Journal of Chromatography, 607 (1992) 25–29
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 328

Rapid fluorimetric assay for the detection of the peptidyl α-amdating enzyme intermediate using high-performance liquid chromatography

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(First received February 25th, 1992; revised manuscript received May 4th, 1992)

ABSTRACT

Peptidylglycine α-amidating enzyme catalyzes the conversion of glycine-extended peptides to their corresponding amidated peptides via a stable α-hydroxyglycine intermediate. Using a new rapid fluorimetric reversed-phase high-performance liquid chromatographic assay, we have demonstrated that the substrate and product of the amidation reaction, as well as both stereoisomers of the α-hydroxyglycine intermediate, can be separated and detected in quantities as low as 1 pmol. The method is highly reproducible and requires less than 11 min for separation and quantification.

INTRODUCTION

Carboxyl terminal amidation is a post-translational modification which is often required for full biological activity of neural peptides and endocrine hormones [1,2]. Peptidylglycine α-amidating enzyme (α-AE) catalyzes the conversion of glycine extended peptides to their corresponding C-terminal amidated peptide products [3,4]. α-AE activity has been identified in and purified from a variety of tissues [5–10]. Early speculation regarding the mechanism of α-amidation suggested that the reaction proceeded through an α-hydroxyglycine intermediate [4,11]. In addition, Young and Tamburini [12] have shown, using a synthetic α-hydroxyglycine tripeptide as a substrate, that α-AE derived from rat medullary thyroid carcinoma cells exhibits a stereobias, where only one stereoisomer of the α-hydroxyglycine peptide is enzymatically converted to amidated product. Recently, an α-hydroxyglycine peptide formed by equine serum α-AE was isolated and characterized [13]. These results support the view that enzymatic amidation proceeds via a two-step mechanism, where formation of a stable peptidyl α-hydroxyglycine intermediate is followed by conversion to a mature α-amidated product. The enzymatic formation of the peptidyl α-hydroxyglycine intermediate by α-AE requires copper, ascorbate [12,13] and molecular oxygen [14]. Conversion of the peptidyl-α-hydroxyglycine intermediate to amidated product is also catalyzed by α-AE, but conversion can occur spontaneously at alkaline pH [13,15,16]. Proteins that possess only one of the two catalytic activities, namely peptidylglycine hydroxylase (PGH) or peptidylamidoglycolate lyase (PAL), have been described recently [13,15–19].

Peptidylglycine α-amidating enzyme

$$\text{R–CONH–CH}_2\text{–CO}_2\text{H} \xrightarrow{\text{PGH}} \text{R–CONH–CH(OH)–CO}_2\text{H} \xrightarrow{\text{PAL}} \text{R–CONH}_2 + \text{OHC–CO}_2\text{H}$$

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0021-9673/92/$05.00

Several rapid sensitive assay systems have been described which employ a variety of separation and detection techniques for measuring amidation activity [3,7,20–24]. All of these assays are based on the conversion of a synthetic glycine-extended peptide to its corresponding peptide amide. None of these commonly used assays, however, was specifically designed to detect the formation of the peptidyl-α-hydroxyglycine intermediate; consequently, the PGH activity cannot be measured. There are only two assays presently available which can selectively detect and measure PGH activity [13,15]. Both of these assays utilize a model peptide substrate and employ reversed-phase high-performance liquid chromatography (RP-HPLC) to separate the enzymatically generated products. Briefly, the corresponding enzymatic products of either Phe–Gly–Phe–Gly–OH or trinitrophenyl–Tyr–Val–Gly–OH are separated by RP-HPLC and detected by monitoring the absorbance at 214 and 344 nm, respectively. Both of these assays are time consuming and possess rather poor detection limits.

We have previously reported the use of a fluorescent RP-HPLC assay system, based on the amidation of a dansyl (Dns) peptide substrate, for measuring enzyme activity [20]. In this communication, we report a modification of the assay that also resolves the α-hydroxyglycine intermediate from substrate and product. This new assay is thus capable of measuring peptidylglycine α-hydroxylase and peptidylamidoglycolate lyase activities.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile (ACN) was obtained from EM Science (Gibbstown, NJ, USA). Sequalog-grade trifluoroacetic acid (TFA) was purchased from Schweizerhall (South Plainfield, NJ, USA). Sodium acetate (NaOAc), tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethane sulfonic acid (MES), piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), 3-[tris (hydroxymethyl)methylamino]-1-propanesulfonic acid (TAPS) and 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxy-1-propanesulfonic acid (AMPSO) were purchased from Sigma (St. Louis, MO, USA).

Peptide synthesis

DNS–Tyr–Val–Gly–OH and Dns-Tyr–Val–NH_2 were synthesized as previously described [20,25]. Synthesis of Dns–Tyr–Val–α-hydroxyglycine–OH and purification of the resulting stereoisomers were carried out as previously described [12].

RP-HPLC assay

RP-HPLC assays were performed using a Hewlett-Packard (Paramus, NJ, USA) 1090 liquid chromatograph equipped with a binary solvent system (DR5), heated column compartment, LUSI controller, auto sampler and auto injector. Separations were achieved on a Keystone (Bellefonte, PA, USA) Hypersil ODS column (100 × 4.6 mm; 5 μm particle size) fitted with a Brownlee (Santa Clara, CA, USA) RP-18 guard column. The substrate, Dns–Tyr–Val–Gly–OH, both stereoisomers of α-hydroxyglycine intermediate, Dns–Tyr–Val–α-hydroxyglycine–OH and amidated product, Dns–Tyr–Val–NH_2, were resolved by gradient elution between 200 m*M* NaOAc pH 6.5 (buffer A) and ACN (buffer B). Elution was carried out at 50°C with a shallow linear gradient from 22–27% B over 8 min, followed by a steep linear gradient to 80% B over 3 min. The column was operated at a flow-rate of 1.2 ml/min. Dansylated peptides were detected using a Gilson (Middleton, WI, USA) 121 fluorimeter equipped with an excitation filter of 352–360 nm and emission cut-off filter of 482 nm. Each peptide was quantified by peak height with reference to standards of known concentration using a Hewlett-Packard 3392A integrator.

Preparation of peptidylglycine α-amidating enzyme

Recombinant type A, relative molecular mass, M_r = 75 000 (A75) α-AE was obtained from stably transformed Chinese hamster ovary (CHO) cells [26] and purified as previously described [27].

RESULTS AND DISCUSSION

RP-HPLC based assay systems utilizing dansylated peptides have proven to be extremely valuable for the study of proteolytic enzymes and other post-translational processing events [20,25,28,29]. Our previously reported fluorescent RP-HPLC α-AE assay, measuring the conversion of Dns–Tyr–Val–Gly–OH to Dns–Tyr–Val–NH_2, would not resolve

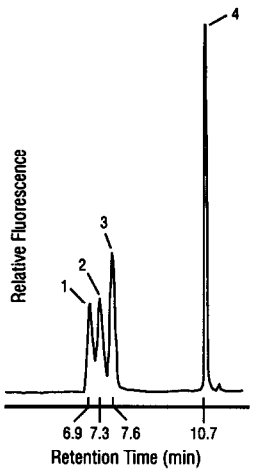

Fig. 1. RP-HPLC separation of dansyl peptides. Each peak represents an equimolar amount (140 pmol) of dansyl peptide. Peaks: 1 = Dns–Tyr–Val–α-hydroxyglycine–OH (stereoisomer No. 1); 2 = Dns–Tyr–Val–α-hydroxyglycine–OH (stereoisomer No. 2); 3 = Dns–Tyr–Val–Gly–OH; 4 = Dns–Tyr–Val–NH_2.

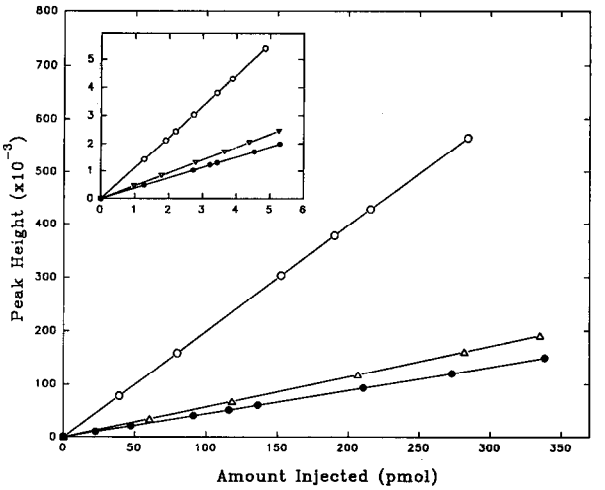

Fig. 2. Typical standard curves for the fluorometric detection of Dns–Tyr–Val–Gly–OH (▽, △), Dns–Tyr–Val–α-hydroxyglycine–OH (●) and Dns–Tyr–Val–NH_2 (○) during RP-HPLC analysis. Each peptide was subjected to analysis in triplicate. Peaks heights are reported in arbitrary units. The inset illustrates the assay sensitivity in the low picomol range.

the substrate from any potential α-hydroxyglycine peptide intermediates. Therefore, we have developed a RP-HPLC assay capable of separating Dns–Tyr–Val–Gly–OH, Dns–Tyr–Val–NH_2 and both stereoisomers of Dns–Tyr–Val–α-hydroxyglycine–OH. Separation of these peptides is achieved by gradient elution between 200 m*M* NaOAc, pH 6.5 and ACN (Fig. 1). The elution is monitored by fluorescence detection and the amount of each dansylated peptide can be quantified by either peak area or peak height. Fluorometric detection allows the direct assay of crude tissue homogenates without interference from other components of the assay mixtures.

Serial dilutions of an equimolar mixture containing both diastereomers of Dns–Tyr–Val–α-hydroxyglycine–OH were analyzed to determine the detection limits of the assay. For either isomer the limit of detection was found to be approximately 1 pmol. A linear relationship between peak height and amount of peptide injected was obtained for each component of the assay (Fig. 2). This relationship allows accurate quantification of the α-hydroxyglycine intermediate, glycine-extended substrate and amidated product in a single assay (Fig. 3). Spontaneous (*i.e.* non-enzymatic) conversion of the peptidyl α-hydroxyglycine compounds, which occurs significantly at alkaline pH [13,15,16], is negligible during the time required for the separation. It should be noted that the RP-HPLC assay reported by Tajima *et al.* [13] employs an ammonium hydrogencarbonate buffer at pH 9.0 to achieve separation of its glycine-extended substrate and α-hydroxyglycine intermediate. Although the α-hydroxyglycine intermediate can be detected using this assay, non-enzymatic conversion of the α-hydroxyglycine intermediate at pH 9.0 precludes the use of this assay for rigorous enzymological studies.

While examining the formation of the α-hydroxyglycine intermediate by A75 α-AE derived from recombinant CHO cells, we noted that the accumulation of intermediate was dependent on assay conditions (Fig. 3). These data show that there is an accumulation of intermediate under both assay conditions, but that the extent of accumulation at pH 5.5 (Fig. 3A) is more than twofold higher than the level attained at pH 7.5 (Fig. 3B). This increase in accumulation occurs despite the fact that the rate of product formation is substantially increased at pH 5.5. The increased formation of amidated peptide in reactions of A75 α-AE at acidic pH has been reported previously [6,27] and is consistent with the

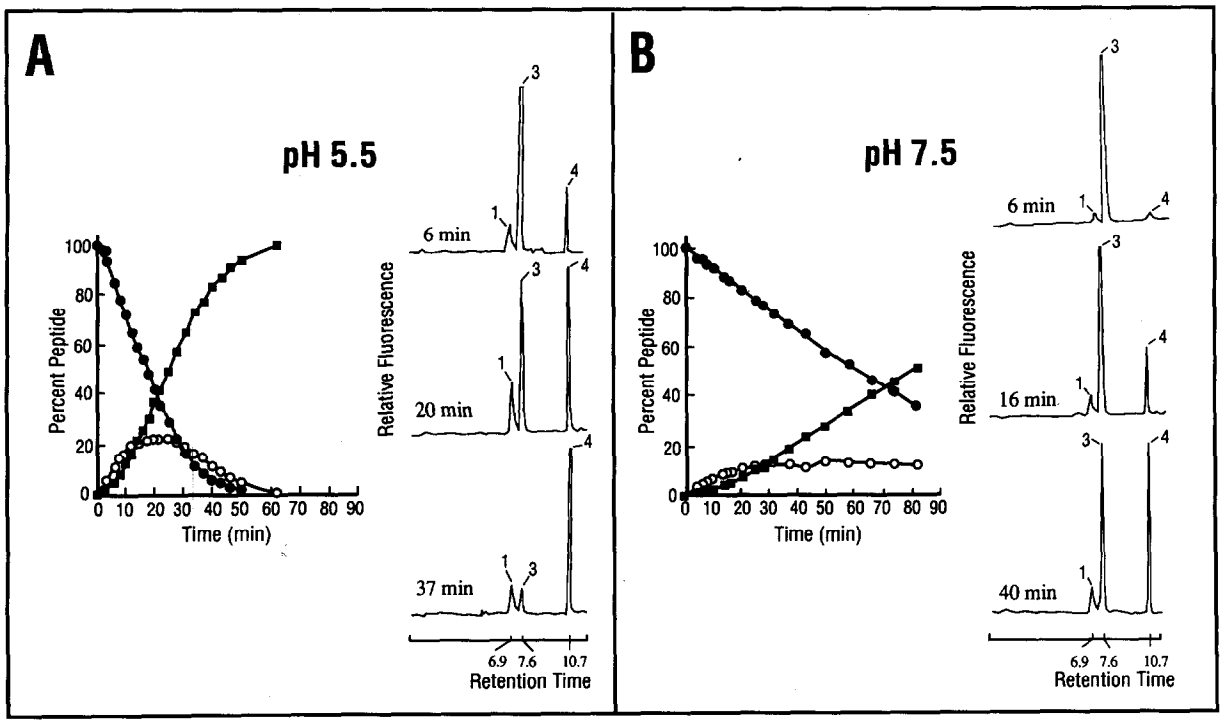

Fig. 3. Time course for the reaction of Dns–Tyr–Val–Gly–OH with A75 α-AE at pH 5.5 and pH 7.5. ● = Dns–Tyr–Val–Gly–OH (substrate); ○ = Dns–Tyr–Val–α-hydroxyglycine–OH (intermediate); ■ = Dns–Tyr–Val–NH_2 (product). Enzyme (0.1 μg, specific activity of 4.6 · 10^6 pmol/min/mg) was added to 900 μl of 25 m*M* mixed buffers, 0.001% Triton X-100, 1 μ*M* $CuSO_4$, 15 μ*M* Dns–Tyr–Val–Gly–OH, 3 m*M* ascorbate and incubated at 37°C. At the indicated time, an aliquot (10–15 μl) was removed and the reaction was terminated by addition of TFA to a final concentration of 1%. Aliquots were analyzed directly using the RP-HPLC assay. The mixed buffers consisted of MES, PIPES, HEPES, EPPS, TAPS and AMPSO adjusted to pH 5.5 (A) or pH 7.5 (B). In each panel (A and B) the time course of the reaction is shown to the left and selected chromatograms at the indicated time points are shown to the right.

fact that the enzyme functions in acidic secretory granules [30]. The RP-HPLC assay clearly demonstrates the ability to efficiently monitor both the PGH and PAL activities.

In the assay, we have included both stereoisomers of the α-hydroxyglycine intermediate, Dns–Tyr–Val–(*R*,*S*)-α-hydroxyglycine–OH, in order to permit further investigation into the mechanism of α-amidation. As mentioned above, Young and Tamburini [12] have shown that only a single stereoisomer of Dns–Tyr–Val–α-hydroxyglycine–OH is converted to α-amidated product. We further substantiated this result by incubating both stereoisomers (*R*,*S*) of Dns–Tyr–Val–α-hydroxyglycine–OH with A75 α-AE and monitoring the conversion to Dns–Tyr–Val–NH_2 using the RP-HPLC assay (data not shown). In addition, the chromatograms in Fig. 3A and B clearly illustrate the stereospecificity of the A75 α-AE catalyzed amidation. With time, only a single stereoisomer of the α-hydroxyglycine intermediate is detected (Fig. 3, peak 1) and subsequently converted to amidated product. We have isolated the α-hydroxyglycine intermediate catalyzed by the A75 α-AE in an attempt to assign absolute configuration (*R* or *S*). However, crystallization of the isolated intermediate necessary to perform the X-ray experiments has been problematic. Since it has been shown that α-AE abstracts the *pro*-S hydrogen of glycine-extended substrates during oxidation [31], it is likely that the stereospecific α-hydroxylation exhibited by the A75 α-AE yields Dns–Tyr–Val–(*S*)-α-hydroxyglycine–OH. In support of this view, α-AE is unable to catalyze the conversion of peptide substrates when the C-termi-

nal glycine residue is replaced with L-amino acids, but is does amidate substrates terminating with D-alanine [32].

In summary, we have developed a rapid and sensitive assay capable of measuring both the PGH and PAL activities which encompass the catalytic activity contained in bifunctional α-amidating enzymes. The assay is ideally suited for the analysis of a large number of samples and can be used for precise measurements of kinetic parameters. This novel assay is extremely useful for the study of multiple forms of α-AE in order to probe their differences and may help to further elucidate the mechanism of α-amidation. The assay is also being used to evaluate the modification of catalytic domains by site-directed mutagenesis.

ACKNOWLEDGEMENTS

We would like to thank Raviraj Kulathila for the purification of the CHO A75 α-AE and Dr. P. P. Shields for helpful discussions in the preparation of this manuscript.

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