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Measurement of the enzymatic specificity of carboxypeptidase A by capillary zone electrophoresis

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Abstract

The development of a novel method using capillary zone electrophoresis for monitoring enzymatic assays involving carboxypeptidase A and methotrexate-α-peptides is reported. Since the hydrolysis product, methotrexate, and the substrate have the same absorption spectrum, spectrophotometry could not be used to monitor the reaction. Eleven methotrexate amino acid prodrugs were synthesized and tested as substrates for carboxypeptidase A. Since the product has a charge difference with the substrate, the reaction could be monitored easily by capillary zone electrophoresis. This method may be adapted to various enzyme kinetics.

1. Introduction

Capillary electrophoresis techniques are increasing in popularity due to their versatility and their powerful analytical capacity. They are used for the separation and characterization of a large variety of biomolecules. Many techniques involving different separation mechanisms have already been described [1,2] showing their advantages in comparison with other common separation procedures such as HPLC and supercritical fluid chromatography [3]. Capillary zone electrophoresis (CZE) is often used for the separation of small peptides or drug molecules and their metabolites. CZE is characterized by rapid analysis times (5–30 min), small sample volumes (10 nl–10 μl), a sensitivity at the nanogram level and a high resolution [4]. These features combined with a high efficiency and reproducibility lead us to use CZE instead of HPLC for monitoring an enzymatic assay in which carboxypeptidase A (CP-A) hydrolyses methotrexate-α-peptides leading to the release of free methotrexate (MTX).

Since MTX-α-peptides and MTX share comparable absorption spectra and a λmax at 372 nm, the spectrophotometry could not be used for monitoring the reaction. It is possible with CZE to separate the substrate and the product according to the electrophoretic mobility. In this case, the lysis of the C-terminal amino acid changes the mass-to-charge ratio and therefore the electrophoretic mobility of the product with respect to the substrate.

In order to test the method and get the most promising MTX prodrug for CP-A, a range of 11 MTX-α-peptides were selected on the basis of their polarity and structure, and then synthesized. Enzymatic assays were then performed on all of them and the hydrolytic potential of

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carboxypeptidase A was evaluated for each MTX analogue using CZE.

2. Experimental

2.1 Materials

Chemicals used for buffers were ACS reagent grade material. Bovine pancreatic carboxypeptidase A Type II-DFP (EC 3.4.17.1) and hippuryl-L-phenylalanine were obtained from Sigma (St. Louis, MO, USA). 0.1 *M* phosphate buffer pH 2.5 (cat no. 148-5010), capillary wash solution (cat. no. 148-5022) and the 24 cm × 25 μm coated capillary cartridge (cat. no. 148-3031) were supplied by Bio-Rad (Mississauga, Ontario, Canada). 0.45-μm nylon filters were purchased at Micron Separations (Westborough, MA, USA).

2.2 MTX-α-peptides synthesis

The solid-phase synthesis of MTX-Arg, MTX-Asn, MTX-Cys, MTX-Gln, MTX-His, MTX-Lys, MTX-Met, MTX-Phe, MTX-Pro, MTX-Trp and MTX-Tyr will be described elsewhere. Briefly, they were synthesized on the BioLynx 4175 Peptide Synthesizer (LKB Biochrom, Pharmacia, Baie d'Urfée, QC, Canada) using the Fmoc method.

2.3 Carboxypeptidase A enzymatic assay

Hippuryl-L-phenylalanine

The CP-A activity was measured using the following modifications of the procedure described by Haenseler *et al.* [5]. 1.0 m*M* hippuryl-L-Phe contained in 2.95 ml of 0.1 *M* Tris-HCl buffer pH 7.3–0.2 m*M* $ZnSO_4$ was incubated at 37°C. The enzymatic reaction was initiated by adding 50 μl of CP-A in order to obtain a final reaction volume of 3.0 ml. The increase in absorbency was monitored with a Philips UV/Vis Scanning Spectrophotometer (Model PU 8720) at 254 nm for 15 min. In accordance with the Worthington Enzyme Manual [6], a value of $\Delta\epsilon mM = 13.6$ was used for the reaction and the CP-A concentration was calculated from $\epsilon mM = 64.2$ at 278 nm.

MTX-α-peptides

The same reaction conditions as described above were set for the CP-A assay with the 11 MTX-α-peptides. Fourteen identical reaction mixtures containing 1.0 m*M* MTX-Phe were prepared in separated test tubes in order to stop the enzymatic reaction at 0, 1, 2, 3, 4, 5, 7, 10, 13, 15, 20, 25, and 30 min. No enzyme was added to the 14th tube which contained only the substrate used as a control. When the reaction time had elapsed for each respective vial, a 200-μl aliquot was transferred into 400 μl of boiling water in a microcentrifuge tube for 10 min to stop the reaction and the aliquots were then centrifuged (100 *g*) to discard the enzyme in the pellet. 500-μl aliquots of the 14 supernatants containing the unreacted MTX-Phe and the MTX freed by the reaction were then evaporated to dryness in vacuo with a Savant SpeedVac Model SC200 (Savant Instruments, Farmingdale, NY, USA). The same procedure was repeated for the 10 other MTX derivatives. The value of $\epsilon mM = 33$ for the reaction at 204 nm was determined experimentally.

2.4 Capillary zone electrophoresis

Sample preparation

The 14 MTX-Phe/MTX containing pellets obtained above, each corresponding to a reaction time ranging from 0 to 30 min, were dissolved in 200 μl of 0.1 *M* phosphate buffer pH 2.5 mixed with 100 μl of the same buffer diluted 1:10. Prior to analysis, samples were filtered through a 0.45-μm nylon filter.

Sample analysis

A BioFocus 3000 Automated Capillary Electrophoresis System (Bio-Rad) was used for all CZE experiments. A minimum of 20 μl of each of the 14 samples was put in specially adapted microcentrifuge tubes. The samples were introduced into a 24 cm × 25 μm I.D. coated capillary cartridge by an 8-s electrophoretic injection.

The injection and the analysis were performed

under a 10 kV constant voltage with a current limit of 50 μA. The polarity was set from positive to negative. The compounds were detected at 0.01 AUFS by a built-in UV/Vis detector operating at 204 nm. The running buffer was 0.1 *M* phosphate pH 2.5 for the whole 11 min fully automated runs. Wash cycles were carried out with deionized water, the capillary wash solution and a nitrogen gas purge. Data were obtained using the BioFocus 3000 Spectra and BioFocus 3000 Integrator software provided with the apparatus computer system.

3. Results and discussion

Only data regarding the reaction of CP-A on MTX-Phe are presented in this section. The other MTX-α-peptides were poorly or not at all hydrolyzed by CP-A except for MTX-Tyr and MTX-Gln which showed a weaker activity than MTX-Phe.

The difference in the respective migration of MTX and MTX-Phe is mainly due to their mass-to-charge ratio. Since the polarity is set from + to −, MTX has the tendency to move faster than MTX-Phe which is in turn larger than MTX. Thus, prior to sample analysis, the CZE apparatus was standardized with MTX and MTX-Phe to check their peak positions. MTX had a retention time (RT) of 8.0 min (data not shown) and MTX-Phe was detected after an average of 9.8 min (see Table 1).

Hydrolysis of MTX-Phe by CP-A was easily followed by capillary zone electrophoresis showing the substrate disappearance and the product appearance. As shown in Fig. 1, the progressive increase of MTX was paralleled by the decrease of MTX-Phe. During previous testings, the formation of a small peak (RT 13 min) was also noticed (data not shown), probably corresponding to phenylalanine which is the secondary reaction product. As Kuefner *et al.* have already reported [7], no cleavage of the MTX peptide bond between the glutamyl and the pteroyl moieties by CP-A was observed.

At time = 0 min of the enzymatic assay (see Fig. 1B), the electropherogram showed that the reaction has already started while the substrate MTX-Phe should be the only compound present.

Table 1

Parameters generated by the BioFocus 3000 Integrator

Reaction time (min)	No. of peaks	Retention time (min)		Peak area (%)		Peak height (%)		Absorbance (204 nm)	
		MTX	MTX-Phe	MTX	MTX-Phe	MTX	MTX-Phe	MTX	MTX-Phe
Control (MTX-Phe)	1	–	10.7	–	100	–	100	–	0.0077
0	2	8.39	9.90	16.84	83.16	21.04	78.96	0.0020	0.0076
1	2	8.22	9.77	35.44	64.56	40.64	59.36	0.0039	0.0057
2	2	8.14	9.72	60.48	39.52	65.17	34.83	0.0067	0.0036
3	2	8.12	9.76	81.01	18.99	82.32	17.68	0.0085	0.0018
4	2	8.04	9.70	92.79	7.21	92.93	7.07	0.0089	0.0007
5	2	8.06	9.74	96.54	3.46	96.52	3.48	0.0089	0.0003
7	1	8.04	–	100	–	100	–	0.0098	–
10	1	8.02	–	100	–	100	–	0.0102	–
15	1	8.03	–	100	–	100	–	0.0098	–

Values are related to the peaks corresponding to MTX-Phe and MTX, respectively. The progression of the enzymatic reaction may then be monitored. The separation conditions are described under Experimental.

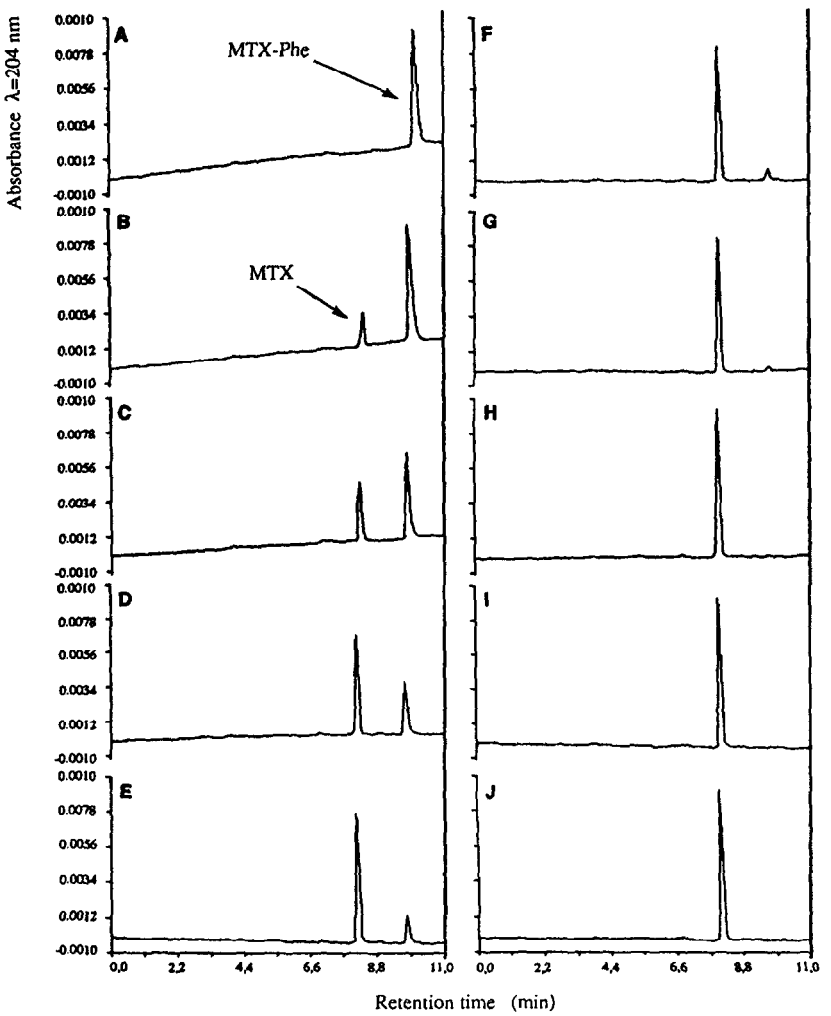

Fig. 1. Electropherograms showing the increase of MTX paralleled by the decrease of MTX-Phe in relation to the progression of the enzymatic hydrolysis. (A) MTX-Phe as a control, and after (B) 0 min, (C) 1 min, (D) 2 min, (E) 3 min, (F) 4 min, (G) 5 min, (H) 7 min, (I) 10 min, (J) 15 min reaction. The separation conditions are described under Experimental.

This was probably due to the method used to stop the enzymatic reaction. Actually, when an aliquot of the reaction mixture was transferred into boiling water, it may have taken a few seconds before the enzyme denaturation occurred.

Many attempts have been made to find a method by which the enzymatic reaction could be stopped instantly. Acids and bases were rejected because of their harmful effects on the compounds and the same is true for any inhibitors such as 1,10-*o*-phenanthroline that could have left undesired residues mixed with MTX which could have made electropherograms difficult to read.

The wavelength selected for monitoring the enzymatic assay by CZE was 204 nm because it is one of the four major peaks in MTX absorption spectrum along with 258, 302 and 372 nm. It is also the most adequate wavelength for measuring the presence of peptides and smaller molecules which are best detected in the range of 190

to 220 nm. The use of CZE is advantageous in this instance because other separation techniques often use solvents that have UV cutoff which prevents the use of these lower wavelengths. Nevertheless, a few assays were carried out at 372 and 302 nm but the peaks appeared to be much smaller for the same amount injected and, combined with a very noisy baseline, an important loss of sensitivity was observed.

As shown in Fig. 2 and as mentioned earlier, CP-A was totally non-responding to MTX-Lys, MTX-His, MTX-Cys and MTX-Arg as substrates and the activity still remained very weak for MTX-Trp, MTX-Pro, MTX-Met and MTX-Asn. Carboxypeptidase A catalyses the hydrolysis of acidic and neutral amino acids from the C-terminal end of a peptide except for proline [8] which is in accordance with the above results. Since lysine, histidine and arginine are basic amino acids and cysteine is an inhibitor of CP-A [6], the non-reactivity of the enzyme towards the corresponding MTX derivatives was not surprising. Also, compounds containing a free carboxyl group or an heterocyclic ring are generally stated as competitive inhibitors [9]. In our study, tryptophan was hydrolysed much more slowly than phenylalanine; in addition, we found that asparagine and glutamine with their amide groups were also poorly hydrolysed by CP-A. Since the structures of tyrosine and phenylalanine are alike, their similar activity was predictable.

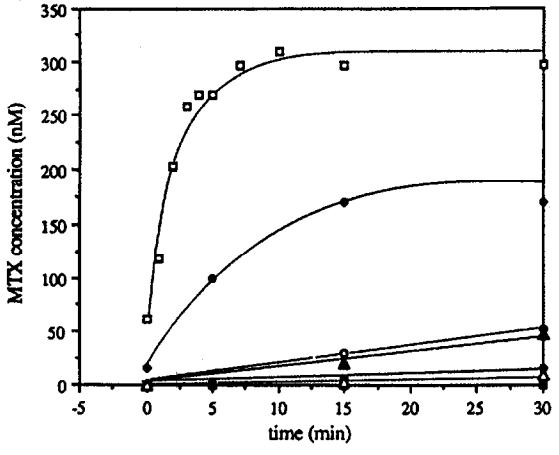

Fig. 2. Enzymatic activity of CP-A on the 11 MTX-α-peptides: MTX-Phe (□), MTX-Tyr (◆), MTX-Gln (○), MTX-Met (▲), MTX-Trp (●), MTX-Asn (△), MTX-Lys, MTX-His, MTX-Cys, MTX-Arg and MTX-Pro (■). (See Experimental for the reaction conditions.)

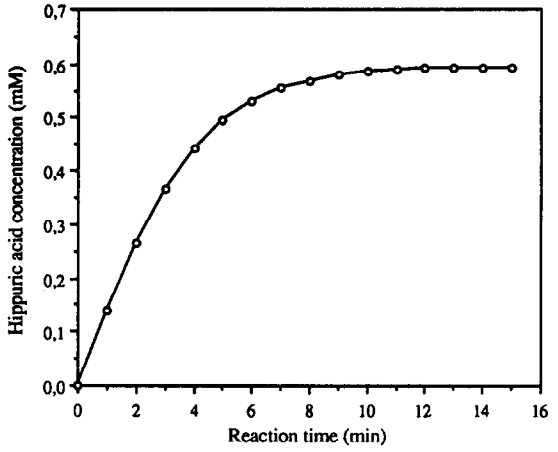

Fig. 3. Enzymatic activity of CP-A on hippuryl-L-phenylalanine. (See Experimental for the reaction conditions.)

As a reference, an enzymatic assay was performed using another CP-A substrate, hippuryl-L-phenylalanine, in conditions used for MTX-Phe (see Fig. 3 and Experimental).

4. Conclusion

In summary, the purpose of this paper was to report a new approach for monitoring enzymatic assays involving MTX-α-peptides hydrolyzed by CP-A. Presently, the synthesis of this type of MTX derivative has an application in the field of cancer research. They may be used as prodrugs which are activated on contact with CP-A thus releasing MTX, an anticancer drug [5,7,10]. These prodrugs are now tested for a more selective and localized chemotherapy [11,12].

The CP-A enzymatic assays monitored by CZE could have an application for the development and the selection of the most suitable MTX prodrugs. Here, MTX-Phe was found to be the best substrate for CP-A out of the eleven tested. The hydrolysis of MTX-Phe by CP-A was com-

parable to the hydrolysis of hippuryl-L-Phe which is a natural substrate for CP-A.

Compared to HPLC and other separation techniques, the CZE method is very rapid, reproducible and requires minimal amounts of material. The progression of the reaction can be visualized easily, combined with a high sensitivity.

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