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Mass spectrometry assay for studying kinetic properties of dipeptidases: Characterization of human and yeast dipeptidases

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ABSTRACT

Chemical modifications of substrate peptides are often necessary to monitor the hydrolysis of small bioactive peptides. We developed an electrospray ionization mass spectrometry (ESI–MS) assay for studying substrate distributions in reaction mixtures and determined steady-state kinetic parameters, the Michaelis–Menten constant (K_m), and catalytic turnover rate ($V_{max}/[E]_t$) for three metallodipeptidases: two carnosinases (CN1 and CN2) from human and Dug1p from yeast. The turnover rate ($V_{max}/[E]_t$) of CN1 and CN2 determined at pH 8.0 (112.3 and 19.5 s^{-1}, respectively) suggested that CN1 is approximately 6-fold more efficient. The turnover rate of Dug1p for Cys-Gly dipeptide at pH 8.0 was found to be slightly lower (73.8 s^{-1}). In addition, we determined kinetic parameters of CN2 at pH 9.2 and found that the turnover rate was increased by 4-fold with no significant change in the K_m. Kinetic parameters obtained by the ESI–MS method are consistent with results of a reverse-phase high-performance liquid chromatography (RP–HPLC)-based assay. Furthermore, we used tandem MS (MS/MS) analyses to characterize carnosine and measured its levels in CHO cell lines in a time-dependent manner. The ESI–MS method developed here obviates the need for substrate modification and provides a less laborious, accurate, and rapid assay for studying kinetic properties of dipeptidases in vitro as well as in vivo.

Numerous small bioactive peptides play very important roles in many physiological processes [1–3]. Metallopeptidases regulate the homeostasis of these small bioactive peptides, and many of the well-known metallopeptidases have been classified in the family of metalloaminopeptidases [4–7]. Metallopeptidases are found in all types of organisms, but interest in studying human enzymes as well as enzymes of pathogenic organisms is increasing due to their association with many pathophysiological conditions, including cancer, malaria, and diabetes [8,9]. Due to their involvement in a range of disease conditions, metallopeptidases (including dipeptidases) are promising targets for designing therapeutic molecules for controlling pathophysiology of the disease [10]. The design of suitable drugs against these metallopeptidases requires detailed understanding of their kinetic mechanism and obtaining reliable kinetic parameters on substrate hydrolyzes.

Most of the naturally occurring di- and tripeptides do not exhibit significant levels of optical properties. Kinetic characterizations of catalytic mechanisms of peptidases rely mostly on indirect methods such as chemical modification of the substrate peptides with chromogenic molecules and derivatization of product and monitoring of the reaction indirectly [11–13]. The introduction of optically active functional groups synthetically has been shown to improve signal change accompanied by peptidase activity and allows continuous monitoring of reactions spectrophotometrically [12,14]. Not only are derivatized pure substrate peptides expensive, but also chemical modifications may alter the enzyme–substrate interactions, resulting in erroneous estimation of kinetic parameters [15]. Alternatively, chromatographic techniques such as the high-performance liquid chromatography (HPLC)[1] method can be used to resolve reactants and products and to determine kinetic parameters [16,17]. Resolution times of reactants and products are often unpredictable, and multiple runs are needed to obtain one steady-state kinetic curve. In addition, peak broadening at higher substrate concentrations may reduce accuracy [18]. In general, chromatographic methods are more laborious and less accurate compared with optical methods. The development of a sensitive, robust, and rapid assay is very important for studying the kinetic features of a large number of metallopeptidases.

Electrospray ionization mass spectrometry (ESI–MS) has found its way into enzymology and is being increasingly used to study

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; ESI–MS, electrospray ionization mass spectrometry; CN, carnosinase; OPA, *o*-phthaldialdehyde; CHO, Chinese hamster ovary; NTA, nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; UV, ultraviolet; TFA, trifluoroacetic acid; RP–HPLC, reverse-phase HPLC; LC–MS/MS, liquid chromatography tandem MS; TIS, TurboIonSpray; AU, arbitrary units; Q, quadruples; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid.

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the kinetic properties of enzymes [19]. MS with its continuous technological advancement is a viable alternative method for studying enzymatic reactions quantitatively [20]. Although MS methods have been used to identify proteolytically cleaved peptides both in vitro and in vivo, they have not been used to characterize kinetic properties of peptidases. An MS-based assay offers many advantages over conventional approaches for studying peptide hydrolysis by peptidases. First, it does not need any reporter group to be present, and hence no chemical modification of reactants is needed. Second, very small amounts of reactants are needed. Third, assay time is short and results are more accurate compared with results from other methods such as chromatography-based methods. To develop a rapid and sensitive ESI–MS assay, we selected three metallopeptidases: carnosinases (CN1 and CN2) from human and Dug1p from yeast (*Saccharomyces cerevisiae* for this study).

The majority of M20 family peptidases are dipeptidases that hydrolyze a variety of bioactive peptides in many organisms. Carnosinase and Dug1p are metal-dependent M20 family dipeptidases that participate in the degradation of two important bioactive peptides. Dug1p is shown to play a very important role in glutathione degradation and hydrolyzes the Cys-Gly dipeptide, an intermediate in glutathione degradation [21]. Both CN1 and CN2 hydrolyze an important bioactive dipeptide, namely carnosine (β-alanyl-l-histidine) [22,23]. A modified ninhydrin-based assay was used for studying the kinetic properties of Dug1p by estimating the amount of cysteine released during hydrolysis [24]. This assay is an indirect method and cannot be used for studying the kinetic properties of Dug1p for non-cysteine-containing peptides if detailed kinetic mechanism needs to be elucidated. Similarly, a fluorescence-based assay that employs derivatizing the released l-histidine with *o*-phthaldialdehyde (OPA) has been used for monitoring the kinetics of carnosinases [25]. We show here that the fluorescence-based assay has very high background and the fluorescence signal is not stable when monitored as a function of time. Therefore, this method is unlikely to provide accurate kinetic parameters and cannot be used for studying the kinetic mechanism of carnosinases. We have also developed an HPLC-based method for carnosinase and compared kinetic parameters with the results of the MS assay. We found that both the HPLC and MS assays yield reliable parameters within error, but the MS assay is fast and less laborious compared with the HPLC-based assay. We extended this approach for studying kinetic properties of all three dipeptidases and also compared the activities of CN2 at two different pH conditions. In addition, we characterized the time-dependent degradation of carnosine in vivo using Chinese hamster ovary (CHO) cells. In summary, we present a sensitive, accurate, and fast ESI–MS method for studying the kinetic properties of peptidases both in vitro and in vivo.

Materials and methods

Carnosine (99% purity) and Cys-Gly were obtained from Sigma. OPA was obtained from Fluka Analytical. Ni^{2+}–nitrilotriacetic acid (NTA) agarose was purchased from Qiagen. All other reagents were purchased from Sigma–Aldrich and were of analytical grade.

Expression and purification of proteins

CN1, CN2, and Dug1p (pET23a) constructs were a kind gift of Bachhawat [24]. Protein expression and purification were done as described previously [24]. Briefly, CN1, CN2, and Dug1p were induced (A_{600} = 0.8 with 0.1 mM isopropyl-β-thiogalactopyranoside) at 18 °C for 16 h. Cells were harvested by centrifugation (6000*g*), and the pellets were resuspended in lysis buffer (300 mM NaCl, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM Tris–HCl, pH 8.0). Cells were lysed by sonication, and the soluble fraction was recovered by centrifugation (10,000*g* for 20 min). His-tagged proteins were purified using Ni^{2+}–NTA affinity chromatography. Purified proteins were dialyzed against the buffer (100 mM NaCl and 20 mM Tris–HCl, pH 8.0) and further purified by size exclusion chromatography (Sephacryl 26/60, S300, GE Healthcare). Peak fractions were pooled and monitored by running on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Circular dichroism (CD) measurements for all three dipeptidases were performed with a J-810 spectropolarimeter (Jasco) equipped with a Peltier-type temperature controller (PTC-348W). Far-ultraviolet (UV) spectra were obtained in a quartz cuvette with a 1-mm light path length, and each spectrum obtained was an average of 10 scans. The ellipticity of protein CD spectra is reported as mean residue ellipticity (MRE) in deg/cm^2/dmol units.

Enzyme assays

OPA derivative-based fluorescence assay

We employed an OPA-based CN1 activity assay with some modifications [24]. Here 0.33% stock of OPA was prepared in 5% final *n*-propanol solution containing 20 mM Tris–HCl (pH 8.0) and 50 mM NaCl. The final concentration of OPA in reaction was 0.93 mM. OPA was added to carnosine (1 mM) and histidine (0.2 mM) separately. Fluorescence intensities were monitored using a Cary Eclipse spectrofluorometer (Varian) with excitation at 350 nm and emission at 428 nm (5 nm bandwidth).

HPLC-based assay

The recombinant CN1 (0.5–6 μg) was mixed with varying concentrations of L-carnosine (0.3–10 mM) in buffer containing 25 mM Tris–HCl (pH 8.0) and 50 mM NaCl and incubated for 20 min at 37 °C, and the reaction was terminated by adding 0.1% trifluoroacetic acid (TFA). Reactants were resolved by reverse-phase HPLC (RP–HPLC) on an analytical Dionex C18 column (120 Å, 5 μM, 4.6 × 250 mm) using a multistep gradient (see Table 1S in supplementary material). Mobile phase for the CN1 assay included water in 0.1% TFA (eluent B) and 80% acetonitrile in 0.1% TFA (eluent A). The flow rate was 0.5 ml/min, with UV registration at 220 nm.

MS-based assay

Assay conditions were similar to RP–HPLC assay conditions. Reactions were stopped by adding 0.2% formic acid, mixed, and immediately placed on ice for analysis. The same protocol was applied as for the CN2 assay, and it was also performed in the presence of metal ions at higher pH (25 mM Tris–HCl [pH 9.2], 50 mM NaCl, and 0.1 mM $MnCl_2$) for comparison. Kinetics of Dug1p was studied using Cys-Gly as substrate in buffer containing 20 mM Tris–HCl (pH 8.0), 50 mM NaCl, and 50 μM $MnSO_4$ as reported in a previous study [24].

The liquid chromatography tandem MS (LC–MS/MS) system (Shimadzu LC system interfaced with Applied Biosystems QTRAP mass spectrometer) equipped with a TurboIonSpray (TIS) electrospray ion source was used. Source parameters were set as described: curtain gas, 10 arbitrary units (AU); source gas 1, 20 AU; collision-activated dissociation, high; interface heater, on. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.1) was used to control sample acquisition and data analysis. The QTRAP mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. Dipeptides were infused into the QTRAP system with a syringe pump at 10 μl min^{-1}. Carnosine and Cys-Gly MS spectra of both reference and enzymatic product were run for each substrate concentration, and data were acquired in Q1 MS mode with the same parameters. The mass range for the Q1 MS scan was set to *m*/*z* 200 to 300 for

carnosine and 100 to 200 for Cys-Gly with a 1-s scan time, and the quadrupole was set to unit mass resolution.

For quantification, a series of reference samples containing different concentrations of carnosine and Cys-Gly were run. Peak areas for dipeptides were calculated using Bioanalyst software, and area values were taken in triplicates for each concentration. The standard curve was plotted using reference values after data normalization. The peak area in enzymatic product was first normalized in the same way as was done for the reference samples and then quantified according to the standard curve. The ratio of peak intensities of substrates at varied concentrations with calibration standard peak intensities multiplied with concentration of the control should give the concentration of the substrate in the sample:

$$C_s = I_s/I_c * C_c * (V_f/V_i), \quad (1)$$

where C_s is the concentration of substrate in the sample, I_s and I_c are the intensities of sample and control, respectively, C_c is the concentration in the control or concentration estimated from the calibration curve, and V_f and V_i are the final and initial volumes, respectively, and their ratio yields the dilution factor.

Determination of initial velocities from ESI–MS data

Obtaining initial velocities during the linear phase of the enzyme reaction is the key to determining kinetic parameters. Translation of peak intensities into substrate concentrations as in Eq. (1) and estimation of the initial velocities from the amount of substrate used represent a two-step procedure for determining kinetic properties of the enzyme. Concentration of the substrate peptide (C_s) before reaction time ($t = 0$) and after reaction time (t) can be stated as $C_{s,0}$ and $C_{s,t}$, respectively. Both $C_{s,0}$ and $C_{s,t}$ can be calculated using Eq. (1), and initial velocity normalized to enzyme concentration is obtained using the following equation:

$$V_0 = (C_{s,0} - C_{s,t})/(t \times [E]_t)[(C_{s,0} - C_{s,t}) = \Delta S], \quad (2)$$

where t is the reaction time and $[E]_t$ is the total enzyme concentration. Initial velocities calculated were fit to a normal Michaelis–Menten model, which is expressed in terms of V_0 as in Eq. (3):

$$V_0 = k_{cat}[E]_t C_{s,0}/K_m + C_{s,0}, \quad (3)$$

where k_{cat} is the turnover rate constant, $[E]_t$ is the total enzyme concentration, and K_m is the Michaelis–Menten constant.

Cell culture study

CHO cells (CHO-K1) used for in vivo study were grown on RPMI 1640 medium with 10% fetal bovine serum (FBS) in a 75-cm^2 T-flask. After reaching 70 to 80% confluency, cells were harvested by trypsin treatment. Cells were counted by trypan blue dye exclusion method. Then 2×10^5 cells were seeded into four wells of a six-well plate, and 4 ml of 5% FBS containing RPMI 1640 medium was added to each well. Carnosine was added into three wells to the final concentration of 10 mM, and one well was kept as a control. Samples were taken at intervals of 30 min, 1 h, and 2 h. Cells from each well were harvested by trypsin treatment, washed with phosphate-buffered saline, and lysed by hypotonic treatment (1.5 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris, pH 7.9). Then 10 µl of 100× protease inhibitor cocktail was added to 1 ml of lysis buffer, which also contained 1 mM phenylmethylsulfonyl fluoride, 4 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol. Cell pellets were resuspended in 250 µl of lysis buffer and incubated on ice for 10 min with intermittent mixing. Cell lysate was centrifuged at 18,000*g* for 20 min. Protein precipitation was done by perchloric acid treatment at a final concentration of 6% and again centrifuged at 18,000*g* for 20 min. Supernatant was subjected to MS analysis.

Results and discussion

Protein expression and characterization

All three metallopeptidases are expressed as fusion proteins with histidine tags and purified as described in Materials and methods. Size exclusion profiles indicate that all three enzymes elute as a single peak, with peak volumes centered at 160 to 170 ml (see Fig. 1S in supplementary material), and are found to be homodimers. Gel filtration purified fractions were run on 12% SDS–PAGE, and all three dipeptidases were found to be more than 98% pure (Fig. 2S). We used CD spectroscopy to assess the native-like secondary structural properties and found that all three purified proteins are folded (Fig. 1A). The structural model of homologous Dug1p reveals that three-dimensional structures of Dug1p would be very similar to the structure of carnosinase [26]. Both CN1 and CN2 act on carnosine and hydrolyze it into β-alanine and histidine, whereas the physiological substrate for Dug1p is Cys-Gly dipeptide. Chemical structures and reaction schemes for hydrolysis are shown in Scheme 1.

Derivatization of released histidine with OPA is known to increase the fluorescence of OPA-adduct at 428/460 nm when excited at 340/360 nm [27]. An increase in fluorescence intensity has been shown to be associated with the amount of histidine released, and quantitation of released histidine is used for estimating initial veloc-

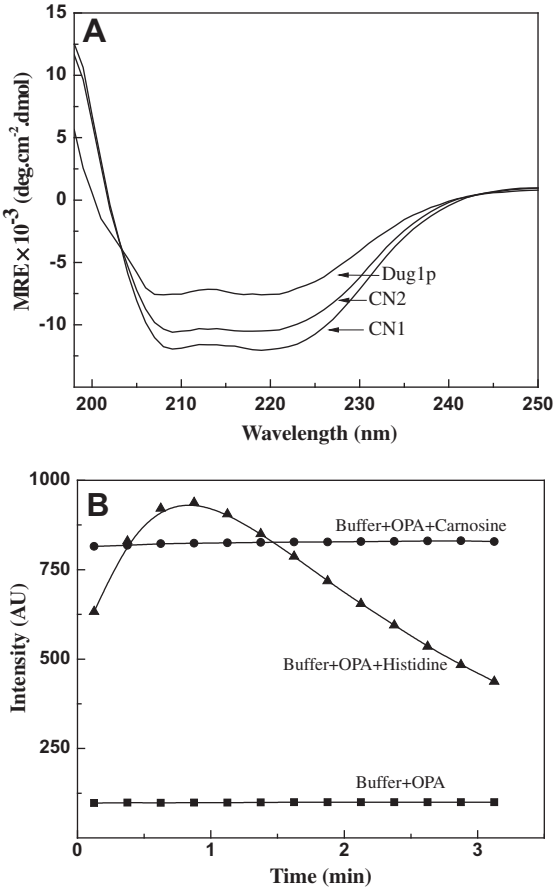
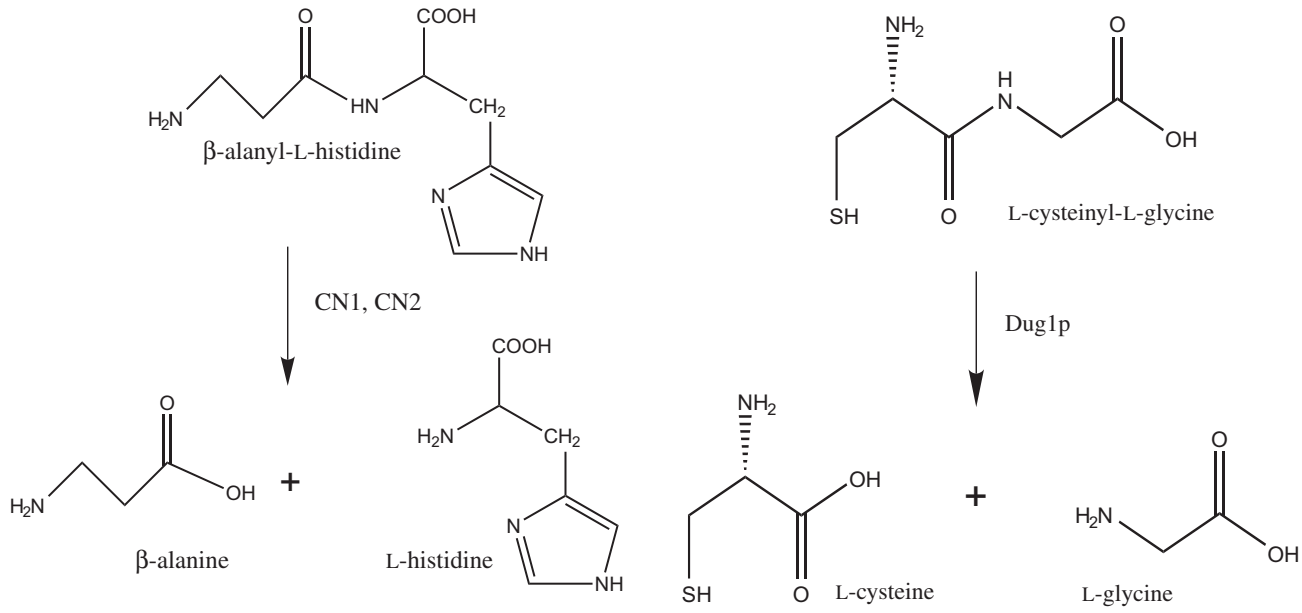


Fig.1. (A) CD spectra of CN1, CN2, and Dug1p at pH 8.0 and 25 °C. CN1, CN2, and Dug1p concentrations were 2.65×10^{-6}, 2.8×10^{-6}, and 3.5×10^{-6} M, respectively. (B) OPA-based fluorescence assay for monitoring carnosinase activity. Carnosine (1.0 mM) and histidine (0.2 mM) were added separately in OPA solution, and fluorescence was measured with increase in time.



Scheme 1. Reaction scheme for dipeptide hydrolysis for CN1, CN2, and Dug1p.

ities. We tested this OPA method for studying kinetics of carnosine hydrolysis. Fluorescence readings of OPA in the presence of either histidine (product) or carnosine (substrate) were compared with OPA fluorescence in the absence of reactants. The steady-state fluorescence properties monitored at 428 nm on excitation at 340 nm are shown for OPA and OPA/reactant mixtures (Fig. 1B). Surprisingly,

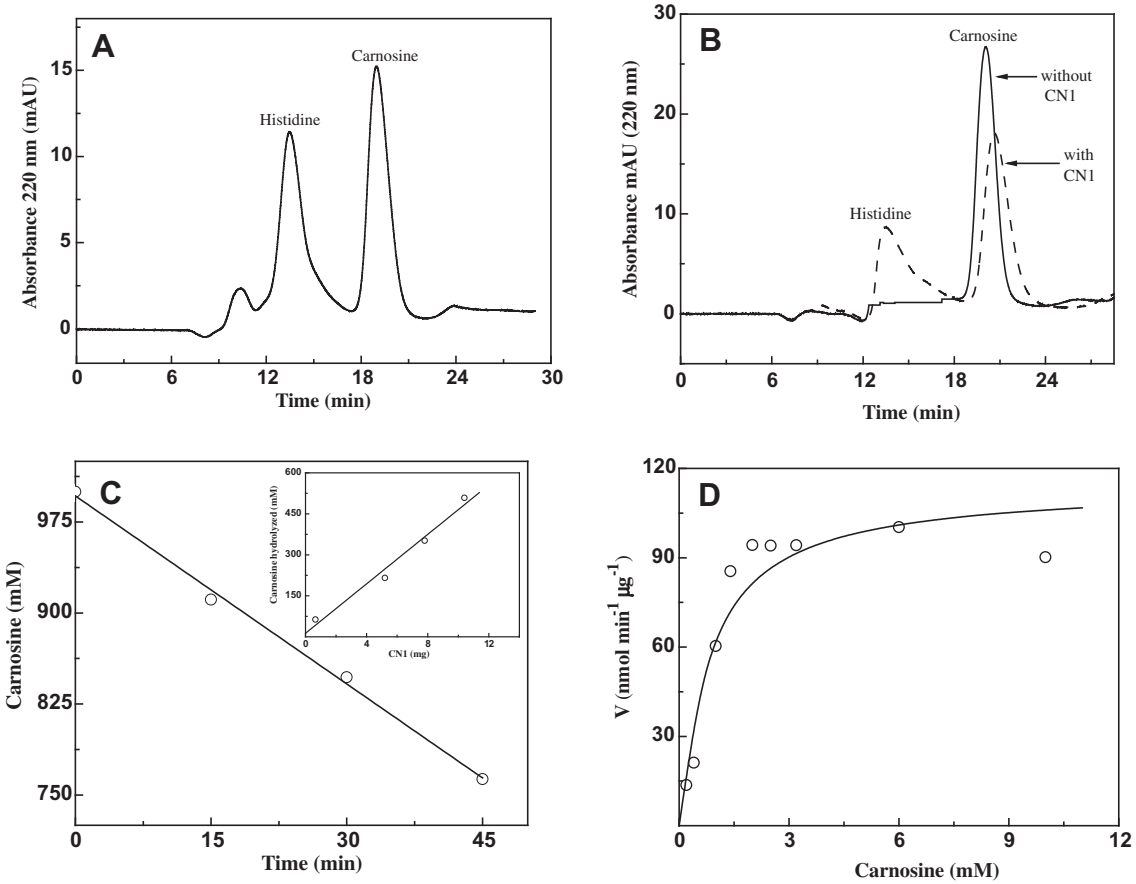


Fig.2. (A) RP–HPLC profile of carnosine and histidine. Reactants were mixed in an equimolar ratio and run on HPLC. A multistep gradient was applied for resolving both of the peaks (see Table 1S in supplementary material). (B) Resolution of substrate (carnosine) and product (histidine) before and after enzyme reaction using RP–HPLC. (C) Time-dependent hydrolysis of carnosine determined from HPLC assay. The amount of carnosine remaining in the reaction was plotted with time. The inset shows hydrolysis of carnosine as a function of CN1 concentrations under defined conditions (20 min of incubation). (D) HPLC-based steady-state kinetics of CN1. Reactions were performed as described in the text. Initial velocities were estimated from the linear phase and plotted against carnosine concentrations.

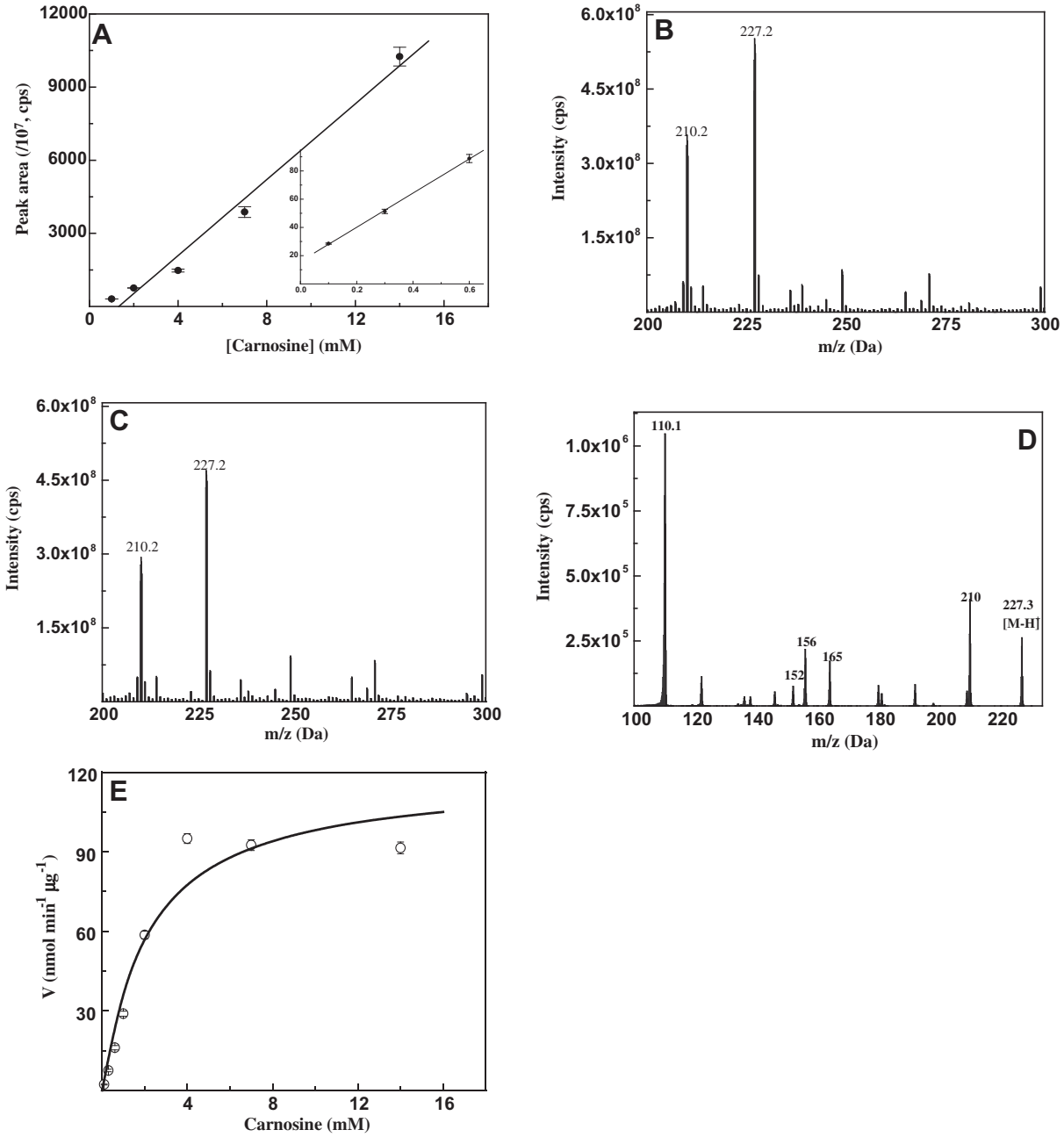


Fig.3. (A) Carnosine standard curve. The calculated peak area plotted against dipeptide concentrations, and peak area was normalized with factor 10^7. The inset shows the data points for lower concentrations. (B and C) MS intensity profiles of carnosine: (B) control; (C) reaction. Carnosine (4 mM) was incubated with 2.4 µg of CN1 for 20 min at 37 °C. Samples were run on Q1MS mode, and intensities (counts per second [cps]) were plotted against the *m/z* value of charged species. (D) MS/MS analysis of carnosine. Ion MS/MS spectra of carnosine and identified species are shown in Scheme 2. MS/MS parameters are mentioned in Table 1. (E) MS-based steady-state kinetics of CN1. Enzymatic reactions were performed and analyzed as described in the text. Initial reaction velocities are plotted against the concentrations of carnosine.

OPA fluorescence increased significantly when carnosine was added in low quantities. The increase in fluorescence in the presence of carnosine (1.0 mM) was similar to the increase in OPA fluorescence in the presence of high amounts of histidine (0.2 mM). However, the fluorescence increase due to the formation of OPA–histidine adduct was not stable and showed nonlinear behavior as a function of time. A short nonlinear rise in the fluorescence during the initial 25 to 60 s and then a steady decrease in fluorescence suggest that reliable kinetic parameters cannot be calculated. These results indicate that OPA–carnosine adduct can yield high background signal that can interfere with the quantitation of OPA–histidine adduct. A recent study showed that OPA can react with carnosine, giving high background fluorescence as observed in this study [28].

RP–HPLC assay for studying kinetics of carnosinases

Separation of substrate and product by RP–HPLC would allow us to monitor the changes in their concentrations and, thus, to determine the kinetic parameters of the enzyme. Equimolar amounts of carnosine and histidine (1 mM each) were mixed and analyzed by the RP–HPLC method. Using simple isocratic or linear gradients, separation of histidine from carnosine was not achievable. A multistep gradient (Table 1S) was used to resolve carnosine and histidine mixtures into two well-separated peaks: one centered at 19.4 min for carnosine and the other centered at 14.2 min for histidine (Fig. 2A). In an enzymatic reaction, 2 mM carnosine was incubated with 2.4 µg of CN1 for 20 min at 37 °C. An aliquot of the reaction

Table 1
Optimized dipeptide-dependent MS/MS parameters.

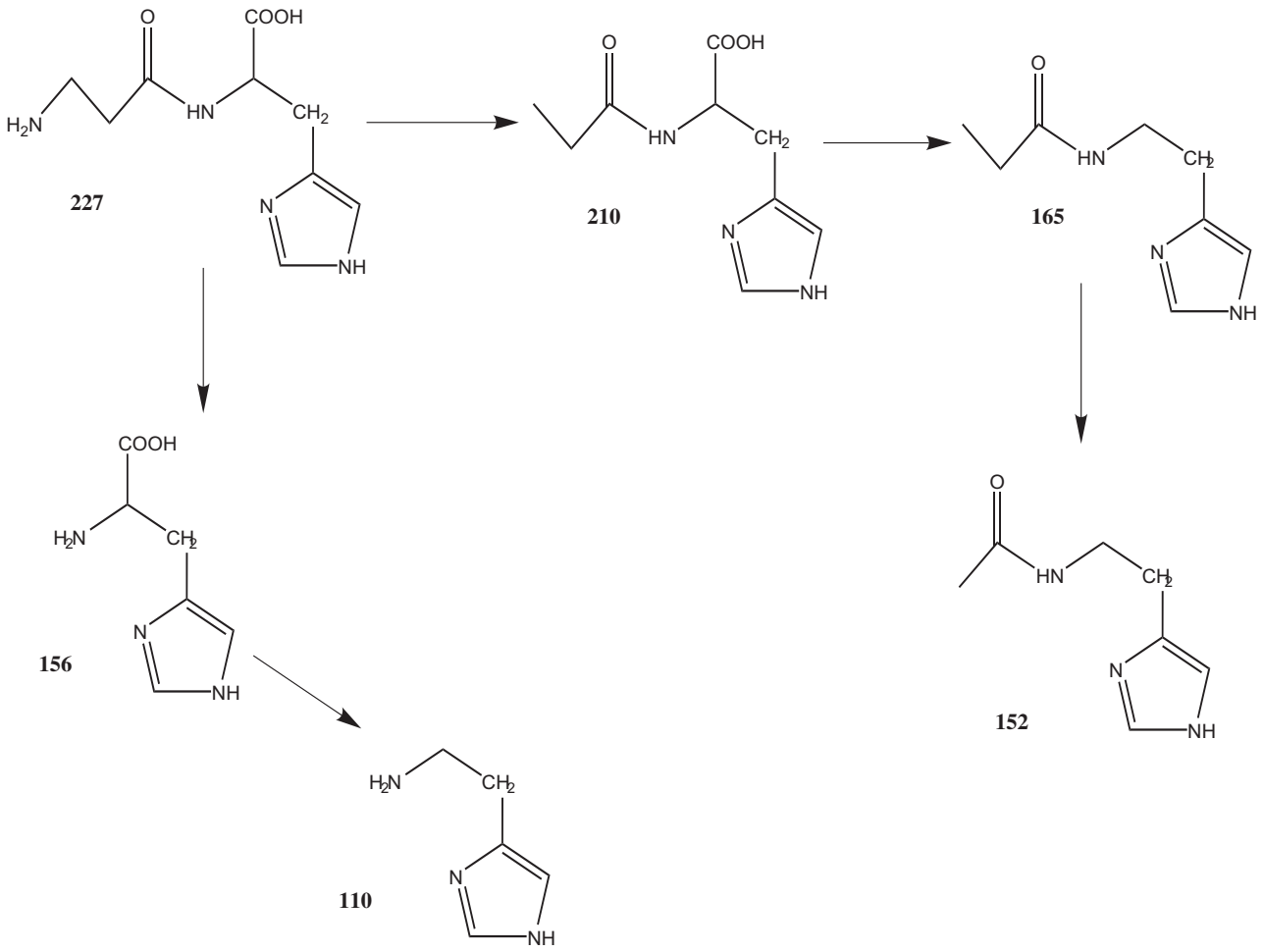
Compound	PM (amu)	CE (au)	DP (V)	EP (V)	Q1R	CEM (V)
Carnosine	227.2	15	60	10	Unit	2400
Cys-Gly	178.1	20	70	10	Unit	2400

Note: PM, precursor ion mass in *m*/*z* of respective dipeptide; CE, collision energy; DP, declustering potential of TIS source; EP, collision cell entrance potential; CEM, continuous electron multiplier.

mixture was injected into the RP–HPLC column and eluted using the multistep gradient. As control, 2 mM carnosine was run and the elution time was compared with retention times of reaction mixture (Fig. 2B). The profile of CN1 hydrolyzed reaction mixture shows two well-resolved peaks that are identified as histidine and carnosine. A linear response of carnosine peak area as a function of concentrations allowed us to estimate the amount of carnosine hydrolyzed after the reaction. We studied the hydrolysis of carnosine as a function of enzyme concentration (at 37 °C, 20 min of incubation) and found a linear response with a correlation coefficient of 0.98 (Fig. 2C, inset). We then studied the time-dependent hydrolysis at a fixed enzyme concentration (2.4 μg) and found that incubation of 20 min would provide accurate estimation of initial velocity (Fig. 2C). We studied rates of hydrolysis at varied substrate concentrations. Kinetic parameters determined for CN1 were K_m = 0.8 mM and $V_{max}/[E]_t$ = 107.5 s^{-1} (Fig. 2D). The HPLC-based assay is more accurate as compared with OPA-based fluorescence methods but is very laborious and time-consuming.

Quantitation of substrate dipeptides and standard curve generation using MS method

We developed the ESI–MS-based assay for studying the steady-state kinetic properties of CN1, CN2, and Dug1p. In the current study, we chose to monitor peak intensities of substrate dipeptides due to the resolution limits of many mass spectrometers for detecting *m*/*z* values below 100 for singly charged species. One of the inherent problems of using ESI–MS for quantitative studies is the lack of a linear relationship between peak intensity and target concentrations. This is because ionization and transmission efficiencies of the target compound in different biological samples are different. Therefore, translating peak intensities into respective concentrations is not likely to yield a linear relationship. Using an internal standard (isotopically labeled) in reaction mixtures provides a reference point [29]. The ratio of peak intensity of internal standard to the signal intensity of the target at any point multiplied by the concentration of internal standard is likely to neutralize any possible errors arising from different sample conditions [30]. However, including isotopically labeled internal standard peptides in the reaction mixture is not a viable option for studying kinetics of peptidases because labeled peptides can also be cleaved by peptidases. Another possible option is to include compounds with very similar chemical properties (analogue dipeptides) with similar ionization and transmission efficiencies as internal standards [31]. In the case of studying peptide hydrolysis by peptidases, spiking another dipeptide as internal standard is a rather critical issue because of the promiscuous nature



Scheme 2. Proposed fragmentation pathway for carnosine. MS/MS spectrum peaks are analyzed for their *m*/*z* values.

of peptidases or the abilities of other peptides to bind to active sites without getting hydrolyzed. Our previous study showed that a potential "nonsubstrate peptide" could compete with the "substrate peptide" and, thus, inhibit the peptidase activity [26].

A more conventional method is the use of a calibration curve for the quantification of substrate and products. This method can be applied successfully to quantitate substances when assay conditions are not different from dilute aqueous conditions. In addition, quantitation can be more rigorously checked if each set of experiments performed at known concentrations is accompanied by a reference run (reaction mixture without enzyme) and calculation of peak intensities. Therefore, we standardized a normalization method to neutralize any variability arising from the concentration-dependent effect. To avoid any concentration-dependent changes in ionization and transmission efficiencies, we diluted samples with high substrate concentrations to a low concentration range where both ionization and transmission efficiencies are similar and scaled their intensities to determine the initial concentration. To cover the dynamic range of substrates and increase the sensitivity in quantification, we used two standard curves separating the high and low concentration ranges of substrates (Fig. 3A; see also Table 1). The r^2 for both of the standard curves is greater than 0.99, and the signal-to-noise ratio at the lowest concentration (0.025 mM) is more than 4.0. Standard curves for both carnosine and Cys-Gly were generated, and each experiment was performed with an additional reference experiment in the absence of enzyme. The concentration of the substrate in the assay sample can be quantitated from the calibration curve and control sample, as described in Materials and methods (Eqs. (1)–(3)). We show here that this method allows us to study the steady-state kinetics of peptidases over a dynamic range of concentrations.

Determination of kinetic parameters for CN1, CN2, and Dug1p

All measurements were performed as described in Materials and methods. The optimal enzyme concentration and reaction time were estimated from Fig. 2C. ESI–MS spectra for carnosine hydrolysis by CN1 are shown in Fig. 3B and C. Comparison of different peak intensities between control experiments where no enzyme is present with peak intensities from reaction sample indicates that intensities of two major peaks, one with an m/z ratio of 227.3, the expected value for singly charged species of carnosine, and an additional peak at 210.3, are significantly reduced in the reaction sample. A previous study reported that the 210.3 peak is the fragment of carnosine in which the amino group is removed [32]. To confirm this, we used MS/MS analyses of carnosine and studied its fragmentation pattern under our experimental conditions. The MS/MS spectrum of carnosine and chemical structures of identified fragmented products are shown in Fig. 3D and Scheme 2. As shown in Scheme 2, we confirmed the identity and structure of the 210.3 peak, which is a fragmented intermediate resulting from the cleavage of β-amino group of β-alanine. Observation of the 210.3 peak in the ESI–MS spectra indicates that the C–N bond linking the β-amino group of β-alanine is very sensitive to lower ionization energies applied in the Q1 mode. Therefore, the total carnosine concentration in the sample is calculated from adding both peak intensities. The reaction was initiated by adding enzymes to reaction sample, and initial velocities were calculated using Eqs. (1)–(3). Initial velocity data (nmol min^{-1} μg $protein^{-1}$) were plotted against varied substrate concentrations and analyzed using nonlinear regression fitting to Eq. (3) (Fig. 3E).

The kinetic parameters (K_m and turnover rate, $V_{max}/[E]_t$) determined for CN1 and CN2 at pH 8.0 were as follows: for CN1,

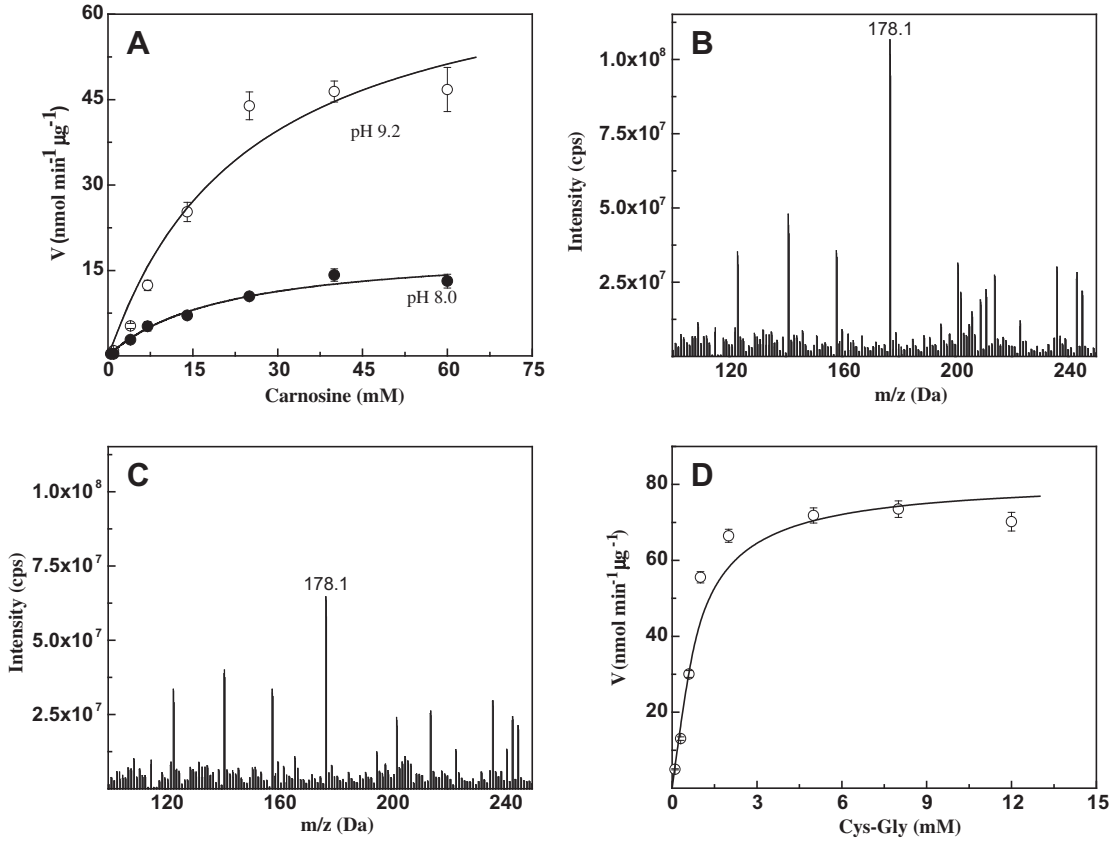


Fig.4. (A) MS-based steady-state kinetics of CN2 at pHs 8.0 and 9.2. Enzyme (2.4 μg) was incubated with different concentrations of carnosine. Reaction was performed at 37 °C for 20 min. Initial reaction velocities are plotted versus the concentrations of carnosine. (B,C) MS intensity profiles of Cys-Gly: (B) control; (C) reaction. Here 2 mM Cys-Gly was incubated with 0.5 μg of Dug1p for 12 min at 37 °C. Samples were run on Q1MS mode. The m/z value of charged species was plotted against the intensity (counts per second [cps]). (D) Steady-state kinetics of Dug1p. Initial reaction velocities are plotted against the concentrations of Cys-Gly.

Table 2
Kinetic parameters for CN1, CN2, and Dug1p.

Enzyme	K_m (mM)	V_{max} (nmol min^{-1} μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
CN1	2.12 ± 0.48	119.02 ± 4.32	112.32 ± 5.46	52.97 ± 12.27
CN2 (pH 8.0)	20.5 ± 5.66	19.06 ± 2.12	19.48 ± 0.83	0.95 ± 0.26
CN2 (pH 9.2)	25.0 ± 5.11	72.6 ± 3.62	74.18 ± 5.11	2.97 ± 0.64
Dug1p	0.78 ± 0.12	81.5 ± 3.12	73.8 ± 4.42	94.6 ± 15.6

K_m = 2.12 mM and $V_{max}/[E]_t$ = 112.3 s^{-1}; for CN2, K_m = 20.5 mM and $V_{max}/[E]_t$ = 19.48 s^{-1} (Fig. 4A and Table 2). The turnover rate for CN1 and CN2 at pH 8.0 suggested that CN1 is approximately 6-fold more efficient for carnosine hydrolysis (Table 2). The K_m reported in this study for CN1 is comparable to the previously reported value (1.27 mM) determined by the HPLC-based method, but details of kinetic parameters were not reported in the previous study [23]. Reported K_m values for CN1 vary from 0.07 to 8.0 mM, but comparisons of details of kinetic parameters of CN1 and CN2 are limited [25,33]. Variation of kinetic parameters indicates that results are assay dependent due to the modification of substrate or difficulties associated with quantifying derivatized product (OPA–histidine adduct). Teufel and coworkers [23] reported that carnosine cannot be hydrolyzed by CN2 at physiological pH. In this study, we observed that although the rate of catalysis is lower, CN2 can degrade carnosine with a turnover rate of 19.5 s^{-1}. Thus, ESI–MS is more sensitive and can be used to determine kinetic parameters for peptidases.

Previous studies showed that activity of CN2 increases at higher pH (>8.5) [23,27]. To extend our ESI–MS analyses for pH-dependent study, steady-state kinetics of CN2 was monitored at pH 9.2 (Fig. 4A). Surprisingly, there was no significant change in K_m for CN2 at pH 9.2, but the turnover rate increased approximately 4-fold ($V_{max}/[E]_t$ = 74.2 s^{-1}). Although previous studies showed that CN2 activity is increased at pH 9.2, our study suggests that the activity increase is not due to increased affinity for the substrate but rather is due to increased catalytic turnover. Next, we studied the kinetics of Dug1p dipeptidase using Cys-Gly as substrate. ESI–MS spectra show that hydrolysis of Cys-Gly peptide can be monitored by following the changes in the 178.1 peak intensity (Figs. 4B and 4C). We characterized the 178.1 peak as a singly charged species of Cys-Gly peptide and determined the kinetic parameters (K_m = 0.78 mM and $V_{max}/[E]_t$ = 73.8 s^{-1}) (Fig. 4D). Kaur and coworkers characterized the Dug1p using a modified ninhydrin method that monitors the release of cysteine [24]. Comparison of kinetic parameters shows that the kinetic constants obtained from MS are comparable to previously reported values, but considering the process steps involved in the ninhydrin assay, it is easier to operate the MS-based assay. Besides, the substrates without cysteine cannot be monitored through the ninhydrin assay. To test whether the ESI–MS assay is amenable for studying the effect of buffer conditions on peptidase activity, we studied the effect of EDTA on the activity of CN1. Hydrolysis of carnosine by CN1 in the presence of EDTA was checked at two different EDTA concentrations (0.1 and 1.0 mM) (Fig. 5A). CN1 is a metallopeptidase, and the presence of EDTA in the buffer will reduce the CN1 activity. ESI–MS study shows that the rate of carnosine hydrolysis is reduced in the presence of EDTA, indicating that our approach described in this study can be used for studying kinetic properties of metallodipeptidases.

Determination of carnosine level in CHO cells

CHO cells were grown and prepared as described in Materials and methods. Samples were run on MS after appropriate dilution

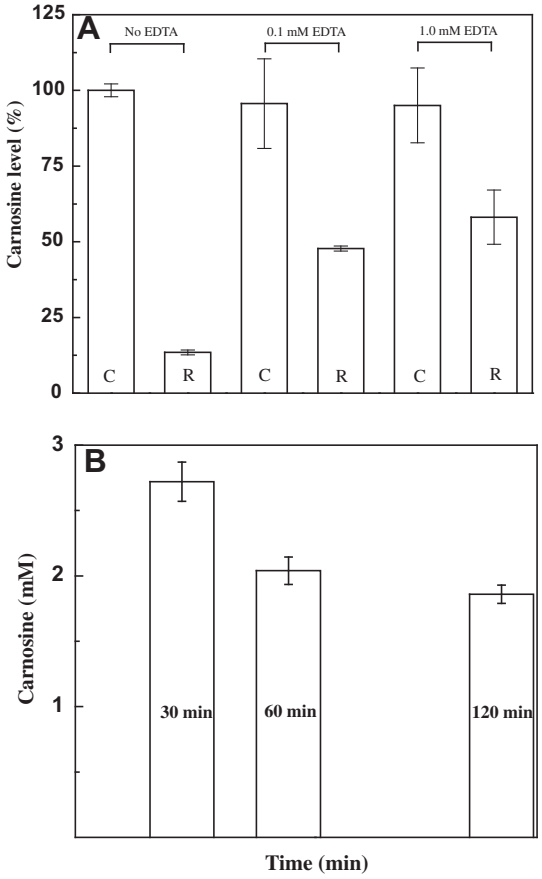


Fig. 5. (A) Effect of EDTA on CN1 activity. The concentration of EDTA added to the reaction mixture is indicated, and the amount of carnosine remaining in the reaction was compared with total carnosine at the beginning of the reaction by MS with the same method. C, control–no enzyme; R, reaction–2.4 μg of CN1. (B) In vivo quantitation of carnosine uptake by CHO cells at different time intervals. The experiment was performed as described in the text. Cells were harvested at different time points, and levels of intracellular carnosine were quantified by ESI–MS.

using the same parameters. After the initial characterization of the observed 227 peak as carnosine by MS/MS analysis (Scheme 2), the amount of carnosine at different time periods was determined using a standard curve. While growing CHO cells in the presence of 10 mM carnosine, we observed that carnosine was taken up by the cells and the level of carnosine increased with time, reaching its highest level (2.75 mM) at 30 min (Fig. 5B). We took 0.2 million cells for the analysis and quantitated the total carnosine concentration. Normalizing by total number of cells used for analysis, we estimate that on average 13.8 nM carnosine is present in the single cell. The kinetics of carnosine influx suggests that after an initial increase, carnosine levels drop to approximately 10.0 nM and reach a steady state. This new steady state may indicate that the rate of carnosine uptake may be equal to rate of carnosine hydrolysis by carnosinase present in the cell. It was shown that extracts of recombinant CHO cells show carnosinase activity [23]. However, the amount of carnosinase present inside the cells cannot be estimated from this study.

Conclusion

MS can be operated in different modes as per the experimental requirements and is becoming a powerful analytical technique in enzymology for monitoring reaction rates and determining kinetic parameters [34–37]. Due to the difficulties associated with

studying kinetics of hydrolysis of small peptides by peptidases, the development of a simple and rapid MS method for determination of kinetic parameters for peptidases is of great interest because many peptidases are drug targets. In this study, we used ESI–MS to characterize kinetic parameters of three peptidases and also quantitated carnosine levels in vivo. Carnosine is an important bioactive peptide, and development of a rapid quantitative assay is useful for detecting the amount of carnosine in clinical samples. Compared with previously described fluorescence and HPLC-based assays that have sensitivity and time issues, the MS system provides a rapid, robust, and accurate method that can be further extended for studying kinetic properties of other dipeptidases. The reported HPLC-based system needs to resolve the peaks of substrates and products, which is sometimes difficult, and also needs a time-intensive gradient run (50–60 min per HPLC run vs. 5–6 min per run with this assay). The developed ESI–MS analysis technique displays linearity over a wide range of analyte concentrations, and the intensity normalization procedure followed can be easily adopted for any peptidase assay. Considering that a large number of assays are needed to study the kinetic mechanism of peptidases, the ESI–MS technique is more suitable in terms of both accuracy and time, and nearly 15 times more assays can be performed in a day without sacrificing reliability of reactant detection and quantification. The HPLC and spectroscopy methods are difficult to implement for biological samples. Time-dependent quantitation of carnosine in CHO cells using the ESI–MS method indicates that ESI–MS can be used in a wide range of clinical and biological studies for discerning the role of dipeptides in different biological processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.06.029.

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