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Monitoring the three enzymatic activities involved in posttranslational modifications of Ras proteins

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Abstract

Ras proteins are important oncogenes that are involved in more than 30% of human cancers. To become functional, Ras proteins have to undergo at least three posttranslational modifications: farnesylation, endoproteolysis, and carboxyl-methylation. The enzymes, which catalyze the three reactions, are under investigation as potential anti-cancer targets. Here we developed a highly sensitive capillary electrophoresis (CE) method for monitoring these three enzymatic activities. In this method, a fluorescently labeled pentapeptide is used as a substrate. The substrate is sequentially converted to three products by the enzymes: protein farnesyltransferase, endoprotease, and methyltransferase. The three products retain the fluorescent label. The substrate and the three products are separated by CE and detected by laser-induced fluorescence. The method is characterized by: (i) the efficiency of greater than 400,000 theoretical plates, (ii) the resolution of greater than 7, and (iii) the limit of detection of as low as 800 molecules of the enzymatic product. Using the new method we measured kinetic parameters of endoprotease-catalyzed cleavage of three carboxy-terminal amino acids from the farnesylated substrate: $V_{max} = 7\,nmol\,min^{-1}\,mg^{-1}$ of the protein and $K_m = 1.3\,\mu M$. The method will find its applications in studies of inhibitors of the three enzymes in search for new Ras-targeting anti-cancer agents.

Keywords: Ras proteins; Protein farnesyltransferase; CAAX-specific prenyl-protein endoprotease; Isoprenylcysteine carboxyl methyltransferase; Capillary electrophoresis; Analysis of enzymatic activities

1. Introduction

Ras proteins are among key players in a signal transduction pathway that controls cell proliferation. Mutated *ras* genes are found in 30% of human cancers, with the highest incidence in the pancreatic (90%) and colon (50%) cancers. Ras mutations, which are present in human tumors, encode single amino acid defects at residues 12, 13 or 61. These mutations lead to Ras being activated even in the absence of external stimuli, which makes cells proliferate indefinitely [1]. To become a functional oncogene, mutated Ras undergoes three posttranslational modifications: farnesylation, endoproteolysis, and carboxyl-methylation. These modifications make Ras hydrophobic and targets the protein to the inner surface of the outer membrane [2–4].

Enzymatic farnesylation is the initial step in a series of posttranslational modifications of Ras proteins [5]. Farnesylation is catalyzed by protein farnesyltransferase (PFTase) enzyme, which transfers a 15-carbon farnesyl moiety from farnesyl diphosphate (FPP) to the sulfhydryl group in cysteine [6]. To be farnesylated, the cysteine residue must be in a carboxy-terminal Cys-a_1a_2X motif, where a_1 and a_2 are small aliphatic amino acids and X is one of the following amino acids: Met, Ser, Gln or Ala [6]. PFTase is specific for the carboxy-terminal Cys-a_1a_2X motif, so that small peptides containing this motif are efficiently farnesylated by the enzyme [7].

Abbreviations: CE, capillary electrophoresis; PFTase, protein farnesyltransferase; Epase, endoprotease; Mtase, methyltransferase; S, 2′,7′-difluorofluorescein-5-carboxyl-Gly-Cys-Val-Ile-Ala; P_1, the product of farnesylation of Cys in S; P_2, the product of Val-Ile-Ala cleavage from P_1; P_3, the product of carboxyl-methylation of Cys in P_2; FPP, farnesyl diphosphate; AdoMet, *S*-adenosyl-L-methionine; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol

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Upon farnesylation, Ras proteins become substrates for an endoprotease (EPase), which cleaves the a_1a_2X tripeptide at the carboxy-terminus [8]. A human gene encoding for specific Ras-targeting EPase activity (hRCE1 gene) was recently discovered, expressed in insect cells, and its product was characterized [9]. This Ras-specific EPase is localized in the endoplasmatic reticulum membrane and not in the plasma membrane. It is a cysteine protease that selectively cleaves the a_1a_2X tripeptide from proteins with the farnesylated (or geranylgeranylated) carboxy-terminal Cys-a_1a_2X motif. The enzyme is specific to this motif only so that it hydrolyses not only proteins but also short peptides [10,11].

Endoproteolysis is followed by methyltransferase (MTase)-catalyzed carboxyl-methylation of the farnesyl-cysteine residue; *S*-adenosylmethionine (AdoMet) is a donor of the methyl group. The recent discovery of the gene, which encodes MTase (corresponding proteins are named Ste14p in yeast and pcCMT or Icmt in mammals) [12,13], intensified studies of the reaction catalyzed by this enzyme. The MTase enzyme is a membrane protein but, like EPase, is restricted to the endoplasmatic reticulum [13]. The substrate specificity of MTase is limited to the farnesylated (or geranylgeranylated) Cys residue, thus allowing the use of small prenylated peptides as substrates [14,15].

Due to a critical role of oncogenic Ras in carcinogenesis, the inhibition of Ras signaling is considered an extremely attractive approach to cancer therapy. The anti-cancer potency of Ras signaling inhibition has been demonstrated by interfering with the signaling in different points of the signal transduction pathways [16–20]. The most successful approach proved to be the one that prevents Ras proteins from their association with the plasma membrane, which is critical to Ras functioning properly. Inhibiting any of the three reactions of Ras posttranslational modifications can block Ras association with the plasma membrane and thus, interfere with Ras signaling.

The inhibition of farnesylation has attracted most attention of cancer researchers so far [21]. A number of peptide and non-peptide inhibitors of FTase have been synthesized as potential anti-cancer therapeutics [22]. The rational design of new FTase inhibitors is stimulated by recent progress in understanding the structure of FTase and its mechanism of catalysis [23,24]. With better understanding of the functional role of Ras endoproteolysis, the inhibition of this reaction started to attract attention of researchers as a potential anticancer approach [8,25]. It has been demonstrated with *ras*-gene-transformed rodent and human cells, that the inhibition of EPase suppresses cell growth and induces apoptosis [26]. Encouraging data on the suppression of tumor growth were obtained in an animal model with a conditional allele of the gene encoding for EPase [3]. It is difficult, however, to forecast the therapeutic future of the approach since essentially all the therapeutic efforts are still at the stages of compound design and early evaluation. Carboxyl-methylation of farnesylated and proteolysed Ras is just beginning to attract attention as a potential anti-cancer target, and there is still only a limited amount of published data on the subject [27].

The development of effective inhibitors of PFTase, EPase, and MTase requires analytical tools for the evaluation of their *in vitro* and *in vivo* activities. This, in turn, requires highly sensitive methods for measuring the activities of the three enzymes. Traditional methods for monitoring these activities are based on labeling a substrate with radioactive or fluorescent tags and separation of the product from the substrate by different kinds of chromatography (see Ref. [28] for the overview of analytical methods). The traditional methods suffer from relatively low sensitivity and the need to use more than one type of analyses if all three activities are to be monitored simultaneously. We have recently reported a new capillary electrophoresis (CE)-based method for the analysis of PFTase, which allows the detection of 10^{-19} mol of the farnesylated product. Here we further develop this method to facilitate simultaneous quantitation of the substrate and the three products of its sequential farnesylation, endoproteolysis and carboxyl-methylation. With the sensitivity, which exceeds those of the other methods by orders of magnitude, this method has the potential to become an important tool in the development of new Ras-targeting anti-cancer agents.

2. Material and methods

2.1. Materials

Yeast *Saccharomyces cerevisiae* PFTase, FPP, AdoMet, PMSF, DTT, Tris, $ZnCl_2$ and $MgCl_2$ were purchased from Sigma-Aldrich (Oakville, Ont., Canada). Membrane preparations containing EPase (yRCE1p) and MTase (Ste14p) were obtained as described earlier [29,30].

2.2. Enzymatic procedures

2.2.1. Substrate (S)

We used a fluorescently labeled pentapeptide, 2′,7′-difluorofluorescein-5-carboxyl-Glu-Cys-Val-Ilu-Ala as an initial substrate. S was synthesized as described elsewhere [28]. S was converted to three products in enzymatic reactions described below (see Fig. 1 for the reaction scheme).

2.2.2. Enzymatic product 1 (P_1)

The first enzymatic reaction was farnesylation of the cysteine residue of S. S, FPP, and PFTase were mixed in the assay buffer (50 mM Tris–HCl, pH 7.4, containing 1 μM $ZnCl_2$, 10 mM $MgCl_2$, and 2 mM DTT) at the final concentrations of 0.3 μM, 30 μM, and 7.0 nM, respectively. The mixture was incubated at 37 °C for 60 min, which ensured the conversion of 90% of S to P_1. After the incubation, this reaction mixture was directly used to obtain the second enzymatic product.

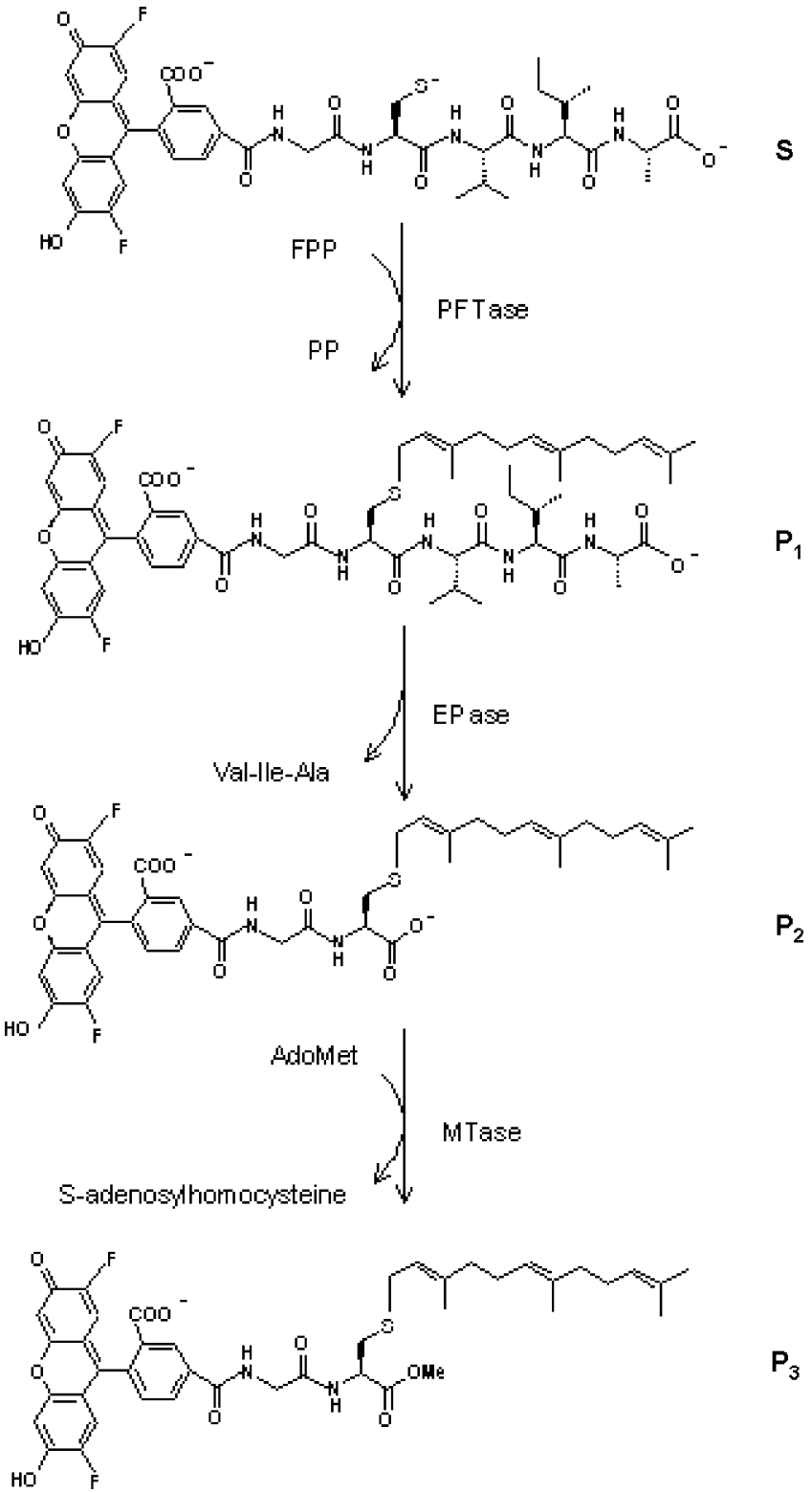

Fig. 1. Enzymatic conversion of a fluorescein-labeled pentapeptide substrate (S) into P_1–P_3. PFTase, EPase and MTase catalyze the three steps, respectively.

2.2.3. Enzymatic product 2 (P_2)

The second enzymatic reaction was endoproteolysis of P_1, in which the Val-Ilu-Ala tripeptide was cleaved from P_1. A 1 μL portion of the stock solution of PMSF (30 mM in 2-propanol) and 1.5 μL of the stock solution of EPase-containing membrane preparation (370 μg/mL of total protein in 0.1 M Tris–HCl buffer, pH 7.4) were added to 27.5 μL of the reaction mixture containing P_1 (see the previous paragraph). The final concentrations of PMSF and EPase-containing preparation were 1 mM and 18.5 μg/mL, respectively. The reaction mixture was incubated at 37 °C for 90 min to yield more than 90% of P_2. This reaction mixture was used directly for obtaining the third product.

2.2.4. Enzymatic product 3 (P_3)

The third enzymatic reaction was carboxyl-methylation of the cysteine residue of P_2. A 1 μL portion of the stock solution of AdoMet (45 mM in H_2O) and 4 μL of the stock solution of the MTase-containing yeast membrane preparation (11.3 mg/mL of total protein in the 5 mM Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl and 0.3 M sorbitol) were added to 25 μL of the reaction mixture containing P_2 (see the previous paragraph). The final concentration of AdoMet and MTase-containing yeast membrane preparation were 1.5 mM and 1.5 mg/mL, respectively, of total protein. The reaction mixture was incubated at 37 °C for 60 min to reach the maximum yield of P_3.

2.3. Capillary electrophoresis

CE analyses of the reaction mixtures were performed using a laboratory-built CE instrument, equipped with a post-column LIF-detector as described elsewhere [31]. The positive electrode was at the injection end of the capillary and the detection end of the capillary was grounded. The difluorofluorescein label was excited by a 488 nm line of an Ar-ion laser (Melles Griot, Ottawa, Ont., Canada). Fluorescence was filtered from stray and scattered laser light with a band pass filter centered at 520 nm (Omega Optical, Brattleboro, VT, USA). An R477 photo multiplier tube (Hamamatsu, Middlesex, NJ, USA) was used as a fluorescence light detector. Fused-silica capillary of 49 cm length × 20 μm ID × 150 μm OD (Polymicro Technologies, Phoenix, AZ, USA) was used for all experiments. Ten different buffer compositions were tested as background electrolytes to optimize the CE method: (i) 50 mM TES/50 mM SDS, pH 7.3; (ii)–(iii) 25 mM tetraborate, pH 8.3, containing either 1% Triton X-100 or 1% Tergitol; (iv)–(vi) 25 mM tetraborate, pH 9.3, containing SDS of 25 or 50 or 100 mM, and (vii)–(x) 25 mM tetraborate/25 mM SDS, pH 9.3, containing either 3 M urea or 2.85 M acetonitrile or 2.46 M methanol or 1% Triton X-100. As all buffers contained surfactants with concentrations higher than critical micellar concentrations, the separation method can be referred to as micellar electrokinetic chromatography (MEKC). The samples were injected by a suction pulse of 9.1 kPa × 1 s resulting in the injection volume of approximately 70 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 HCl, 100 mM NaOH for 2 min followed by a rinse with deionized water for 2 min.

2.4. Kinetic analyses

Kinetic analyses of enzymatic products' formation were conducted in the following way. Five-microliter aliquots were taken from the reaction mixture at different times during the course of the reaction. To stop the reaction, 5 μL of the run buffer (25 mM tetraborate/25 mM SDS/3 M urea at pH 9.3) was added to the aliquot immediately. SDS and urea facilitated the denaturing of the enzymes and thus interrupted enzyme-catalyzed reactions. Each aliquot was placed on ice and then was subjected to the CE analysis, described in Section 2.3. The amounts of the substrate and products were quantitated according to the corresponding peaks' areas.

The determination of the kinetic parameters for endoproteolysis was carried out as follows. EPase (final concentration of 18.75 μg/mL) was incubated with the P_1-containing reaction mixtures (final concentrations of P_1 were in the range of 0.14–1.12 μM) at 37 °C for 15 min. The progress of the reaction was monitored as described in the previous paragraph. The initial reaction rates were determined using linear parts of kinetic curves.

In another series of experiments we simultaneously monitored the depletion of the substrate and the accumulation of three enzymatic products. The initial reaction mixture consisted of 0.63 μM S, 4.6 nM PFTase, 30 μM FPP, and 18.5 μg/mL EPase in the assay buffer (50 mM Tris, pH 7.4, containing 1 μM $ZnCl_2$, 10 mM $MgCl_2$, 2 mM DTT and 1 mM PMSF). This reaction mixture was incubated for 10 min at 37 °C to progress through the farnesylation step. MTase-containing yeast membranes and AdoMet were then added (as described in Section 2.2.4) and the new reaction mixture was further incubated for 40 min.

3. Results and discussion

3.1. General approach

The aim of this work was to develop a CE-based method for simultaneous quantitation of the fluorescent pentapeptide substrate (S) and three products (P_1–P_3) of its sequential farnesylation, endoproteolysis, and carboxyl-methylation (see Fig. 1 for the reaction scheme). The method development required S and P_1–P_3, We synthesized S and enzymatically produced P_1 using the procedures developed earlier [28]. To obtain and characterize P_2 and P_3 we had to have an appropriate method for their analysis. We did not choose to use traditional HPLC-based methods, but rather try the

CE-method in development for such analyses. We expected that the conditions we previously optimized for the CE separation of S and P_1 (25 mM borate/25 mM SDS, pH 9.3 or 50 mM TES/50 mM SDS, pH 7.3 as a run buffer) [28], would be satisfactory for the separation of S and P_1–P_3, and thus, for optimizing the production of P_2 and P_3. When P_2 and P_3 are obtained, they could be used for refining the analytical method itself.

3.2. Synthesis and separation of P_1–P_3

We first found conditions at which P_1–P_3 could be produced from S by three enzymes (PFTase, EPase, and MTase) in the same vial without changing the buffer. Commonly used buffers for PFTase contain Mg^{2+} and Zn^{2+} [32,33], while those for EPase and MTase typically contain EDTA or phenanthroline [29]. These buffers are not compatible as Zn^{2+} is an inhibitor of EPase [30], whereas EDTA and phenanthroline are chelators of Mg^{2+} and Zn^{2+}. To compose a buffer that would suite all three enzymes we decreased the concentration of Zn^{2+} to 1 μM, which is much below the level of the K_i value for Zn^{2+}-induced inhibition of EPase [29], and excluded EDTA and phenanthroline. The buffer was based on 50 mM Tris–HCl, pH 7.4 and contained 1 μM $ZnCl_2$, 10 mM $MgCl_2$, 2 mM DTT (stabilizer of S), and 1 mM PMSF (protease inhibitor, which does not inhibit EPase). We dissolved S in this buffer, added PFTase and FPP (the donor of the farnesyl group). After a 50 min incubation, S was almost completely converted into P_1, confirming that the buffer was suitable for FTase (Fig. 2, panel A, traces 1 and 2). Then, EPase was added to the reaction mixture containing P_1. After the incubation, the peak of P_1 disappeared and another peak appeared with the migration time shorter than that of P_1 and longer than that of S (Fig. 2, panel A, trace 3). This peak was ascribed to P_2, which was consistent with P_2 being less hydrophobic than P_1 and more hydrophobic than S. P_2 is more hydrophobic than S because it has a farnesyl group and less hydrophobic than P_1 because it lacks a highly hydrophobic Val-Ile-Ala moiety. Finally, we added MTase-containing yeast membrane preparation and AdoMet (the donor of the methyl group) to the reaction mixture containing P_2 and observed the decreased peak of P_2 and the appearance of a new peak with a migration time between those of P_2 and P_1 (Fig. 2, panel A, trace 4). This peak was ascribed to P_3, which migrates very close to P_1. We could not achieve more than a 50% conversion of P_2 to P_3 that might be connected with the formation of S-adenosylhomocysteine, a potent inhibitor of MTase [34].

To confirm the separation of S, P_1, P_2, and P_3, the reaction mixture containing all four species was analyzed. It was obtained by simultaneous addition of PFTase and EPase to S and 10 min delayed addition of MTase to this reaction mixture. The four species were baseline separated by CE using either of the two run buffers optimized in [28] (Fig. 2, panel B). Thus, were able to find conditions at which the three products could be obtained from S by a sequence of three

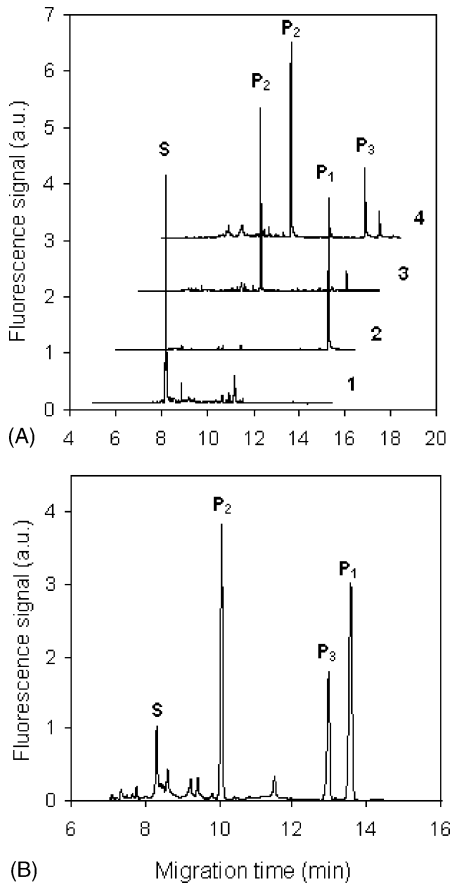

Fig. 2. Separation of the fluorescent pentapeptide substrate (S) and products of its sequential farnesylation (P_1), endoproteolysis (P_2) and methylation (P_3). Panel A shows electropherograms of the reaction mixture obtained by the conversion of S (trace 1) to P_1 (trace 2), P_2 (trace 3) and P_3 (trace 4). Panel B shows the electropherogram of the reaction mixture after a 10 min incubation of S with both PFTase and EPase and followed by adding MTase and incubating for additional 10 min. The run buffer was 25 mM tetraborate, 25 mM SDS at pH 9.3. Other conditions are described in Section 2.

enzymatic reactions performed in the same buffer. Moreover, we showed that the CE-method developed for the separation of S from P_1 in [28] could facilitate baseline separation of S, P_1, P_2, and P_3.

3.3. Optimization of separation conditions

As a next step, we optimized the separation conditions for the four species in an attempt to improve the resolution of P_1 and P_3 ($R \approx 2$). P_1 and P_3 have similar high hydrophobicity and tend to be efficiently solubilized in SDS micelles so that their migration times are close to each other and to that of the micelles. As a result of this, neither the increase of SDS concentration of up to 100 mM nor the use of other surfactants led to improved separation (data not shown). The resolution of P_1 and P_3 improved to up to 7 when the mixture of S, P_1, P_2, and P_3 was separated in the running buffers containing either organic modifiers or urea (Fig. 3). Such

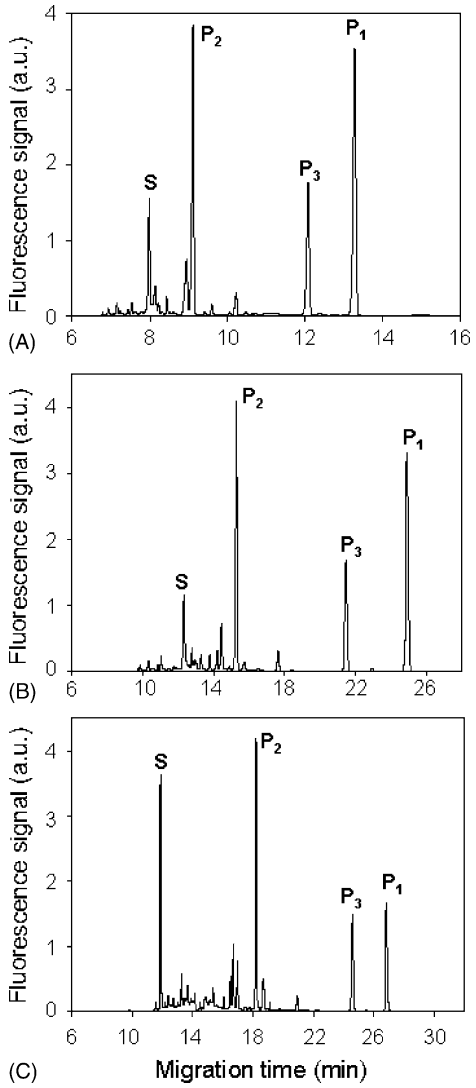

Fig. 3. CE separation of the fluorescent pentapeptide substrate (S) and products of it sequential farnesylation (P_1), endoproteolysis (P_2) and methylation (P_3). The reaction mixture, which was obtained in the way described in Fig. 2, was subjected to CE in 25 mM borate/25 mM SDS (pH 9.3) run buffer supplemented with: 3 M urea (panel A), 2.85 M acetonitrile (panel B), and 2.46 M methanol (panel C). Other conditions are described in Section 2.

additives are widely used for CE of hydrophobic peptides and proteins [35–37]. Urea was preferable because of shorter analysis time and therefore, the urea-containing buffer was used in all our further experiments.

3.4. Reaction kinetics

We applied the developed method for quantitation of S, P_1, P_2, and P_3 to study the kinetics of the enzymatic reactions. Simultaneous farnesylation and endoproteolysis and subsequent methylation were achieved by the simultaneous addition of PFTase and EPase to S and 10 min delayed addition of MTase to this reaction mixture. We choose not to add the three enzymes simultaneously as the addition of

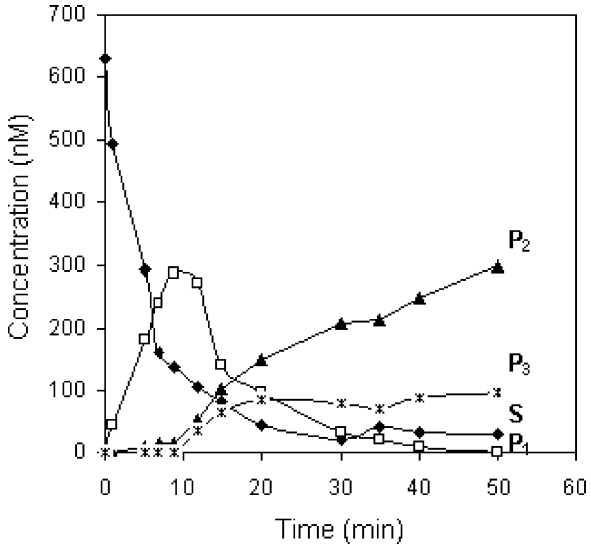

Fig. 4. Reaction kinetics of S (◆), P_1 (□), P_2 (▲) and P_3 (∗) in the reaction mixture described in Fig. 2. The concentrations of the species were determined by measuring the areas of corresponding peaks in CE separation of the species. The run buffer was 25 mM borate/25 mM SDS/3 M urea at pH 9.3. Other conditions are described in Section 2.

MTase-containing crude membrane preparation to S inhibited the formation of P_1. The mechanism of this inhibition is not clear due to the complex content of the membrane preparation used. The kinetics of the depletion of S and the production of P_1–P_3 are shown in Fig. 4. This is the first time when the kinetics of this 3-step conversion was measured.

Moreover, we used our method to determine the kinetic parameters of the endoproteolysis reaction using the Lineweaver–Burk equation. The K_m values of 1.2 ± 0.3 and $1.4 \pm 0.5\,\mu M$ were obtained from the formation of P_2 and the depletion of P_1, respectively. The values for V_{max} were found to be 3.0 ± 1.5 and $12 \pm 4\,nmol\,min^{-1}\,mg^{-1}$ obtained from the formation of P_2 and the depletion of P_1, respectively (the units contain mg of total protein in the EPase preparation). These results are in good agreement with each other as well as with the literature data obtained by other methods (K_m = 1–6 μM, V_{max} = 1–50 nmol min^{-1} mg^{-1}) [29,38]. We determined the limit of detection of our CE-LIF method using fluorescein as a reference and an S/N ratio of 5 as a criterion of detectability [39]. The mass limit of detection was 10^{-21} mol and the concentration limit of detection was 20 pM.

4. Conclusions

To conclude, we developed a CE-based method for the analysis of three enzymatic reactions involved in posttranslational modifications of Ras proteins. The method consumes only sub-nanoliter volumes of the sample per analysis and has a limit of detection of as low as 800 molecules of enzymatic products. Using the new method we measured kinetic

parameters of the endoproteolysis reaction. This method will find applications in search for and characterization of Ras-targeting anti-cancer therapeutics.

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