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# The Dynamics of Protein Decomposition in Lakes of Different Trophic Status - Reflections on the Assessment of the Real Proteolytic Activity *In Situ*

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**Abstract** The aim of this paper is to discuss the methodology of our investigation of the dynamics of protein degradation and the total *in situ* proteolytic activity in meso/eutrophic, eutrophic, and hypereutrophic freshwater environments. Analysis of the kinetics and rates of enzymatic release of amino acids in water samples preserved with sodium azide allows determination of the concentrations of labile proteins ($C_{LAB}$), and their half-life time ($T_{1/2}$). Moreover, it gives more realistic information on resultant activity *in situ* ($V_{T1/2}$) of ecto- and extracellular proteases that are responsible for the biological degradation of these compounds. Although the results provided by the proposed method are generally well correlated with those obtained by classical procedures, they better characterize the dynamics of protein degradation processes, especially in eutrophic or hypereutrophic lakes. In these environments, processes of protein decomposition occur mainly on the particles and depend primarily on a metabolic activity of seston-attached bacteria. The method was tested in three lakes. The different degree of eutrophication of these lakes was clearly demonstrated by the measured real proteolytic pattern and confirmed by conventional trophic state determinants.

**Keywords:** Proteolysis, extracellular enzymes, labile proteins, lake water

Dissolved proteins (DCAA, dissolved combined amino acids) and their degradation products (DFAA, dissolved free amino acids) constitute the important pool of organic nitrogen occurring in aquatic environments [2, 31, 41]. Whereas DFAA concentrations in natural waters are relatively low and do not exceed 1.0 μM, concentrations of DCAA (expressed as amount of amino acids released from DCAA after complete chemical hydrolysis) are generally much higher and vary between 1.0 and 6.0 μM [18]. Numerous studies have shown that both DFAA and DCAA fractions effectively stimulate secondary production [20, 26, 29] and found that the uptake of DFAA and DCAA degradation products by heterotrophic bacteria covers up to 25% of carbon and up to 50% of nitrogen demand in marine environments.

Contrary to DFAA, the majority of proteins and peptides from the DCAA fraction cannot be taken up through the cell membrane. Therefore, they have to be hydrolyzed outside the bacterial cell by periplasmatic, membrane-bounded, and perhaps extracellular (free) proteases to amino acids or oligopeptides (<7 monomers) that can be assimilated directly [9]. However, only 20–40% of the DCAA fraction in natural waters is prone to enzymatic hydrolysis. The rest is less susceptible to enzymatic degradation and can be protected from rapid enzymatic hydrolysis by adsorption to organic particles and complexation (especially humic components), being enclosed inside mesopores (pores 2–50 nm in diameter), incorporation into cell membrane structures, or glucosylation of their amino groups [27, 37]. In order to establish the role of extracellular proteins in aquatic environments and characterize the dynamics of their transformations in natural waters, the effect of these processes on resultant proteolytic activity *in situ* has to be considered.

The majority of methods for studying proteolytic activity and DCAA assimilation need $^3$H-labeled protein addition to the analyzed water sample [10, 14, 18]. Other techniques permit determination of maximal potential peptidase activity ($V_{max}$) by measuring the amounts of fluorescent or colored products released during incubation of the sample enriched with an artificial analog of natural substrates (*e.g.*, L-leucine-4-methyl-7-cumarinylamide or L-leucine *p*-nitroanilide) [15, 43]. Both of these methods, however, give only relative values and do not permit realistic approximation of real rates of protein hydrolysis *in situ*. Therefore, the hydrolysis rate of a given radiolabeled protein (or protein mixture) depends on their molecular structure and molecular mass.

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**Table 1.** Basic limnological parameters of the studied lakes, average (*n*=6) concentrations (±standard deviations) of total phosphorus, chlorophyll *a*, dissolved organic carbon (DOC), and number of bacteria (BN) in the surface water of the studied lakes during the study period.

| Lake | Surface area (ha) | Depth mean /Max. (m) | Secchi disc (m) | Total phosphorus (μmol P/l) | Chlorophyll a (μg/l) | BN ($\times 10^6$ cells/ml) | DOC (mg C/l) | Trophic conditions |
|---|---|---|---|---|---|---|---|---|
| Kuc | 99 | 8.0/28.8 | 4.5±0.2 | 0.3±0.1 | 3.3±1.4 | 5.83±0.88 | 13.06±0.21 | Meso/eutrophic |
| Mikołajskie | 498 | 11.2/25.9 | 1.4±0.6 | 1.9±0.4 | 33.1±3.6 | 8.63±1.02 | 12.25±0.19 | Eutrophic |
| Tałtowisko | 327 | 14.0/39.5 | 0.5±0.3 | 2.8±0.4 | 87.3±7.2 | 10.49±1.21 | 13.06±0.19 | Hypereutrophic |

In practice, the results obtained by these techniques never reflect the actual hydrolysis rate of the natural DCAA pool, which is commonly characterized by great complexity, resulting in varying susceptibility of each of its components to biodegradation [2]. Fluorometric or colorimetric methods using artificial analogue of natural substrates (*e.g.*, L-leucine-4-methyl-7-cumarinylamide) are also limited with respect to their ecological significance for two important reasons: (1) they demand the assumption that the resultant affinity of natural proteases to a given "artificial" substrate is identical or very similar to the resultant affinity of these enzymes to all natural substrates present in the analyzed water sample and (2) they require the use of such artificial substrate concentrations that fully saturate the natural enzyme set or concentrations approximately equal to the overall concentrations of DCAA occurring in the water sample. In practice, compliance with the above requirements is rather unrealistic, because the concentrations and composition of natural substrates are always unknown. Therefore, the results obtained using these methods only permit to determine relative rates (or rather, the measure that characterizes quantity and quality) of proteolytic enzymes presented in an analyzed water sample and do not describe the real rate of enzymatic hydrolysis of the DCAA and PCAA (particulate combined amino acids) pool *in situ*.

In this paper, we propose and discuss a new, direct approach to description of protein decomposition dynamics in lake water and to determination of the real rate of protein hydrolysis *in situ*. The proposed methodology was successfully applied to three lakes of different trophic status that varied from meso/eutrophy to hypereutrophy. This technique provides an alternative method to standard ones, based on "artificial" substrates. Contrary to these, it permits to quantify the combined activity of both ecto- and extracellular proteases *in situ* in a better way, and to determine natural labile proteins ($C_{LAB}$) concentrations and their half-life time ($T_{1/2}$) in lake water.

## Material and Methods

### Study Area and Sampling

The studies were carried out during summer 2001 (July, August) and 2002 (July, August, September) in three lakes of the Mazurian Lake District (North-Eastern Poland). Lakes chosen for investigations represented different trophic status, which varied from meso/eutrophic (L. Kuc) through moderately eutrophic (L. Mikołajskie) to heavily eutrophic (L. Tałtowisko). Basic limnological parameters that characterize the studied lakes are presented in Table 1. Subsamples (1 l) of pelagic water were taken once a month from the surface (0.2 m depth) of five sampling sites situated along the longest transect of the lake. The subsamples were mixed vol/vol in polyethylene containers, in order to obtain one integrated sample that was representative for each studied lake, and transported to the laboratory immediately (within 1 h).

### Protein Hydrolysis *In Situ*: Assay Procedure and Data Analysis

The proposed technique is a further development of our earlier idea [8, 32] that the best way to assess the real ectoenzyme activity *in situ via* is direct chemical determination of product(s) released during hydrolysis of all constituents of the natural substrate pool by a whole set of nonspecific iso/allozymes presented in a natural, unchanged water sample. This consists of analysis of the dynamics of enzymatic release of amino acids (AA) and short-chain oligopeptides (SCP) from natural proteins in lake water samples preserved chemically against assimilation of enzymatically released products (AA+SCP) by aquatic microorganisms.

Analyzed water samples (2 l) were preserved with sodium azide (to 0.3% $NaN_2$ of final concentration) and incubated 4–5 days at 20±2°C (temperature of lake water during investigations) in darkness. Every 1–3 h (during incubation), three 5-ml portions of the preserved sample were taken and centrifuged (20 min, 6,000 RPM, 4,000 ×*g*, EBA 12, Hettich) in order to eliminate large organic particles, which could affect the fluorescence reading. Increase in AA+SCP concentrations was measured with the *o*-phthaldialdehyde method [30], modified as follows: triplicates of the water sample (3.9 ml) were supplemented with 0.05 ml of borate buffer (0.4 M, pH=9.0) and 32 μl of 2-mercaptoethanol, mixed and, when added of 0.05 ml of *o*-phthaldialdehyde (OPA) solution (1.25 mg/ml in ethanol), mixed again immediately. Final concentrations of reagents in each replicate were 1.0 μM, 114.4 mM, and 116.5 μM, respectively. The fluorescence of samples was measured in a Shimadzu RF 1501 Spectrofluorimeter (Ex=330 nm, Em=455 nm) within

15 min. The concentration of amino acids and short-chain oligopeptides detected by the OPA method (AA+SCP) was calculated from a linear-regression calibration curve prepared with L-leucine as a standard. The detection limit of the method was 0.2 μM [23]. Subsamples taken from the preserved sample at time t=0 served as controls.

To test whether the decreasing rate of AA+SCP release from the total combined amino acids (DCAA+PCAA) pool during incubation of the lake water sample with $NaN_3$ was an effect of exhaustion of labile (enzymatically hydrolyzable) combined amino acid (LCAA) or a decline in proteolytic activity, we analyzed the dynamics of the enzymatic release of AA+SCP from particulate substrate HPA (hide powder azure) added (0.25 mg HPA/ml) to preserved water sample taken from L. Mikołajskie and incubated at 20°C in darkness. HPA is a mixture of various scleroproteins coupled with Remazol Brilliant Blue R dye through covalent bonds. Particulate substrate was used in order to avoid possible inhibition of proteases by excess of dissolved proteins [11, 14, 36]. The rate of AA+SCP release from HPA was determined by the same method, as the real protein hydrolysis rate. The possibility of abiotic HPA degradation was excluded by analysis of AA+SCP release in an autoclaved, HPA-enriched, and $NaN_2$-preserved control.

### Standard Methods

The number of bacteria (BN) was determined by direct counting under an epifluorescence microscope (Nikon Eclipse 450) in slides stained with DAPI, according to Porter and Feig [28]. For L-leucine-aminopeptidase activity ($V_{max}$ AMP) determination, 0.1 ml of appropriate substrate - L-leucine-4-methyl-cumarinylamide hydrochloride (Leu-MCA)] solutions in deionized water were added to 3.9 ml of water samples yielding final Leu-MCA concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 μM. Fluorescence of the product [7-amino-4-methylcoumarin (AMC)] was determined spectrofluorometrically (380 nm Ex and 460 nm Em) in a Shimadzu RF 1501 spectrofluorometer at zero time and after 0.5--1.0 h of sample incubation. The tested enzyme-substrate system followed first-order Michaelis-Menten kinetics. Plot of the reaction velocity (v) against substrate concentration [S] always displayed a rectangular hyperbola relationship, according to the equation

$$v=V_{max}\times[S]/(K_m+[S])$$

Nonlinear regression analysis was applied to calculate the kinetic parameters of enzymatic reactions by means of PC software Origin 6.1.

The total protein concentrations (DCAA+PCAA) were determined as the concentration of AA+SCP released from labile proteins after chemical hydrolysis (6 M NaOH for 24 h at 30°C). Concentrations of AA+SCP were measured by the *o*-phthaldialdehyde method according to Roth [30], modified as was described above.

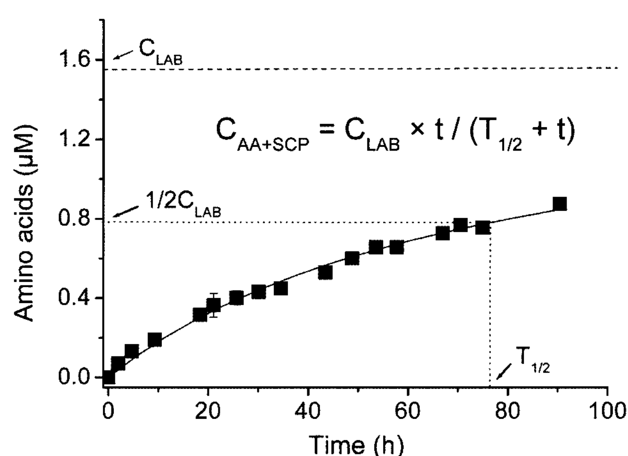


**Fig. 1.** The time course of AA+SCP (amino acids+short-chain oligopeptides) release from labile proteins (LCAA).
$C_{LAB}$, LCAA concentration; $T_{1/2}$, half-life time of LCAA.

## RESULTS AND DISCUSSION

The typical pattern of increase in AA+SCP concentrations in $NaN_2$-preserved water samples taken from the surface layer of all types of the studied lakes are presented in Fig. 1. The relationship shown in the figure can be described mathematically by the following nonlinear regression equation:

$$C_{AA+SCP}=C_{LAB}\times t/(T_{1/2}+t)$$

where $C_{AA+SCP}$ is AA+SCP concentration, t is incubation time, $C_{LAB}$ is labile protein concentration, and $T_{1/2}$ is half-life time of labile protein ($C_{LAB}$) in the sample. In addition, the standard differentiation of this function gave the f'(t) value for $t=T_{1/2}$, which described the rate of enzymatic release of AA+SCP from labile proteins at time equal to their half-life period in the analyzed lake water sample ($V_{T1/2}$). We assumed that this value is a reasonable measure of a protein hydrolysis rate that permits the direct comparison of proteolytic activity in lakes of different trophic status. Some illustrations of the time course of enzymatic AA+SCP release in samples taken in July 2002 from the three lakes of differing trophic status are shown in Figs. 2A, 2B, and 2C.

The most critical and disputable points of the proposed approach are limitations that may affect amino acid determination by the OPA method, the relatively long incubation time (3–4 days), and the necessity for preservation of the sample against microbial growth. Many chemicals commonly used for inhibiting the metabolic activity of bacteria may disturb the fluorescence reading and influence the proteolytic ecto- and egzoenzymes activities.

The following criteria motivated us to choose sodium azide as a fixing agent: (1) even in small (0.3%) concentrations, it completely inhibits the metabolic activity of aerobic

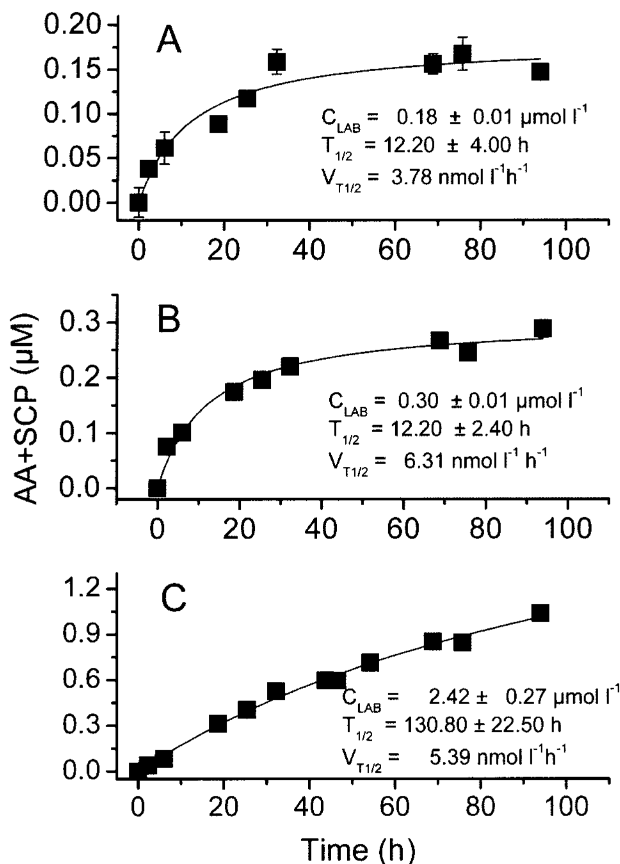


**Fig. 2.** Enzymatic release of AA+SCP in lakes of different trophic status: A, meso/eutrophic Lake Kuc; B, eutrophic Lake Mikołajskie; C, hypereutrophic Lake Tałtowisko.

$C_{LAB}$, LCAA concentration; $T_{1/2}$, half life time of labile proteins; $V_{T1/2}$, the rate of enzymatic release of AA+SCP from labile proteins at $T_{1/2}$ time.

microorganisms [1, 22, 39] including processes of AA+SCP assimilation; (2) it does not damage cell walls or cell membranes, and therefore, prevents the leakage of cellular content into the external environment [25]; (3) it does not cause nonenzymatic degradation of natural proteins, (4) it does not (or only slightly) affect the activity of proteolytic enzymes [12]; and (5) it does not disturb quantification of AA+SCP by the OPA method.

The other disputable point of the proposed method is the possibility that ectoproteases lose at least a part of their initial activity during the relatively long incubation of lake water fixed with sodium azide. However, in azide-preserved natural lake water samples enriched with HPA, we observed a linear increase in AA+SCP concentrations. The constant HPA hydrolysis rate ($V_{HPA}$) noted during the experiment strongly supports the assumption that the decrease of the rate of AA+SCP release was more the effect of decreasing of the LCAA pool than the reduction in proteolytic activity (Fig. 3). In a sample taken in August 2002 from Lake Ta³towisko, a linear increase in AA+SCP concentration (released from LCAA) was observed up to four days from the moment of sample preservation (rate=9.6±0.4 nmol $l^{-1}$ $h^{-1}$, r=0.99, $p$<0.0001, n=12). This additionally supports the assumption that proteolytic ectoenzymes are stable enough for a successful determination of *in situ* protease activity as well as $C_{LAB}$ and $T_{1/2}$ parameters by the proposed method. Strong theoretical support for our method is also provided by the suggestions of Vives-Rego *et al.* [40], Bright and Fletcher [3], and others, that ectoenzymes and extracellular enzymes remain active for several days after the death of microorganisms that produce them, and that enzymes attached to dead bacterial cells may constitute an important part of the total proteolytic enzyme pool in water environments.

Inhibition of proteolytic enzymes by amino acids released from LCAA during incubation may be, at least theoretically, another factor affecting the results provided by the proposed method. Hałamejko and Chróst [13] showed that L-leucine-aminopeptidase is subject to competitive inhibition by L-leucine, but this process becomes significant only when leucine concentrations exceed 10.0 µM. However, in samples supplemented with HPA and preserved by sodium azide, enzyme inhibition by amino acids was not observed, even when the AA+SCP concentration was as high as 7.80 µM (Fig. 3). Consequently, since even in highly eutrophicated waters amino acid concentrations do not exceed 8.00 µM [10], the assumption that they may inhibit the activity of natural proteases during the experiment should be rejected.

The sensitivity of the AA+SCP assay limits the suitability of the proposed method to lakes with relatively high LCAA concentrations. According to Lindroth and Mopper [23], the limit of AA+SCP detection by the OPA method is

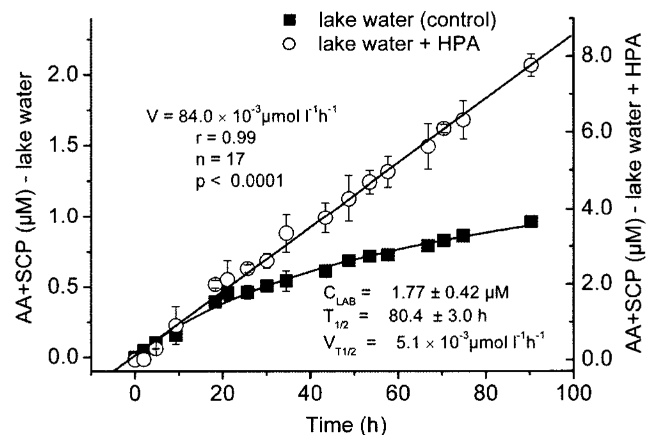


**Fig. 3.** Dynamics of the enzymatic release of AA+SCP from labile proteins occurring in the surface water of L. Mikołajskie (left axis, squares) and from artificial protein HPA (right axis, open circles).

$T_{1/2}$, half life time of labile proteins; $V_{T1/2}$, the rate of enzymatic release of amino acids from labile proteins at $T_{1/2}$ time; $V_{HPA}$, the rate of enzymatic release of amino acids from HPA. Further explanations in the text.

about 0.20 μM. Since in oligotrophic environments LCAA concentrations seldom exceed this value, application of our method to these environments is not possible. Inversely, the proposed method can be successfully applied to meso/eutrophic, eutrophic, and hypereutrophic waters where the LCAA pool is much larger and concentrations of enzymatically released AA+SCP considerably exceed 0.20 μM. An additional source of potentially significant measurement errors was adsorption of AA+SCP on membrane filters and on the surface of laboratory vials [14]. To decrease the adsorption phenomenon, we centrifuged our samples instead of filtering them prior to the AA+SCP. Centrifuging not only reduced the enzymatically liberated adsorption of DFAA but also removed large seston particles that affected fluorescence reading.

Ambient protease activity depends, on among other factors, both enzyme and substrate quantities. Although $T_{1/2}$ and $C_{LAB}$ characterize well the rate of protein hydrolysis *in situ* in a particular lake, they usually do not permit the comparison of real rates of LCAA hydrolysis in lakes of different trophic status, which often exhibits significant variations of labile protein concentrations. As a possible solution of this problem, we propose to differentiate the function that describes increase in AA+SCP concentrations in $NaN_2$-preserved water samples (Fig. 1), resulting in

$$f'(t)=T_{1/2}\times C_{LAB}/(T_{1/2}+t)^2$$

The differential, f'(t), describes changes in the rate of AA+SCP during sample incubation and allows to compare precisely the efficiency of protein decomposition processes in various lakes (Fig. 4). We propose to use the value of f'(t) for $t=T_{1/2}$, ($V_{T1/2}$) as a useful parameter, characterizing both the

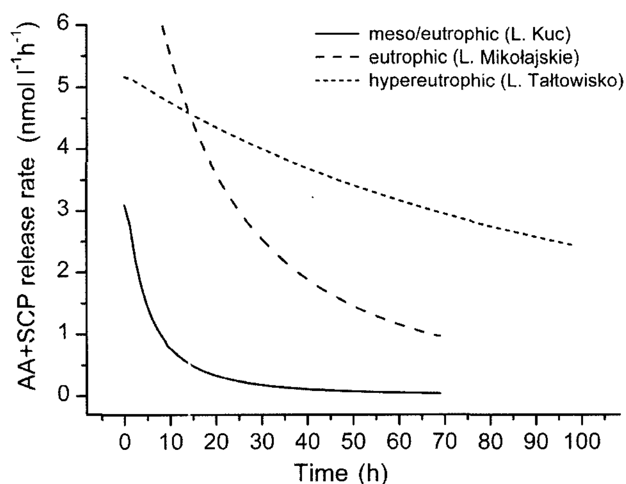


**Fig. 4.** Changes of the rate of AA+SCP release (differential of function $f'(t)=T_{1/2}\times C_{LAB}/(T_{1/2}+t)^2$) during incubation of samples taken from three lakes of different trophic status in September in meso/eutrophic L. Kuc, eutrophic L. Mikołajskie, and hypereutrophic L. Tałtowisko.

labile protein pool and the real protein hydrolysis rate in an examined lake water sample. However, as a further development of the method, we recommend to calculate also the f'(t) value for $t\rightarrow0$ ($V_{T0}$). It should be strongly pointed out that the $V_{T0}$ value may turn out to be the most realistic approximation of the real proteolytic activity *in situ.*

The usefulness of $V_{T1/2}$ for the description of protein hydrolysis *in situ* was verified by environmental studies. In 15 out of 18 cases, $V_{T1/2}$ significantly correlated ($r=0.8$, $p<0.0033$, $n=15$) with the value of maximal potential L-leucine-aminopeptidase activity ($V_{max}$ AMP), expressed as the maximal potential rate of Leu-MCA hydrolysis, determined according to the method proposed by Hoppe [15]. It should be pointed out that for the above calculations, data for Lake Tałtowisko in July, August, and September 2002 were not included. In these cases, extremely high $V_{max}$ AMP (795.7±60.2, 912.0±151.5, and 601.0±79.8 nmol $l^{-1}$ $h^{-1}$, respectively) did not correspond to expected high $V_{T1/2}$ (5.4, 5.7, and 6.1 nmol $l^{-1}$ $h^{-1}$, respectively). This could be an effect of the presence of relatively high concentrations of natural labile proteins more resistant to hydrolysis than the artificial substrate (Leu-MCA). The above hypothesis can also be confirmed by the fact that in all analyzed cases, the correlation between the number of bacteria (BN) that were major producers of proteolytic enzymes [6, 17, 24] and $V_{T1/2}$ was significantly higher ($r=0.63$, $p<0.005$, $n=18$) than the correlation between BN and $V_{max}$ AMP ($r=0.55$, $p<0.018$, $n=18$). This suggests that the proposed method offers a better description of protease activity than methods based on simple artificial substrates, particularly in the presence of natural labile proteins more resistant to hydrolysis than Leu-MCA. An additional interesting conclusion supported by the analysis of the presented results is the fact that L-leucine-aminopeptidase is probably one of the most important ectoenzymes that participate in protein decomposition processes in aquatic environments, as it was postulated earlier by Hoppe *et al.* [16] but not confirmed definitely until now.

The Leu-MCA hydrolysis rate expressed as L-leucine-aminopeptidase activity ($V_{max}$ AMP) is generally much higher than the approximated rate of protein degradation ($V_{T1/2}$). In comparison to artificial substrate (Leu-MCA), proteins occurring commonly in lake water have a much more complex chemical structure. Therefore, their complete hydrolysis requires, beside L-leucine-aminopeptidase, cooperation of a variety of other proteolytic enzymes. DCAA and PCAA are also less susceptible to enzymatic degradation, perhaps due to possible inhibition of proteases by various inhibitors always present in natural waters [18]. Moreover, the application of high concentrations of artificial substrate leads to full saturation of L-leucine-aminopeptidase, whereas the concentrations of natural protein in lake water are far too inadequate to saturate all of the constituents of the protease pool [29].

**Table 2.** Concentrations (±standard deviations) of total and labile proteins (LCAA) in water of different trophic status and participation of the LCAA fraction to the total protein pool.

| Lake | Date | Total proteins (DCAA+PCAA) | Labile proteins (LCAA) | % |
|---|---|---|---|---|
| | | μM | μM | |
| L. Kuc | Jul 2001 | 3.11±0.14 | 0.56±0.16 | 17.9 |
| | Aug 2001 | 4.00±0.16 | 0.62±0.11 | 15.4 |
| | Apr 2002 | 0.69±0.02 | 0.47±0.06 | 68.2 |
| | Jul 2002 | 1.77±0.03 | 0.18±0.02 | 10.3 |
| | Aug 2002 | 1.80±0.05 | 0.15±0.01 | 8.2 |
| | Sept 2002 | 1.29±0.02 | 0.18±0.02 | 14.2 |
| L. Mikołajskie | Jul 2001 | 4.07±0.01 | 1.77±0.42 | 43.5 |
| | Aug 2001 | 3.97±0.07 | 2.61±1.20 | 65.7 |
| | Apr 2002 | 1.94±0.03 | 1.68±0.22 | 86.9 |
| | Jul 2002 | 2.07±0.04 | 0.31±0.02 | 14.8 |
| | Aug 2002 | 2.24±0.03 | 0.47±0.03 | 21.0 |
| | Sept 2002 | 1.37±0.04 | 0.02±0.00 | 1.4 |
| L. Tałtowisko | Jul 2001 | 3.78±0.18 | 1.56±0.11 | 41.3 |
| | Aug 2001 | 7.50±0.01 | 7.31±1.25 | 97.4 |
| | Sept 2002 | 1.54±0.04 | 1.10±0.07 | 71.0 |
| | Jul 2002 | 3.84±0.24 | 2.42±0.27 | 63.1 |
| | Sept 2002 | 2.34±0.03 | 0.67±0.25 | 28.5 |

The values of $V_{max}$ AMP observed in samples taken from surface water of the examined lakes varied from 60 to 912.0±151.5 nmol $l^{-1}$ $h^{-1}$, and were situated within the range of activities measured by other authors [7, 33, 42]. $V_{T1/2}$ calculated for the same samples changed from 1.1 to 13.2 nmol $l^{-1}$ $h^{-1}$. Although $V_{T1/2}$ values differ by one–two orders of magnitude from $V_{max}$ AMP, they are closely related to the rates of uptake of AA+SCP released from DCAA, which varies most often from several to tens nmol $l^{-1}$ $h^{-1}$ [26].

The concentrations of LCAA in lake water varied from 0.02 μM to 7.31 μM and were usually proportional to their trophic status. The lowest concentrations (0.15–0.62 μM) were found in meso/eutrophic lake Kuc, higher (0.02–2.61 μM) in eutrophic lake Mikołajskie, and the highest (0.67–7.31 μM) in hypereutrophic Lake Tal/towisko (Table 2). It should be noted that although the level of labile proteins in meso/eutrophic Lake Kuc remained relatively stable, in more eutrophicated lakes, substantial seasonal variations of labile protein concentrations were observed. Moreover, LCAA concentrations in meso/eutrophic Lake Kuc were not at all or only loosely related to the total protein concentration ($r$=0.61, $p$<0.193, $n$=6), whereas in eutrophic Lake Mikołajskie and in hypeutrophic Lake Tałtowisko, the relationship between total proteins and LCAA was more evident ($r$=0.8, $p$<0.05, $n$=6 and $r$=0.95, $p$<0.01, $n$=5, respectively). Generally, the contribution of labile proteins to the total proteins pool in the meso/eutrophic lake was lower than in the other two studied lakes (Table 2). These results may suggest that either microbial communities from meso/eutrophic Lake Kuc used proteins as C and N source less efficiently than those living in lakes of higher trophy, or that proteins synthesized by primary producers in this lake were particularly susceptible to enzymatic decomposition. In highly eutrophicated environments, intensive growth of algae producing easily degradable proteins was observed by Keil and Kirchman [19] and by Williams [41].

A statistically significant correlation between BN and $C_{LAB}$ ($r$=0.7, $p$<0.0019, $n$=17) proves that regardless of the trophic status of the lake, LCAA fractions constitute an important C and N source for heterotrophic bacteria and effectively stimulate secondary production. The relationship between protein degradation and growth rates of heterotrophic bacteria was shown by Hollibaugh and Azam [14]. Carlson *et al.* [5] postulated that labile proteins are intensively mineralized by microorganisms in marine surface water, whereas the degradation-resistant proteins (*e.g.*, glucosylated or immobilized in cellular membranes) are either hydrolyzed in deeper parts of the water column or accumulate in the bottom sediments.

LCAA concentrations as well as their hydrolysis rate ($V_{T1/2}$) were generally lower in meso/eutrophic Lake Kuc than in the eutrophic lakes (L. Mikołajskie and L. Tałtowisko) (Fig. 5). This was certainly due to a commonly higher bacterial abundance in eutrophic than in meso/eutrophic environments. A high rate of protein hydrolysis and considerably higher LCAA concentrations in hypereutrophic Lake Tal/towisko suggests that, compared with meso/eutrophic L. Kuc, the DCAA there were probably a primary organic C and N source for heterotrophic bacteria. Such a situation may also occur in other highly eutrophic lakes when other easily assimilated inorganic N sources are limited [21] and

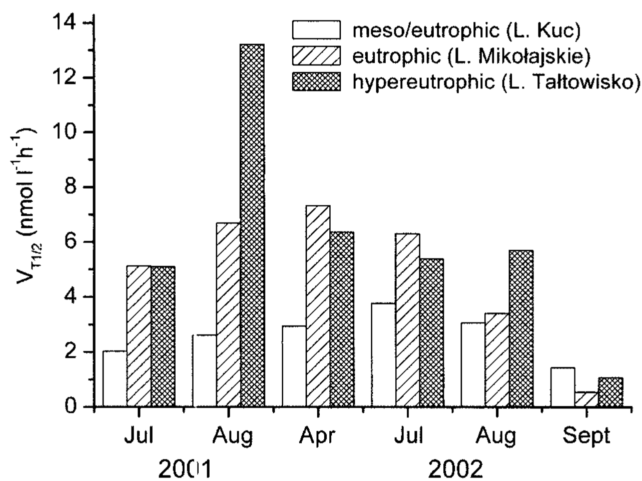


**Fig. 5.** Hydrolysis rate of labile proteins at $T_{1/2}$ ($V_{T1/2}$) in lakes of different trophic status.

the majority of nitrogen assimilated by bacteria originates from organic sources [4, 35], perhaps from LCAA.

The proposed method enables the determination of three basic parameters that describe the dynamics of protein decomposition processes in natural aquatic environments: the concentrations of labile proteins ($C_{LAB}$), their half degradation time ($T_{1/2}$), and realistic measure of resultant activity *in situ* ($V_{T1/2}$) of both ecto- and extracellular proteases responsible for hydrolysis of the labile part of DCAA and PCAA fractions. The results obtained by this technique were fully consistent with those acquired by classical fluorometric methods [16]. Moreover, they describe the complicated processes of protein degradation in greater detail than classical methods based on artificial analog of natural protease substrates. The essential disadvantage of the method is the relatively long time required for each set of assays (up to 4–5 days), which makes it rather useless for routine measurements. Although the proposed technique may be carefully applied for meso/eutrophic waters, it is especially recommended for eutrophic and hypereutrophic environments where the concentrations of proteolysis-resistant proteins and/or metabolic activity of attached bacteria in lake water are relatively high. For such environments, it should be possible to calculate not only $V_{T1/2}$ but also the absolute value of the protein decomposition rate *in situ* ($V_{T0}$). Moreover, our method also allows studies on protein degradation rates in fully saturated enzyme substrate systems; *e.g.*, in the matrix of biofilm covering the particles suspended in lake water where penetration of artificial substrates is probably extremely difficult or even impossible [3, 11]. The knowledge on the real enzyme activities connected with these structures seems to be particularly important, because they substantially affect not only proteolytic activity but also the overall metabolic activity of the bacteria in all aquatic environments [2, 11, 33, 34].

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