used immunoblotting and immunoprecipitation to compare the ability of DNS, CTAB, NP40, and Emp to solubilize vimentin. As shown in Fig. 5, all four detergent systems resulted in similar levels of vimentin solubilization, as determined by immunoblotting. In parallel with our findings with keratins, NP40 and Emp appeared to be the gentlest in preserving the antibody to allow for vimentin immunoprecipitation (Fig. 5). The thick band marked by an arrow corresponds to the rabbit-anti-mouse antibody (under non-reducing conditions) used during immunoprecipitation. The band marked by an arrowhead (M_r approximately 105 kD) was nonspecific and is related to the rabbit antibody used.

Discussion

There is an accumulating number of diseases involving mutations in keratin and neurofilament intermediate filaments (reviewed in 4,19,20). Some of the mutations involve potential alteration of phosphorylation sites, as for example the introduction of a putative protein kinase C phosphorylation site in K5 (21), or the deletion of a lysine that forms part of a consensus sequence for a cdc-2-like kinase in neurofilament-H (22). The ability to perform biochemical studies using immunoprecipitation methods should facilitate studying phosphorylation and other potential modifications of the epidermal keratins, and determine if indeed alterations in these modifications have a pathophysiological role. Although isolation of a relatively pure and insoluble keratin preparation can be done with relative ease using high salt extraction (e.g. 8, 9), immunopurification of detergent solubilized material has several advantages. These advantages include purity of the immunopurified proteins, the ability to carry out *in vitro* modifications in an aqueous environment (e.g. 23), and potentially, the identification of associated proteins (e.g. 14,24).

A number of studies support the use of Emp as an efficient solubilizing detergent that maintains proteins in an antigenically active form, and that preserves the enzymatic activity of isolated proteins. This includes the solubilization of erythrocyte membrane proteins (25), and the solubilization of significant quantities of proteins from pathogens including the major outer gonococcal membrane protein (26) and herpes simplex viral proteins (27). Furthermore, short of using strongly denaturing high concentrations of SDS or urea, Emp was found to be highly effective in solubilizing nuclear matrices (28). Despite its high protein-solubilizing capacity, Emp is a relatively mild detergent that compares, in terms of maintaining proteins in their native conformation, with nonionic detergents such as Triton X-100. For example, the enzymatic activity of 5-nucleotidase and alkaline phosphatase in the presence of Triton X-100 and Emp was either similar or better than the corresponding enzyme activities in the absence of detergent, and far better than the corresponding enzyme activity in the presence of deoxycholate (29).

The mechanism of Emp solubilization is not known but its zwitterionic and amphipathic nature (29) are potential factors that are important in its ability to stabilize keratins. The charge distribution of this zwitterionic detergent is similar to phospholipids such as phosphatidylcholine (29), and several studies have shown that intermediate filaments including keratins bind to lipids (reviewed in 30). For example, glycosphingolipids such as globoside co-localized by double immunofluorescent staining with vimentin, desmin, keratins (epidermal and simple epithelial) and glial fibrillary acid protein in cells that express these IF (31). Similarly, keratins in mammary gland primary cultures appear to noncovalently associate with polar lipids that may represent phospholipids (32), and several nonepithelial IF proteins were shown to interact *in vitro* with synthetic phospholipids (33).

In conclusion, Empigen BB is a relatively mild zwitterionic detergent that improves on our current ability to solubilize keratins while simultaneously allowing for their study using immunological methods. It should prove to have practical uses in biochemical studies of keratins in normal as well as diseased epithelia.

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