Discussion

Comment to Sherr and Sherr (1999): “Is there any appropriate way to distinguish different β-N-acetylhexosaminidase activities in aquatic environments?”

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Abstract

The recent paper of Sherr and Sherr on detecting low-affinity β-glucosaminidase activity in several marine microbes extends current knowledge about hydrolytic enzyme activities in natural aquatic systems. However, their conclusions regarding the whole-cell assay with MUF-N-acetyl-β-D-glucosaminide (MUF-[GlcNAc]) cannot be accepted. First, we explicitly demonstrate a strong correlation between extracellular activities of the high-affinity enzymes and grazing rates of bacterivorous protists. Therefore, the assay can still be recommended for the estimation of total protistan grazing on prokaryotic picoplankton. Second, the ability of many aquatic organisms to produce enzymes which cleave fluorogenic substrates, such as MUF-[GlcNAc] and/or MUF-β-D-N,N,N',N'-triacetylchitotriose (MUF-[GlcNAc]3), has been well-documented during the last decade. Thus, neither of the two substrates may be considered as exclusively specific for targeting either lysozymes or β-N-acetylhexosaminidases. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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In a recent paper, Sherr and Sherr [1] concluded that the use of MUF-N-acetyl-β-D-glucosaminide (MUF-[GlcNAc]) in marine microbial assemblages was compromised by the following facts: (1) all classes of marine microbes tested, bacteria, protists and phytoplankton, exhibit a low-affinity β-N-acetylglucosaminidase activity; (2) the pH maximum for the enzyme activity is in the range of 6–8 for most of the microbes and (3) several of the phytoplankton species have both cell-specific and volume-specific β-N-acetylglucosaminidase activities comparable to those of the bacterivorous protists at both pH 4.5 and 7.0. While Vrba et al. [2] added MUF-[GlcNAc] directly to intact microbial cells in water samples (a whole-cell assay), Sherr and Sherr [1] based their criticisms on another method (derived from an acid lysozyme assay [3]) measuring enzymatic activities in sonicated samples (a cell-free assay).

One can respond to the aforementioned objections as follows. The first point is irrelevant because the whole-cell assay with MUF-[GlcNAc] is based on the measurement of the high-affinity enzyme, which seems to be specifically connected with protistan phagotrophy on prokaryotes [2,4]. Therefore, in the whole-cell assay, protistan enzymes are distinguished kinetically and not according to their pH optima (point (2) of Sherr and Sherr [1]), which need not differ from other microbial enzymes. The reported nature of phytoplankton activities (point (3) of Sherr and Sherr [1]) is not surprising (cf. e.g. [5,6]), but these activities concern only the low-affinity enzymes. The activities of high-affinity enzymes, which are the target of whole-cell assays [2,4], were not detected by Sherr and Sherr [1].

For their studies, Sherr and Sherr [1] used a cell-free assay, i.e. they added MUF-[GlcNAc] to sonicated samples. This treatment means that they measured the activities of all enzymes, both extracellular and intracellular, produced by the microorganisms they tested. As a consequence, Sherr and Sherr could hardly controvert without a careful comparison the whole-cell assay suggested by Vrba et al. [2], which was principally based on measuring extracellular enzyme activities. Nevertheless, Sherr and Sherr claimed [1] that both protist cultures tested (Cafe-0168-6496/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
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teria sp. and Uronema sp.) exhibited only a low-affinity ($K_m > 45 \mu M$) enzyme. This disagrees with conclusions of Vrba et al. [2,4] that digestive enzymes of bacterivorous protists appear to have a high affinity ($K_m < 1 \mu M$) for MUF-[GlcNAc].

I argue that Sherr and Sherr [1] could not estimate kinetic parameters of any high-affinity enzymes (sensu [2,4]) using a concentration range as limited as 2.5–25 $\mu M$. It is worth noting in this context that all the concentrations Sherr and Sherr used were far below the half-saturation constants ($K_m$) for MUF-[GlcNAc] [1]. Yet a simple rule (derivable from the Michaelis–Menten equation but commonly neglected) says that concentrations as high as 10 times $K_m$ should be used for measuring activity at saturating concentrations. Therefore, routine measurements at 20 $\mu M$ hardly provide an adequate basis for comparing MUF-[GlcNAc] hydrolysis (presumably) in mixtures of cellular enzymes (neither on pH nor on per cell scales).

Sherr and Sherr might have measured the kinetics of some intracellular enzymes rather than extracellular ones because the samples were sonicated prior to substrate addition. The same is true for all pH profiles presented in Figs. 2 and 3 [1], perhaps with one exception (Fig. 2C) based on experiments with bacterial cells that might be more resistant to sonication. Therefore, criticisms of the whole-cell assay based on pH profiles are dubious. The similarity in pH profiles must not be generally accepted as evidence of similarity of enzymes since pH profiles of even pure enzymes may differ when acting on different substrates [7].

In addition, a careful inter-comparison of the whole-cell [2] and cell-free [3] assays with both MUF-[GlcNAc] and MUF-[GlcNAc3] (MUF-$\beta$-D-$N,N',N''$-triacetylchitotriose) showed the highest sensitivity of the whole-cell assay with MUF-[GlcNAc] for freshwater bacterivorous protist [4]. In contrast, Sherr and Sherr [1] do not compare either the two MUF-substrates or both assays and thus their conclusions do not reflect very precisely the state of art in this field.

Jenkins et al. [8] showed that interpretation of the acid lysozyme assay [3] is not simple and many metazoan enzymes may interfere, in particular in freshwater samples. Vrba et al. [2,4–6,9] have always considered other potential inhabitants of aquatic environments which release enzymes cleaving the MUF-[GlcNAc]. Vrba and Macháček [10] described the release of moulting enzymes by Daphnia (9 $\mu M$ as was indeed verified by Espie and Rolf [11]). In addition to high-affinity enzymes [3], we distinguished kinetically low-affinity enzymes in lake and reservoir samples [6]. The latter enzymes, however, correlated either with diatom biomass or with crustacean abundance [6]. Suggestions of similar relationships have been recently described for extracellular chitinolytic activity from marine environments [12–14].

Scigelova and Crout [7] reviewed data on microbial enzymes releasing GlcNAc monomer and compared amino acid sequence data, biochemical characteristics and catalytic properties of these enzymes. The enzymes cleaving MUF-[GlcNAc] should be correctly named $\beta$-N-acetylhexosaminidases (EC 3.2.1.32) [15] owing to the common catalytic capacity of the enzymes to cleave many natural and artificial substrates (for review see [7] and references therein). Actually we [2] quoted that fact while keeping the term $\beta$-N-acetylglucosaminidase, commonly used for the enzymes releasing the GlcNAc monomer from polymers. Numerous authors have suggested that some microbial $\beta$-N-acetylhexosaminidases may also cleave oligomers including MUF-[GlcNAc] (cf. Tables 1 and 2 in [7]).

Using MUF-[GlcNAc] and the whole-cell assay, Oosterveld et al. [14] have recently found in marine environments all the types of extracellular enzymes described by our group from freshwater. On the contrary, Sherr and Sherr based their criticisms in particular on the assumption that the MUF-[GlcNAc] and MUF-[GlcNAc3] are highly specific substrate analogues for targeting chitin and peptidoglycan degradation, referring to Gooday [16] and Mulisch [17], respectively. In my opinion, such a serious misinterpretation contradicts the state of art. Neither Gooday’s [16] nor Mulisch’s [17] reviews are consistent with interpretations of Sherr and Sherr [1]. Indeed contrary conclusions have been reached: “Lysozymes (endo-$\beta$-N-acetylmuramidases) degrade the glycan of the bacterial cell wall, but a number of them (e.g. egg white lysozyme) may also hydrolyze chitin, thus overlapping in their specificities with chitinases” [17,18]. I wish also to emphasize in this context that MUF-[GlcNAc3], named of course MUF-chitotriose, much better imitates natural substrates for chitinolytic enzymes than those for lysozymes. Bacterial peptidoglycan, unlike homopolymeric chitin, is a macromolecule in which GlcNAc alternates with $N$-acetylmuramic acid having side peptide chains.

Moreover, enzyme sequence data reveal a high degree of similarity between most microbial, invertebrate and vertebrate $\beta$-N-acetylhexosaminidases, which suggests a common evolutionary ancestor for these enzymes [7]. The same is likely true for many lysozymes, chitinases and even chitosanases of phage, bacterial, plant or vertebrate origin [18–21]. The hen egg white lysozyme, which was a prototype of the acid lysozyme assay for protistan bacterivory [3], is in fact an unusual enzyme, which probably has undergone substantial modification from the ancestral protein [19].

Summarizing correctly numerous observations in the literature [7,8,16–22], one can hardly argue for the particular substrate specificity of any enzymes under debate as Sherr and Sherr [1] do. The purified enzymes can be distinguished by using defined natural substrates or inhibitors [7]. Appropriate inhibition experiments may be accepted as a final evidence of the substrate specificity of enzymes but never selective citation.

An intriguing question, not yet answered, remains the actual allocation of the high-affinity digestive enzyme(s) in
or on the cells of bacterivorous protists. The cell-free assays [1,3,23] in fact cannot answer this question at all; pH profiles or pH adjustment of sonicated samples can hardly add any reasonable information (see above). The whole-cell assay [2,4] appears valid for measuring the activity of both free extracellular enzymes and ectoenzymes due to the addition of a soluble MUF-substrate to native samples of a living microbial community. Phagocytous protists, however, accumulate digestive enzymes primarily in lysosomes and then their content is released into food vacuoles. Thus, this hydrolytic potential, when measured by the cell-free assay, may not correspond with an instant actual ingestion [3] but rather with a potential of digestion. From this point of view, I consider the suggestion of Zubkov and Sleigh [23] to use the enzyme activity as a measure of protozoan biomass as reasonable and appropriate if there is negligible interference of other enzymes in the cell-free assay with MUF-[GlcNac] (cf. [8]).

From a strictly cellular point of view, the protistan digestive hydrodases should not be called intracellular (i.e. plasmatic); however, they certainly are not extracellular either. Thus, the following question must be addressed: how can we measure their activities extracellularly with the whole-cell assay [2,4]? In my opinion, we may hypothesize the following explanation. A dissolved MUF-substrate can be ingested along with ingested bacterial cells or any kind of food particles into food vacuoles. Then the MUF-substrate is efficiently cleaved in the food vacuoles and the MUF perhaps is partly released into the sample environment again if incubation times are long enough. All MUF, irrespective of extracellular or intracellular production, is finally liberated after an alkalization with sodium hydroxide (40 mM final concentration) prior to reading fluorescence [2,9].

The simplest explanation of the apparent controversy between the acid lysozyme assay [3] and our [2,4] ‘seriously-compromized approach’ [1] is most probably the following one: neither MUF-[GlcNAC] nor MUF-[GlcNAC] is actually cleaved by lysozymes. Monitoring of the lysozyme action with the MUF-[GlcNAC] probably requires a coupled reaction involving β-N-acetylhexosamidase [22]. Both my [4] and Zubkov’s (personal communication) experience with adequate comparisons of both MUF-[GlcNAC] and MUF-[GlcNAC] favors the former for routine detection of protistan biomass [23] or ingestion rate [2,4] under well-controlled conditions. We still can recommend the whole-cell assay with MUF-[GlcNAC] for the estimation of total protistan grazing on prokaryotic picoplankton [4] because as far as we know [2,4,6,14] the extracellular high-affinity enzymes have never been observed in the total absence of bacterivorous protists. Although measuring kinetics does not sound very simple, the method can be significantly simplified by using an advanced microplate fluorescence reader [24] and software, which compares directly two fits of different complexity (e.g. Prism 3.0, www.graphpad.com). Moreover, a concentration range may be reduced by half for screening protistan grazing only; alternatively a single low concentration (≤1 μM) may be used for routine measurements [4].

References


