Bacterial expression and characterization of human pancreatic phospholipase A₂

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Abstract

Mammalian pancreatic phospholipases A₂ (PLA₂) have recently been implicated in cell surface receptor-mediated inflammation. As a first step toward understanding how human pancreatic PLA₂ (hp-PLA₂) interacts with membranes and other biological targets including cell-surface receptors, we constructed its bacterial expression vector which can be used for the mutagenesis and protein over-expression. The expression vector (pSH-hp) was constructed using a synthetic hp-PLA₂ gene whose transcription is controlled by T7 promoter. hp-PLA₂ was expressed as a mature protein in high concentration in Escherichia coli cells and formed inclusion body. The solubilization of inclusion body protein followed by the refolding and purification produced ca. 5 mg of pure protein from one liter of growth medium. Kinetic studies of recombinant human, bovine and porcine pancreatic PLA₂s using polymerized mixed liposomes and micelles as substrates showed that despite their highly homologous structures these mammalian pancreatic PLA₂s have distinct phospholipid head group specificity and different activity toward various lipid substrates.

Keywords: Human pancreatic phospholipase A₂; Bacterial expression; Substrate specificity

1. Introduction

Mammalian pancreatic phospholipase A₂ (PLA₂) are highly homologous proteins the main function of which is to digest dietary phospholipids emulsified with bile juice [1]. Recently, several lines of evidence have indicated that mammalian pancreatic PLA₂s are present in different tissues and might play other physiological roles including cell surface receptor-mediated inflammation [2]. It is therefore becoming more important to understand how exactly they interact with membranes and other biological targets including cell-surface receptors. All known mammalian pancreatic PLA₂s show strong sequence homology. Also, tertiary structures of bovine (bp-PLA₂) and porcine pancreatic PLA₂ (pp-PLA₂) are nearly iden-
tical except for the difference in a surface loop region [3]. Accordingly, it has been thought that all mammalian pancreatic PLA₂s have similar structural and functional properties and readily available bp-PLA₂ and pp-PLA₂ have been used for most in vivo and in vitro studies. As shown in Fig. 1, however, human pancreatic PLA₂ (hp-PLA₂) has some noticeable amino acid substitutions in key locations. For instance, it has a larger number of cationic residues in the amino terminus and has aspartate in place of lysine at position 53 which was shown to be important for the substrate binding of bp-PLA₂ and pp-PLA₂ [4,5]. Although much is known about the structure and function of bp-PLA₂ and pp-PLA₂, little is known about the structure and function of hp-PLA₂. As a first step toward extensive structure–function studies on hp-PLA₂, we set out to construct its bacterial expression vector. Previously, hp-PLA₂ gene was cloned [6] and the protein was expressed in yeast [7]. However, it has never been over-expressed in amounts sufficient for biophysical and structural analyses. Herein, we describe the construction of highly efficient expression vector for hp-PLA₂, the bacterial expression, the refolding and the purification of recombinant hp-PLA₂. We also describe differences in kinetic properties among human, bovine and porcine pancreatic enzymes.

2. Materials and methods

2.1. Materials

1-Hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (pyrene-PC), -ethanolamine (pyrene-PE) and -glycerol (pyrene-PG) were purchased from Molecular Probes (Eugene, OR). 1,2-bis[12-(lipoxyloxy)-dodecanoyl]-sn-glycero-3-phosphoglycerol (BLPG) was prepared as described elsewhere [8]. Large unilamellar liposomes of BLPG were prepared by multiple extrusion of phospholipid dispersion in 10 mM Tris–HCl buffer (pH 8.4) through 0.1 μm polycarbonate filter (Millipore) in a microextruder Liposofast (Avestin, Ottawa, Ont.) and then polymerized in the presence of 10 mM dithiothreitol as described [9]. Phospholipid concentrations were determined by phosphate analysis [10]. Triton X-100, sodium deoxycholate and dodecylsucrose were from Pierce (Rockford, IL), Sigma and Calbiochem (San Diego, CA), respectively. Fatty acid-free bovine serum albumin was from Bayer Inc. (Kankakee, IL). 5,5’-Dithiobis(2-nitrobenzoic acid) and sodium sulfite were obtained from Aldrich. 2-Nitro-5-sulfothiobenzoate was synthesized from 5,5’-dithiobis(2-nitrobenzoic acid) as described [11]. All restriction enzymes, T4 ligase, T4 polynucleotide kinase and isopropyl β-D-thiogalactopyranoside (IPTG) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNA polymerase I and Klenow fragment were from Amersham (Arlington Heights, IL). AmpliTaq polymerase and pfu DNA polymerase were from Perkin Elmer and Stratagene (La Jolla, CA), respectively. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and used without further purification. Recombinant bp-PLA₂ was prepared as described previously [4]. Recombinant pp-PLA₂ was expressed in E. coli using an expression vector pBL-pp constructed by sub-cloning into pET21a vector (Novagen; Madison, WI) the coding region of pp-PLA₂ gene which was kindly provided by Professor H.M. Verheij of Utrecht University.

2.2. Construction of human pancreatic PLA₂ expression vector

The scheme for constructing an expression vector for hp-PLA₂ (pSH-pp) is illustrated in Fig. 2. The synthetic gene for hp-PLA₂ was made from two

| bp-PLA₂ | ALWQFNMKCKIPSSEPLL—CYKQAKKLDSS |
| pp-PLA₂ | ALWQFRSMIKCAIPGSHPLM—CYRDAKNLDS |
| Hp-PLA₂ | AYWQFRKMIKCVPIPGSDPFGL—CYDQAKKLDSS |

**Fig. 1.** The comparison of amino acid sequences of bovine, porcine and human pancreatic PLA₂s. The amino-terminal twenty residues and residues 51 to 60 are shown to highlight key substitutions. Unique human PLA residues are shown in bold.
fragments each containing 208 base pair (fragment 1) and 210 base pair (fragment 2). Each fragment has two unique restriction sites at 5' and 3' end; EcoRI and HindIII for fragment 1 and HindIII and BamHI for fragment 2, respectively. The unique HindIII site at the junction was created by modifying the DNA sequence of hp-PLA₂ gene, which was eventually mutated to the original sequence for protein expression. As shown in Fig. 2, each gene fragment was prepared from four partially overlapping single-stranded oligonucleotides (64 to 73 base long) whose complementary sequences are ca. 20 base long.

Oligonucleotides used were as follows: Oligo-1 (68-mer), 5'-CTC GAA TTC CAT ATG GCG GTT CAG TTC CGC AAG ATG ATC AAG TGC GTT ATT CCT GGT AGT GAT CC-3'; Oligo-2 (64-mer), 5'-CAC CTA AGC CAC AGT AGC CGT AGT TGT TGT ATT CTA AGA ACG GAT CAC TAC CAG GAA TAA C-3'; Oligo-3 (65-mer), 5'-CTG CTA CTG TGG CTT AGA AGG TTC TGG CAC TCC TGT AGA TGA ACT GGA CAA GTG CTG CCA GAC TC-3'; Oligo-4 (71-mer), 5'-GCA GAA GCT TAC AGC TGT CCA GCT TCT TCG CTT GAT CGT AGC AGT TGT CAT GAG TCT GGC

Fig. 2. The strategy for constructing the bacterial expression vector for hp-PLA₂ (pSH-hp). See the text for details.
AGC ACT TGT CC-3'; Oligo-5 (67-mer), 5'-CTG TAA GCT TCT GCT GGA CAA TCC GTA CAC TCA CAC CTA CTC CTA CTG GTG CTC TGG TTC TGC TAT-3'; Oligo-6 (72-mer), 5'-GGT CGC AGT TGC AAA TGA ACG CTT CAC ACT CTG TGT TCT TGC TAC AGG TGG TTG CCG AGC CAG AGC ACG-3'; Oligo-7 (73-mer), 5'-GTT CAT TTG CAA CTG CGA CCG CAA TGC TGC CAT CTG TTT CTC TAA GGC CCT TCC GTA TAA CAA GGC ACA CAA GAA C-3'; Oligo-8 (70-mer), 5'-GCA GGA TCC TAC ATA TTA TTA ACT CTG GTG CTC TGG TAA GCT TCT GCT GGA CAA TCC GTA CAC ACT TGT CTT GGT GTC CAG GTT CTT GTG CTC TTT ATA CTT CTT GGT GTC CAG GTT CTT GTG TGC CTG GTT ATA C-3'. To obtain each double-stranded gene fragment, four oligonucleotides (1 µg each) were mixed, annealed, and extended with T7 DNA polymerase (0.8 U) or Klenow DNA polymerase (4 U) for 30 min. The products were analyzed on a 2% agarose gel and the major band for each fragment was excised, purified and amplified by the polymerase chain reaction (PCR) using AmpliTaq DNA polymerase and two flanking primers (oligo 1, 4 for fragment 1 and oligo 5, 8 for fragment 2). The PCR consisted of 23 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 1 min. The PCR products were subjected to a 2% agarose gel and the major band for each fragment was excised and purified. The PCR amplified fragment 1 and 2 were digested with EcoRI/Hind III and HindIII/BamHI, respectively, subcloned into pGEM-7Zf(+) vector (Promega; Madison, Wisconsin) and transformed into JM109 cells. To combine two DNA fragments, the two pGEM-7Zf(+) vectors were digested with HindIII/BamHI and a larger fragment from fragment 1 and a smaller one from fragment 2 were purified on an agarose gel. These fragments were then ligated to produce a pGEM-7Zf(+) vector containing the whole hp-PLA2 gene. From this vector, the hp-PLA2 gene (408 base pair) was digested with NdeI and BamHI and subcloned into pET21a vector. Finally, the unique HindIII site introduced for combining the two DNA fragments was removed by site-directed mutagenesis to yield pSH-hp which contains the complete coding sequence for hp-PLA2. The site-directed mutagenesis was performed using a single-stranded DNA purified from pET21a vector and a commercial mutagenesis kit from Amersham [12]. The DNA sequence of coding region was verified by the sequencing analysis using a Sequenase 2.0 kit (Amersham).

2.3. Expression and purification of human pancreatic PLA2

E. coli strain BL21(DE3) was used as a host for the protein expression. A 3 l Luria broth containing 100 µg/ml of ampicillin was inoculated with 30 ml of overnight culture from a freshly transformed single colony and was grown at 37°C. When the absorbance of medium at 600 nm reached 0.2, additional ampicillin was added to a final concentration of 1 mM. The culture was induced by the addition of 0.5 mM IPTG when the absorbance of medium at 600 nm was 0.8. After the culture was incubated for 4 h at 37°C, cells were harvested at 3000 × g for 10 min at 4°C and resuspended in 50 ml of 0.1 M Tris–HCl buffer, pH 8.0 containing 5 mM EDTA, 0.5% (v/v) Triton X-100. The sonication was performed using a Sonifier 450 (Branson) by a pulse mode for 10 × 15 s. The inclusion body pellet was obtained by centrifugation at 10 000 × g for 10 min. The pellet was resuspended in the same buffer and the sonication and centrifugation was repeated. The pellet was solubilized in 10 ml of 8 M urea solution containing 0.3 M sodium sulfite (pH 8.0) and stirred vigorously at room temperature for 30 min. Two ml of 2-nitro-5-(sulfothio)-benzoate solution (25 mM) was then added and the modification was monitored spectrophotometrically at 412 nm. After the modification was complete (ca. 20 min), the mixture was further stirred for 30 min and any insoluble matter was removed by centrifugation at 35 000 × g for 1 h at room temperature. The reaction mixture was loaded onto a Sephadex G-25 column (2.5 × 20 cm) equilibrated with 25 mM Tris–HCl buffer, pH 8.0 containing 5 M urea and 5 mM EDTA, and the first major peak was collected (45 ml). To this solution of sulfonated protein, 100 ml of 25 mM Tris–HCl, pH 8.0 containing 5 mM dodecylsulfoxide, 10 mM CaCl2, 8 mM reduced glutathione and 4 mM oxidized glutathione was added dropwise over 2 h. The solution was kept at room temperature for 20 h, at which point protein solution was dialyzed overnight against 2 × 4 l of 25 mM Tris–HCl buffer, pH 8.0. To remove any precipitates, the solution was filtered through a small (2.5 × 2 cm) Sephadex G-25 column equilibrated with 25 mM HEPES buffer, pH 8.0. The clear solution was loaded onto a HiLoad 16/10 S Sepharose column (Pharmacia) attached to a FPLC system (Pharmacia).
The folded protein was eluted with a gradient of NaCl from 0 to 0.5 M in 25 mM HEPES buffer, pH 8.0. The major protein peak, eluted with ca. 0.25 M NaCl, was collected, dialyzed against water, lyophilized and stored at −20°C. Purity of protein was confirmed by SDS–polyacrylamide electrophoresis. Protein concentration was determined by the bicinchoninic acid method Pierce. The amino-terminal amino acid sequence of recombinant hp-PLA2 was determined by Edman degradation using Applied Biosystems 477A Protein Sequencer. Fragments were sequentially separated with reverse phase HPLC and compared to standards.

2.4. Kinetic measurements

PLA2-catalyzed hydrolysis of polymerized mixed liposomes was carried out at 37°C in 2 ml of 10 mM HEPES buffer, pH 7.4, containing 0.1 μM pyrene-containing phospholipids (1 mol%) inserted in 9.9 μM BLPG, 2 μM bovine serum albumin, 0.16 M NaCl, and 10 mM CaCl2. Enzyme concentrations were adjusted to keep the half-life of the reaction below 5 min. The progress of hydrolysis was monitored as an increase in fluorescence emission at 378 nm using a Hitachi F4500 fluorescence spectrometer with the excitation wavelength set at 345 nm. Spectral band width was set at 5 nm for both excitation and emission. Pseudo-first-order rate constants were calculated from the non-linear least-squares analysis of reaction progress curves from which \( k_{cat}/K_m \) values were calculated by dividing them by enzyme concentrations.

3. Results and discussion

3.1. Construction of expression vector for hp-PLA2

The nucleotide sequence of the synthetic hp-PLA2 gene is shown in Fig. 3. The degenerate DNA sequence of the synthetic gene was inferred from the amino acid sequence of hp-PLA2 and the codons were selected based on the preferred codon usage of E. coli [14]. A long stretch of guanines and cytidines and internal repeats were avoided to prevent the formation of secondary structures. As illustrated in Fig. 2, our strategy for the construction of synthetic hp-PLA2 gene was to divide the gene into two DNA fragments of comparable length (ca. 200 base pair) which could be joined by ligation through a unique restriction site. Each DNA fragment was prepared from the extension of four overlapping single stranded oligonucleotides using several DNA polymerases, including T7 DNA polymerase, Klenow fragment, Sequenase and DNA polymerase I. Among these, only DNA polymerase I did not successfully perform the extension reaction. The resulting DNA fragments were amplified by PCR using either AmpliTaq or pfu DNA polymerase. The agarose gel electrophoresis of amplified DNA fragments showed that only AmpliTaq DNA polymerase yielded DNA bands with expected size. Presumably, pfu DNA polymerase due to its exonuclease activity could not effectively amplify nicked DNA templates. The synthetic DNA fragments were initially subcloned into EcoRI/HindIII and HindIII/BamHI sites of cloning vector pGEM-7zf(+) and eventually the whole coding region of hp-PLA2 was subcloned into NdeI/BamHI site of pET21a vector for protein expression and mutagenesis. The pET21a was selected as an expression vector because it allows both the protein expression and the preparation of a phagemid for mutagenesis [12]. Overall, our approach is faster and more economical than a conventional method of sequentially ligating several double stranded DNA segments. The DNA sequencing of entire coding region of hp-PLA2 from several transformants revealed ca. two random muta-
3.2. Expression and purification of hp-PLA₂

bp-PLA₂ and pp-PLA₂s have been over-expressed in E. coli cells as pro-enzymes from which mature PLA₂s are generated by the enzymatic digest using trypsin [15,16]. In general, the tryptic digest is difficult to control and, as a result, significantly lowers the overall protein yield. It also entails an extra purification step. To circumvent these difficulties, pSH-hp was designed to express the mature hp-PLA₂ in T7 promoter-based pET21a vector. As shown in Fig. 3, the coding sequence of hp-PLA₂ is inserted in frame with initiation codon ATG in the NdeI site of pET21a. In E. coli, the initiator Met is rapidly removed by methionine amino peptidase when the second amino acid has a small side chain, such as Gly, Ala, Ser, Cys, Pro and Thr [17]. Thus, it was expected that hp-PLA₂ would be expressed with Ala at its amino terminus.

When BL21(DE3) cells containing pSH-hp were induced by IPTG, production of a major 14 kDa protein band could be detected in whole cell extracts on a SDS–polyacrylamide gel (Fig. 4, lane 3), which was not present in uninduced controls (Fig. 4, lane 2). As is the case with other secretory PLA₂s expressed in E. coli, hp-PLA₂ was expressed as an inclusion body (Fig. 4, lane 4). The inclusion body containing some bacterial proteins was thoroughly

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Fig. 3. Nucleotide sequence of the synthetic hp-PLA₂ gene. The gene is shown in the 5’ to 3’ direction. The amino acid sequence of expressed protein and unique restriction sites are also shown.

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Fig. 4. SDS–polyacrylamide gel electrophoresis of recombinant hp-PLA₂ expression and purification. Lane 1, purified recombinant hp-PLA₂ as a molecular weight marker; lane 2, whole cell lysate of non-induced BL21(DE3) cells harboring pSH-hp; lane 3, whole cell lysate of IPTG-induced BL21(DE3) cells harboring pSH-hp; lane 4, solubilized inclusion body after washing; lane 5, refolded recombinant hp-PLA₂ eluted from HiLoad 16/10 S Sepharose column as a major peak.
washed, sulfonated and solubilized in 8 M urea. By selectively enhancing the solubility of hp-PLA₂, the sulfonation not only helped solubilize hp-PLA₂ but also removed the bacterial proteins which interfered with subsequent refolding steps. The refolding and disulfide bond formation procedure is similar to a published protocol for bp-PLA₂ [4,15]. The addition of refolding buffer containing a glutathione mixture to the urea solution of sulfonated hp-PLA₂ resulted in protein precipitation, which greatly reduced the refolding yield. The precipitation was prevented by slowly adding the urea solution of protein to the refolding mixture containing a non-ionic detergent, dodecanoylsucrose, which was shown to improve the refolding efficiency of recombinant bp-PLA₂ [12]. Once the refolding solutions were mixed, the protein precipitation was not significant during incubation and dialysis. The progress of refolding was monitored by measuring the enzyme activity of aliquots of refolding mixture using pyrene-PG/BLPG as substrate. After the enzyme activity of refolding mixture reached a maximum (after ca. 20 h), it was dialyzed to remove urea and other reagents. The refolded protein was purified by ion exchange chromatography using a HiLoad 16/10 S Sepharose column. The active protein emerged from the column as a sharp peak with a smaller shoulder peak (Fig. 5). The protein from the major peak was electrophoretically pure (lane 5 in Fig. 4 and showed the highest enzyme activity (data not shown). The amino-terminal amino acid sequence analysis clearly showed that it was pure hp-PLA₂ with the amino-terminal Met fully removed. On the other hand, the protein collected from the minor shoulder peak exhibited lower PLA₂ activity and contained a mixture of improperly processed proteins with Met, Val and Trp, respectively, as first amino acid. Taken together, these results demonstrate that hp-PLA₂ can be expressed and refolded as a fully mature protein and purified to homogeneity by a single step of ion exchange chromatography. Overall yield of purified protein was ca. 5 mg of protein per 1 liter culture.

3.3. Kinetic properties of hp-PLA₂

To systematically compare kinetic properties of hp-PLA₂, bp-PLA₂, and pp-PLA₂, we used three types of phospholipid substrates; anionic polymerized mixed liposomes, anionic mixed micelles and electrically neutral micelles. Apparent second-order constants, \( \left( \frac{k_{cat}}{K_m} \right)_{app} \), determined for various polymerized mixed liposomes and micelles are summarized in Table 1. In polymerized mixed liposome system, it is possible to accurately determine the head group specificity of PLA₂ by varying the head group struc-

<table>
<thead>
<tr>
<th>enzyme</th>
<th>pyrene-PC/BLPG</th>
<th>pyrene-PE/BLPG</th>
<th>pyrene-PG/BLPG</th>
<th>diC(_8)PC micelles</th>
<th>Triton X-100/DC/diC(_8)PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>hp-PLA₂</td>
<td>0.6 ± 0.1</td>
<td>33.0 ± 4.0</td>
<td>20.0 ± 2.5</td>
<td>1.3 ± 0.1</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>bp-PLA₂</td>
<td>0.4 ± 0.1</td>
<td>2.6 ± 0.3</td>
<td>14.0 ± 2.0</td>
<td>3.6 ± 0.4</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td>pp-PLA₂</td>
<td>4.4 ± 1.0</td>
<td>7.2 ± 0.3</td>
<td>73.0 ± 8.0</td>
<td>1.4 ± 0.1</td>
<td>8.4 ± 0.6</td>
</tr>
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See Experimental section for experimental conditions and methods to calculate rate constants. Values of \( \left( \frac{k_{cat}}{K_m} \right)_{app} \) represent (mean values ± standard errors) determined from a minimum of three measurements. Enzyme concentrations were adjusted from 1 nM to 0.1 μM to keep the half-life of the reaction below 5 min. DC: sodium deoxycholate.
ture of hydrolyzable phospholipids in an inert polymerized matrix [4,8,18]. Two zwitterionic phospholipids, pyrene-PC and pyrene-PE, and an anionic phospholipid, pyrene-PG, were used as inserts in the BLPG polymerized matrix. Anionic BLPG was used as a polymerized matrix because pancreatic PLA_2s strongly prefer anionic lipid surfaces to electrically neutral ones. From Table 1, it is obvious that the three pancreatic PLA_2s have distinct phospholipid head group specificity. As reported previously, bp-PLA_2 preferred anionic pyrene-PG to zwitterionic pyrene-PE and pyrene-PC while exhibiting the lowest activity toward pyrene-PC [4]. When compared to bp-PLA_2, pp-PLA_2 was three- to ten-fold more active toward polymerized mixed liposomes which might derive from its higher binding affinity for anionic lipid surfaces. pp-PLA_2 also preferred anionic phospholipids, as observed with bp-PLA_2, but it did not distinguish between pyrene-PE and pyrene-PC as much as bp-PLA_2 did. In contrast, hp-PLA_2 showed a quite different specificity pattern, exhibiting the highest activity toward pyrene-PE. Previous mutagenesis studies indicated that Lys-53 of bp-PLA_2 and Arg-53 of pp-PLA_2 are involved in their anionic head group specificity presumably by electrostatically repelling cationic head groups of zwitterionic phospholipids [4,5]. For instance, the K53E mutant of bp-PLA_2 showed much enhanced activity toward pyrene-PE. Thus, the high activity of hp-PLA_2 toward pyrene-PE appears to be due to the presence of Asp at position 53 of hp-PLA_2. Toward pyrene-PG/BLPG polymerized mixed liposomes, hp-PLA_2 was 1.4-fold more active than bp-PLA_2 but was 3.7-fold less active than pp-PLA_2. This implies that extra cationic residues in the amino-terminal α-helix of hp-PLA_2 do not significantly enhance its interfacial enzymatic activity. To further compare kinetic properties of these pancreatic enzymes, we measured their relative activity toward electrically neutral diC_8 PC micelles and anionic Triton X-100/sodium deoxycholate/diC_8 PG mixed micelles. Of note, bp-PLA_2, which was the least active toward polymerized mixed liposomes, was more active than hp-PLA_2 and pp-PLA_2 toward micellar substrates regardless of their surface charge. Presumably, bp-PLA_2 is more efficient than other enzymes in extracting a substrate from loosely packed micellar surfaces to which all pancreatic PLA_2s readily bind. Taken together, our kinetic measurement demonstrate that despite highly homologous structure, the three mammalian pancreatic PLA_2s have distinct phospholipid head group specificity and different activity toward various lipid surfaces. A comprehensive structure–function study on pancreatic PLA_2s is in progress to understand the molecular origin of these differences.

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References