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Note

Simultaneous analysis of different species involved in hexaglycine hydrolysis

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Due to advances in biotechnology and modern methods of synthesis, there is a great interest in developing therapeutic agents that are peptide and protein based[1]. One of the important requirements towards using peptides and proteins as therapeutic agents is an understanding of their nature such as physicochemical properties, enzymatic reactions and transport across membranes. Glycine peptides of different chain lengths have been used as model peptides by many researchers to investigate transport across various membranes[2–5]. Our group is using peptides of 2–6 glycine units to study physicochemical properties and transport behaviour across membranes. The high-performance liquid chromatography (HPLC) assays of various glycine peptides using C_8 bonded phase[6] and NH amino column[7] were found unsatisfactory due to poor separations and long retention times. Another assay for 2–6 amino acid peptides using a C_{18} column with 0.1 *M* phosphate buffer at pH 2.1 or 0.6 *M* perchloric acid at pH 0.2 showed extremely poor retention times for glycine peptides[8].

One of the properties under investigation was the acid-catalyzed hydrolysis which involved the starting material hexaglycine (HG) and different intermediates such as pentaglycine (PG), tetraglycine (FG), triglycine (TG) and diglycine (DG). In order to study the kinetics of this peptide, an effective HPLC assay was required to analyze all intermediates and final products. There were two requirements for the assay. First, the use of an aqueous mobile phase, since the hydrolysis studies were conducted in aqueous media; and second, analyses with HPLC without pretreatment of the samples. This was particularly important because pretreatment could alter the composition of different species in hydrolysis samples.

EXPERIMENTAL

Standard materials

Glycine (G), DG, TG, FG, PG and HG were of high-purity grade and were obtained from Sigma. Pentanesulfonic acid, sodium dihydrogenphosphate and phosphoric acid were used as supplied by Aldrich.

Standard solutions

Stock solutions for all glycine peptides were prepared by using 20 mg of peptide

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in 100 ml of doubly distilled water. A standard solution containing 0.01 mg/ml of HG, PG, FG, TG, DG and 0.1 mg/ml of glycine was prepared by mixing different volumes of stock solutions.

Apparatus

The analytical system consisted of an LDC/Milton Roy ConstaMetric III metering pump with a 20-μl fixed-loop injector and a SpectroMonitor 3000 variable-wavelength detector set at 205 nm and 0.1 a.u.f.s with a signal routed to a Perkin-Elmer programmable integrator for peak area integration.

Columns and HPLC eluents

A Val-U-Pak HP analytical column (Regis, Morton Grove, IL, U.S.A.), 250 × 4.6 mm I.D., packed with 5-μm spherical ODS silica (reversed phase) was used. The eluent consisted of 10 m*M* sodium dihydrogenphosphate and 5 m*M* sodium pentanesulfonate (ion pairing agent) in water which was adjusted to pH 2.7 with phosphoric acid. The flow-rate was adjusted to 1.0 ml/min.

Hydrolysis studies

The hydrolysis studies were conducted at various temperatures (80, 85 and 90°C) in 0.1 *M* HCl. The reaction mixture consisted of a mixture of 50 mg of peptide and 50 ml of 0.1 *M* HCl. Samples of 1 ml were withdrawn at various time intervals (0, 4, 8, 12, . . .100 h). The samples were mixed with 1 ml of ice-cold 0.1 *M* sodium dihydrogenphosphate to stop the reaction by increasing the pH to approximately 3 and decreasing the temperature to below 30°C. (Under these conditions the peptide hydrolysis was negligible for the duration of the analysis.)

RESULTS AND DISCUSSION

A typical chromatogram representing the separation of different compounds is shown in Fig. 1. It can be seen from the chromatogram that the retention time of each component increased with an increase in the number of amino acids in the peptide.

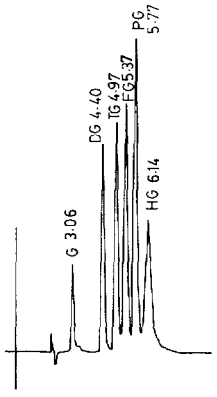

Fig. 1. Chromatogram showing peaks for glycine (G), diglycine (DG), triglycine (TG), tetraglycine (FG), pentaglycine (PG) and hexaglycine (HG).

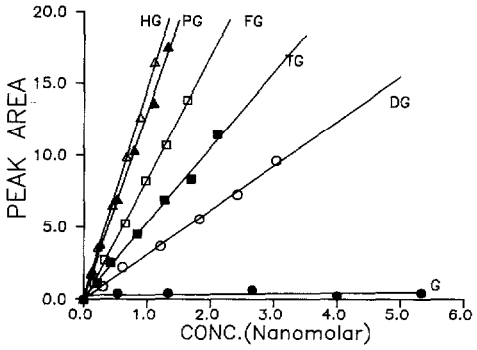

Fig. 2. Calibration curves for different species for peak area *versus* concentration.

The peaks were calibrated with both peak area and peak height as shown in Figs. 2 and 3. The calibration plot of peak area *versus* concentration (Fig. 2) shows that the slope gradually increases as the number of amino acids in the chain increases. On the other hand, the calibration plots of peak height *versus* concentration (Fig. 3) do not show a uniform pattern. This is probably due to broadening of the peaks and a decrease in peak height at longer retention times.

A comparative analysis indicated that both peak height and area under the peak were equally reproducible. The calibration constants obtained by linear regression ($r^2 \geq 0.99$) in area per nmol were as follows: G = 110.12; DG = 3093.84; TG = 5277.08; FG = 8355.2; PG = 12 950.83; HG = 14 648.4; and in height per nmol were G = 962.3; DG = 37 982.1; TG = 63 894.6; FG = 101 520.2; PG = 174 103.9; HG = 89 297.2. A plot of capacity factor, k', *versus* chain length is shown in Fig. 4. The relationship is linear for all peptides except glycine. This is probably due to the zwitterionic and highly polar nature of glycine. The pK_a of glycine is 2.4 and the pH of the mobile phase is 2.7. Therefore, approximately 66% of glycine will be present as zwitterion which has a relatively higher polarity as compared to the other peptides.

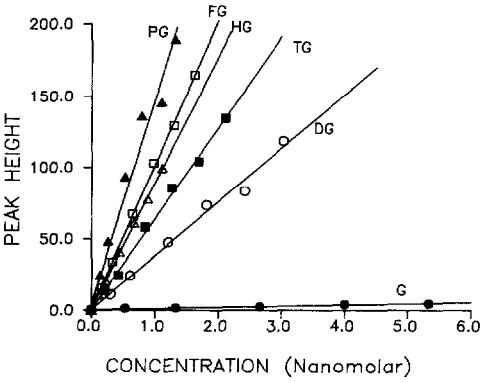

Fig. 3. Calibration curves for different species for peak height *versus* concentration.

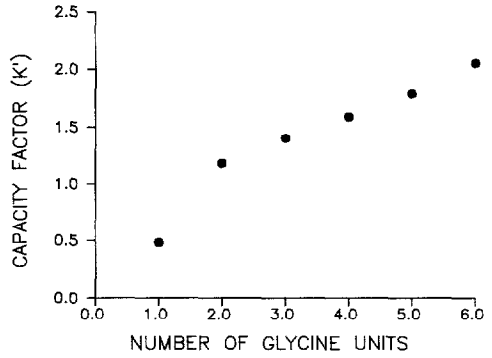

Fig. 4. Capacity factor (k') *versus* number of glycine units.

Application to peptide hydrolysis

The above assay was used to analyze different kinetic samples obtained from hydrolysis studies. A typical set of kinetic data is shown in Fig. 5. The plot shows that the concentration of HG decreases exponentially, whereas the concentrations of PG, FG and TG increase initially, reach a maximum and then decrease exponentially. This indicates that the above compounds are present as intermediates during hydrolysis. The rate of diglycine hydrolysis was found to be much smaller than other rates. Therefore, the concentration profil for diglycine reached a maximum at longer times (approximately 120 h, not shown in the figure) and then decreased exponentially. The concentration of glycine increased exponentially as a function of time. The glycine data were not used in the determination of the rate constants, and hence not shown in the figure. This assay was used for extensive hydrolysis studies of various glycine peptides and results will be reported in a subsequent paper[9]. It was found that for the duration of the assay all species were stable.

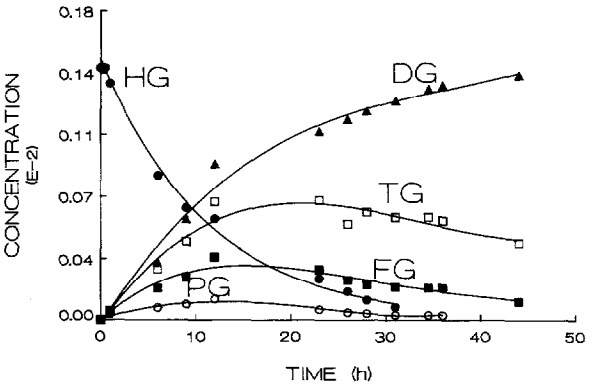

Fig. 5. Concentration *versus* time plots for various species during acid-catalyzed hydrolysis.

CONCLUSIONS

A fast and efficient HPLC assay was successfully developed to analyze glycine peptides. All species were stable during the analysis. The assay was successfully used to study hydrolysis kinetics of hexaglycine. This assay can also be used to study the kinetics of various intermediates independently.

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