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Application of fluorescence markers for diagnosis of bacterial assemblage: hydrolytic enzyme activity in aquatic ecosystem

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Abstract
Most of existing methods to monitor enzymatic activity in microbial culture cannot be applied directly to study hydrolytic activities in aquatic environments. The low natural substrate concentrations as well as the sub optimal *in situ* environmental conditions (unsuitable temperature, pH and the presence of interfering substrates) make the existing laboratory methods often inadequate to measure enzymatic activity. Recently, molecular fluorosensors are commonly used for sensitive assays involved in examining hydrolytic activity in aquatic environment *in situ*. These allow for simpler and more rapid measurement of hydrolytic enzyme activity. Furthermore, the stability of the artificial substrates produces very low fluorescence background and can be used without any loss in sensitivity at high concentration (mM) that are sometimes required to measure enzyme saturation.

Keywords: fluorosensor, bacteria, aquatic, hydrolase, organic matter.

INTRODUCTION

Microheterotrophs, particularly the heterotrophic bacteria are the main biological components of aquatic ecosystem that control the movement of the majority of organic compounds that are mostly formed as Dissolved Organic Matter (DOM). The major step of the microbial loop concept is the conversion of uDOM (utilisable DOM) into bacterial biomass and production which is strongly dependent on enzymatic capacities of the microbial community (Azam *et al.*, 1983). Therefore, it is suggested that the microbial loop model is amended to include the initial and obligatory hydrolytic step of the microbial nutrient regeneration (Chróst 1990; Hoppe 1991). The coupling between hydrolytic activities and uptake systems greatly increases the efficiency of DOM utilization by free living bacteria in aquatic environments. Most of bacterial production appears to be utilized by protozoa and very small metazoans (Sherr and Sherr, 1987). The high respiratory potential of heterotrophic bacteria enables oxidation of organic matter thereby releasing CO_2, PO_4^{3-}, NH_4^+ and other small molecules that are important nutrients for phytoplanktons. Therefore, the microbial loop is an important contributor to the steady state of algal nutrients and promotes a steady state of algal biomass and production in the absence of other sources of nutrients (Chróst, 1990). Considering the importance of hydrolytic enzymes within the microbial loop, they operate at the molecular level in aquatic environments and affect the function of the whole aquatic ecosystem.

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It has been widely assumed that most extracellular hydrolytic activities in aquatic systems except phosphatase originate predominantly from bacteria (*e.g.* Hoppe *et al.*, 1988; Chróst, 1992; Sala and Güde, 1996). Bacterial enzymatic activities are important for mobilization, transformation, and turnover of organic and inorganic compounds in aquatic environments (e.g. Sala and Gude, 2004; Arnosti *et al.*, 2005; Misic and Fabiano, 2006). These data provide information for understanding basic processes of decomposition in both freshwater and marine ecosystems.

Molecular fluorosensors have been used as artificial substrates to study hydrolytic enzymes *in situ* (Hoppe 1983). This article evaluates the application of fluorescence markers as substrates for different hydrolytic enzyme activities in natural samples, and to localise their activity, i.e. intracellular, periplasmatic space or free dissolved and to evaluate the hydrolytic activity as a tool to differentiate living and active from dead and inactive bacteria.

Choice of substrate type and methods to measure hydrolytic enzyme activity *in situ*

There are three methods to measure enzymatic activity, i.e. photometrically, fluorometrically and by use of radio-labelled substrates. Short incubation times for the analysis, high sensitivity and stability of substrates are the prime factors in choice of the method. However, each of the above methods has some shortcomings. For example, longer incubation time and lower sensitivities are the major problems in the photometric analysis. Using radio-labelled substrates can actually overcome these problems. However, there are not enough commercially available radio-labelled polymeric substrates. Fluorescent substrates on the other hand are artificial substrates, therefore, several problems concerning substrate specificity, fluorescence background (*i.e.* chemical hydrolysis of fluorescent marker that could be detected as hydrolytic activities) and about them being representatives of natural substrates must be considered. Chromogenic substrates, which can be easily measured photometrically, need long incubation times of up to 72 to 96 h (Stevens and Parr, 1977), because high concentration of substrates is needed. Consequently, longer incubation of time is required to measure hydrolytic activities. This causes difficulties in maintaining the *in situ* conditions during incubation. Microbial proliferation, changes in community structure and ectoenzyme(s) synthesis may occur (Chróst *et al.*, 1986). Thus, growth is stopped by plasmolytic or antiseptic agents, which are not harmful for proteins, i. e. the hydrolases themselves (Halemejko and Chróst, 1986). Additionally, this approach provides the possibility to concentrate the enzymes. However, total activity may be significantly overestimated by change in permeability of cell surface (Frankenberger and Johanson, 1986). This leads to altered substrate transport rates, conformation changes in membrane bound enzymes and/or liberation of intracellular hydrolases, which in intact cells should not be included in the extra- and ectoenzyme activity (Hoppe *et al.*, 1986; Martinez and Azam, 1993b). Moreover, intracellulary accumulated competitive substrates or end product can be released and affect the cleavage of the chromophoric substrate.

Radio-labelled polymeric substrates are a promising option to study hydrolytic enzyme activity in aquatic environment (Hollibaugh and Azam, 1983; Cunningham and Wetzel, 1989). These substrates are actually natural substrates that are only slightly modified during radiolabelling with ^{14}C, ^{3}H, ^{32}P or ^{125}I (Molano *et al.*, 1977). Thus, the degradation of radiolabelled substrates can be measured within short incubation time (min). However, due to high cost and the health risks of radio-labelled substrates, this technique is not used widely.

Fluorometric assays are reported to be suitable for the measurement of hydrolytic activities in plankton communities (Somville and Billen, 1983). Molecular fluorosensors provide very sensitive tools for detecting and quantifying hydrolytic enzymes in aquatic environments (Hoppe 1983; Chróst and Krambek 1986). Their short incubation time (minutes to hours) is advantageous (Somville, 1984). The stability of the substrate produces very low background fluorescence. Therefore, it can be applied in high concentrations (mM) without any loss in sensitivity, which is sometimes needed to measure enzyme saturation (Münster *et al.*, 1989). This is because the enzyme should be substrate saturated to avoid the possible competition with co-occurring natural substrates and competitive inhibition of substrates by inhibitor in the samples, and thus, enable the calculation of maximum reaction velocity. Low concentrations of fluorescent artificial substrates are comparable to natural substrates concentrations, which are often in the pico to nanomolar range (Chróst 1991).

Methodological considerations of using fluorescent substrates

Fluorogenic substrates have been verified to be representative for natural substrates (with a similar enzyme affinity) through competitive inhibition experiments by naturally occurring substrate analogs (Hoppe, 1983; Somville, 1984). Fluorescent substrates are enzyme specific because enzyme activity is decreased by their own competitor. For example, cellobiose is an appropriate competitor only to β-glucosidase measured by MUF-β-glucosid (Somville, 1984) indicating that MUF-β-glucosid is an appropriate substrate. Also, Methylumbelliferyl substrates are cleaved specifically by the respective enzyme (Koester *et al.*, 1997). Glycosidases are very specific, because they only cleave structurally homologous substrates, e.g. β-glucosidase does not hydrolyse maltose, sucrose or MUF-α-glucosid.

Hydrolytic enzymes in the aquatic environment comprise a complex enzyme system that is not specific for one substrate. For example, both polymeric and oligomeric 1,4-β-linked glucose can be substrates for β-glucosidases. Thus, a variety of carbohydrate molecules (e.g. cellobiose, cellotriose, cellulose) can be hydrolyzed by β-glucosidases. Therefore, the artificial substrates used in the analysis should be representative for all naturally occurring substrates in water samples. Also, L-leucine-4-methyl-7-coumarinylamide hydrochloride (leucine-AMC) is actually a non-specific substrate for aminopeptidase. The occurrence of leucine in the structure suggests that this substrate should be cleaved only by a leucine aminopeptidase. However, studies on leucine-AMC hydrolysis using different natural proteins (albumin, casein, globulin etc), dipeptides (leucyl-alanine, leucyl-leucine) and

polypeptides as competitive inhibitors indicate that leucine-AMC is also hydrolyzed by many other proteolytic enzymes (Chróst, 1992). Thus, leucine-AMC can be used to measure leucine-aminopeptidase as well as an overall aminopeptidase activity in water samples. Therefore, the term "aminopeptidase" is frequently used in the study of hydrolases in aquatic environments to represent all aminopeptidase activities in samples although leucine aminopeptidase is used as a substrate.

Another important factor is the overestimation of esterase activity caused by the instability of esterase substrates in water. Several esterase substrates are reported to be relatively unstable, e.g. fluorescein diacetate, 5(6)carboxy-fluoresceindiacetate, 2'7' dichloro- fluoresceindiacetate. The instability increases with pH, alkalinity and salinity (Riegman *et al.*, 2002). 4-MUF-acetate is reported to be unstable with increasing pH from 4.0 to 8.0 (Niemi and Vepsalainen, 2005). Therefore, the measure of high esterase activity in water samples and in isolates, in addition to hydrolytic enzymes investigated, may also be acquired from the instability of fluorescent substrate. To overcome this potential problem, a correction for non-enzymatic hydrolysis of MUF-acetate using control as well as confirmation of the linearity of hydrolytic reaction should be performed in the esterase study.

Kinetics of hydrolytic enzymes

In aquatic and marine environmental studies, kinetics of hydrolytic enzymes are not frequently estimated, as these studies are time consuming (Chróst and Overbeck, 1987; Sebastian and Niel, 2004). Using one model substrate concentration at saturation to measure V_{max} as a proxy for enzyme amount is more common (Murrel *et al.*, 2003; Taylor *et al.*, 2003; Sala *et al.*, 2005). However, in oligotrophic systems only a narrow range of substrate concentrations yielded alkaline phosphatase activity estimates comparable to alkaline phosphatase V_{max} determined by kinetic assays (Sebastian *et al.*, 2004). Therefore, elucidation of the *in situ* rates of hydrolysis in aquatic systems, should include kinetic assays. Kinetic analysis by means of Michaelis Menten equation enables the calculation of maximum reaction velocity (V_{max}) and Michaelis Constant (K_M), which is an indication of enzymatic affinity of substrates. This approach allows the determination of substrate concentration required to saturate the enzyme. This is important to measure the maximum hydrolytic enzyme velocity. Thus, hydrolytic activity is analogous to the realistic rates of hydrolysis *in situ*.

The wide range (multiphasic) of enzyme kinetics have indicated a coexistence of different esterases, aminopeptidases and β-glucosidases (Talbot and Bianchi 1997; Tholosan *et al*., 1999; Unanue *et al*., 1999) Since a plankton sample is a pool of organisms (not only bacteria) there might be some molecules and/or particles interfering with the hydrolytic reactivity such as humic substances, colloidal organic matter and detritus. In spite of the limitations discussed above, fluorosensors are well suited to characterize hydrolytic enzymes in the aquatic environment and to measure the activity of selected important hydrolytic enzymes involved in the degradation of polymers typical for DOM in aquatic systems.

Bacterial cell specific hydrolases

Fluorosensors are also widely applied to measure cell specific hydrolytic enzyme activities in different organisms. Different species (*e.g. Bacillus globigii* ATCC 9372, *B. pumilis* ATCC 7061, *E. coli* ATCC 4352, *Pseudomonas stutzeri*) have contrasting sets of extracellular and ectohydrolases that vary in their inducibility and have different substrate sensitivities (Snyder *et al.*, 1986). Cell specific activities of protease, β-glucosidase, α-glucosidase, alkaline phosphatase, lipase and chitinase from 44 marine isolates is reported to vary over a broad range (Martinez *et al*., 1996). Variability in constitutive leucine aminopeptidase in Gram negative bacterial isolate is also found by Martinez and Azam (1993a). This indicates that each organism displays different enzyme activities and has a high degree of "specialization" for different types of polymeric substrates. Thus, hydrolytic activity measurements in bacterial communities might not represent the activity of a particular or each organism in the community.

Discussions are ongoing on whether the total hydrolytic activity measured in field samples represents only extracellular and ectoenzyme or includes intracellular enzyme as well. Nonetheless, three fluorescent substrates i.e. 4-methylumbelliferyl-acetate (MUF-acetate), 4-methylumbelliferyl-β-D-glucoside (MUF-glucoside) and L-leucine-4-methyl-7-coumarinylamide hydrochloride (leucine-AMC) are able to measure and/or recognize extracellular, intracellular and ectoenzymes. This is extremely depended on the cell loading by the fluorogenic substrates. This approach estimates the sum of extracellular, cell surface and periplasmic (but not cytoplasmic) enzyme activity (Hoppe, 1983).

Most of leucine aminopeptidase measured in *Synechococcus* (cyanobacteria) by means of leucine-AMC was periplasmic or ectoenzymes. The complete inhibition of peptidase by peptone in *Synechococcus* WH 7803 suggested that intracellular enzymes did not contribute to the cleavage of leucine-AMC (Martinez & Azam, 1993a). Moreover, in Gram negative marine bacterial isolates, leucine-AMC and MUF-phosphate measured the periplasmic enzyme activity and not the cytoplasmic enzyme activity. Also, after destruction of

bacterial cell membrane, activities of hydrolase increased by up to 26%. These activities were accounted to be cytoplasmic because leucine-AMC was assumed to be impermeable (Martinez & Azam 1993b). Thus, hydrolytic enzyme measurements using the fluorogenic substrates represent the sum of cell surface and periplasmic (but not cytoplasmic) pools of the enzymes. However, epifluorescence microscopy investigation shows that fluorescent model substrates (MUF and AMC) are cleaved outside bacterial cytoplasm (Hoppe *et al.*, 1986). These results report no stained bacterial cells in the experiments. Thus, it is suggested that fluorescence substrates should not be used as the only tool to differentiate living, active from dead or inactive bacteria.

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