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# Measuring the activity of farnesyltransferase by capillary electrophoresis with laser-induced fluorescence detection

Enzymatic farnesylation of oncogenic forms of *Ras* proteins is the initial step in a series of posttranslational modifications essential for *Ras* activity. The modification is catalyzed by the enzyme, protein farnesyltransferase (PFTase), which transfers a farnesyl moiety from farnesyl diphosphate to the protein. We employed capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection to develop a rapid and sensitive method for the determination of PFTase activity *in vitro*. The limited substrate specificity of PFTase allowed us to use a fluorescently labeled pentapeptide instead of a *Ras* protein as a substrate for the enzyme; the product of the enzymatic reaction was the farnesylated pentapeptide. The product was separated from the substrate by CE and quantified with LIF detection. Under optimal conditions, the separation was achieved within 10 min with a resolution of 86. The mass and concentration limits of detection for the farnesylated product were $10^{-19}$ mol and 0.28 nM, respectively. By measuring the rate of accumulation of the farnesylated product, we were able to determine the kinetic parameters of the enzymatic reaction. For yeast PFTase as an enzyme and difluorocarboxyfluorescein-labeled GCVIA peptide as a substrate, the values of $k_{cat}$ and $K_M$ were found to be $(3.1 \pm 0.3) \times 10^{-3}$ $s^{-1}$ and $(12.0 \pm 1.2)$ μM, respectively. Our results suggest that CE-LIF can be efficiently used for the determination of enzymatic activity of PFTase *in vitro*. After minor modifications, the developed method can be also applied to other reactions of enzymatic prenylation of proteins.

**Keywords:** Capillary electrophoresis / Farnesyltransferase / Fluorescently labeled peptide / Laser-induced fluorescence
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## 1 Introduction

Protein farnesyltransferase (PFTase) catalyzes the alkylation of a cysteine residue in a substantial number of proteins found in eukaryotes [1]. This reaction is the first step in a series of post-translational modifications that enhance the association of the proteins with the membrane and are required for their proper biological function. The *Ras* proteins are prominent among those modified with farnesyl moieties. They play a critical role in the signal transduction pathway that controls cell division. When mutated, *Ras* proteins can become oncogenic; approximately 30% of human cancers are associated with mutations in *Ras* proteins. These cancers include: pancreatic adenocarcinomas (90%), colon adenocarcinomas and adenomas (50%), thyroid carcinomas and adenomas (50%), lung adenocarcinomas (30%), myeloid leukemias (30%), and melanomas (20%) [2, 3]. Because farnesylation of mutated *Ras* is essential for its ability to oncogenically transform cells, PFTase is under intense investigation as a potential target for cancer therapy [4].

PFTase catalyzes transferring a 15-carbon farnesyl moiety from farnesyl diphosphate (FPP) to the cysteine residue of a protein. To be farnesylated, the cysteine residue has to be within a carboxy-terminal $Ca_1a_2X$ motif, where C is cysteine, $a_1$ and $a_2$ are typically small aliphatic amino acids and X is one of the following amino acids: methionine, serine, glutamine, or alanine (Fig. 1) [5]. PFTase appears to be specific only to the carboxy-terminal $Ca_1a_2X$ motif, so that small peptides containing this motif are efficiently farnesylated by the enzyme [6]. Although most substrates of PFTase *in vivo* are proteins, small peptides are widely used as substrates for the enzyme in *in vitro* reactions due to the simplicity of their synthesis and analysis [7].

Various methods for the determination of PFTase activity have been reported. Most of them rely on labeling of one of the substrates (protein/peptide or farnesyl diphos-

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**Abbreviations: A**, alanine; **C**, cysteine, **dff-GCVIA**, 2',7'-difluorofluorescein-5-carboxyl-glycinyl-cysteinyl-valinyl-isoleucinyl-alanine; **DIEA**, diisopropylethylamine; **Fmoc**, $N^{\alpha}$-9-fluorenylmethyloxycarbonyl; **FPP**, farnesyl diphosphate; **HBTU**, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; **PFTase**, protein farnesyltransferase; **TES**, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; **TIS**, triisopropylsilane; **V**, valine


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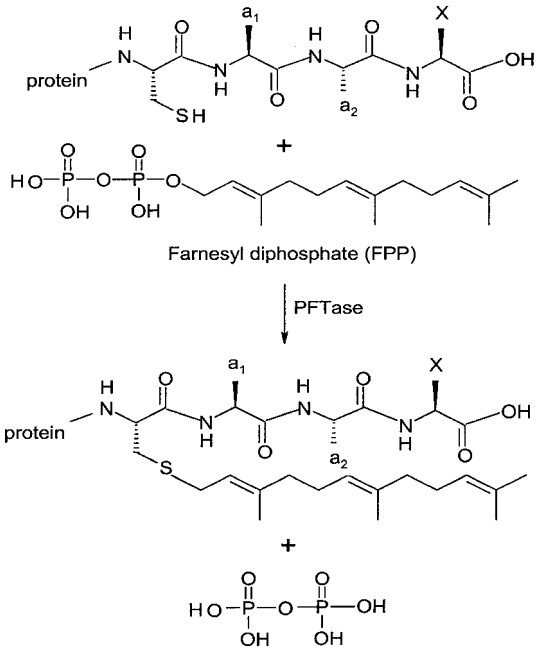



**Figure 1.** PFTase-catalyzed farnesylation of the cysteine residue in a $Ca_1a_2X$-containing protein.

phate) and the separation of the labeled substrate from the labeled product. A widely used approach involves radiolabeled FPP. This method suits both the *Ras* protein and small peptides as the first substrate. If *Ras* protein is used as a substrate, then the separation of FPP from the farnesylated *Ras* protein can be achieved by filtration through glass-fiber filters [8], gel sizing on Sephadex G-50 [9], phosphocellulose filter-binding assay [10], or TLC after protein digestion using a proteolytic enzyme [11]. If a small peptide is utilized as a substrate of PFTase, FPP can be separated from a farnesylated peptide using TLC [12], HPLC [13, 18–21], or phosphocellulose paper [14]. Radioactive detection can be used in the on-line mode of HPLC, although at the expense of a considerable loss in sensitivity [13]. Relatively high sensitivity can be achieved only in an off-line mode with long exposure times [15]. Fluorescence detection is used as an alternative to radioactivity in the analyses of PFTase activity [22]. A peptide substrate of PFTase is typically labeled with a fluorescent dye in a way that does not disturb the recognition of the peptide substrate by the enzyme. The labeled substrate is farnesylated in the enzymatic reaction and later on, the nonreacted substrate is separated from the farnesylated product by HPLC [23]. Fluorescence detection has a number of advantages. First, it is characterized by outstanding sensitivity and high speed. Second, fluorescently labeled substrates are more stable than radioactive ones and do not require high safety standards typical for handling radioactive materials. Third, it is possible to design fluorescently labeled substrates that do not requite the separation stage, thus making the analysis simpler and faster. An example of such a substrate is a peptide, modified with a dansyl dye [16, 17]. When a dansylated peptide is farnesylated, the emission spectrum of the dansyl moiety shifts 10–15 nm due to the increased hydrophobicity of the dye's environment. This shift is sufficient for the quantitation of the farnesylated product without separating it from the substrate. The use of dansylated peptides is a very powerful tool for the analysis of PFTase activity, especially when dealing with samples containing the substrate and the product in moderate amounts.

If the activity of PFTase is low, the amount of farnesylated product formed is also low. None of the described methods are suitable for rapid analysis of samples containing less than $10^{-13}$ mol of the farnesylated product. Much lower limits of detection can be achieved, however, if CE with LIF detection is used. CE-LIF was successfully applied to monitoring the activities of glycosyltransferases [24], phosphatases [25], and kinases [26]. Extremely low limits of detection achieved allowed detecting enzymatic products formed by a single enzyme molecule [27]. In this work, we used CZE with LIF detection to develop a rapid and highly sensitive method for the analysis of PFTase activity *in vitro*.

## 2 Materials and methods

### 2.1 Materials and reagents

FPP, farnesyl bromide, and yeast *Saccharomyces cerevisiae* farnesyltransferase were purchased from Sigma-Aldrich (Oakville, ON). Wang resin, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), and $N^{\alpha}$-9-fluorenylmethyloxycarbonyl (*N*-Fmoc)-protected amino acids were purchased from Advanced ChemTech (Louisville, KY, USA) except for Fmoc-A, which was purchased from Acros/Fisher Scientific (Tustin, CA, USA). Diisopropylethylamine (DIEA) and piperidine were obtained from Sigma-Aldrich. DMF was from Fisher Scientific and dried over 3Å molecular sieves. Oregon Green 488 carboxylic acid, succinimidyl ester (5-isomer) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma-Aldrich or BDH (Toronto, ON).

### 2.2 Synthesis of 2',7'-difluorofluorescein-5-carboxyl-glycinyl-cysteinyl-valinyl-isoleucinyl-alanine (dff-GCVIA)

A mixture of Wang resin (0.998 g, 1.0 mmol/g, 1.0 eq.), Fmoc-A (1 558 g, 5.0 eq.), HBTU (1 855 g, 4.9 eq.), DIEA (1.74 mL, 10 eq.) and DMF (10 mL) was placed in a cen-

trifuge tube and rotated end-over-end for 21 h. The resin was collected and washed with DMF (10 mL × 1 min × 3), capped with acetic anhydride (3 mL)/DIEA(1 mL)/DMF(10 mL) for 30 min, and washed with DMF (10 mL × 1 min × 3). The Fmoc group was removed with 20% piperidine/DMF (10 mL × 5 min × 3), and the resin was washed with DMF (10 mL × 1 min × 3). The coupling cycle for enlogation of the peptide consisted of (i) coupling the Fmoc-amino acid (2.0 eq.)/HBTU(1.95 eq.)/DIEA(4.0 eq.)/DMF(10 mL), (ii) a washing step with DMF (10 mL × 1 min × 3), (iii) removal of the Fmoc group with 20% piperidine/DMF (10 mL × 5 min × 3), and (iV) a final washing with DMF (10 mL × 1 min × 3) to add I,V,C, and G in succession. A mixture of the peptide resin (50.8 mg, 5.1 eq.), which had been washed with methanol and dried under vacuum, Oregon Green 488 carboxylic acid succinimidyl ester (5.0 mg, 1.0 eq.), DIEA (25 μL, 14.6 eq.) and DMF (1 mL) was rotated end-over-end overnight. The resin was collected, washed with DMF (10 mL × 1 min × 3) and methanol (10 mL × 1 min × 3), and dried under vacuum. The dried resin was treated with a cocktail (3 mL) of 94% TFA /2.5% EDT/2.5% $H_2O$/1% triisopropylsilane for 18 h. The solvent was removed under vacuum, and the residue was rinsed with hexane (× 3) and dissolved in 30% ACN/0.1%TFA/$H_2O$. It was purified by HPLC using Vydac $C_{18}$ protein and peptide column (300 Å, 10 μm, 22 × 250 mm) and eluted with a gradient of 30% to 35% ACN/0.1% TFA/water for 5 min, followed by isocratic elution with 35% ACN/0.1%TFA/$H_2O$ (flow rate: 10 mL/min). Fractions between 26 to 36 min were collected. The combined fractions were lyophilized to give 2.5 mg (30%) of an orange powder that was 94% pure as judged by analytical HPLC; electrospray MS (+ ion mode) *m/z* at: 856.4, (− ion mode) *m/z* at 854.3.

### 2.3 Synthesis of dff-GCVIA-farnesyl

The farnesylated product, dff-GCVIA-farnesyl, was synthesized essentially as described by Dolence *et al.* [23]. Farnesyl bromide (1.2 eq.) was allowed to react with dff-GCVIA in the presence of DIEA (3.0 eq.) in DMF. After 1–3 h, the reaction was quenched with water, and the resulting mixture was lyophilized. The residue solid was dissolved in 1:1 v/v acetic acid/ACN and purified by HPLC (Hewlett-Packard model 1050) on a Sephasil Peptide C18 column (Amersham Biosciences, Baie D'urfe, QC) with a linear gradient from 50% ACN/50% water/0.1% TFA to 100% ACN/0.1% TFA over 20 min at 1.0 mL/min. The farnesylated product was eluted at 8.5 min and detected at 520 nm in an on-line mode; the excitation wavelength was 488 nm. The product was used as an authentic standard in CE method development.

### 2.4 Enzymatic reaction

The enzymatic buffer contained 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM DTT, and 0.04% w/v dodecyl-β-D-maltoside in 50 mM Tris buffer at pH 7.0 [21]. The reaction mixture consisted of 0.3–30 μM dff-GCVIA, 0.5–30 μM FPP, and 46–130 nM PFTase in a total of 150 μL of the enzymatic buffer. The reaction was initiated by the addition of the enzyme, incubated at 30°C for 5 min to 48 h, quenched with 10 μL of 1.2 M HCl, and placed on ice (final pH 1). Control experiments were conducted with adding the enzymatic buffer instead of the enzyme.

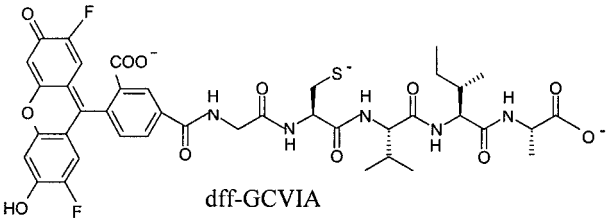
### 2.5 Analysis of the enzymatic reaction

CE analyses of the reaction mixtures were performed using a laboratory-built CE instrument, equipped with a post-column LIF detector as described elsewhere [28]. Electrophoresis was run in a positive-polarity mode, positive electrode was at the injection end of the capillary and the detection end of the capillary was grounded, using a Spellman CZE 1000 power supply (Plainview, NY, USA) as a source of high voltage. A 488 nm line of an Ar-ion laser (Melles Griot, Ottawa, ON) was utilized to excite fluorescence. Fluorescence was filtered from stray and scattered laser light with a band pass filter centered at 520 nm (Omega Optical, Brattleboro, VT, USA). An R1477 photomultiplier tube (Hamamatsu, Middlesex, NJ, USA) was used as a light detector. Fused-silica capillaries of 40 cm × 20 μm ID × 150 μm OD (Polymicro Technologies, Phoenix, AZ, USA) were used for all experiments. Four different running buffers were utilized in CE: (i) 2.5 mM borax at pH 9.1, (ii) 25 mM borax/25 mM SDS at pH 9.3, (iii) 50 mM borax/20 mM β-cyclodextrin at pH 9.5, and (iv) 50 mM TES/50 mM SDS at pH 7.3 (TES = *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid). The samples were injected electrokineticly at 50 V/cm × 2 s resulting in the injection volume of 65 pL. The electrophoresis was carried out at 400 V/cm at ambient temperature. The capillary was rinsed with the run buffer solution for 2 min before each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

## 3 Results and discussion

### 3.1 Analytical method

In this work, we used a fluorescently labeled peptide, dff-GCVIA, as a substrate of PFTase (Fig. 2). The farnesylated product was synthesized chemically and used as an authentic standard. The product was considerably more hydrophobic than the substrate; all our analyses were



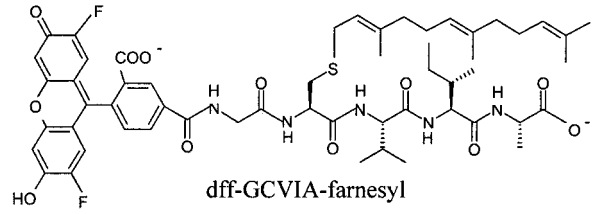




**Figure 2.** Structural formulas of the fluorescently labeled substrate, dff-GCVIA and its farnesylated product, dff-GCVIA-farnesyl.

based on this difference. We developed a CZE method for rapid and sensitive quantitation of the farnesylated product. Initially, bare borate, phosphate, and TES buffers were explored. Bare buffers proved not to resolve the farnesylated product from the substrate (see *e.g.*, Fig. 3A). Therefore, we used β-cyclodextrin or SDS that are both known to enhance the resolving power of separation for substances with different hydrophobicities [29]. Both additives dramatically improved the quality of the separation of the farnesylated product from the substrate. In the presence of cyclodextrin at pH 9.5, the migration time of the substrate was longer than that of the product (Fig. 3B). The hydrophobic product has two negative charges while the substrate has three negative charges at basic pHs (see Fig. 2). Moreover, the product is larger, and in addition it forms an inclusion complex with neutral β-cyclodextrin. The charge to size ratio of the complex is considerably lower than that of the substrate resulting in decreased electrophoretic mobility of the complex. Thus, the complex is barely retained by the electric field and migrates faster, while the substrate is strongly retained by the electric field and migrates slower.

In the presence of SDS micelles, the farnesylated product exhibited longer migration times than the substrate (Figs. 3C and D). The hydrophobic product is solubilized into negatively charged SDS micelles and its mobility controlled by that of the micelles. The negative charge-to-size ratio of SDS micelles is higher than that of the substrate, and as the result, the solubilized product is

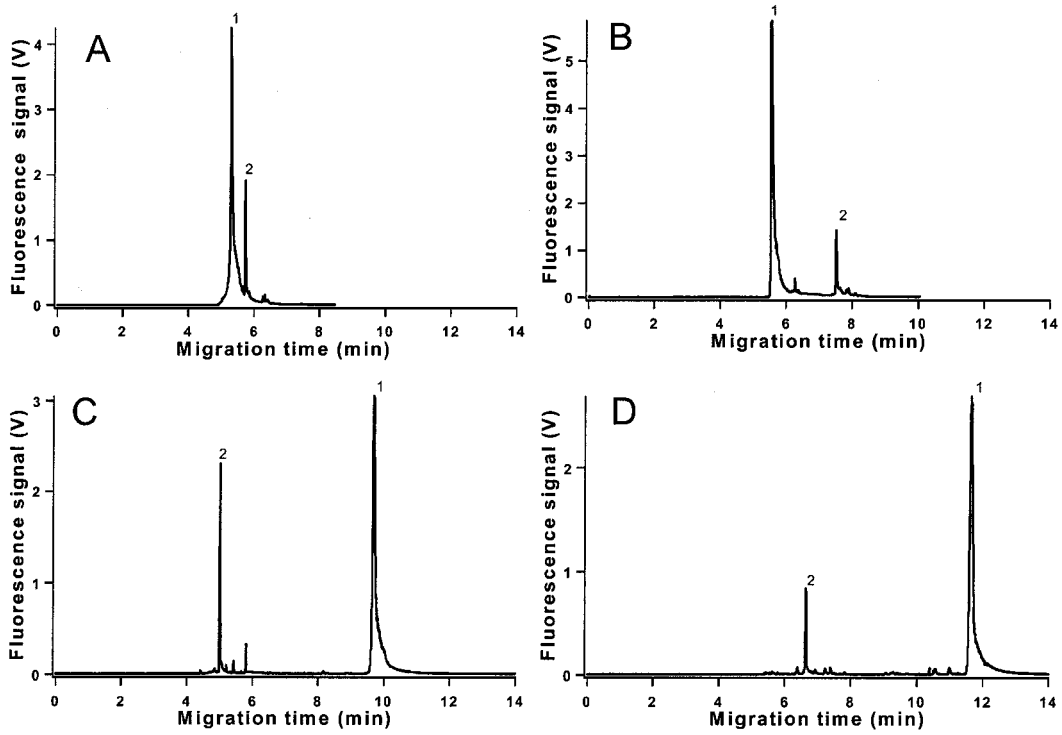



**Figure 3.** CE separation of the farnesylated product (peak 1) from the substrate (peak 2). The reaction mixture contained 0.8 μM dff-GCVIA, 20 μM FPP and 46 nM PFTase after 48 h incubation at 30°C in the enzymatic buffer. Running buffers: (A) 25 mM borax, pH 9.1; (B) 50 mM borax, 20 mM β-cyclodextrin, pH 9.5; (C) 50 mM TES, 50 mM SDS, pH 7.3; (D) 25 mM borax, 25 mM SDS, pH 9.3.

retained longer and elutes later than the substrate. MEKC with SDS-containing buffers proved to provide better separation quality (Table 1) and, therefore, we chose them for our further study. The migration time was shorter in the TES-SDS buffer resulting in lower diffusion and better resolution than in the borax-SDS buffer. We found that the effect of the buffer nature on the migration time and thus on the resolution was more pronounced than that of pH. We determined the limit of detection of the CE-LIF method with respect to the farnesylated product using an S/N ratio of 3 as a criterion [24]. The mass limit of detection was $10^{-19}$ mol and the concentration limit of detection was 0.28 nM. The mass limit of detection of our method exceeds those in all previously used methods by a few orders of magnitude. The mass limit of detection in CE-LIF with a sheath-flow design is typically better than $10^5$ molecules due to elimination of light scattering and associated with it background [30]. It should be noted, that this low detection limit can be achieved without compromising the speed of the assay; the analysis takes no longer than 15 min.

**Table 1.** Resolution and efficiency for the separation of the farnesylated product from the substrate in CE with different separation buffers

| Running buffer | Resolution | Efficiency (Number of theoretical plates) | |
|---|---|---|---|
| | | Product | Substrate |
| 2.5 mM Borax, pH 9.1 | 2.1 ± 0.1 | 4 200 ± 200 | 5 000 ± 200 |
| 25 mM Borax, 25 mM SDS, pH 9.3 | 27.1 ± 0.2 | 28 000 ± 1000 | 9 000 ± 500 |
| 50 mM Borax, 20 mM β-cyclodextrin, pH 9.5 | 9.8 ± 0.1 | 5 600 ± 200 | 11 000 ± 400 |
| 50 mM TES, 50 mM SDS, pH 7.3 | 86.4 ± 0.3 | 19 400 ± 900 | 4 800 ± 400 |

### 3.2 Kinetic parameters

Next we used the developed method to determine the kinetic parameters of PFTase-catalyzed farnesylation of the dff-GCVIA substrate. The kinetic scheme of the reaction is depicted in Fig. 4. We determined the catalytic rate constant, $k_{cat}$, and Michaelis constant for the peptide, $K_M$, using the Lineweaver-Burk equation:

$$\frac{1}{v_0} = \frac{1}{k_{cat} \times [PFTase]_0} + \frac{K_M}{k_{cat} \times [PTFase]_0} \times \frac{1}{[Pep]_0} \quad (1)$$

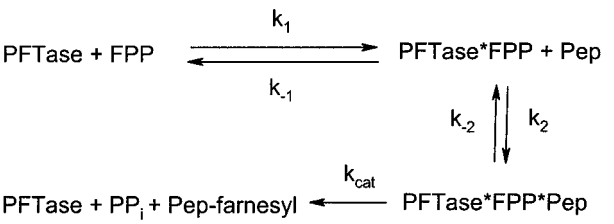



**Figure 4.** Kinetic mechanism for PFTase-catalyzed farnesylation of the peptide substrate, Pep, in the presence of FPP.

where $v_0$ is the initial rate of the enzymatic reaction, $[PFTase]_0$ is the total concentration of the enzyme and $[Pep]_0$ is the initial concentration of the peptide substrate. The initial reaction rate for different concentrations of the peptide substrate was determined by quantifying the amount of farnesylated product after 5 min incubation. After this short incubation only 0.5% of the substrate was converted to the product, Pep-farnesyl. The slow conversion allowed us to use a linear approximation in finding the reaction rate:

$$v_0 = \frac{[Pep - farnesyl]}{time} \quad (2)$$

The kinetic parameters of our enzymatic reaction were calculated to be $k_{cat} = (3.1 \pm 0.3) \times 10^{-3}$ $s^{-1}$ and $K_M = 12.0 \pm 1.2$ μM.

## 4 Concluding remarks

In conclusion, we developed a very sensitive method for rapid determination of PFTase activity *in vitro*. Classically, the kinetic parameters of PFTase-catalyzed farnesylation are determined by using an unlabeled protein and a radioactive FPP substrate. The mobility shifts are observed by gel electrophoresis with autoradiography. The developed CZE-LIF method is a powerful alternative with improved resolution, a less sample load, a low level of mass limit of detection and a short operation time. The method will be of great value in studies of inhibitors of PFTase *in vitro*. Moreover, a subject to slight modification, the method can be also used for the analysis of other protein prenylation reactions.

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