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KINETIC ANALYSIS OF THE CARBOXYPEPTIDASE A HYDROLYSIS OF OLIGOPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC)-based method was developed to follow the time course of the hydrolytic action of pancreatic carboxypeptidase A on oligopeptide substrates of the general formula benzoyl-(glycyl)$_n$-L-Phe, n being in the range 1–5. The proposed procedure is sensitive at the nanomolar level of substrate, but also allows the kinetics of substrate hydrolysis to be investigated over a wide range of concentrations. The prior quenching of the carboxypeptidase activity and the use of isocratic conditions for the rapid separation of the substrates and their products (in less than 8 min for the slowest one), allow the automation of the procedure, which could become an easy and versatile alternative to the commonly used spectrophotometric methods. A comparative evaluation of the use of a spectrophotometric method to measure carboxypeptidase A activity on the same substrates indicated that the latter is not valid for long oligopeptides ($n \geqslant 2$) at concentrations higher than 5 mM owing to an interference of a physico-chemical nature. For benzoylglycyl-L-Phe ($n = 1$), the apparent kinetic parameters were calculated by means of the HPLC method and by a well established spectrophotometric procedure, and both yielded similar values.

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INTRODUCTION

Pancreatic carboxypeptidase A (CPA, peptidyl-L-amino acid hydrolase, E.C. 3.4.17.1) catalyses the cleavage of the terminal bond of peptides and proteins with an aromatic or branched-aliphatic amino acid at the C-terminus[1]. The enzymic properties of CPA are usually studied by measuring the hydrolysis of synthetic dipeptide derivatives, either by continuous methods (spectrophotometric[2–4], fluorimetric[5] and conductimetric[6]) or by discontinuous spectrophotometric procedures[7–9]. A modified ninhydrin method for the determination of the C-terminal amino acid released during the hydrolysis[10] is the only one described for the analysis of the CPA-catalysed cleavage of longer oligopeptides such as those of the general formula benzoyl(Gly)$_n$-L-Phe, n being 1–5[11]. As this procedure requires large amounts of substrate and is also subject to interference from other ninhydrin positive substances[12], we became interested in finding an alternative procedure to quantify the CPA kinetics for the hydrolysis of the above-mentioned oligopeptides, which could be useful in a range of concentrations from 50 μM to 50 mM. Our aim is to use these substrates and analytical procedures to characterize further the enzymic properties of porcine CPA and its zymogens, which have been extensively studied in our laboratory[13–15].

In an initial attempt, an spectrophotometric assay was used to follow the decrease in absorbance in the peptide UV band. This had to be performed in the region of 220 nm for oligopeptides with $n \geqslant 2$, thus making necessary the use of very thin cells (0.1 mm optical path). Under these conditions we found an interference, of a physico-chemical nature, with the measurement of the enzymic activity that made its correct determination very difficult. Several tests were carried out in order to identify and to eliminate this interference, none of which was successful.

Because of these problems, we decided to develop a semi-automated method based on the instantaneous quenching of the action of CPA on peptide substrates by addition of trifluoroacetic acid (TFA), followed by separation and quantification of the components of the reaction mixture by reversed-phase high-performance liquid chromatography (HPLC). This method provides reliable kinetic measurements for a wide range of substrate sizes and concentrations in comparison with control spectrophotometric assays carried out at low substrate concentrations. The feasibility of this procedure is illustrated by the determination of the apparent kinetic parameters for the CPA hydrolysis of benzoylglycyl-L-Phe (BGP) by both spectrophotometric and HPLC methods. The results are also compared with those given in the literature.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system, with a WISP 710B automatic injector, a Model M730 data module and an Altex Ultrasphere C_{18} ODS (particle size 5 μm) column (4.5 cm × 4.6 mm I.D.) was used. A double-beam Hitachi Model 220 thermostated spectrophotometer was used to calculate peptide and protein concentrations and to record CPA activity.

Chemicals

All chemicals were of analytical-reagent grade. Tris (Trizma-base) (Sigma),

sodium chloride (Merck) and zinc chloride (Panreac, Spain) were used for buffer preparation. Dithizone (Merck) and carbon tetrachloride (Merck) were used for metal extraction. Norganic-filtered Millipore water, HPLC-grade acetonitrile (Carlo Erba) and TFA (Aldrich) were used for all HPLC experiments.

Enzymes

Porcine pancreatic CPA_α was obtained from partial tryptic activation of pro-carboxypeptidase A (PCPA) by the method described previously[13]. The enzyme concentration was calculated spectrophotometrically using $A^{1\%}_{280} = 19.6\ l\,mol^{-1}\,cm^{-1}$ (ref. 2).

Oligopeptide substrates and products

Hippuric acid (BG), BGP, N-(2-furanacryloyl)-L-phenyalanyl-L-Phe (FAPP) (Sigma), benzoylglycyl-Gly (BG2) and benzoyl(glycyl)$_2$-Gly (BG3) (Bachem) were used without further purification. Benzoyl(glycyl)$_4$-Gly (BG5) and benzoyl(glycyl)$_5$-L-Phe (BG5P) were synthesized by the procedure of Spilburg *et al.*[16] with minor modifications. The same method was adapted to the preparation of benzoyl(glycyl)$_2$-L-Phe (BG2P) and benzoyl(glycyl)$_3$-L-Phe (BG3P) via reaction of L-Phe with the N-hydroxysuccinimido esters of BG2 and BG3, respectively.

All the synthesized compounds were purified by recrystallization (ethanol–water) until chromatographic homogeneity [thin-layer chromatography on silica gel plates using chloroform–methanol–water (83:15:2) as solvent] and characterized by infrared and ^1H (200 MHz) and ^{13}C (50 MHz) NMR spectroscopy. The amino acid composition and concentration of the above oligopeptides in solution were checked by amino acid analysis following a dabsyl chloride–HPLC method[17].

Buffer preparation and metal extraction

A 50 m*M* Tris–HCl–0.5 *M* NaCl buffer (pH 7.5) was used for enzymic experiments. Zinc chloride up to 1 μM was always added before carrying out any CPA activity measurement in order to preserve the enzyme integrity[11]. When indicated, buffer and oligopeptide solutions were extracted with dithizone dissolved in carbon tetrachloride in order to remove metal ions[11].

Spectrophotometric-based kinetic analysis

BGP hydrolysis was measured by following the decrease in absorbance in the region of 280 nm, according to the method of Whitaker *et al.*[3]. For BGP, BG2P, BG3P and BG5P hydrolysis at low concentrations (less than 1 m*M*), the decrease in absorbance at 220 nm was recorded. Changes in the molar absorptivities ($\Delta\varepsilon$) were calculated from absorbance values when substrate hydrolysis was completed. For all four substrates, 2-mm optical-path cells (Hellma) with a reaction volume of 0.5 ml were employed and 50–100 n*M* enzyme concentrations were used. This allowed straight progress curves to be obtained for the first 3–5 min of the reaction (less than *ca.* 10% hydrolysis), from which initial velocities were calculated. Demountable micro-cells (Hellma) of 0.1-mm optical path with a volume of 50 μl were used to determine the kinetics of hydrolysis of BGP, BG2P, BG3P and BG5P at higher concentrations (greater than 1 m*M*) at 220 nm by the following procedure: the substrate solution was placed in an eppendorf tube and the reaction was initiated by the addition of 5–10 μl of

CPA to complete a reaction volume of 60 μl, which was immediately mixed in a vortex stirrer; 50 μl of the reaction mixture were transferred to the cell, which was closed and placed immediately in the spectrophotometer cell compartment. In this way recordings could begin less than 30 s from mixing. Initial velocities were calculated as described above. All experiments were performed at 25°C. Assay of FAPP hydrolysis by CPA was performed as described by Peterson *et al.*[4].

HPLC-based kinetic analysis

All HPLC analyses were carried out using isocratic elution at a flow-rate of 0.5 ml/min with different water–acetonitrile proportions in 0.1% TFA, according to the substrate being analysed, and monitored at 280 nm. Solvents were previously degassed. An automatic injector and a plotter with integrating built-in capabilities were always employed for the quantitative determination of the chromatographic peak areas of both peptide substrates and their products. The samples for the kinetic determinations were prepared as follows: 300 μl of each substrate (BGP, BG3P or BG5P) concentration were placed in an eppendorf tube and 10 μl of CPA (final concentration around 50 n*M*) were added to initiate the reaction; sample aliquots of 45 μl were withdrawn at fixed time intervals (0.5 min) for 3 min (less than *ca.* 10% hydrolysis) and the reaction was quenched by adding 5 μl of TFA (final concentration 0.1–1% TFA, depending on the substrate employed). Samples from the kinetic experiments were kept frozen (−20°C) if not analysed immediately. Standards of known concentrations of the different peptide substrates and products were analysed and used as references to calculate, by interpolation, the amount of each product as a function of the integration of the peak area corresponding to the sample.

Calculation of kinetic parameters

A linear regression program was used for the calculation of the apparent kinetic parameters, K_m and V, from v *versus* v/S plots, where K_m is the Michaelis–Menten constant, V the maximum velocity, v the initial velocity and S the substrate concentration. These parameters were refined by a non-linear regression procedure[18].

RESULTS AND DISCUSSION

Fig. 1 shows that the difference spectra for BGP, BG2P, BG3P and BG5P after their complete hydrolysis by CPA have a strong negative absortion band at 220 nm. BGP also exhibits two other cleavage-sensitive bands at 254 and 280 nm. Because the initial absorbances were higher than 1.8, even when using 2-mm optical-path cells, 0.1-mm optical-path cells had to be used in order to follow the reaction at 220 nm at substrate concentrations above 1 m*M* for BG2P, BG3P and BG5P.

In order to check the stability of the assayed oligopeptide substrates (BGP, BG2P, BG3P)[a] in the absence of enzyme, the UV absorbance at different wavelengths was recorded for some time. Surprisingly, a sigmoidal perturbation of the absorbances in the 220–270 nm range was apparent in the first 8 min after placing the sample in the cell (Fig. 2). This effect was noticeable for oligopeptide concentrations above 4–5 m*M*. Although the decrease in absorbance accounts for less than 5% of the absolute initial

[a] BG5P could not be tested because of its low solubility at concentrations higher than 5 m*M*.

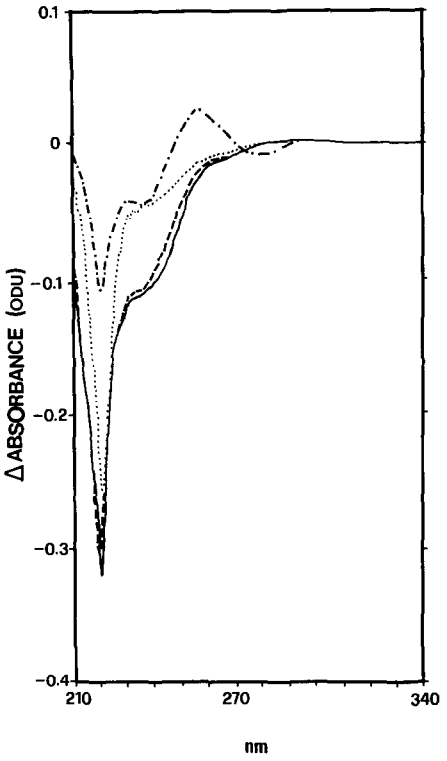

Fig. 1. Difference spectra for 0.5 m*M* solutions of benzoylglycyl-L-Phe (BGP) (· – · – ·), benzoyl(glycyl)$_2$-L-Phe (BG2P) (———), benzoyl(glycyl)$_3$-L-Phe (BG3P) (– – –) and benzoyl(glycyl)$_5$-L-Phe (BG5P) (·····) after complete hydrolysis by CPA in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C in 2-mm optical-path cells. ODU = Optical density units.

values, this perturbation makes the spectrophotometric assay difficult to apply. The rate and value of the perturbation increase both with increasing substrate concentration and increasing oligopeptide length, achieving stabilization in 4–8 min (being slower for the longer substrates). We also observed that this perturbation is independent of the exposure of the substrate solution to UV light, an indication that it is not a photochemical effect. When the assay is performed in the presence of enzyme, the above perturbation precludes a correct determination of the decrease in absorbance due to the enzymic activity. The possibility of allowing the substrate solution to stabilize before the addition of the enzyme is not feasible because of the closed design of the 0.1-mm optical-path cells.

Several unsuccessful attempts were made to eliminate the above interference. Prior degassing of all solutions and careful control of the temperature were carried out to avoid bubble formation and eliminate possible changes in the ε value of the substrates. Also, initial solubilization of the oligopeptide in acetonitrile–water solutions followed by ultracentrifugation was performed in order to avoid substrate aggregation. Finally, exhaustive metal extraction of all solutions was carried out to eliminate the possibility of formation of metal complexes, which could induce spectrophotometric perturbations. No improvements were observed after taking the

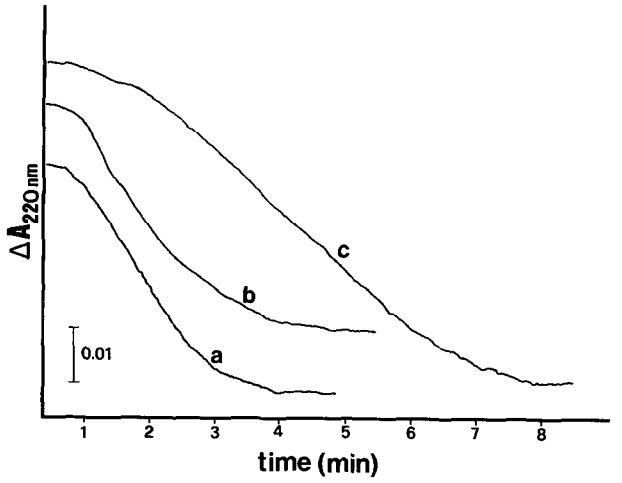

Fig. 2. Recordings of the absorbance perturbation at 220 nm of different peptide substrates when placed in a 0.1-mm optical-path microcell; (a) benzoylglycyl-L-Phe (BGP); (b) benzoyl(glycyl)$_2$-L-Phe (BG2P); (c) benzoyl(glycyl)$_3$-L-Phe (BG3P) in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C. All substrate concentrations were 15 m*M*. See Experimental for details.

above precautions. We also found a similar sigmoidal interference for unrelated proteins at high concentrations, such as chymotrypsin at 100 mg/ml or FAPP at 10 m*M*, when using the microcells. As a complementary test we recorded the sigmoidal interference for 5 m*M* FAPP in 0.1- and 0.2-mm optical-path cells at 330 nm, the absolute decrease in absorbance of the former being twice that of the latter. In addition, we recorded the sigmoidal interference for 10 m*M* BGP at 25 and 37°C, the value at the former temperature being 1.2 times higher than that at the latter.

We suggest that the UV spectrophotometric perturbation observed in ultra-thin cells with concentrated solutions of oligopeptides can originate from the co-operative interaction of molecules of a thin lamina of the solution with the walls of the cell, resulting in a particular alignment with respect to the walls, changing the isotropic nature of the solution into a partially anisotropic one. A comparative phenomenon has been described for polybenzoyl-L-glutamate, giving homeotropic liquid crystal preparations[19,20], which together with other features makes it partly similar to that described above[19–22].

To avoid the above problems, an HPLC-based method was developed for the analysis of the CPA hydrolysis of these oligopeptides. In Fig. 3, typical elution profiles for the reversed-phase HPLC analysis of the CPA-catalysed hydrolysis of BGP, BG3P and BG5P are shown. The two chromatographic peaks that appear in front of the corresponding product peak are due to optical interferences produced by sample injection. The concentration of acetonitrile had to be decreased with increasing peptide length in order to achieve a satisfactory and rapid (in less than 5, 6 and 8 min, respectively) separation between substrates (BGP, BG3P, BG5P), products (BG, BG3, BG5) and interference peaks. The other product, L-phenylalanine, common for all three oligopeptides, does not interfere in their determination at 280 nm. There was no detectable hydrolysis of BG3 or BG5 during the assay as they are very poor substrates of CPA[1].

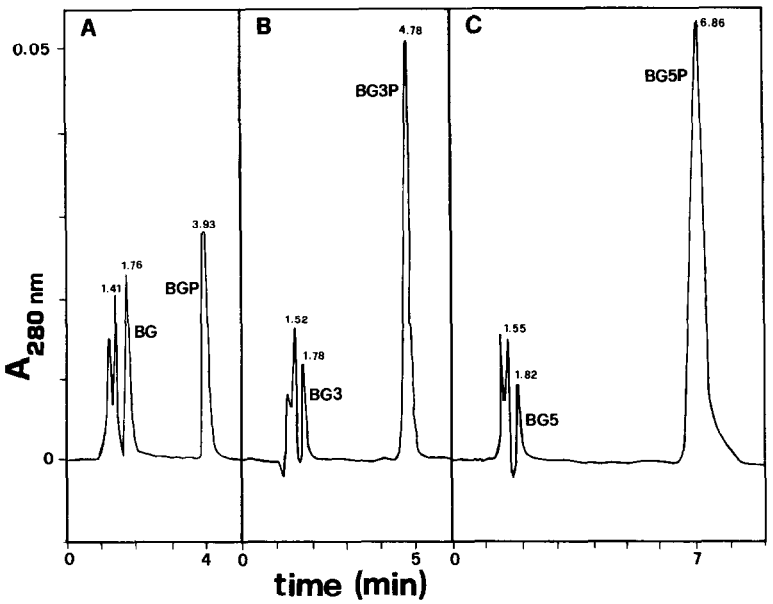

Fig. 3. Reversed-phase HPLC profiles of TFA-quenched samples of CPA-hydrolysed solutions of 0.5 m*M* of (A) benzoylglycyl-L-Phe (BGP), (B) benzoyl(glycyl)$_3$-L-Phe (BG3P) and (C) benzoyl(glycyl)$_5$-L-Phe (BG5P). Elution was carried out with 32%, 25% and 23% acetonitrile in water, respectively, in 0.1% TFA. CPA concentration, 57 n*M*; final TFA concentration in quenched samples, 1% for BGP and 0.1% for BG3P and BG5P.

The hydrolysis rate could be measured by following either the decrease in the peak of the substrate or the increase in the peak of the product. We adopted the latter approach because more accurate results were obtained and also the precision was lower when the peak of the substrate was used, especially at high concentrations. The calibration graphs for BG, BG3 and BG5 (with correlation coefficients from 0.997 to 0.999) that were used to calculate the corresponding amounts of products formed from the integration areas of the peaks are shown in Fig. 4. Chromatographic runs were always performed in duplicate over the range 0–0.1 absorbance and showed good reproducibility. Under the assay conditions described, unperturbed and well defined chromatographic elution profiles were obtained with 20-μl injection volumes; samples can be injected directly up to substrate concentrations of 15 m*M*. Above this concentration it is necessary to dilute the samples for kinetic determinations. In order to test concentrations of substrate below 0.1 m*M*, the injection volume and/or the sensitivity of the HPLC detector have to be increased. The CPA concentrations used in this work (*ca.*50 n*M*) can be reduced approximately 250-fold employing the same procedure.

The linearity of the peptide hydrolysis rate as a function of the enzyme concentration in the range used (20–200 n*M*), as determined by the HPLC method, was tested and yielded good results (correlation coefficients between 0.9903 and 0.9989). The proportionality between substrate concentration and integration areas before and after the addition of TFA was also verified for BGP, BG3P and BG5P.

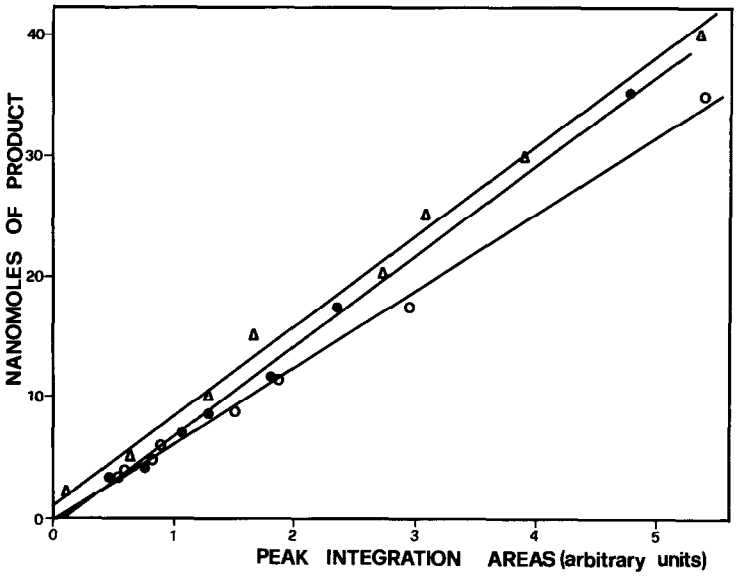

Fig. 4. Calibration graphs for hippuric acid (BG) (●), benzoyl(glycyl)$_2$-Gly (BG3) (○) and benzoyl-(glycyl)$_4$-Gly (BG5) (△) as a function of peak-integration areas of HPLC elution profiles, and nanomoles of each eluted product. Correlation coefficients, 0.9980, 0.9969 and 0.9985, respectively. HPLC conditions as in Fig. 3. Products were in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C. All measurements were made in duplicate.

together with the efficiency of TFA in quenching the enzymic activity (data not shown). It is very important to note that the concentration of TFA added for quenching had to be carefully selected in order to avoid substrate aggregation at very low pH values. A final value of 0.1% TFA can be generally used for all the oligopeptide substrates analysed over a wide range of concentrations (we tested up to 50 m*M* for BGP and BG3P and up to 5 m*M* for BG5P), although at low BGP concentrations higher TFA values can also be used (up to 1%).

The results for the quantification of the hydrolysis of the above-mentioned substrates by CPA employing both spectrophotometric and HPLC methods are summarized in Table I. It is seen that the initial velocities obtained with the two methods are in good agreement, although the spectrophotometric-derived values are slightly higher, probably owing to an underestimation of the substrates $\Delta\varepsilon$ values[23].

In Fig. 5, typical progress curves for the CPA-catalysed hydrolysis of BGP in the concentration range 0.5–6.5 m*M*, as obtained from HPLC data, are shown. The apparent kinetic parameters calculated by both the HPLC and the spectrophotometric methods are given in Table II, and agree well with those in the literature for bovine CPA[3,10]. This determination was restricted to the first part of the *v*–*S* curve for BGP and assuming Michaelis–Menten kinetics. A more complete kinetic analysis would be more complicated because anomalies at higher concentrations have been reported[3,10].

The method described in this work shows comparable sensitivity and speed to the HPLC method recently proposed by Galdes *et al.*[24] for the analysis of reaction mixtures of the CPA-catalysed hydrolysis of dansylated dipeptides and depsipeptides

TABLE I

COMPARISON OF THE CPA-CATALYSED HYDROLYSIS OF OLIGOPEPTIDES AS DETERMINED BY THE SPECTROPHOTOMETRIC AND HPLC METHODS

All assays were performed in duplicate at 0.5 m*M* of oligopeptide concentration in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C. CPA concentration, 57 n*M*. Specific activities are given in μmol min^{-1} $nmol^{-1}$. Values are not significantly different for $t_{0.95}$ (bounderies of the central interval of the Student *t* value).

Substrate	*Specific activity*	
	Spectrophotometric method[a]	*HPLC method*[b]
BGP	1.090	0.958[c]
BG3P	0.563	0.432[d]
BG5P	1.773	1.597[d]

[a] Specific activities calculated from spectrophotometric data using $\Delta\varepsilon_{280} = 0.072\,l\,mmol^{-1}\,cm^{-1}$ for benzoylglycyl-L-Phe (BGP), $\Delta\varepsilon_{220} = 1.28\,l\,mmol^{-1}\,cm^{-1}$ for benzoyl(glycyl)$_3$-L-Phe (BG3P) and $\Delta\varepsilon_{220} = 1.53\,l\,mmol^{-1}\,cm^{-1}$ for benzoyl(glycyl)$_5$-L-Phe (BG5P).

[b] Specific activities calculated from HPLC data at different proportions of solvent A (water–0.1% TFA) and solvent B (acetonitrile–0.1% TFA): 68:32 (BGP), 75:25 (BG3P) and 77:23 (BG5P).

[c] Withdrawn aliquots were quenched by adding TFA up to 1%.

[d] Withdrawn aliquots were quenched by adding TFA up to 0.1%.

in order to identify reaction intermediates in cryokinetic studies. It is also comparable to the method proposed by Grimwood *et al.*[25] for the quantification of carboxypeptidase N following the hydrolysis of furanacryloyl derivatives of Ala–Lys or Ala–Arg. In both instances the analysis is performed at fixed and low substrate concentrations. In this present work we use different peptide substrates for the

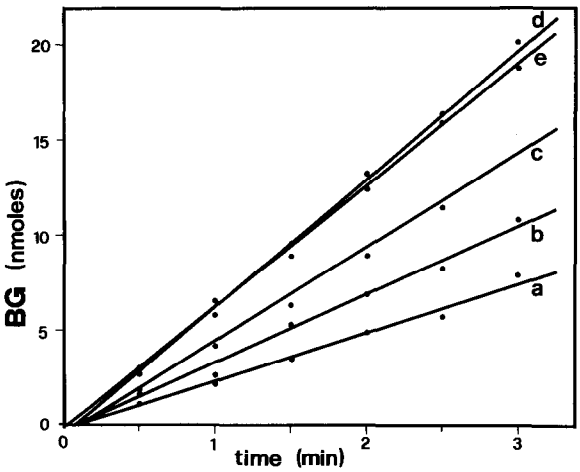

Fig. 5. HPLC-determined progress curves for the CPA-catalysed hydrolysis of benzoylglycyl-L-Phe (BGP) at (a) 0.65, (b) 1.62, (c) 3.23, (d) 4.85 and (e) 6.47 m*M*, in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C. CPA concentration, 56 n*M*; final TFA concentration in quenched aliquots, 1%. Correlation coefficients were 0.9925, 0.9949, 0.9948, 0.9978 and 0.9988, respectively. HPLC conditions as in Fig. 3. All measurements were made in duplicate.

TABLE II

APPARENT KINETIC PARAMETERS FOR THE CPA-CATALYSED HYDROLYSIS OF BGP DETERMINED BY THE SPECTROPHOTOMETRIC AND HPLC METHODS

Benzoylglycyl-L-Phe (BGP) range of concentrations analysed, 0.5–6.5 m*M*. All measurements were carried out in duplicate in 50 m*M* Tris–HCl–0.5 *M* NaCl–1 μM $ZnCl_2$ (pH 7.5) at 25°C. CPA concentration, 56 n*M*. Parameter values are not significantly different for $t_{0.95}$. Confidence limits for each parameter are also indicated.

Kinetic parameter	*Spectrophotometric method*[a]	*HPLC method*[b]
K_m (m*M*)	1.68 ± 0.02	1.39 ± 0.04
V (μmol min^{-1} nmol^{-1})	3.15 ± 0.01	2.95 ± 0.03

[a] According to the method described by Whitaker *et al.*[3].

[b] HPLC eluent, water–acetonitrile (68:32), both in 0.1% TFA. Withdrawn aliquots were quenched by adding TFA up to a final concentration of 1%.

determination of the CPA activity. Also, different conditions for its quenching (TFA instead of either hydrochloric or phosphoric acid) are applied in order to increase both the solubility of the peptide substrates and products (particularly when working with long oligopeptides) and the lifetime of the column. The more comprehensive design of our method, which is intended to be used in kinetic studies covering a wide range of substrate sizes (dipeptides to hexapeptides) and concentrations, required a careful selection of chromatographic conditions for their analysis. It is also interesting that the use of a very short HPLC column (and very cheap, as it is commercially available as a guard column) shortens the time that would be required to separate long oligopeptides and products in an HPLC column of standard length.

To summarize, we have developed a sensitive and flexible method for the analysis of the CPA kinetic properties with oligopeptides as substrates by means of an isocratic reversed-phase HPLC separation of quenched samples. The method can be automated and allows the sequential analysis of multiple samples by the use of an autosampler. This technique combines reproducibility together with accuracy and requires short times and small amounts to complete the analysis. Using oligopeptide substrates of the general formula benzoyl(glycyl)$_n$-L-Phe, n being 1–5, their hydrolysis products can be detected at the nanomole level. The practical CPA concentration range is from 0.2 n*M* to 0.2 μM. The range of peptide substrate concentrations that can be determined by the HPLC method described is very wide and the method is clearly much more convenient than currently used spectrophotometric procedures, particularly at high concentrations (no limit when combined with accurate dilutions prior to the HPLC analysis).

The application of this method can facilitate more detailed studies on the complex kinetic behaviour that was observed for CPA with N-benzoylated peptide derivatives either in solution or in crystals[11,16], and to provide further information about the configuration of the active site in several subsites[26–28], which could more easily accommodate longer substrates. It has already been described for bovine CPA in solution that non-Michaelis–Menten kinetics apparently disappear when longer substrates are employed[11,16], a fact that could be confirmed by the method described here. The proposed method can also be used in enzymological studies of carboxypeptidases in the presence of different salts and solvents, a field of growing interest[24,29].

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