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Model toxicity assay for amino acid derivatives using green paramecia: Comparison of natural amino acids and *N*-acetylated non-protein amino acids

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ABSTRACT

Amino acid toxicity is a wide spread phenomenon in biological systems observed in both prokaryotes and eukaryotes. Especially, non-protein amino acids and metabolites can be toxic to humans through food chains. Therefore, development of a simple model assay system for assessing the toxicities of both natural and non-protein amino acids is required and cultured protozoan cells would fulfill the requirements. In this study, we propose the use of green paramecia (*Paramecium bursaria*) for assessing the acute toxicity of natural amino acids and non-protein *N*-acetylated amino acid derivatives. The cell survival rates of *P. bursaria* exposed to solutions of 20 free amino acids and commercially available 16 *N*-acetylated amino acid derivatives for 12 h of incubation were assessed. The LC_{50} obtained for natural amino acids are all above mM level. In contrast, we observed that *N*-acetylation of most amino acids resulted in highly elevated toxicity. Enhancement of toxicity by *N*-acetylation was observed in glycine, alanine, leucine, proline, aspartic acid, glutamic acid, asparagines, glutamine, cysteine, methionine, phenylalanine, tryptophan, and tyrosine with LC_{50} ranging between *ca*. 100 and 200 μM.

KEYWORDS: bioassay, green paramecia, *N*-acetylated amino acids

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INTRODUCTION

Amino acid toxicity is a wide spread phenomenon in biological systems observed in both prokaryotes and eukaryotes [1]. Some non-protein amino acids and metabolites can be toxic to humans, e.g. *Lathyrus* species contain a neurotoxic oxalyl-amino acid, thus, some potential toxins may be passed along a food chain through animal intermediates [2]. Non-protein amino acids are common in plants and are present in widely consumed animal feeds and human foods such as alfalfa (*Medicago sativa*), which contains canavanine, and lentil (*Lens culinaris*), which contains homoarginine [3, 4]. Recent studies have shown that such toxicity can be attributed to misincorporation of amino acid analogues into proteins by biosynthesis [2, 5].

Amino acid toxicity can be also observed with natural amino acids. Excitement in living cells can be induced by free amino acids such as glutamate and this would occasionally result in acute cell toxicity. Such response can be found in mammalian cells [6, 7], plant cells [8, 9], and also in protozoan cells such as *Paramecium* cells [10].

Taken together, development of a simple model assay system for assessing the toxicities of both natural and non-protein amino acids is required and cultured protozoan cells would fulfill the requirements.

Paramecium species including green paramecia (*P. bursaria*) have been widely used as facile assay materials for eco-toxicity tests [11, 12] and

food toxicity assays such as toxic impact of food-contaminating acrylamide [13]. Since green paramecia are symbiotic complexes [14], good models for chemical impacts to both algae (symbiotically growing within the ciliate host cells) and protozoan microorganisms, could be provided. It is well known that green paramecia receives the photosynthetic products from the green algae, therefore, culturing and long term maintenance of the cells are relatively easier under experimental light sources compared to other model ciliates [15]. This is another advantage of the use of this material.

N-acetylated amino acids are non-protein amino acid derivatives present in various organisms, and therefore, physiological roles for *N*-acetylated amino acids especially those during metabolism have been documented to date [16]. Some of *N*-acetylated amino acids, chiefly *N*-acetylcysteine are expected to play some protective roles against metal toxicity in various organisms including mammals [17]. In addition, *N*-acetylcysteine is now clinically applied to protect the patients from the acute toxicity of organophosphate [18], over-dose acetaminophen [19], and overdose foliate (given to children [20]). Therefore, assessment of any hidden impact of *N*-acetylcysteine must be carefully examined. Recently, possible toxic impacts of *N*-acetylated amino acids such as *N*-acetylglycine [21] and *N*-acetylglutamate [22] naturally present in animal-derived foods have been discussed and finally proven safe based on the toxicity assays using bacterial and mammalian cell lines. Development of a novel simple assay may accelerate such safety assessment process.

Apart from *N*-acetylated amino acids, the roles for free amino acids as natural osmo-regulators or cell volume regulating agents have been documented in various organisms including ciliates. For an instance, cell volume regulation by free amino acids in *Paramecium calkinsi* has been reported [23]. Furthermore, free amino acids are known to induce various cytological events in several *Paramecium* species [24], as classical reports described that high dose of free L-lysine induces the breakdown of macronuclear in some paramecium species [24]. However, effect of non-protein amino acids in ciliate models has not been documented to date.

In this study, we propose the use of green paramecia for assessing the acute toxicity of natural amino acids and non-protein *N*-acetylated amino acid derivatives.

MATERIALS AND METHODS

Organisms used

The green paramecium strain INA-1 (Fig. 1a; syngen 1, mating type I) was originally collected from the Ongagawa River (Kama-city, Fukuoka Prefecture, Japan) at the sampling point INA as described by Nishihama *et al.* [25].

Since the cell line was established after single cell isolation, all the cells in the culture were clones sharing identical genetic background. Green cells of *P. bursaria* (INA-1 cells) were cultured as described by Kadono *et al.* [12]. Briefly, the culture medium was prepared with a yeast extract-based nutrition tablet (1 EBIOS tablet/l; Asahi Food & Healthcare, Tokyo, Japan). Culture medium was renewed with 2-week intervals. One nutrition tablet (250 mg) contains 94.2% (w/w) dried yeast homogenates and 5.5% (w/w) carbohydrates. The bacterized nutrition medium was prepared by inoculating the medium with the food bacterium *Klebsiella pneumoniae* 1 day prior to the subculturing of ciliate cells. The ciliate culture was initiated with *ca.* 10-20 cells/ml and propagated to the confluent level (over 1000 cells/ml) under a light cycle of 12 h light and 12 h dark with *ca*. 3500 lux (30 cm from the light source) of natural-white fluorescent light at 23°C.

Toxicity assays

Paramecia in the stationary phase were washed once with the yeast extract-based medium described above. The toxicity assay using green paramecium was carried out on 12-well microplates and the median lethal concentration (LC_{50}) was determined for each of amino acids and their derivatives. Each well of the microplates was filled with 900 µl of the culture media containing 100 paramecium cells and 100 µl of amino acid solutions was added onto each well. Then the microplates were incubated for 12 h at 23°C under continuous dark condition, and the number of living paramecium cells were counted at the end of the incubation under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan).

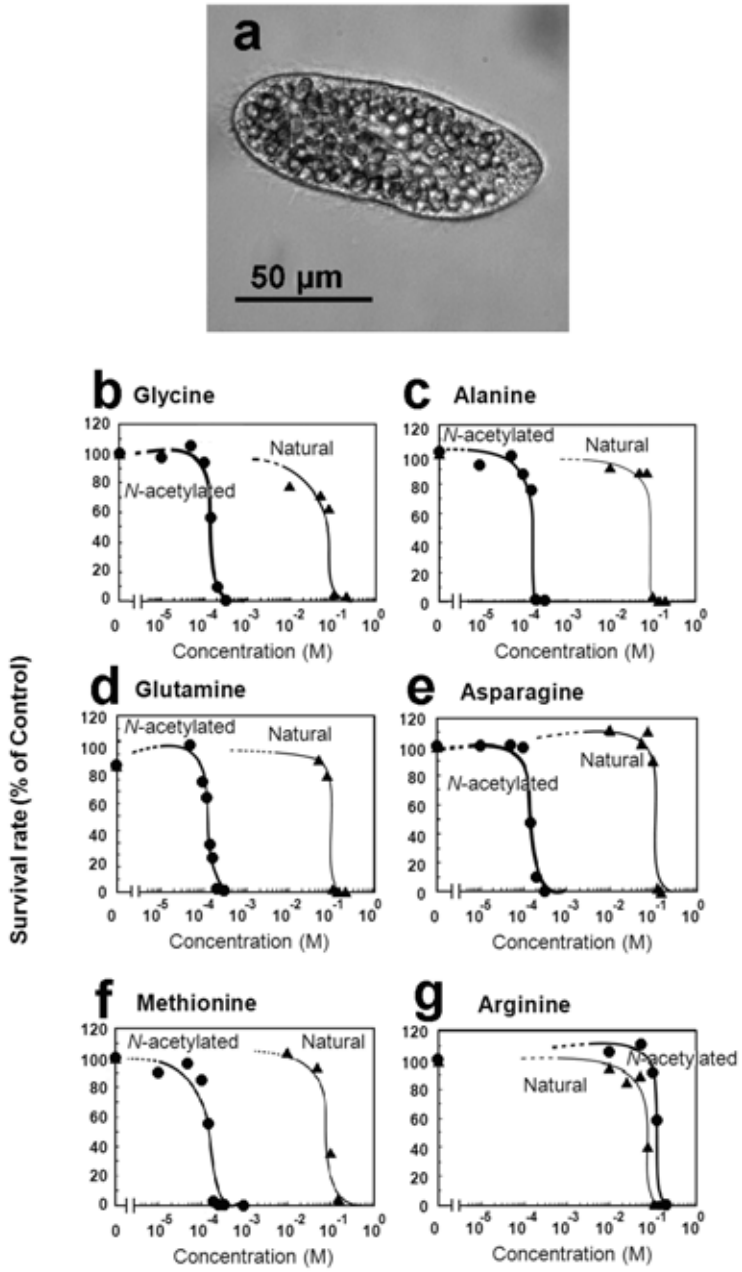

Fig. 1. Typical results from toxicity assays comparing the impacts of natural and *N*-acetylated amino acids. (a) Typical image of *P. bursaria*. (b-g) typical results for free and *N*-acetylated glycine, alanine, glutamine, asparagine, methionine and arginine.

RESULTS AND DISCUSSION

Toxicity assay for 20 natural amino acids

The cell survival rates (measured as acute toxicity) of *P. bursaria* exposed to free amino acid solutions for 12 h of incubation were assessed. Typical results (toxicity curves) for free glycine, alanine, glutamine, asparagine, methionine and arginine are shown in Fig. 1(b-g). Among the amino acids examined (totally 20 different amino acids), toxicity of leucine, tyrosine, and tryptophan could not be elucidated since high concentration up to some ten mM was shown to be inert. The LC_{50} obtained for natural amino acids are all above mM level

(Table 1). Therefore as expected, we can conclude that natural amino acids are not toxic at physiological concentrations.

Toxicity of *N*-acetylated amino acids

Acute toxicities of commercially available 16 *N*-acetylated amino acids were elucidated for comparison with natural amino acids using green paramecia (Table 2). We found that *N*-acetylation of most amino acids results in highly elevated toxicity as examples of *N*-acetylated glycine, alanine, glutamine, asparagine, and methionine are shown in Fig. 1(b-f). Enhancement of toxicity by *N*-acetylation was observed in glycine, alanine, leucine, proline, aspartic acid, glutamic acid, asparagines, glutamine, cysteine, methionine, phenylalanine, tryptophan, and tyrosine. Few amino acids showed inertness regardless of *N*-acetylation as the typical toxicity curve for arginine is presented in Fig. 1g. This type of amino acids, of which the toxic profile is not affected by *N*-acetylation, include arginine, histidine, and lysine.

Kato and Imamura have conducted a series of study revealing the mechanism of amino acid transport within the symbiotic algae from green paramecia [26-28]. However, our knowledge on the amino acid transport by the hosting ciliate in *P. bursaria* is largely limited at present. Understanding of the mode of amino acid toxicity in green paramecia would require consideration on the mechanism of amino acid transport. The hydrolysis of given amino acids in ciliate models would be surprisingly high compared to the rate of uptake by protozoan cells as the cells of *Tetrahymena pyriformis* reportedly show 240-fold higher rate of extracellular amino acid degradation than the rate of uptake [29].

Table 1. LC_{50} for 20 natural amino acids in *Paramecium bursaria*.

Category	Amino acids	LC_{50}[a]	Note
Non-polar	Glycine	84 mM	
	Alanine	87 mM	
	Leucine	ND[b]	NOEL[c] > 100 mM
	Proline	87 mM	
Basic	Arginine	70 mM	
	Histidine	142 mM	
	Lysine	78 mM	
Acidic and related	Aspartate	262 µM	Acute cell death[d]
	Glutamate	250 µM	Acute cell death[d]
	Asparagine	113 mM	
	Glutamine	87 mM	
Sulfurous	Cysteine	121 mM	
	Methionine	88 mM	
Aromatic	Phenylalanine	90 mM	
	Tyrosine	ND[b]	NOEL[c] > 1.25 mM
	Tryptophan	ND[b]	NOEL[c] > 50 mM
Branched chained	Valine	108 mM	
	Isoleucine	108 mM	
Hydroxyl	Serine	125 mM	
	Threonine	100 mM	

[a], median lethal concentration; [b], not determined; [c], non-observable effect levels (NOEL); [d], two acidic amino acids induced the cell death within 5-10 min after treatments. Some amino acid were hardly soluble in the culture media, thus NOEL instead of LC_{50} are shown.

Table 2. LC_{50} for *N*-acetylated amino acids in *Paramecium bursaria*.

N-Acetylated amino acids tested	LC_{50} [a]	Note
N-acetylglycine	157 µM	Acute cell death[b]
N-acetylalanine	168 µM	Acute cell death[b]
N-acetylleucine	148 µM	Acute cell death[b]
N-acetylproline	175 µM	Acute cell death[b]
N-acetylarginine	116 mM	Acute cell death[b]
N-acetylhistidine	88 mM	Acute cell death[b]
N-acetyllysine	104 mM	Acute cell death[b]
N-acetylaspartate	108 µM	Acute cell death[b]
N-acetylglutamate	128 µM	Acute cell death[b]
N-acetylasparagine	150 µM	Acute cell death[b]
N-acetylglutamine	142 µM	Acute cell death[b]
N-acetylcysteine	162 µM	Acute cell death[b]
N-acetylmethionine	155 µM	Acute cell death[b]
N-acetylphenylalanine	140 µM	Acute cell death[b]
N-acetyltyrosine	145 µM	Acute cell death[b]
N-acetyltryptophan	200 µM	Acute cell death[b]

[a], median lethal concentration; [b], all *N*-acetylated amino acids tested here induced the cell death within 5-10 min after treatments although LC_{50} for each chemical was evaluated 12 h after incubation.

It is well documented that the presence of intact amino groups prevents the rapid permeation of the amino acids across the cell membrane. Therefore the likely feature of *N*-acetylation is the enhanced cell permeability. The best known example of *N*-acetylation-mediated cell permeation is of *N*-acetylcysteine often used as a cell-permeable scavenger of reactive oxygen species [30]. Therefore, it is tempting to speculate that the elevated toxicity found in 13 *N*-acetylated amino acids, is due to enhanced cell permeability. The presence of additional basic groups in *N*-acetylated arginine, histidine, and lysine may contribute to impermeability into the cells, and therefore toxicity of these amino acids could not be fully elevated by *N*-acetylation.

The key difference between the present study carried out in green paramecia and the previously reported assays with bacterial and mammalian cell cultures, is the *N*-acetylation-dependently enhanced toxicity of the amino acids. The reports by Harper *et al.* concluded that two *N*-acetylated amino acids (*N*-acetyl glutamate and *N*-acetyl glycine) tested by them are not genotoxic or acutely toxic in bacteria, mice and rats [21, 22]. At present, our knowledge on the toxic nature of the *N*-acetylated amino acids is still limited. Therefore further toxicity tests using other organisms are highly encouraged.

PERSPECTIVES

In addition to the impacts of free amino acid derivatives, toxicity of peptides can be tested using our system. Such peptides of interest to be tested in our future research include the neurotoxic peptides derived from prion proteins [31].

The present study proposed the use of green paramecia as materials for toxicity assay evaluating the lethal impacts of free amino acids and their derivatives. For assessment of the impacts of amino acid derivatives at sub-lethal lower concentrations, much more sensitive assay model is required. One of possible approaches is to examine the chemical impacts at low concentrations, on the movement of living cells. Green paramecia reportedly show galvanotactic migration towards anodic electrode in the capillary system [32], upon stimulation of T-type calcium ion channel [33].

Such capillary assay system may provide a rapid and sensitive platform for toxicity assay.

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