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Notes & Tips

High-performance liquid chromatography method for detecting prostate-specific membrane antigen activity

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One of the glutamate carboxypeptidase II (GCPII)[1] enzymes of recent medical interest is the membrane-bound prostate-specific membrane antigen (PSMA; EC 3.4.17.21). This enzyme was originally characterized as the complementary ligand to the monoclonal antibody 7E11.C5 which exhibited immunohistochemical staining specific among human tissues for the prostate epithelium [1]. It has been shown that PSMA is expressed in normal and neoplastic prostate epithelial cells and in prostatic tumor metastases; however, the importance of this enzyme is due to its strong expression in prostate cancer cells and it was discovered during the development of the LNCaP cell line, one which retains most of the known features of prostate cancer [2]. Although a cytosolic form of PSMA is predominant in normal cells, the membrane-bound form predominates in prostate cancer cells. Moreover, a 100-fold difference in expression of the ratios of the mRNA messages encoding the two forms of PSMA has been observed, which may be indicative of a disease state [3].

PSMA possesses a high sequence homology to the membrane-bound *N*-acetylated α-linked acidic dipeptidase (NAALADase), which is characterized by its ability to hydrolyze the neuropeptide *N*-acetylaspartylglutamate (NAAG) as shown in Fig. 1 [4]. PSMA also exhibits such activity and is commonly assayed by monitoring the hydrolysis of NAAG [2]. In contrast to NAALADase which has been extensively studied due to its presumed regulatory role in glutamate neurotransmission [4], questions of medical interest remain to be answered for PSMA, including its possible role in folic acid metabolism and its poorly understood NAALADase-type activity. It is expected that the acquisition of inhibitors and substrate probes of PSMA may help to further the current understanding of the biological role of this metallocarboxypeptidase and serve to elucidate germane active-site features.

Our primary interest in developing a general HPLC-based assay for PSMA arose from our interest in screening a library of inhibitors for this enzyme. A previously established radioenzymatic assay for monitoring NAALADase continues to be employed [4–6]. This conventional method involves in vitro incubation of [^{3}H]NAAG in the presence of PSMA that is usually obtained from cell lysates from the LNCaP cell line or expressed in transfected PC3 tumor cells [4,7–9]. Once the incubation is quenched, the enzyme reaction mixture is subjected to ion-exchange chromatography to selectively elute [^{3}H]glutamic acid. The eluate is collected and the amount of liberated glutamic acid is determined by scintillation counting. Although a fluorimetric method using OPA has been recently employed to monitor GCPII-mediated hydrolysis of *N*-acyldipeptides, K_m values could not be determined below 5 μM [10]. With an interest to reduce the time and labor associated with the conventional radioenzymatic assay we recently developed a convenient HPLC assay for monitoring PSMA enzymatic activity

Materials and methods

Chemicals. Preparations of prostate-specific membrane antigen were obtained as a generous gift from Alan Brunelle (Northwest Biotherapeutics, Bothell, WA) and was prepared as described previously [11]. [^{3}H]NAAG and [^{3}H]glutamic acid were purchased from

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[1] *Abbreviations used:* GCPII, glutamate carboxypeptidase II; PSMA, prostate-specific membrane antigen; NAALADase, *N*-acetylated α-links acidic dipeptidate; NAAG, *N*-acetylaspartylglutamate; [^{3}H]NAAG, *N*-acetyl-L-aspartyl-L-[3,4-^{3}H]glutamate; [^{3}H]glutamic acid, [3,4-^{3}H]glutamate; OPA, *o*-phthaladehyde; HFBA, heptaflyorobutyric acid.

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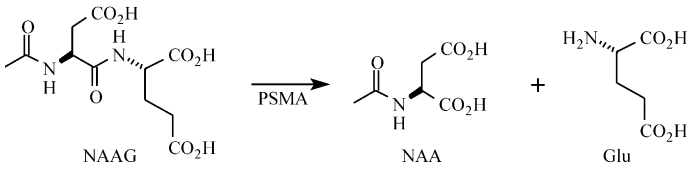

Fig. 1. PSMA-mediated hydrolysis of NAAG.

NEN Life Science Products (Boston, MA). Monoflow 3 scintillation fluid used with the radioisotope HPLC detector was purchased from National Diagnostics (Atlanta, GA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO) and were used without further purification.

Incubation conditions and analytical methods. A typical incubation mixture (final volume 250 μL) was prepared by the addition of 10 μL radiolabeled substrate [^{3}H]NAAG (125 μM, 0.8 Ci/mmol) to 230 μL Tris buffer (50 mM, pH 7.4, 50 μM $CoCl_2$). The enzyme reaction was initiated by the subsequent addition of 10 μL PSMA preparation (0.384 mg protein/mL). The reaction was allowed to proceed for 15 min with constant shaking at 37 °C and then terminated by the addition of 25 μL cold NaH_2PO_4 (1.0 M, pH 7.4) followed by vortexing and centrifugation (7000*g*). A 20-μL aliquot of the resulting supernatant was then analyzed by HPLC as follows. [^{3}H]NAAG and its hydrolytic product, [^{3}H]glutamic acid, were separated and quantified by analytical reversed-phase HPLC (Sphereclone 5 μm ODS(2), 4.6 × 250 mm, Phenomenex, Torrence, CA) using a Hitachi 7000-series HPLC system outfitted with an autosampler and radioisotope detector (β-RAM Model 2 with Scintflow B2 ver. 3.16 software, IN/US Systems, Tampa, FL). The β-RAM detector was configured with a scint./eluate ratio of 2.5 using a cell volume of 1.0 mL. Analog output from the β-RAM detector was recorded on the Hitachi 7000-series integrator. Using a mobile phase of glycine buffer (50 mM, pH 2.4, with 0.25% HFBA) at a flow rate of 2.0 mL/min, NAAG and its hydrolytic product glutamic acid were detected with retention times of 4.70 and 2.95 min, respectively.

Results and discussion

It was envisioned that NAAG and glutamate could be efficiently separated by reversed-phase HPLC. To our surprise, no such method or application existed in the accessible scientific literature. Based upon the acidic nature of both analytes, low pH conditions were expected to maintain the carboxylates in a protonated state, thus allowing for greater retention by the nonpolar medium of a C-18 stationary phase. Although NAAG would likely be neutral under such conditions and be suitably retained by a C-18 column, glutamate would exist as the ammonium ion, possibly precluding its retention by a nonpolar stationary phase. However, it was posited that the addition of an acidic hydrophobic agent such as heptafluorobutyric acid to the mobile phase could serve as an ion-pairing agent for glutamate's amino terminus.

Based upon the constraints and strategies noted above, the mobile phase was initially composed of glycine buffer (pH 2.5), methanol, and heptafluorobutyric acid. It was noted that reducing the proportion of methanol in the mobile phase resulted in enhanced resolution of the analytes and, ultimately, this trend led to the complete exclusion of methanol from the mobile phase. The percentage of HFBA added to the mobile phase was varied to reveal that 0.25% optimized the resolution of NAAG and glutamate. Once established with authentic standards under controlled conditions, this method was tested with in vitro incubations of [^{3}H]NAAG in the presence of PSMA.

Following incubation with PSMA obtained from cell lysates of the LNCaP cell line, the labeled substrate ([^{3}H]NAAG) was separated from its PSMA-mediated

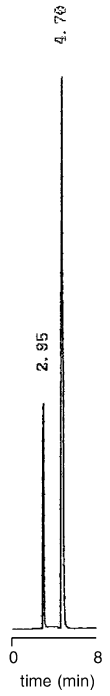

Fig. 2. Typical HPLC chromatogram for PSMA assay.

product ([^{3}H]glutamic acid) by analytical reversed-phase (C-18) chromatography. [^{3}H]NAAG and [^{3}H]glutamic acid elute with retention times of 4.70 and 2.95 min, respectively. A representative chromatogram of an incubation sample in which [^{3}H]NAAG was hydrolyzed to glutamic acid according to the method described under Materials and methods is shown in Fig. 2. Under the conditions of the HPLC analysis, the capacity factors k' for the analytes [^{3}H]NAAG and [^{3}H]glutamic acid were determined to be, respectively, 2.67 and 1.30, with a selectivity factor for their separation of 2.05.

In summary, a simple isocratic reversed-phase HPLC method for monitoring PSMA activity that employs an in-line radioisotope detector has been established. Although the analysis of individual samples can be completed under 10 min, modifications of this method using short-format columns may further reduce analysis time and waste. This method is rapid and convenient and allows for automated analysis when an HPLC system is outfitted with an autosampler. It is anticipated that this method will now allow a more rapid analysis of PSMA and NAALADase activity and will facilitate the screening of inhibitors of these enzymes.

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