

Current Methods for Analysis of Enzymatic Peptidyl-tRNA Hydrolysis

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Abstract

Understanding peptidyl-tRNA and the enzymes responsible for recycling them has come from the ability to detect and quantify enzymatic peptidyl-tRNA hydrolysis. The methods available to study removal of peptides from tRNA have evolved considerably. Radioactive [¹⁴C] amino acids were first implemented to monitor cleavage of the peptide-nucleotide ester bond of uniform peptidyl-tRNA substrates. Later, Northern blots with radiolabeled oligonucleotide probes were used to observe cleavage of specific peptidyl-tRNAs or individual tRNA from bulk peptidyl-tRNA populations. Finally, the use of fluorescently labeled amino acids was introduced, which could be coupled to anisotropy or PAGE readouts. Here we review the methods for quantification and analysis of enzymatic peptidyl-tRNA hydrolysis and summarize their inherent advantages and disadvantages.

Keywords: Peptidyl-tRNA; Peptidyl-tRNA hydrolase; Enzyme hydrolysis; Functional assay; Pth1; Pth2

Introduction

Peptidyl-tRNAs are thought to be inconspicuous molecules found in all cells. This is mainly due to cells having developed efficient means to recover tRNA from the unusable peptide bound form. However, the significance of peptidyl-tRNA and its enzymatic hydrolysis is finally being discovered, particularly in regard to impact on protein biosynthesis and cell viability.

In the course of translation, ribosomes stall on average 10% of the time [1]. If not rescued, disassembly of the translation machinery results in the release of peptidyl-tRNA. Peptidyl-tRNA is also generated from translation of minigenes or short ORFs [2-4]. Build-up of peptidyl-tRNA is lethal due to tRNA starvation [5]. The importance of preventing peptidyl-tRNA accumulation is apparent from the multiple mechanisms cells employ to recycle them [6,7]. These mechanisms depend on whether peptidyl-tRNA is part of the stalled ribosome or has been released. In stalled ribosomes, peptidyl-tRNA is recycled by ribosome associated proteins with peptidyl-tRNA hydrolase function [8,9]. If the ribosome is disassembled and peptidyl-tRNA is released, the complexity of tRNA recycling mechanisms differs greatly between prokaryotes and eukaryotes. In most bacteria there is a single, essential peptidyl-tRNA hydrolase enzyme, Pth1, responsible for removing the peptide from peptidyl-tRNA [10]. In certain bacterial species, a Pth2 homolog has been identified [11] but has not been structurally or functionally characterized. In eukaryotes, there is an emerging network of enzymes that recycle peptidyl-tRNAs, including mitochondrial associated Pths and Pth domain containing proteins [12-14].

From a biochemical point of view, peptidyl-tRNA is somewhat of an enigma having dual character – part peptide, part nucleic acid, Figure 1. This dual character leads to tremendous heterogeneity. Peptidyl-tRNA isolated from any given cell is a mixture of more than 40 tRNA molecules and considerably larger number of peptides [15]. Even with all of the possible differences, the character of the tRNA component is fairly uniform – a typical “clover leaf” structure with large negative charge. Each has an attached peptide of variable length and composition. The nature of the peptide component is much more heterogeneous and largely determined by size and composition. The presence of a shorter peptide does not change the properties of the molecule much from the properties of tRNA itself. A larger peptide chain can, however, significantly alter the molecular properties and

have implications for the choice of methods to analyze peptidyl-tRNA enzymatic hydrolysis.

Herein we review the methods that have been developed to monitor the cleavage of the peptide-tRNA ester bond of peptidyl-tRNA. The methods are separated into three categories based on the underlying principle used to determine the degree of hydrolysis using: (1) radioactive labeled amino acids, (2) electrophoretic separation of tRNA, and (3) fluorophore conjugated amino acids. The methods differ in their requirements for peptidyl-tRNA uniformity. While most employ a specific peptidyl-tRNA, it should be noted that production of uniform specific peptidyl-tRNA molecules is rather challenging. Therefore, specific peptidyl-tRNA tends to be replaced by a modified aminoacyl-tRNA for *in vitro* experiments. Moreover, there are instances in which observation of a heterogeneous sample of peptidyl-tRNA is advantageous. The selection of an appropriate method for peptidyl-tRNA hydrolysis should take into account several factors: hydrolysis product of interest (peptide or tRNA), amount of peptidyl-tRNA necessary to accomplish the study, and feasibility of the method given constraints on time, equipment, and resources.

Radioactive Labeled Amino Acids

The first reported methods for study of peptidyl-tRNA hydrolysis utilized [¹⁴C] labeling. Initially a [¹⁴C] labeled amino acid was attached to the tRNA using the appropriate tRNA synthetase. Peptidyl-tRNA was then formed by condensation of an additional peptide with the [¹⁴C]-aminoacyl-tRNA [16,17]. The preparation of such peptidyl-tRNA was formidable but yielded a natural and very relevant substrate for enzyme studies. To measure activity, tRNA was precipitated with TCA after incubation with Pth1. Soluble hydrolysis products, the

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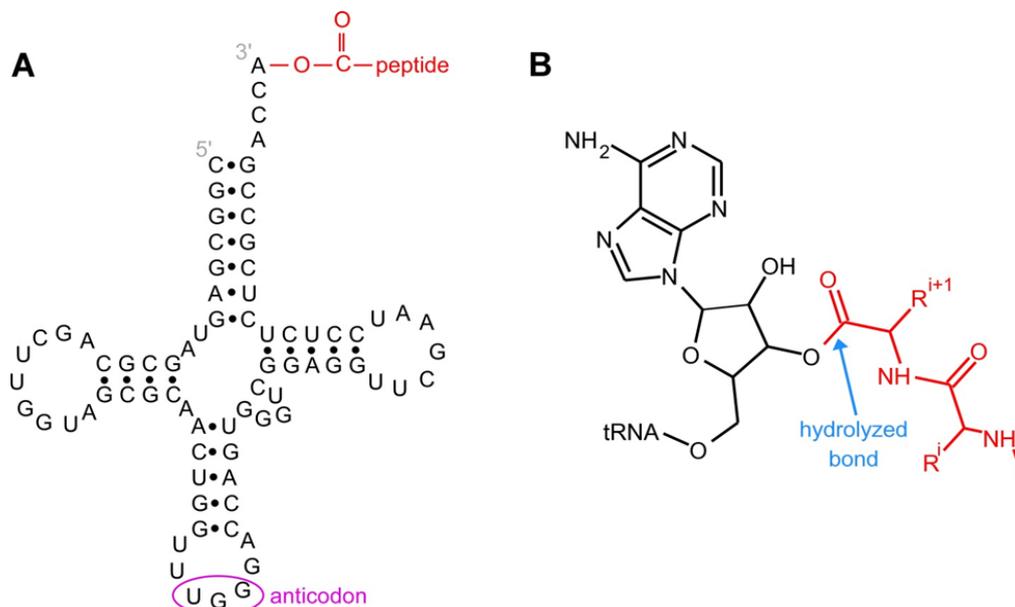


Figure 1: A. An example of peptidyl-tRNA. The peptide (red) is attached through an ester bond to the 3' end of tRNA (black), in this case tRNA^{Phe}. B. Close up of the ester bond between ribose 3'-hydroxyl group and C-terminus of the peptide chain. Note that the ester bond can also be formed between ribose 2'-hydroxyl group.

[¹⁴C] peptides, were separated with paper chromatography, visualized using ninhydrin staining, and quantified by scintillation counting of the excised peptide band. Early initial insight into the enzymatic activity of bacterial Pth1 was gained using this methodology [16,17]. Several subsequent modifications have been reported. The amount of hydrolyzed peptide was determined using scintillation of the supernatant obtained by centrifugation of the TCA quenched reaction mixture omitting the paper chromatography separation step [18] and N-acetyl-[¹⁴C]-aminoacyl-tRNA was used instead of peptidyl-tRNA [19]. While Pth1 is not able to hydrolyze aminoacyl-tRNA, it does catalyze the cleavage of N-acetyl-aminoacyl-tRNA. Introducing N-acetylated-aminoacyl-tRNA thus simplified the preparation of the substrate and, like the omission of the paper chromatography step, expedited the assay.

Radioactively labeled peptidyl-tRNA has also been generated using an *in vitro* translation system with [³⁵S]-Methionine incorporated into TnaC peptide [20]. This peptidyl-tRNA has not been used as a substrate for Pth activity assays but, made in sufficient quantity, would be an excellent specific substrate. In addition, this system could be readily modified for production of other specific peptidyl-tRNA as in the case of fluorescently labeled peptidyl-tRNA below. Upon enzymatic hydrolysis, the remaining nonhydrolyzed substrate as well as the hydrolyzed peptide could be precipitated, separated using PAGE, and visualized by autoradiography. Quantification of hydrolysis would then be determined based on band intensity.

The methods employing radioactively labeled amino acids are well suited for determination of Pth enzyme kinetic parameters. They are extremely sensitive, can be used for a wide range of signal intensities (i.e. substrate concentrations), and quantification can be readily performed. On the other hand, they require the use of a specific peptidyl-tRNA, production of which is a multistep process that may be unnecessarily demanding for certain applications. The use of radioactivity may also be undesirable or problematic. It has also been shown that the rate of Pth1 hydrolysis depends on peptide composition [18] and length [21]

which is not accounted for by using N-acetyl-aminoacyl-tRNA or a single peptidyl-tRNA species.

Electrophoretic Separation of tRNA

Separation of tRNA using low pH urea polyacrylamide gels was introduced to study the levels of aminoacylation in *E. coli* [22]. Total RNA isolated from the cell was separated on an acid-urea polyacrylamide gel and tRNAs (free, aminoacyl, and peptidyl) detected using a specific nucleotide probe labeled with [³²P]-ATP (i.e. a Northern blot), Figure 2. This technique was further simplified by using minigels instead of large gels traditionally used for nucleic acid separation [23]. The analysis could be further expedited by employing nonspecific nucleotide staining [24,25]. Compared to those employing radioactively labeled substrate, this method makes observation of both the hydrolyzed product (tRNA) and the nonhydrolyzed peptidyl-tRNA possible on one gel. However, it is limited to the use of peptidyl-tRNA with very short peptides because, in general, larger peptides tend to have more positive charge at lower pH. This prevents peptidyl-tRNA from entering the gel.

Methods using electrophoretic separation of tRNA are well suited for determination of Pth enzymatic activity. They allow the use of heterogeneous peptidyl-tRNA directly isolated from cells and hydrolysis for the entire population. Specific peptidyl-tRNAs can also be used. The fact that these methods are relatively nondemanding in terms of substrates, yet work with natural substrates, makes them very attractive for many applications. The ability to perform enzymatic hydrolysis with heterogeneous, bulk peptidyl-tRNA isolated from bacterial cells, separate the reaction products on a minigel, and stain for detection makes the analysis of peptidyl-tRNA enzymatic hydrolysis achievable in any biochemistry laboratory.

Fluorophore Conjugated Amino Acids

The most recent development in the analysis of enzymatic peptidyl-tRNA hydrolysis has been the introduction of fluorophores conjugated

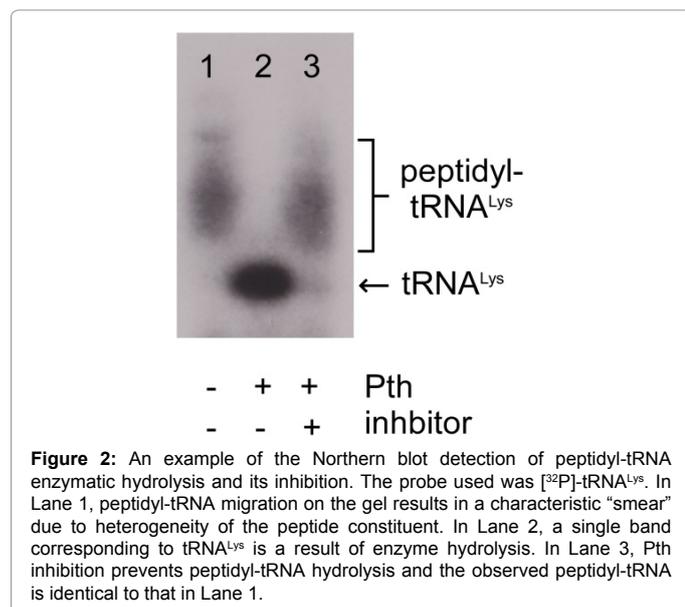


Figure 2: An example of the Northern blot detection of peptidyl-tRNA enzymatic hydrolysis and its inhibition. The probe used was [³²P]-tRNA^{Lys}. In Lane 1, peptidyl-tRNA migration on the gel results in a characteristic “smear” due to heterogeneity of the peptide constituent. In Lane 2, a single band corresponding to tRNA^{Lys} is a result of enzyme hydrolysis. In Lane 3, Pth inhibition prevents peptidyl-tRNA hydrolysis and the observed peptidyl-tRNA is identical to that in Lane 1.

to amino acids. The concept is the same as for [¹⁴C] studies but is less hazardous and has extended utility from the ability to detect changes in fluorescence anisotropy. Initial reports of fluorescent peptidyl-tRNA hydrolysis used BODIPY^{FL} succinimidyl ester for the labeling of lysyl-tRNA^{Lys} used in downstream applications [26]. Similarly, Oregon Green was used for labeling of methionyl-tRNA^{Met} [27]. Later, introduction of fluorophore labeled methionine to initiator tRNA was utilized to introduce a fluorescent label to any peptide produced using an *in vitro* translation system [28].

Several possibilities exist to determine the amount of hydrolyzed peptidyl-tRNA using fluorescent amino acids. Peptides could be

separated from peptidyl-tRNA via HPLC with a fluorescence detector measuring output [26]. Also, changes in fluorescence anisotropy could be used as an indicator of hydrolysis [26], a method adaptable to higher throughput screening. In addition, separation of hydrolysis products can also be achieved using three-layer low pH Tris-Acetate SDS-PAGE [29]. The advantage of these gels is that they enable use of peptidyl-tRNA with longer peptide constituents. Like the previous methods employing electrophoretic separation, this technique allows observation of both hydrolysis product (in this case the peptide) as well as the substrate (nonhydrolyzed peptidyl-tRNA) (Table 1).

The development of fluorescent labeling techniques resulted in the first high throughput, anisotropy based assay for screening of potential Pth1 inhibitors [26]. Fluorescent approaches rely on fluorescently labeled specific peptidyl-tRNAs or aminoacyl-tRNA. Disadvantages to this method are high cost and batch to batch variability. On the other hand, thanks to the high sensitivity of fluorescent probes, relatively small amounts of substrate are required for detection, partially offsetting the cost. Fluorescent probes enable straightforward quantification of peptidyl-tRNA hydrolysis and Pth activity or inhibition provided the equipment (fluorescence reader, spectrofluorimeter capable of anisotropy measurements) is available. It should be taken into account that fluorescence background from natural products and synthetic small molecules can be problematic for Pth inhibitor screening.

Conclusions

The development of Pth functional assays has allowed implementation of several significant applications. Having a functional assay can be used to determine whether a potential Pth gene or putative Pth-like domain can hydrolyze or even bind peptidyl-tRNA (i.e. fluorescence anisotropy). This ability has led to the definitive determination that plant CRS2 (a putative Pth) can bind, but not hydrolyze peptidyl-tRNA (McFeeters, unpublished), in agreement with *in vivo* complementation studies [30].

Detected Molecule	[Ref.]	Detection Method	Separation Technique	Sensitivity/Quantification	Advantages and Disadvantages
Methods employing radioactively labeled amino acids					
[¹⁴ C]-peptide/ N-acetyl-[¹⁴ C]-leucine	[16,17]	Ninhydrin staining/ Scintillation	Paper electrophoresis	High/Yes	(+) low amount of substrate (-) requires synthetic steps to prepare substrate or uses acetylated aminoacyl-tRNA
[¹⁴ C]-peptide/ [¹⁴ C]-diacetyl-lysine	[18,19]	Scintillation	Centrifugation	High/Yes	(+) low amount of substrate (-) requires synthetic steps to prepare substrate or uses acetylated aminoacyl-tRNA
Methods employing electrophoretic separation of tRNA					
tRNA	[22,23]	[³² P]-ATP (Northern blot)	PAGE	High/Yes	(+) allows use of specific and heterogeneous substrates (-) time consuming (-) requires short peptide length
tRNA	[24,25]	Methylene blue Silver staining	PAGE	Moderate/Yes High/Yes	(+) allows use of specific and heterogeneous substrates (+) fast (-) sensitivity (-) requires short peptide length
Methods employing fluorescently labeled amino acids					
Fluorophore (conjugated to peptide)	[29]	Fluorescence	SDS-PAGE	High/Yes	(+) sensitivity (-) involves extra step of fluorophore conjugation
Fluorophore (conjugated to amino acid)	[26]	Fluorescence anisotropy Fluorescence	(HPLC) ^a	High/Yes	(+) sensitivity (+) high throughput (-) involves extra step of fluorophore conjugation (-) uses aminoacyl-tRNA substrate

^aHPLC with a fluorescent readout was used to validate the enzymatic hydrolysis of peptidyl-tRNA while fluorescence anisotropy was used for determining the activity of Pth1.

Table 1: Summary of methods for analysis of peptidyl-tRNA hydrolysis presented here.

The first functional radioactive assay allowed for the determination of Pth kinetic parameters [16,21] providing insight into mechanism and comparison between different Pth enzymes or mutants [19]. A more readily producible probe for Northern blot readout enabled initial screening of Pth1 inhibitors [31,32]. Further methodological improvements, in particular the ability to use fluorescence readouts [26] has led to higher throughput screening and more potential to advance Pth1 as an antibacterial target.

In conclusion, multiple methods exist for the measurement of peptidyl-tRNA cleavage and Pth enzyme activity. Each has distinct advantages, which should be taken into account for new applications. Improvements in generation of specific peptidyl-tRNA, including synthetic approaches [33] and *in vitro* translation [20,28], continue to advance. With ever improving bioanalytical techniques, even simpler, more robust, and relatively quicker methods with higher throughput are on the horizon.

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