Contractile protein biochemistry in the Pollard Lab in Baltimore

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Abstract

We describe our search for the molecular mechanisms of cell motility with personal recollections of bucket biochemistry in Tom Pollard's Lab at the Johns Hopkins, circa 1980.

Keywords Biochemistry · Myosin · Protein

From 1979 to 1984, we worked in Tom Pollard's lab at the Johns Hopkins Medical School, located in the heart of East Baltimore. This was a very exciting time in the field of non-muscle cell motility. Biochemistry was one of the major productive approaches used by our lab and others. We were guided by following the paths laid out by previous work on muscle—the Huxleys, the Szent-Györgyi, Annemarie Weber, and others were our heroes.

Tom's vision, acquired while he was a postdoc in the lab of Ed Korn, a renowned membrane biochemist, was to use a non-muscle cell source that could be grown in the kilogram quantities that characterized muscle sources. *Acanthamoeba castellanii*, was the organism of choice. The approach was “bucket” biochemistry—grow up 100s to 1000s of grams of this eukaryotic cell, disrupt the cells and purify proteins and protein complexes that displayed properties that showed them to be actin, myosin, or some novel protein that affected one or the other. *Acanthamoeba* was a wonderful organism for this purpose, being easy and inexpensive to culture and grow in large amounts, not pathogenic, and easy to disrupt. Our recollection is that 1 kg cost us ~\$100 in supplies.

Ironically, at the time, we questioned whether yeast and fungi would be suitable for an approach like this one, because we wondered whether the sheer amounts of cell wall material and the difficulties in disrupting the cells would be problematic. Also, we knew that yeast were not motile organisms, so we assumed yeast cells would have little need for much in the way of muscle-like contractile proteins. Of course, in a few years, we realized that our concern was unfounded, and yeast and fungi were indeed also wonderful systems for biochemistry, ones that allowed for genetics and cell biology.

One of the fun things about the lab and this approach was our determination, under Tom's leadership, to purify as many proteins as possible from one prep (Fig. 1). We worked together to sterilize large quantities of rich media in large carboys, to which we added some essential heat-labile vitamins after they cooled. Tom himself kept the stocks of *Acanthamoeba* cells growing between preps. When we planned for a large prep, we would grow larger amounts of the stock cultures in liter quantities in Fernbach flasks, and then inoculated three or four glass carboys that each contained about 20 l of medium. The carboys were fitted with stoppers and glass tubing through which we pumped filtered air, from the inexpensive sort of pumps used for fish tanks, to provide oxygen. Each carboy had a large stir bar (a luxury at the time) and sat on a large solid stir plate, at room temperature. The cells grew over the course of several days and each day, we would monitor cell numbers and look for contamination. Our team of postdocs and MD/PhD students planned carefully for the big day of the prep.

On the morning of what would be a long day, we arrived early and starting spinning down the 60–80 L of *Acanthamoeba* cell suspension in a centrifuge that could handle 6 one L bottles. Fifteen minutes was sufficient to pellet the cells, the supernatant was discarded, and the bottle was filled

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Fig. 1 A young(er) Tom Pollard, then recently appointed Director of Cell Biology and Anatomy, hard at work in the cold room (he was, and still is the youngest Director to head a Hopkins Medical School Department). He is shown loading a gel filtration column designed to size myosin away from actin and KI, which was added to disrupt actomyosin. The ingenious design of the column minimized contact with KI and afforded good separation of the two precious contractile proteins

again. This was repeated multiple times, until the entire 60–80 L had been collected into an enormous cell pellet, frequently weighing in at a kg (wet weight) or more.

The pellet was mixed with three volumes of added buffer—a volume so large that a conventional Dounce homogenizer, which was adequate to disrupt the cells, would have been simply too time-consuming. We were fortunate to have a Parr “bomb” in which the mixture was pressurized with N_2 . When the pressure was released to atmospheric pressure, we gave the unsuspecting creatures the bends—cavitation due to N_2 coming out of solution caused the cells to break. The nitrogen protected our precious proteins from oxidation. The mantra in the lab was to do it cold, do it fast, do it buffered and do it reduced.

This large volume of supernatant was then spun at high speed, 40,000 rpm, in a high-volume rotor, a Ti45, for 45 min. The rotor held six tubes, and that was not sufficient for the volume of lysate. We had to perform several additional spins with same rotor. Having a second rotor would have been an inconceivable luxury due to the cost of the rotor. At this point, the value of the prep, based on our combined hours of work, plus the value of the titanium rotor, and the fact that we were spinning at high speed after balancing the tubes carefully, meant that we were always nervous about accidents. Indeed, tubes occasionally cracked, and material leaked out, trapping the smashed tube in the rotor. The skill of extracting these crumpled and crushed pieces of plastic was a highly valuable one, learned largely through trial and error—a claw hammer and a machine screw, some washers and a nut were the way to go!

We mixed this high-speed supernatant with DEAE-cellulose in bulk—pouring the protein-rich supernatant onto any ion exchange column would have irreversibly clogged the column. About a liter or two of DEAE-cellulose had been charged and washed with the starting buffer. This buffer, and the lysis buffer, contained ATP, which was perhaps the most costly reagent in the preparation. The buffer also contained beta-mercaptoethanol (BME), to provide a reducing

environment. At the time, DTT was commercially available, but was too expensive to use in the necessary quantities. The smell of BME permeated the cold room, as we did all these maneuvers on the lab bench in the cold room. Paraphrasing Colonel Bill Kilgore (played by Robert Duvall in the film *Apocalypse Now*), we would say that as biochemists, “We love the smell of BME in the morning – it smells like victory!” No one dared to complain. The DEAE cellulose was mixed in bulk with the supernatant and then poured into a very large column. The DEAE-cellulose was recycled from the previous prep, washed with acid and base and then equilibrated for pH and with ATP, on the preceding day, in preparation.

The Parr bomb and now the column were in the cold room, where we then destined to work for the next several days. The column was washed with buffer and then a salt gradient, which we constructed manually with large beakers connected by pieces of tubing. The base of the gradient maker was carved out of the Styrofoam that held sterile 50-mL Falcon tubes scarfed (that is a technical term) from the monoclonal culture facility. Our tubing was long enough to include a safety loop for when the gradient was finished at some point in the coming hours (in the middle of the night). We collected the largest fractions possible in the largest fraction collector one could afford, and we finished with over 200 40-mL fractions.

From those fractions, starting on day 2, we then proceeded to do assays that allowed us to find actin, myosin-I, myosin-II, profilin, alpha-actinin, capping protein, and ADF/cofilin (aka actophorin). We took tremendous pride, following Tom’s lead, in how little money we had spent and how many proteins we were going to obtain.

The running of this first large DEAE column, from which all the proteins emerged, was a critical step, and Tom was often standing with us, in the cold room, watching the resin pack, assembling the top piece of the column with the input tubing, setting up the gradient, and getting the fraction collector running. While there was some down time as the column was getting going and these steps were completed, Tom would often run back to his office, where he served as department chair and a senior editor of the *Journal of Cell Biology* (which was the major cell biology journal of the time). Aside from these trips to his office, Tom was often down on his knees on the floor of the cold room, cleaning up a spill or some accumulated debris—sometimes collecting precious fractions that eluded a collection tube or two. Tom was the consummate boy scout, in the best literal sense of that term—having earned the rank of Eagle, he always left the camp site better than he found it, and he never failed to pitch in with the most menial tasks, leading by example and from within the pack.

We stood side-by-side with him, as a group, and we were always proud to have him as our leader. Given our ages, he was more of an older brother than a father figure, and he was a great teacher. As in a family, we often tussled and argued, with each other and with him, but we were ultimately motivated

most strongly by our unflinching desire to do great work and achieve great things.

After these initial 2 days of the big prep, each of several people, having done their personal assay on many of the fractions, took their pool and went forward. We had to wait for each other, and we had to argue about exactly where to draw the line between this pool and that one, with concern for getting all of the fractions that contained our particular protein. Feelings often ran high, but with great camaraderie in the end. We often worked late into the evenings and stopped at one of the great Baltimore bars for a beer and a burger, or at Mamma Mia's on Monument Street, on the way home. The nights ran long because we constantly feared that the proteases were coming up behind us and eating our precious proteins. Protease inhibitors were cruder than today and added as individual components, so keeping everything as cold as possible and purifying the proteins as fast as possible was the mainstay of our defense. For many of us, we worked until we started the next multi-hour column, or we worked until we realized that we were dropping things without a good reason (like valuable glassware—broken pieces were stashed in Doug Murphy's trash so no one had to own up to the error). That was our signal to go sleep and eat.

One important reason that the biochemical approach succeeded was the assays that were used to detect the activities of the proteins. For proteins that either promoted the gelation of actin filaments (such as the filament crosslinker alpha-actinin) or decreased the viscosity of actin filament solutions (such as the barbed-end capping protein discovered by Tom), the lab had developed a simple viscosity assay using a capillary tube and a steel ball that rolled down the tube, held at an angle. Tom had invented this assay, and we also took great pride in how inexpensive it was despite being done on hundreds of fractions. The capillary tubes and the steel balls were simple, used in ways that had never been envisioned previously. The apparatus was plastic, made by Tom and us by cutting and gluing simple inexpensive pieces of plastic sheet together. The apparatus sat in a fish tank, with water and an

inexpensive temperature controller. Through the wall of the fish tank, we timed the movement of the steel ball rolling down the actin-filled capillary tube with a stop watch. For the myosin jocks, ATPase activity was the way to go. Once each of us had a good antibody to our proteins many assays were replaced by SPBAs (pronounced SPEEBAHs), i.e., a solid phase binding assay that required home labeled ^{125}I -protein A (we used the leftover ^{125}I from Vann Bennett's lab—the stuff that was no longer hot enough for Vann's lab to do their thing).

This biochemical approach, used by many other labs and ourselves, was remarkably fruitful, identifying many important proteins. With the purified proteins in hand, we studied their biochemical mechanisms, we made antibodies to localize them in cells, and we ultimately, in later years, cloned cDNAs and genes. To be sure, other approaches at the time were also quite fruitful; genetics, signal pathway elucidation, and electron microscopy made advances that converged with synergy.

At the time, the field was following the great examples of progress made with phage, bacterial and muscle biochemistry, working to define how structures were assembled out of proteins, and how those molecular machines imparted biological function. More than once, someone would say that, following on this path, we would 1 day disrupt the gene the encoded our favorite protein and then observe the resulting phenotype in a moving animal cell, perhaps even a human one. At this, the group would roar with laughter, because such a thing was clearly and absurdly impossible!

We look back fondly at *Acanthamoeba*, bucket biochemistry and our time at “the Johns.”

Compliance with ethical standards

Conflict of interest Daniel P. Kiehart declares that he has no conflict of interest. John A. Cooper declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.