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Isotachophoretic determination of 2–5A phosphodiesterase

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ABSTRACT

Together with 2–5A synthetase and ribonuclease L, 2–5A phosphodiesterase belongs to the 2–5A system, which plays an important role in the action of interferon. Analytical capillary isotachophoresis was used for the determination of 2–5A phosphodiesterease activity. Enzyme assay was optimized using snake venom phosphodiesterase as a source of 2–5A phosphodiesterase activity. The 2–5A trimer core was used as a substrate. Enzyme activity was determined in time- and concentration-dependent reactions. In addition, 2–5A phosphodiesterase activity was determined in lysates of mononuclear blood cells.

INTRODUCTION

The 2–5A system is one of the effector systems known to be involved in the action of interferons [1]. In the presence of double-stranded RNA, the interferon-induced enzyme 2–5A synthetase is activated and forms a number of 2–5 oligoadenylates from ATP (containing 2–14 adenosine residues). These oligoadenylates activate a ribonuclease (RNaseL), which splits viral or messenger RNA, thus preventing protein synthesis. A third enzyme, 2–5A phosphodiesterase (2–5A PDE), splits the 2–5 phosphodiester bonds of the oligoadenylates and returns RNaseL to its inactivated form. The formation of the dimeric form of 2–5 oligoadenylates in the 2–5A synthetase reaction and its degradation by 2–5A PDE is shown in Fig. 1 as an example. The physiological role of 2–5A PDE is to inhibit the diffusion of the 2–5 oligoadenylates from the place where they are formed. In this way, the action of activated RNaseL can be located near the partially double-stranded RNA, and, consequently, the single-stranded areas of this RNA (*e.g.* a viral RNA during the replication phase) are split preferentially [2].

Some assays for 2–5A PDE activity have been described in the literature, most of them using radiolabeled compounds [3–4]. Here, we present a novel and simple assay using analytical capillary isotachophoresis. The optimal assay conditions were

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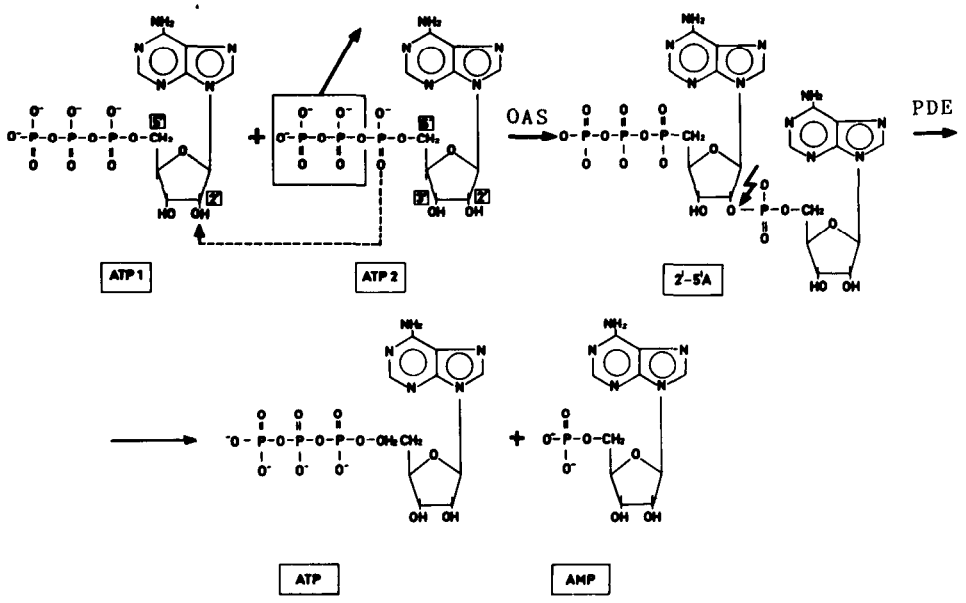

Fig. 1. Synthesis of 2–5 oligoadenylates (dimeric form) by 2–5A synthetase (2,5-oligoadenylate synthetase, OAS) and its degradation by 2–5A phosphodiesterase (PDE).

developed using the 2–5A trimer core (ApApA) as a substrate (the 2–5A cores have the same structure as the 2–5As, except they lack the initial triphosphate group in the 5′-position; 2–5A cores are not able to activate RNaseL but are degraded by 2–5A PDE to the same extent as the corresponding 2–5As [5].) The degradation of ApApA was followed using snake venom phosphodiesterase (SV-PDE), an enzyme known to have 2–5A PDE activity [6], and by lysates of mononuclear blood cells which contain 2–5A PDE activity.

EXPERIMENTAL

Isotachophoresis

The isotachophoretic analysis was performed on an LKB Tachophor 2127. Signals were registered by either UV absorbance at 254 nm or registration of the conductivity (*C*) signal. 0.01 *M* HCl–β-alanine plus 0.3% (w/v) methylcellulose 4000 (Fluka, Switzerland), pH 3.65, was used as leading electrolyte (LE) and 0.01 *M* caproic acid (Merck, Germany) as terminating electrolyte (TE). The current during signal detection was 75 μA. Analysis was carried out at 15°C in a 23 cm × 0.5 mm I.D. capillary. Chart speed during detection was 10 cm/min. Usually, 5-μl samples were injected onto the Tachophor.

2–5A Phosphodiesterase assay

A 10-μl volume of incubation buffer containing 2–5A trimer core [A2′p5′A2′p5′A (abbreviation ApApA), P. L. Biochemicals, U.S.A.] was incubated with 10 μl of 2–5A PDE-containing solution (lysates of mononuclear blood cells

(MNBCs) or SV–PDE (EC 3.1.4.1, Sigma, U.S.A.) in an eppendorf cup at 37°C. At the end of the incubation, the reaction mixture was heated for 2.5 min in a boiling water bath. The samples were centrifuged and the supernatants were frozen at −70°C until isotachophoresis was carried out.

Lysates of mononuclear blood cells

MNBCs were isolated using Lymphoprep (Nyegaard, Norway) from heparinized whole blood samples, washed twice and subsequently lysed using a buffer containing 0.5% Nonidet-P 40 (Sigma).

RESULTS

Isotachophoretic characterization of 2–5A cores

Fig. 2 shows the isotachophoretic pattern of the dimeric (ApA), trimeric (ApApA) and tetrameric (ApApApA) forms of 2–5A cores. Since the commercially available trimeric form is the cheapest, it was chosen as substrate for the 2–5A PDE reaction.

The calibration curve for ApApA, $y = 1.5 + 9.1x$ (x-axis: ApApA, 0–5 mmol/l; y-axis: zone length, 0–47.5 mm), showed strong linearity ($r^2 = 0.990$; S.D. = 2.733). No spontaneous degradation of ApApA occurred during a 2-h incubation period in the absence of 2–5A PDE activity (data not shown).

Degradation of ApApA in the presence of different amounts of SV-PDE

Fig. 3 shows the degradation of ApApA after 5 and 60 min incubation in the presence of different amounts of SV-PDE. Whereas 100% degradation occurred using 0.04 I.U./ml SV-PDE after 60 min, only a very small amount of ApApA was split using 0.0004 I.U./ml SV-PDE.

In order to follow the time course of ApApA degradation more exactly, the following experiments were carried out using 0.01 I.U./ml SV-PDE.

Time course of 2–5A core degradation using SV-PDE

In Fig. 4, the time course of the degradation of ApApA in the presence of 0.01 U/ml SV-PDE is presented. ApApA signals are indicated by arrows. Several reactions

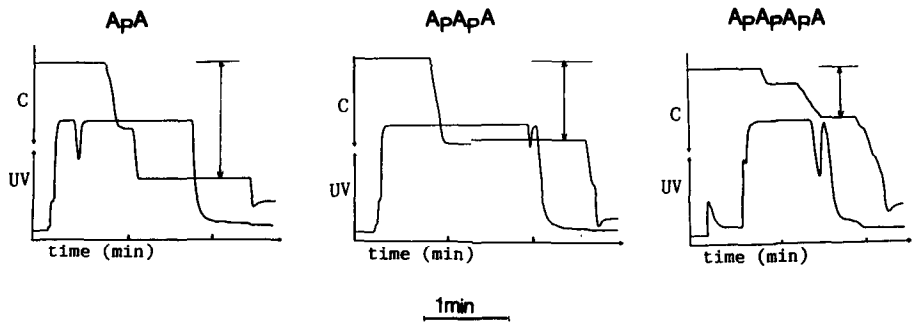

Fig. 2. Isotachopherograms of commercially available 2–5A cores, ApA (74%), ApApA (48%) and ApApApA (34%). Numbers in parenthesis are conductivity signals (C): LE–TE 100%.

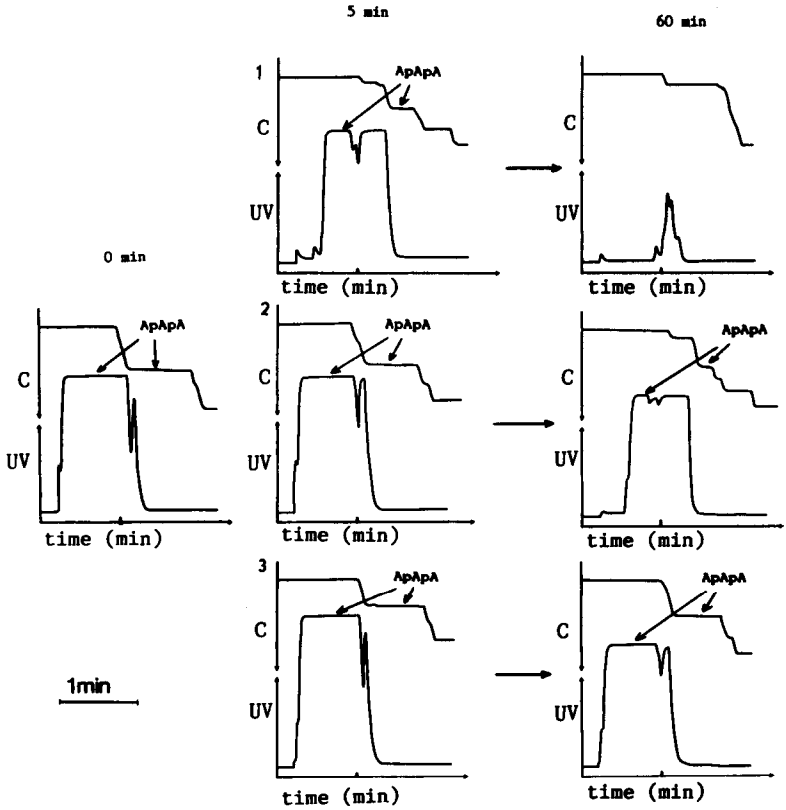

Fig. 3. Degradation of ApApA (3 mmol/l) after 5 and 60 min incubation at 37°C with 0.04 I.U/ml (1), 0.004 I.U./ml (2) and 0.0004 I.U./ml (3) SV-PDE.

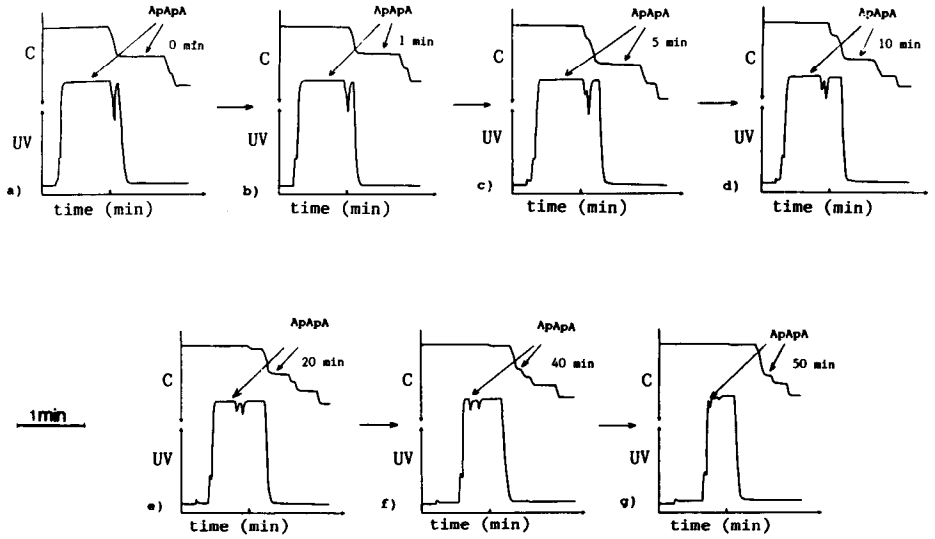

Fig. 4. Time course of ApApA degradation in the presence of 0.01 I.U./ml SV-PDE. Changes in ApApA signals during the incubation are indicated by arrows. Incubation mixture: 10 μl ApApA (3 mmol/l) plus 10 μl SV-PDE (0.01 I.U./ml).

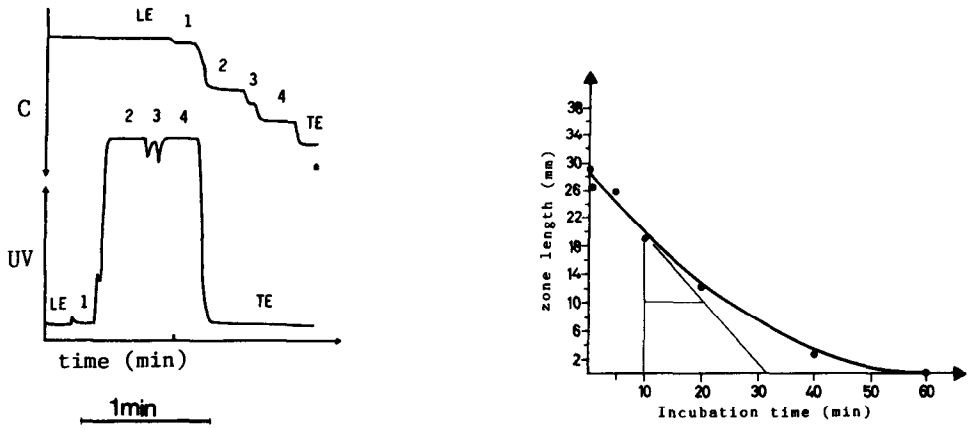

Fig. 5. Identification of products formed during the degradation of ApApA by SV-PDE by comparing the conductivity signals with pure substrates (1) Phosphate: 2–3% (pure substrate 0–5%); (2) ApApA; 54% (pure substrate: 51%); (3) AMP: 60% (pure substrate: 63%); (4) ApA: 76% (pure substrate: 74%).

Fig. 6. Time course of ApApA degradation: 10 µl ApApA (3 mmol/l) plus 10 µl SV-PDE (0.01 I.U./ml) were incubated for 60 min at 37°C.

occur in parallel during this process: SV-PDE activity splits ApApA into ApA and AMP; subsequently, ApA is split into AMP and adenosine, which cannot be detected in the isotachopherogram. In addition, SV-PDE activity leads to the degradation of AMP, forming inorganic phosphates, which are finally the only substances detectable in the isotachopherogram after complete ApApA degradation (see Fig. 3.1). In Fig. 5 the identification of the substances occurring during ApApA degradation is shown by comparing their conductivity signals with those of the corresponding pure substrates (data in parenthesis).

The time course of the degradation of 3 mmol/l ApApA (20 µl reaction volume) over a time period of 60 min using 0.01 U of SV-PDE is shown in Fig. 6. From these data a turnover rate of $0.98 \cdot 10^{-1}$ mmol/l ApApA per min at 37°C can be calculated.

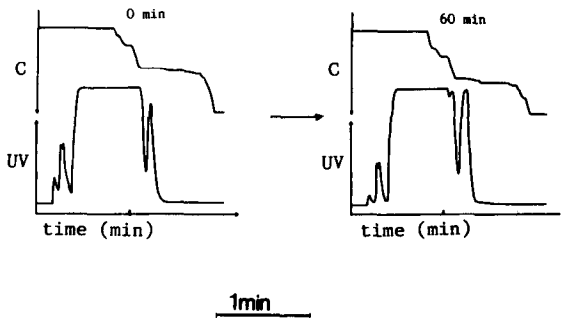

Fig. 7. Degradation of 10 µl ApApA (3 mmol/l) in the presence of 10-µl lysates of $5 \cdot 10^5$ MNBCs after 60 min incubation at 37°C.

2–5A PDE activity in MNBCs

Fig. 7 shows the degradation of ApApA by lysates of $5 \cdot 10^5$ MNBCs after 60 min incubation at 37°C. In the example given, 15.7% of ApApA was degraded. In order to examine the reproducibility of these determinations, two further runs were done with the same sample, yielding exactly the same results (15.7 ± 0% degradation; $n = 3$).

DISCUSSION

The present work shows that analytical capillary isotachophoresis is a very suitable method for the determination of 2–5A PDE activity. It is characterized by high accuracy, indicated by, for example, the high linear regression coefficient in the calibration curve, and by a precision great enough to allow the determination of enzyme activity after a 5–10% decrease of the ApApA concentration. The reliability of this method is remarkably high, as was demonstrated in Fig. 7. Although 2–5 oligoadenylates (ppppApA, pppApApA, pppApApApA, etc) are the natural substrates of this enzyme, ApApA is equally suitable as a substrate for the test. The only reason for its use is that it was the cheapest of all oligoadenylates commercially available.

In cellular systems, 2–5 oligoadenylates are rapidly degraded by 2–5 PDE activities [6], thus leading to reversibility and regional action of the interferon-induced 2–5A system. However, in some cases, *e.g.* in severe virus infection, it may be advantageous to prolong the activation of RNaseL. For this reason, some analogue of 2–5 oligoadenylates that mimic the interferon effects and which are degraded much more slowly by 2–5A PDE than the substrates naturally formed have been developed [7–9]. For testing the resistance of such 2–5 analogues to 2–5A PDE activity, the isotachophoretic 2–5A PDE assay may be a cheap and suitable alternative to radioactive methods.

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