Invertebrate Biology 117(3): 181–185.

A cautionary note on measuring protistan bacterivory by acid lysozyme

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Abstract. Protistan bacterivory is considered important in aquatic ecosystems but difficult to measure. Two recently proposed enzymatic assays of protistan bacterivory rely on lysozyme hydrolysis of the β(1-4) glycosidic bond between N-acetylglucosamine and the fluorogenic compound 4-methylumbelliferyl. We analyzed protists and metazoans for acid-lysozyme (L_{ACID}), and found that 5 of 6 protists and 20 of 21 (95%) metazoan genera had L_{ACID}. Protistan bacterivory estimates based on L_{ACID} activity may be erroneous if micrometazoans are in analyzed samples, as is likely in benthic and littoral samples and without careful sample processing. In addition, lysozymes (e.g., L_{ACID}, β-N-acetylglucosaminidase) hydrolyze both peptidoglycan and chitin, meaning that bacterivory may not be distinguished from chitin digestion by lysozyme analyses. Therefore, lysozyme-based analyses of bacterivory should be applied only to samples that do not contain chitinous organisms, and perhaps predators of chitinous organisms. Finally, dissolved organic compounds interfered with fluorometric analysis of enzymatic substrate hydrolysis, leading to an underestimate of L_{ACID} activity. Lysozyme-based analyses of protistan bacterivory developed for use on pelagic samples will be difficult to apply to some inland waters and benthic or littoral samples without precautions to exclude metazoans, chitinolytic enzymes in organisms, and dissolved organic compounds.

Microbial food webs are important in aquatic ecosystem energetics and cycling of materials (Azam et al. 1983; Cole et al. 1988; Pomeroy & Wiebe 1988). Protozoans are most often considered the major grazers of bacteria in aquatic systems, but metazoans also feed on bacteria and can indirectly regulate bacterial composition and productivity by preying on protozoans (Porter et al. 1985; Berninger et al. 1991; Jurgens et al. 1994). Bacterivory by protozoans and metazoans has most often been estimated by quantifying fluorescently- or radio-labeled bacteria, or by inference from changes in bacterial abundance and/or biomass (e.g., Nygaard & Hessen 1990; Sherr et al. 1992; Jurgens et al. 1994). These techniques entail various problems, and alternative methods based on analysis of lysozymes have been proposed (Gonzalez et al. 1993; Vrba et al. 1993). We use the term "lysozymes" here to include all enzyme forms that hydrolyze the β(1-4) glycosidic bond between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan, regardless of specific nomenclature (Stryer 1981; Cabezas 1989).

Gonzalez et al. (1993) developed the acid lysozyme (L_{ACID}) assay to measure lysozyme activity in protistan cell lysates at acid pH. Because protistan food vacuoles are acidic (Sleigh 1989), and both extracellular and cytoplasmic lysozymes operate at more basic pH, Gonzalez et al. (1993) proposed that the L_{ACID} assay "should be a specific indicator of protistan bacterivory." Gonzalez et al. (1993) demonstrated maximal L_{ACID} activity at pH 4.5 and a strong relationship between L_{ACID} activity and intake of fluorescently-labeled bacteria by protists in several pelagic and nearshore marine water samples and cultures. Finally, Gonzalez et al. (1993) emphasized that quantitative estimates of protistan bacterivory by L_{ACID} activity should be calibrated against an independent measure of bacterivory (e.g., intake of fluorescently-labeled bacteria).

The L_{ACID} assay is clearly a valuable tool for measuring bacterivory by protists. However, some aquatic samples (e.g., those from inland, estuarine, or benthic marine waters) can often contain dense populations of various metazoans that are not easily separated from protists (e.g., rotifers). If metazoans contribute to measured "protistan" L_{ACID} activity in such samples, protistan bacterivory would be overestimated and metazoan importance underestimated. For those systems, the L_{ACID} assay must pass another test for it to be con-

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sidered protist-specific: metazoans must not produce L_{ACID} as well. Gonzalez et al. (1993) argued that the L_{ACID} assay is protistan-specific because marine bacterial exoenzymes are inactive at pH<5 and function maximally at seawater pH. However, metazoans were not tested for L_{ACID} activity.

In addition, protistan L_{ACID} must be shown to differ from protistan and metazoan lysozymes that can act at acid pH but that may indicate activities other than bacterivory. Lysozymes hydrolyze peptidoglycan, but also hydrolyze chitin, which is composed only of NAG molecules joined by β(1-4) glycosidic bonds (Stryer 1981). Therefore, chitin-bearing metazoans that molt, protistan and metazoan predators of chitin-bearing prey, detritivores, and aquatic fungi with chitinous cell walls may all have lysozymes that hydrolyze the analog substrate, 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotriose (MUF-CHT), used in the method of Gonzalez et al. (1993). Chitotriose is a trimer of NAG molecules and an intermediate breakdown product of chitin digestion (Gooday 1990; Vrba et al. 1993). If analyzed samples contain organisms with lysozymes that are not involved in bacterivory but that hydrolyze chitin and react with MUF-CHT, the method of Gonzalez et al. (1993) could overestimate bacterivory by either protists or metazoans.

We began this study with the intent of measuring protistan bacterivory in communities known to include metazoans. To do so, we first wanted to know if the method would discriminate protistan from metazoan bacterivory. Alternatively, we thought that a general measure of community (protistan and metazoan) bacterivory might still prove useful. Provided that the L_{ACID} method is bacterivory-specific, we expected *a priori* that two metazoan genera would not have L_{ACID}: tardigrades have stylet mouthparts and feed by piercing plants or small metazoans (Pennak 1989); and cyclopoid copepods are raptorial feeders (Pennak 1989). In the process of explaining our results, we more closely examined the chemistry underlying the lysozyme-based bacterivory methods, including that of chitin digestion.

Methods

Organisms were obtained from three small ponds on the Merritt Island National Wildlife Refuge, Florida, and from Carolina Biological Supply Co. (CBS). The ponds varied in salinity (<1, 7, and 16 ppt). A pond sample was collected by scooping or pushing submerged aquatic vegetation into a 1-l wide-mouth plastic bottle. The bottle was filled with pond water and placed on ice until return to the laboratory for processing. A second bottle was also filled with pond water and chilled to ensure sufficient water for maintaining organisms in the lab.

Samples were held at room temperature during processing. Each field-sample jar was vigorously shaken to dislodge organisms attached to plants. Aliquots of field-collected and cultured samples were examined in a Petri dish with a dissecting microscope. Organisms were individually isolated for acid-lysozyme analysis by micropipetting them through 3 or 4 sequential transfers in filter-sterilized (0.2 μm) pond or culture water. Six protistan genera and 21 metazoan taxa were isolated and analyzed.

Isolated organisms were sonicated and duplicate extracts were analyzed according to Gonzalez et al. (1993). We measured fluorescence at 360 nm with a Perkin-Elmer LS 50 B luminescence spectrometer (excitation at 330–400 nm, emission filter=470 nm). Readings from boiled controls (1 per extract) were subtracted from results to correct for background fluorescence. Results were expressed as pM MUF·mL^{-1} extract·h^{-1}. We were interested in the presence or absence of L_{ACID} activity among protistan and metazoan taxa, not in quantitative estimates of bacterial grazing rates, which would require calibration with fluorescently-labeled bacteria (FLB) (Sherr et al. 1987). We did not standardize extracted biomass among taxa, which would be expected to affect magnitude of results. An entirely different study is required to compare L_{ACID} activities among taxa (controlled for feeding, molt cycles, etc.); such a quantitative study would be predicated on first demonstrating presence or absence of L_{ACID}, our goal here.

Results

Results are listed in Table 1, with enzyme activity expressed in the same units used by Gonzalez et al. (1993); taxonomy is per Pennak (1989). Genera for which we list 2 sets of values were isolated from two samples (e.g., a pond and a CBS culture). Values in Table 1 should be interpreted as indicating presence or absence of enzyme activity only; extracted samples were not controlled for organism density. Each value was corrected for background fluorescence.

Of the 6 protistan genera tested, only *Stentor* apparently lacked L_{ACID} (Table 1). Values for *Actinosphaerium* were lowest among the other taxa we tested, probably due to low density of isolated organisms in the analyzed extract. Two sets of values are listed for *Paramecium:* the first for organisms isolated in the original brown-colored culture solution, and the second from organisms isolated in an optically-clear, colorless culture solution. Because we expected *Paramecium* to have L_{ACID}, we were surprised at the

Table 1. Acid lysozyme analysis results. Each row lists enzyme rates (corrected for boiled controls) for duplicate extracts of each taxon. Values calculated as negative rates (based on standard curves) are listed as 0.000. CBS = organisms from Carolina Biological Supply Co.; analyzed 3 July 1996; Pond = organisms collected from ponds in Merritt Island National Wildlife Refuge, FL, analyzed 15 June 1996.

		pM MUF·mL^{-1} extract·h^{-1}	
	Source	Analysis 1	Analysis 2
Phylum Sarcomastigophora			
Subphylum Sarcodina			
Class Actinopoda			
Actinosphaerium sp.	CBS	0.458	2.167
Actinosphaerium sp.	CBS	1.021	1.125
Subphylum Sarcodina			
Class Rhizopoda			
Amoeba proteus	CBS	7.042	3.896
Amoeba proteus	CBS	6.438	6.583
Subphylum Mastigophora			
Class Phytomastigophorea			
Peranema sp.	CBS	10.292	10.250
Chilomonas sp.	CBS	43.542	38.708
Phylum Ciliophora			
Paramecium sp. (brown soln)	CBS	0.000	0.000
Paramecium sp. (clear soln)	CBS	173.500	181.042
Stentor sp.	CBS	0.000	0.000
Stentor sp.	Pond	0.000	0.021
Phylum Platyhelminthes			
Class Turbellaria			
Microdalyiella sp.	Pond	5.896	6.562
Stenostomum sp.	CBS	0.167	0.229
Stenostomum sp.	Pond	0.000	0.000
Phylum Rotifera			
Brachionus sp.	Pond	0.083	0.208
Lecane sp.	Pond	0.396	0.438
Lepadella sp.	CBS	0.188	0.812
Philodina sp.	CBS	17.188	15.438
Rotaria sp.	Pond	0.458	0.292
Phylum Nematoda			
Cephalobus sp.	CBS	67.729	35.958
Unidentified nematode	Pond	0.104	0.208
Unidentified nematode	Pond	0.583	0.542
Phylum Tardigrada			
Unidentified tardigrade	CBS	12.500	7.146
Phylum Annelida			
Class Oligochaeta			
Stylaria sp.	CBS	365.646	367.208
Tubifex sp.	CBS	337.354	311.917
Unidentified naidid	Pond	267.375	238.271
Phylum Arthropoda			
Class Crustacea			
Subclass Branchiopoda			
Artemia sp.	CBS	316.188	332.833
Daphnia sp.	CBS	327.354	329.708
Scapholeberis sp.	CBS	21.792	24.458
Subclass Ostracoda			
Unidentified ostracod	Pond	99.229	97.500
Unidentified ostracod	Pond	43.688	40.708
Subclass Copepoda			
Cyclopoid copepod	CBS	116.604	123.271
Harpacticoid copepod	Pond	0.208	0.312
Subclass Malacostraca			
Asellus sp.	CBS	368.375	336.250
Gammarus sp.	CBS	353.417	417.688

negative result for organisms in the original culture solution. Analysis of organisms in clear solution indicated that *Paramecium* did, in fact, have L_{ACID}, and that dissolved organic compounds (e.g., humic and tannic acids) in the culture solution apparently interfered with fluorescence detection at 360 nm.

We analyzed 10 micrometazoan taxa (Platyhelminthes, Rotifera, Nematoda, and Tardigrada). Nine had L_{ACID}, including organisms we expected would not (tardigrades). *Stenostomum* did not clearly have L_{ACID}, based on the mixed results (Table 1) which were probably related to low density of extracted individuals in the pond sample.

All 11 of the macrometazoan taxa (Annelida and Crustacea) were clearly positive (Table 1). Greater biomass for some of these genera was probably responsible for higher mean values compared to the micrometazoans. Cyclopoid copepods clearly had L_{ACID}, contrary to our expectations.

In summary, 5 of 6 protistan genera tested had L_{ACID}; the one lacking L_{ACID} was *Stentor*. Of the 21 metazoan taxa tested, 20 (95%) clearly had L_{ACID}.

Discussion

Acid lysozyme analysis is a promising technique for measuring pelagic protistan bacterivory, as was clearly demonstrated by Gonzalez et al. (1993). However, it may be difficult to extend the method to other aquatic habitats (i.e., benthic, littoral), where samples often contain numerous metazoans. All six of the metazoan phyla tested included species positive for L_{ACID}. Many of the L_{ACID} values obtained in our study were comparable to or greater than the maximum value (0.578 pM MUF·mL^{-1}·h^{-1} for a culture of 2.7×10^6 cells·mL^{-1} of *Micromonas pusilla*) reported by Gonzalez et al. (1993). The values in our study certainly reflect greater biomass in our samples (e.g., macrometazoans). Although enzyme activity values in our study were not standardized per unit biomass, the gen-

erality and magnitude of values in Table 1 suggests that the presence of metazoans in samples analyzed for protozoan L_{ACID} activity could affect results, especially if whole water samples (e.g., Gonzalez et al. 1993) or large-mesh (>35 mm) plankton netting are used in non-pelagic waters. The magnitude of error due to metazoan presence will vary with composition of sampled communities; our results simply indicate the potential for such error.

Likens & Gilbert (1970) found that 35-μm mesh is needed to quantitatively sample rotifers (i.e., remove rotifers from filtrate). Use of mesh >35 μm to exclude metazoans (e.g., Vrba et al. 1993) will permit small metazoans to pass into samples, potentially leading to overestimates of protistan bacterivory. Although it may exclude some large protists, use of 20-μm (e.g., Sherr et al. 1992) or 35-μm mesh will more clearly separate protists from metazoans.

The fact that "acid lysozyme" has maximal activity at pH 4.5 is not by itself indicative of a protist-specific assay. Multiple representatives of six metazoan phyla exhibited lysozyme activity at that pH in our analysis. Gonzalez et al. (1993) did not compare protistan and metazoan lysozyme pH optima, but compared only protistan and bacterial lysozyme pH ranges. Digestive fluids of some invertebrate animals are acidic (e.g., Teo & Sabapathy 1990; Teo & Woodring 1994); it is likely that acidic digestive fluids occur in other taxa as well.

In addition, L_{ACID} analysis does not appear to be bacterivore-specific. Organisms that do not feed on bacteria (e.g., tardigrades, cyclopoid copepods) also have L_{ACID}. Gonzalez et al. (1993) correctly stated that peptidoglycan occurs only in eubacterial cell walls, but lysozymes hydrolyze β(1-4) glycosidic bonds of both peptidoglycan and chitin (Stryer 1981). Any samples that contain organisms with chitinolytic activity will overestimate protistan bacterivory when analyzed for L_{ACID} by the method of Gonzalez et al. (1993). In addition, it is possible that some protists use lysozymes to degrade chitin of captured prey (e.g., rotifers, nematodes, gastrotrichs, etc.) and peptidoglycan of bacterial cell walls. Thus, L_{ACID} cannot be expected to distinguish between protistan and metazoan bacterivory, let alone bacterivory and chitinolysis in general.

Overlap in metazoan and protistan sizes, and the presence of chitinolytic lysozymes, may also affect the method of Vrba et al. (1993), which uses 4-methylumbelliferyl β-N-acetylglucosaminide (MUF-NAG) hydrolysis at pH 7.5 as a measure of protistan bacterivory. The methods of Gonzalez et al. (1993) and Vrba et al. (1993) both analyze enzymatic hydrolysis of the β(1-4) glycosidic bond attaching MUF to NAG. That of Gonzalez et al. (1993) uses NAG trimers (chitotriose), while that of Vrba et al. (1993) uses NAG monomers. The extracellular enzyme assayed by Vrba et al. (1993), β-N-acetylglucosaminidase, is a lysozyme by definition (Stryer 1981). The methods differ (e.g., sonication of cells versus whole-cell analysis, pH), but the basic lysozyme-glycosidic bond reaction is central to both methods. Vrba et al. (1993) considered interference by bacterial chitinolytic enzymes (lysozyme), but did not rule out other sources of chitinolytic lysozyme. Non-pelagic samples may include organisms that either use lysozymes to digest chitin-bearing prey and detritus, or that have chitin and that may release lysozyme during growth. We did not test the effects of metazoans and/or chitinolysis on the method of Vrba et al. (1993), but our results indicate a potential for problems similar to those described above for L_{ACID}, if extended to non-planktonic samples.

Our results indicate one additional problem with extending the L_{ACID} method beyond marine pelagic waters. Dissolved organic compounds (DOC) are known to interfere with fluorescence measurements (Eisenthal & Damon 1992); this occurred in the *Paramecium* culture solution in our study. Gonzalez et al. (1993) did not encounter this interference in their samples, as is likely for many marine pelagic samples. However, some freshwater and estuarine samples are more likely to contain DOC, further complicating extension of the method of Gonzalez et al. (1993) to samples from habitats other than marine pelagic waters.

Enzymatic assays of bacterivory offer the potential to mitigate errors introduced by other methods (Gonzalez et al. 1993), but only if enzymatic assays themselves are carefully used. For some samples (e.g., those likely to contain small metazoans), the L_{ACID} assay may overestimate protistan L_{ACID} activity by including L_{ACID}-bearing metazoans. The metazoan-caused error may weaken the relationship between L_{ACID} assay results and independent measures of protistan bacterivory (e.g., fluorescently-labeled bacteria intake). Seasonal analyses of samples containing L_{ACID}-bearing metazoans could be confounded by seasonal successions of metazoan populations, suggesting repeated calibrations of L_{ACID} assay results and careful treatment of samples to exclude metazoans. Some pelagic samples may be effectively filtered to remove small metazoans, using ≤35 μm mesh (Likens & Gilbert 1970), but sample processing methods should be carefully examined for their efficacy. It will likely be difficult to mitigate error caused by L_{ACID}-bearing metazoans in complex (e.g., benthic or littoral) samples, for which filtration is less effective. In addition, potential chitinolysis by chitin-bearing organisms, by their predators, and by detritivores (e.g., aquatic fungi) may introduce error. Thirdly, dissolved organic com-

pounds can interfere with fluorescence of the analog substrate used in lysozyme analyses. All three of these potential problems are more likely to occur if methods developed for pelagic waters are extended to more complex aquatic habitats (i.e., freshwater and estuarine benthic and littoral sites). Careful handling and examination of samples will be needed to guard against possible errors due to these problems.

Acknowledgments. We thank Ron Geison of Sigma Chemical Company for helpful information, and William Martz for review comments regarding biochemistry. We also thank Vicki Pearse, Robert Heath, and 2 anonymous reviewers for comments that improved the manuscript. This work was supported by Dynamac Corporation and by a NASA/ASEE Summer Faculty Fellowship awarded to DGJ.

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