Total direct chemical synthesis and biological activities of human group IIA secretory phospholipase A_2

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Human group IIA secretory phospholipase A_2 (hGIIA $sPLA_2$) is reported to be involved in inflammation, since its expression level is enhanced under various inflammatory conditions. In this work, we report the total chemical synthesis of this enzyme (124 amino acids) by solid-phase method. The identity of the protein, in denatured or folded (7 disulphide bonds) forms, was confirmed by electrospray MS. Synthetic $sPLA_2$ possesses the same circular dichroism spectrum, enzymic activity in hydrolysing different phospholipid substrates, and inhibitory effect in thrombin formation from prothrombinase complex as the recombinant $sPLA_2$. Furthermore, LY311727, a reported specific hGIIA $sPLA_2$ inhibitor, is able to inhibit the synthetic and the recombinant enzymes with the same efficiency. This study demonstrates that chemically continuous solid phase synthesis is an alternative and less time-consuming approach to producing small, structurally folded and fully active proteins of up to 124 amino acids, such as hGIIA $sPLA_2$. Moreover, this technique provides more flexibility in analogue synthesis to elucidate their physiological functions and pathological effects.

Key words: inflammation, PLA_2, protein folding, solid phase peptide synthesis, SPPS.

INTRODUCTION

Phospholipase A_2 (PLA_2) constitutes an ongoing growing family of enzymes, twelve groups known to date [1,2], which is divided into intracellular PLA_2, including cytosolic PLA_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$), and secretory PLA_2 ($sPLA_2$). It specifically catalyses the hydrolysis of the *sn*-2 ester bond of glycerophospholipids to release free fatty acids and lysophospholipids. In the case of arachidonic acid and/or lyso-platelet-activating-factor (lyso-PAF), the inflammatory cascade can be switched on to produce eicosanoid (prostaglandins and leukotrienes) and/or PAF by different enzymes such as cyclo-oxygenases (Cox-1 and Cox-2), lipoxygenases and PAF acetyltransferase.

Among human $sPLA_2$s discovered, five groups, GIB, GII, GV, GX and GXII, are of low molecular mass (14–18 kDa). They are all cysteine-rich (5–8 disulphides), structurally globular, millimolar calcium dependent and interfacial enzymes [1–3]. Except for the recently cloned and expressed GXII $sPLA_2$ [2], the others are all reported to be involved in inflammation, either through binding to their specific receptors (GIB) [4,5], or by acting directly on the plasma membrane (GII, GV and GX). It has been reported that the GV and GX enzymes can attack the intact membranes on the extracellular face [6–9], while the GII (A, D, E and F) enzymes need to be internalized into the cells [10] to exert their lipolytic functions or act on the cells undergoing apoptosis or activation [11–13].

Because of the importance of the human $sPLA_2$s as potential targets in the development of anti-inflammatory drugs, availability of these enzymes should be very useful in the selection of specific inhibitors of each group or subgroup of $sPLA_2$. In addition to gene expression, chemical synthesis represents an alternative methodology to produce large quantities of small proteins. In the case of $sPLA_2$s, total chemical syntheses of GIIA (124 amino acids) and GV (118 amino acids) have been reported recently by the Kent group, using Boc (t-butoxycarbonyl) chemistry [14–16]. Their strategy was based on native chemical ligation of two large unprotected peptide segments, (^{1}N–^{58}G)-$sPLA_2$ and (^{59}C–^{124}C)$sPLA_2$, or four, (^{1}N–^{27}H)$sPLA_2$, (^{28}C–^{58}G)$sPLA_2$, (^{59}C–^{87}L)$sPLA_2$ and (^{88}C–^{124}C)$sPLA_2$ for GIIA synthesis (Figure 1), and four segments for GV synthesis, through firstly a trans-thioesterification and secondly the formation of the native amide bond by a five-member ring rearrange-

NH_2-^{1}NLVNFHRMIKLTTGKEAALSYGFY^{25}**G**CHCGVGGRGSPK
DA^{40}**T**DRCCVTHDCCYKRLEKRG^{59}**C**GTKFLSYKFSNSGSRITC
AKQDSCRSQLCECDKAAATCFARNKTTYNKKYQYYSNKHCR
GSTPR^{124}C-OH

Figure 1 Primary sequence of hGIIA $sPLA_2$

The ^{59}Cys, ^{40}Thr and ^{25}Gly are shown in boldface type to indicate the last residue of the segments of which the identity was confirmed by MALDI-MS during the synthesis. Seven disulphide bonds are found in the native structure of hGIIA $sPLA_2$ for the following cysteine pairs: 26/117, 28/44, 43/97, 49/124, 50/90, 59/83 and 77/88.

Abbreviations used: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, calcium-independent PLA_2; $sPLA_2$, secretory phospholipase A_2; hGIB, IIA, IID, IIE, IIF, V, X and XII, human group IB, IIA, IID, IIE, IIF, V, X and XII; PAF, platelet-activating factor; Boc, t-butoxycarbonyl; Fmoc, fluorenyl-9-methoxycarbonyl; SPPS, solid-phase peptide synthesis; HMP, 4-hydroxymethylphenoxymethylpolystyrene; tBu, t-butyl; NMP, N-methylpyrrolidone; DCC, *N*,*N*-dicyclohexylcarbodi-imide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; EDT, ethane-1,2-dithiol; ESMS, electrospray MS; β-py-C_{10}-HMP, 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphomethanol, sodium salt; β-py-C_{10}-PG, 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol, ammonium salt; β-py-C_{10}-HPC, 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; MALDI-MS, matrix-assisted laser-desorption ionization-MS; FXa, Factor Xa; FVa, Factor Va; Trt, trityl.

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ment. This method has also been applied successfully for a great number of other peptide and protein preparations from chemically synthetic and/or biochemically expressed constructs; for review, see [17]. However, for small proteins such as $sPLA_2$s, straightforward synthesis should provide a more direct and less time-consuming access to these enzymes. This has already been demonstrated by our chemical synthesis of proteins, such as 72-residue HIV-1 NCp7 and analogues [18–20], 93-residue human synaptobrevin II [21] and 96-residue HIV-1 Vpr [22,23]. Nevertheless, direct stepwise synthesis of proteins of more than 100 amino acids remains to be a big challenge.

In this work, we describe the total direct synthesis of denatured hGIIA $sPLA_2$ using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and biological properties of the folded protein. No difference has been observed between the synthetic and recombinant $sPLA_2$ in our physicochemical and extensive biological studies. This approach is advantageous in that one can rapidly synthesize this protein, analogues, other human $sPLA_2$s and readily introduce modifications by point mutation, incorporation of different types of probes or post-chemically changeable groups; thus allowing for improved analysis of their physiological roles and pathological effects.

EXPERIMENTAL PROCEDURES

Materials

4-Hydroxymethylphenoxymethylpolystyrene (HMP) resin, Fmoc-L-amino acids, solvents and other reagents for solid phase peptide synthesis (SPPS) were purchased from Applied Biosystems (Paris, France), except for Fmoc-L-Asn(MeTrt)-OH, which was from Neosystem (Strasbourg, France). Other chemicals and porcine pancreatic $sPLA_2$ were from Acros, Aldrich Chimie or Sigma (Paris, France). 1-Hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphomethanol, sodium salt (β-py-C_{10}-HMP), 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol, ammonium salt (β-py-C_{10}-PG), and 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β-py-C_{10}-HPC) were from Molecular Probes (Eugene, OR, U.S.A.). Human prothrombin and FXa were from Enzyme Research Laboratories (South Bend, IN, U.S.A.). FVa was from Haematologic Technologies Inc. (Essex J., VT, U.S.A.). The chromogenic substrate for thrombin amidolytic activity, S-2238, was from Chromogenix (Cincinnati, OH, U.S.A.). BSA (fraction V) was from Sigma. LY311727 was a kind gift from the Lilly Company (Lilly Research Laboratories, Eli Lilly and Company, IN, U.S.A).

Peptide synthesis

Assembly of the 124 amino acids of hGIIA $sPLA_2$ (Figure 1) was carried out in a stepwise manner on an Applied Biosystems model 431A automated peptide synthesizer, starting from 0.05 mmol of HMP resin (0.90 mmol/g) as described for synthesis of large peptides, such as Vpr (96 amino acids) and analogues [22]. A 20-fold excess (1 mmol) of Fmoc-protected amino acids was used and coupled in *N*-methylpyrrolidone (NMP) in the presence of *N*,*N*-dicyclohexylcarbodi-imide (DCC)/1-hydroxybenzotriazole (HOBt), using a longer coupling time (70 min). Acetylation with acetic anhydride was applied for ^{91}Asp and ^{89}Glu after coupling and cleavage of Fmoc protecting group of ^{91}Asp by piperidine was repeated four times. This last treatment was also used for the N-terminal 20 amino acids. The following side chain protections were used: t-butyl ether for Ser, Thr, Tyr; t-butylester for Glu and Asp; trityl for Cys, His, Asn, and Gln; 2,2,5,7,8-pentamethylchromane-6-sulphonyl for Arg; t-butoxy-

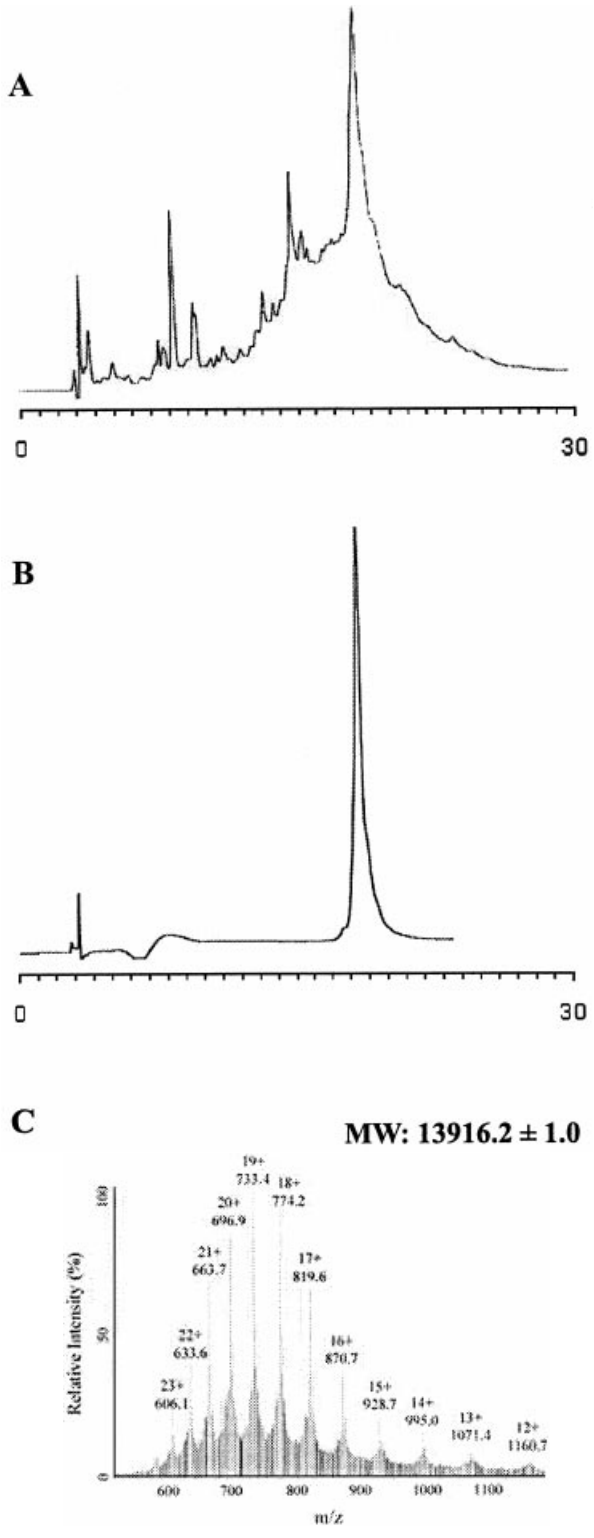
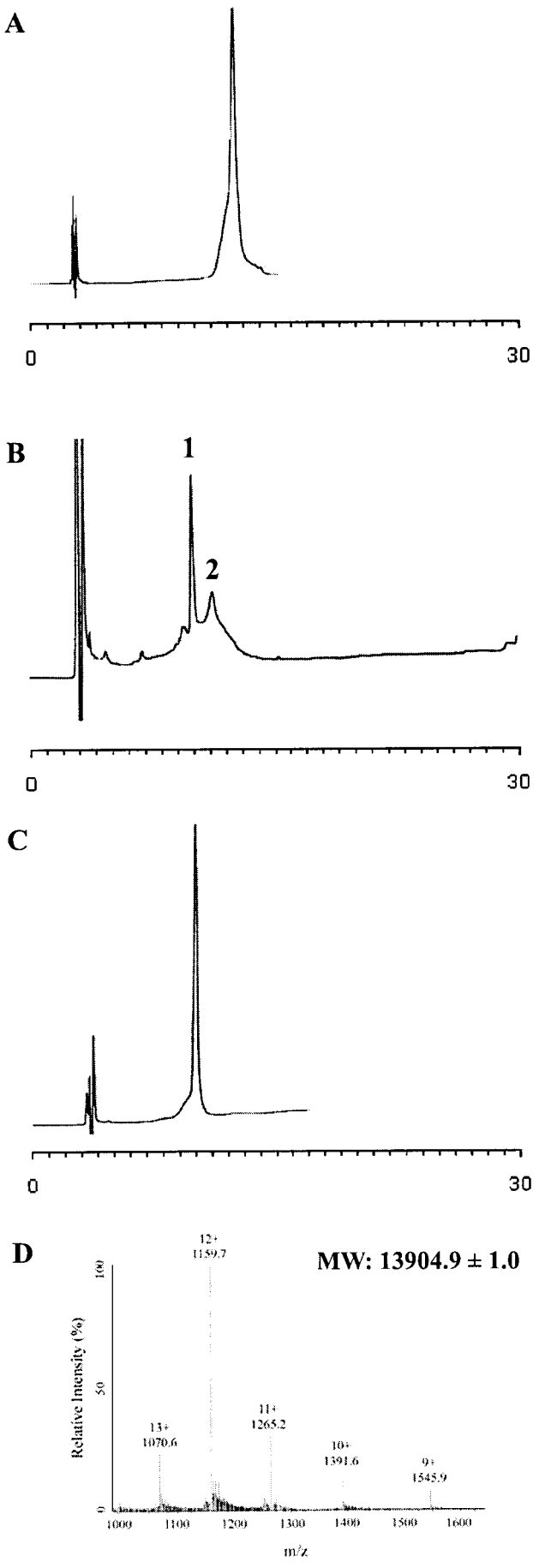


Figure 2 Straightforward synthesis of hGIIA $sPLA_2$

(**A**) HPLC chromatography of crude denatured hGIIA $sPLA_2$ at the end of synthesis. (**B**) HPLC chromatography of twice-purified denatured hGIIA $sPLA_2$. HPLC conditions for (**A**) and (**B**): Vydac C4 column, linear gradient of 10–90% B (see the Experimental procedures section) in 30 min. (**C**) ESMS spectrum of denatured hGIIA $sPLA_2$ in *m*/*z* pattern with molecular mass found: 13916.2 ± 1.0 Da, obtained from all the observed charge states (calculated average isotopic mass, 13918.0 Da).



carbonyl for Lys and 4-methyltrityl for the N-terminal Asn. The peptide was simultaneously cleaved from the resin and side-chain deprotected by treatment with trifluoroacetic acid (TFA, 70 ml) in the presence of phenol (5.25 g), thioanisole (3.5 ml), ethane-1,2-dithiol (EDT, 1.75 ml) and water (3.5 ml) for 2 h at 25 °C. The resin was filtered and the TFA removed under reduced pressure. The peptide was then precipitated with a cold (−20 °C) mixture of ether and n-heptane (1:1, v/v) and collected by centrifugation (2500 ***g*** for 10 min). The pellet was dissolved in TFA (9 ml) and precipitation in the same cold solvent mixture and centrifugation were repeated once more. The peptide was then dissolved in a mixture of CH_3CN (acetonitrile) and water. After removal of CH_3CN in reduced pressure, the peptide was freeze dried and twice purified by semi-preparative HPLC, first using a gradient of 0–60 % B (70 % CH_3CN/30 % H_2O/0.09 % TFA) over 90 min on a C18 column and secondly a gradient of 10–40 % B over 90 min on a C4 column. The fractions were analysed and pooled to finally give 8.0 mg (1 % yield based on the molecular mass of TFA salt and relative to the original HMP resin) of the $sPLA_2$ in reduced form as demonstrated by electrospray MS (Figure 2C).

HPLC

Analytical reversed-phase HPLC was performed on a Shimatzu LC 6A system using Vydac C4 or C18 columns (5 μm, 0.46 cm × 25 cm). Semi-preparative reversed-phase HPLC was performed on an Applied Biosystems 1406A system using Vydac C18 or C4 columns (10 μm, 1.0 cm × 25 cm). Linear gradients of B in A (0.1 % TFA/H_2O) were used in both analytical and semi-preparative HPLC. The flow rates were respectively 1.0 ml/min (analytical) and 2.0 ml/min (semi-preparative) with the detection at 214 nm.

Electrospray MS (ESMS)

Mass spectra were obtained with a BioQ quadrupole electrospray mass spectrometer (Micromass, Manchester, U.K.). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all the observed charge states of a peptide with Mass Lynx software. Theoretical masses were calculated using Mac Pro Mass software (Beckman Research Institute, CA, U.S.A.).

Folding of $sPLA_2$

The purified $sPLA_2$ (4.0 mg) in reduced form (Figure 3A) was dissolved in 5.2 ml of 7 M degassed guanidine hydrochloride buffered with 10 mM sodium borate, pH 7.5, at 4 °C by shaking. This solution was then diluted rapidly to a peptide concentration of 0.1 mg/ml in a degassed buffer, pH 8.5 at 4 °C, with 0.9 M guanidine hydrochloride, 10 mM sodium borate, 10 mM calcium chloride, 2 mM L-cysteine and 1 mM L-cystine as final concentrations. It was shaken with a rotary plate and the formation of the disulphides was monitored by the enzymic $sPLA_2$ activity

Figure 3 Folding of denatured hGIIA $sPLA_2$ and ESMS spectrum of folded hGIIA $sPLA_2$

(**A**) HPLC chromatography of denatured hGIIA $sPLA_2$ at time 0. (**B**) HPLC chromatography of the folding solution after 18 h reaction. (**C**) HPLC chromatography of purified folded hGIIA $sPLA_2$. HPLC conditions for (**A**), (**B**) and (**C**): Vydac C18 column, linear gradient of 35–65 % B in 30 min. (D) ESMS spectrum of folded hGIIA $sPLA_2$ in m/z pattern with molecular mass found, 13 904.9 ± 1.0 Da, obtained from all the observed charge states (calculated average isotopic mass, 13 904.0 Da).

assay (see below) and analytical HPLC. After an 18 h reaction (Figure 3B), the protein solution was dialysed against an aqueous acetic acid solution (0.025 %, v/v; 3 × 2 litres) and then freeze dried. The purification was carried out using semi-preparative HPLC on a C18 column with a gradient 0–40 % B over 90 min. This gave 0.4 mg of a white residue (Figure 3C), of which the ESMS is shown in Figure 3(D), and 2.6 mg of an another white residue corresponding to peak 2 in Figure 3(B) (see the Results section).

Preparation of recombinant hGIIA $sPLA_2$

The expression vector pT7-7 (Amersham Biosciences, Sarclay, France) was transfected into the BL21(DE3) *Escherichia coli* strain. The *Bam*HI/*Hin*dIII fragment encoding hGIIA $sPLA_2$ was cloned in the expression vector to obtain a wild-type primary sequence for hGIIA $sPLA_2$ [24]. hGIIA $sPLA_2$ was expressed as a fusion protein with a 12-amino-acid N-terminal extension ending by an arginine residue for tryptic liberation of the hGIIA $sPLA_2$ with its native N-terminus, as described previously [24]. The active hGIIA $sPLA_2$ protein was obtained from the refolded fusion protein by tryptic cleavage. hGIIA $sPLA_2$ was finally purified sequentially on two SP-Sephadex columns, tested for activity using a fluorescent substrate, and checked for purity by FPLC and SDS/PAGE as reported previously [25].

CD spectroscopy

CD spectra were recorded using a Jobin-Yvon CD6 dichrograph, driven by an IBM PC operating with a CD6 data acquisition and manipulation program. Spectra in the range 190–260 nm were run at 25 °C in 5 mM sodium phosphate, pH 7.5 with a 0.1 cm quartz cell. Protein concentration was set at 15–20 μM and determined using an UV spectrophotometer and the absorption coefficient of 10400 $M^{-1} \cdot cm^{-1}$ at 280 nm, calculated on the basis of absorption of the 8 tyrosines in hGIIA $sPLA_2$. The CD signals are shown in an arbitrary unit, because of a little difference in the sample concentrations, to give the best comparison.

Enzymic $sPLA_2$ activity

$sPLA_2$ activity was assayed with fluorescent phospholipid analogues as described previously [25]. The activity of both recombinant and synthetic hGIIA $sPLA_2$ was compared with three different substrates: 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphomethanol, sodium salt (β-py-C_{10}-HPM), 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol, ammonium salt (β-py-C_{10}-PG), 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β-py-C_{10}-HPC). Briefly, the reaction medium was prepared by sequential addition of: (a) 980 μl of 100 mM Tris/HCl, pH 7.4, 150 mM NaCl and 1 mM EGTA, (b) 1 μM (final concentration) of the indicated fluorescent substrate, (c) 10 μl of 10 % fatty-acid-free BSA, and (d) 2 μl of purified hGIIA $sPLA_2$ at 1 μg/ml. The enzymic reaction was then initiated by adding 10 mM $CaCl_2$. The specific activity of each hGIIA $sPLA_2$ was then calculated and expressed as μmol of substrate hydrolysed per min and per mg of purified hGIIA $sPLA_2$.

Inhibition by LY311727

In a polystyrene cuvette, containing 970 μl of the assay solution [50 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 1 mM EGTA, 0.1 % BSA (fatty acid free), and 2 μM β-py-C_{10}-PG], LY311727 at different concentrations (10 μl of stock solutions in DMSO or ethanol), 1 ng of each $sPLA_2$ (10 μl) and 10 μl of an aqueous solution of calcium chloride (1 M) were added successively. The variation of the fluorescence (λ_{ex} = 342 nm and λ_{em} = 398 nm) was recorded and the initial slope of the curve was used to calculate velocity of the enzymic reaction. The IC_{50} values were determined with three independent sample preparations in two different solvents (DMSO and ethanol) by plotting inhibition percentage obtained from the ratio of the velocities of two assays with or without the inhibitor, versus log concentration.

Measurement of prothrombinase activity

The inhibitory effect of the hGIIA $sPLA_2$ on the prothrombinase activity has been shown to be independent of phospholipids [24,26]. For this reason, the effect of both recombinant and synthetic hGIIA $sPLA_2$ was examined on the prothrombinase complex reconstituted in the absence of phospholipids. Prothrombinase purified components were mixed and subsequently assayed at 37 °C in Tris-buffered saline (0.1 M Tris/HCl, 0.05 M NaCl, 0.5 % BSA, 5 mM $CaCl_2$, pH 7.4) in the following order: 1 nM FXa was incubated with 1 nM FVa for 4 min, then the reaction was started with 1 μM prothrombin. The level of activated prothrombin activity was determined at various time points as described using a chromogenic substrate S-2238 [26]. The hGIIA $sPLA_2$ was added at the beginning of the 4 min preincubation period. The IC_{50} value, which corresponds to a 50 % inhibition of the thrombin generation, was then determined for the recombinant, as well as the synthetic, hGIIA $sPLA_2$ as reported previously [26].

RESULTS

Peptide synthesis

The Fmoc/tBu strategy was chosen to perform the total direct stepwise synthesis of hGIIA $sPLA_2$. Peptide chain elongation was accomplished using DCC/HOBt as coupling reagents in NMP and a 20-fold excess of Fmoc-L-amino acids throughout the synthesis. A longer coupling time was used, without optimization, and a systematic acetylation after each coupling has been found unnecessary. The synthesis was followed with an equipped UV spectrophotometer monitoring system and a systematic analysis of three segments, obtained before delivering the ^{58}G, ^{39}A and ^{24}Y for coupling (Figure 1). The corresponding peptides were cleaved from a small sample of the peptide-resin and side chain de-protected with the same mixture as for the final product. The crude peptides were then purified by HPLC and the identity of the major product was confirmed using a matrix-assisted laser-desorption ionization (MALDI)-MS. The resulting average isotopic masses $[M+H]^+$ (Da) are given as follows: (^{59}C–^{124}C)$sPLA_2$ calculated, 7511.6, observed, 7514.3; (^{40}T–^{124}C)$sPLA_2$, calculated, 9780.2, observed, 9779.2; (^{25}G–^{124}C)$sPLA_2$, calculated, 11162.8, observed, 11163.5. At the end of the synthesis, the crude $sPLA_2$ in reduced form (Figure 2A) was obtained and twice purified by semi-preparative HPLC. The fractions containing the desired peptide were pooled and freeze-dried to give 8.0 mg of the reduced $sPLA_2$ (Figure 2B). Its identity was then confirmed by ESMS, calculated, 13918.0, observed, 13916.2 ± 1.0 Da, as shown in Figure 2(C). The samples were then stored at −20 °C before the folding experiment.

Disulphide formation and folding of $sPLA_2$

The purified $sPLA_2$ in reduced form (4.0 mg) was oxidized in the conditions as described in the Experimental Procedures section. The folding process was followed in parallel with an enzymic assay and analytical HPLC. As reported previously [15], a sharp peak was detected at a retention time 2.4 min less than that

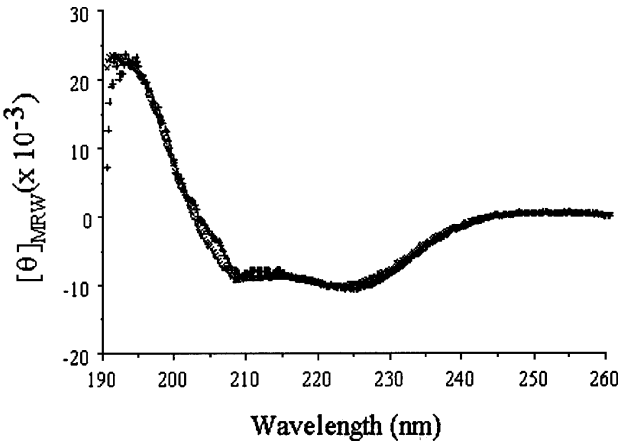


Figure 4 CD spectra of synthetic (+) and recombinant (×) hGIIA $sPLA_2$

The protein concentrations used for spectra recording are 15–20 μM, as determined by UV absorption at 280 nm.

of the reduced $sPLA_2$ under the same HPLC conditions (Figure 3A). Concomitantly, the progressive decrease of the reduced $sPLA_2$ peak on HPLC corresponded to a steady increase of the sharp peak and the enzymic activity of the folding solution (results not shown). Upon completion of the reaction, the reduced sPLA2 peak disappeared completely, along with a maximum enzymic activity (Figure 3B). After dialysis against a large volume (3×2 litres) of aqueous acetic acid solution (0.025 %, v/v) and freeze-drying, the crude folded $sPLA_2$ was dissolved in water and subjected to HPLC for isolation of the sharp peak (Figure 3B, peak 1) from the by-products, appearing mainly as a large peak (Figure 3B, peak 2). Enzymic assay revealed that a high enzymic activity was detected in the fractions corresponding to peak 1 (Figure 3B), by contrast, little enzymic activity (at least 100 times less) was found in the other fractions. Therefore, the fractions were pooled separately and freeze-dried to give 0.4 mg of a white residue for the first pool corresponding to peak 1 (Figure 3B and 3C) and 2.6 mg for the second pool corresponding to peak 2 (Figure 3B). ESMS analysis of the first pool resulted in an experimental mass of 13904.9 ± 1.0 Da (Figure 3D). This is in good accordance with the calculated mass of 13904.0 Da. Taking into account its high enzymic activity, pool 1 was used for the following CD spectrum recording and biological experiences.

It is worthwhile noting that when the second pool was treated with dithiothreitol (50-fold) at 0.14 mM in a Hepes/HCl (5 mM, pH 7.5) buffer overnight at 37 °C, a single and sharp peak was detected at the same retention time as that of the purified reduced $sPLA_2$ under the same HPLC conditions. Co-injection of the pure reduced $sPLA_2$ and the above solution provided only one peak (results not shown). This suggests that the products corresponding to peak 2 (Figure 3B) should be different misfolded $sPLA_2$s, compared with those described previously [14].

It has also been observed that the folding yield was significantly affected by random initial oxidization of the peptide, containing free thiol groups, even at a high concentration of guanidine hydrochloride (7 M) at pH 7.5. The dilution should follow immediately after the dissolution. If delayed, misfolding could occur due to defects or incorrect formation of disulphide bridges. In addition, this was irreversible under the folding conditions, even in the presence of a redox pair, cysteine and cystine (results not shown).

Table 1 Specific enzymic activity (SA) on different substrates, anti-prothrombinase property (APTP) and inhibition by LY311727 of synthetic and recombinant hGIIA PLA_2

PG, phosphatidylglycerol; PA, phosphatidylmonomethyl ester; PC, phosphatidylcholine; APTP, inhibition of the prothrombinase complex formation.

	SA (μmol · min^{-1} · mg^{-1})			IC_{50}	
Enzyme	PG	PA	PC	APTP (nM)	Inhibition by LY311727 (μM)
Synthetic	338 ± 30	180 ± 10	< 1	80 ± 20	0.47 ± 0.04
Recombinant	322 ± 20	175 ± 10	< 1	65 ± 15	0.47 ± 0.04

CD spectra

Secondary structure of the synthetic and recombinant $sPLA_2$ was studied by comparing their CD spectra. As can be seen in Figure 4, good superposition of the two spectra suggests that the synthetic and recombinant $sPLA_2$ possessed the same secondary-structural elements.

Biological activities

It has been well documented that hGIIA $sPLA_2$ hydrolyzes anionic substrates much more efficiently than zwitterionic substrates. For this reason, three glycerophospholipids, β-py-C_{10}-PG, β-py-C_{10}-HPM and β-py-C_{10}-HPC, were used to compare the specific activity of the synthetic and recombinant $sPLA_2$s. The results (Table 1) demonstrated that the zwitterionic β-py-C_{10}-HPC was effectively a bad substrate in both cases when compared with the anionic β-py-C_{10}-PG and β-py-C_{10}-HPM. Moreover, no significant difference was found about the specific activity of the $sPLA_2$s from different preparations on the three phospholipids used.

It is also well known that the hGIIA $sPLA_2$ is capable of inhibiting the formation of prothrombinase complex and this property is independent of its catalytic activity, but critically related to the three-dimensional structure of the enzyme and particularly to the presence of two clusters of basic residues close in space [24]. As shown in Table 1, the synthetic $sPLA_2$ is comparable with the recombinant $sPLA_2$ in their capacity to inhibit formation of the above-mentioned complex. This confirms the idea that the $sPLA_2$s obtained from the two different methods are structurally identical, as deduced from the CD spectra comparison.

Lilly Research Laboratories have reported recently a series of specific inhibitors of the hGIIA $sPLA_2$ [27]. One of these compounds, LY311727, has been used in a great number of biological studies. In this work, we have checked the specificity of this compound to GIIA $sPLA_2$ versus GIB $sPLA_2$ under our enzymic assay conditions. It exhibited effectively a more potent inhibitory activity towards the recombinant hGIIA $sPLA_2$ with an IC_{50} of 0.47 ± 0.04 μM than towards porcine pancreatic $sPLA_2$ (GI) with an IC_{50} of 8 μM. In addition, the same IC_{50} was obtained when using either the synthetic $sPLA_2$ or the recombinant one (Table 1). This result provides further evidence that the catalytic sites of both enzymes are similar.

DISCUSSION

A great number of mammalian $sPLA_2$s have been cloned and expressed in recent years and their tissue distribution is well established. However, their physiological functions remain to be

elucidated and their pathological effects are to be further studied, notably for the new $sPLA_2$s recently discovered. A direct and less time-consuming method to prepare these enzymes, and their mutant analogues, is of great interest for laboratories working in this field.

Innovation in SPPS, such as direct preparation of C-terminal thioester derivatives with Boc and more recently Fmoc chemistry [28], and the application of native chemical ligation using unprotected segments from SPPS allow this technique to be of great use in peptide and protein preparations. It provides a feasible opportunity to synthesize proteins of increasing size, as demonstrated by the recent success in the GIIA and GV $sPLA_2$s syntheses using synthetic segments [14–16], for instance, and in syntheses using synthetic and recombinant ones [17]. Combination of native chemical ligation and desulphurization would significantly broaden the application of chemical synthesis of proteins without cysteine residues [29]. However, more time is spent when using SPPS segments, since supplementary work (segment purification, ligation, intermediate product identification etc.) is needed. Therefore, for small proteins such as $sPLA_2$s, it seems important to know whether direct stepwise synthesis is accessible. Of course, success of stepwise synthesis of large segments in reasonable quantity and purity is of great interest in the extension of chemical ligation methods to obtain larger proteins (more than 200 amino acids).

In this work we have shown, for the first time, that a small protein with 124 amino acids in its sequence, such as hGIIA $sPLA_2$, can be successfully synthesized directly by SPPS, without needing solution coupling of small segments. This expands our collection of proteins by direct chemical synthesis, such as HIV-1 NCp7 and Vpr, and human synaptobrevin II, allowing rapid and simple access to fully active proteins and various mutants, including fluorescent-probe-containing and ^{15}N-enriched ones [19,22]. According to the monitoring diagram that followed each Fmoc deprotection, the synthesis was proceeding very smoothly up to ^{20}Ser, except for ^{89}Glu, of which coupling was a little less efficient and an acetylation was applied, and ^{91}Asp, of which the Fmoc group was less well de-protected and thus four cleavage steps were needed. This was true for the last twenty N-terminal amino acids too, mainly due to increasing peptide size and consequently less efficiency of cleavage. For the other amino acids only two cleavages were sufficient. The global yield of the synthesis before folding is about 1%, better than reported using the chemical ligation method [14,15]. By contrast, the folding yield is only about 10%, much less than those previously reported [14,15], likely due to the rapid random oxidization of the reduced protein during the dissolution. This is to be optimized in our laboratory.

The identity of the reduced hGIIA $sPLA_2$ was confirmed by an electrospray mass spectrometer before performing the folding experiment. We have obtained the same type of m/z pattern as reported previously [14], with the maximal intensity found for the 18th and 19th charged state. After folding, it was shifted to the 12th charged state, with the same character as previously described for the same enzyme [14,15] and other globular proteins [30,31]. The mass obtained agrees well with the theoretical one.

Structural analyses of the synthetic hGIIA $sPLA_2$ have relied on CD spectroscopy and its anti-coagulant activity determination, as compared with the recombinant hGIIA $sPLA_2$. Good superposition of the two spectra provides evidence that the two enzymes are structurally similar. This was confirmed by their same potency for inhibiting the formation of prothrombinase complex, which is dependent of the three-dimensional structure, but not the lipolytic property of the protein.

Enzymic activity of the synthetic hGIIA $sPLA_2$ was studied using 3 different substrates and was compared with that of the recombinant one. The same affinity and discrepancy towards these substrates suggest that both enzymes function in the same manner and their catalytic core is structurally identical. In regard to this point, further evidence was provided by the inhibition experiments, using LY311727. It has been reported as a potent and specific inhibitor of human GIIA $sPLA_2$, since it blocks this enzyme with an apparent K_B of 0.27 ± 0.05 μM and has no effects on porcine pancreatic PLA_2 at 10 μM in a tissue-based assay [27]. Our results on the two human $sPLA_2$s are consistent with the above K_B value. By contrast, we have not found the same specificity of this compound in our experimental conditions as described. This could be due to the difference of the two assay systems and in particular to side-specific effect of the inhibitor on the substrate vesicles in the case of our fluorescent assay.

In conclusion, we have demonstrated in this study that small proteins such as hGIIA $sPLA_2$ can be synthesized directly by continuous SPPS, resulting in a fully biologically active enzyme. This synthetic approach should allow access to analogues with different modifications, such as mono- or multi-site mutation with natural or unnatural amino acids, incorporation of various probes and post-chemically changeable groups, thereby facilitating biological investigations and pharmaceutical development. Ongoing work will further define the scope of the method as applied to other human $sPLA_2$s.

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