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Inhibition of nonspecific polymerase activity using Poly(Aspartic) acid as a model anionic polyelectrolyte

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ABSTRACT

DNA polymerases with strand-displacement activity allow to amplify nucleic acids under isothermal conditions but often lead to undesirable by-products. Here, we report the increase of specificity of isothermal amplification in the presence of poly (aspartic) acids (pAsp). We hypothesized that side reactions occur due to the binding of the phosphate backbone of synthesized DNA strands with surface amino groups of the polymerase, and weakly acidic polyelectrolytes could shield polymerase molecules from DNA and thereby inhibit nonspecific amplification. Suppression of nonspecific polymerase activity by pAsp was studied on multimerization as a model side reaction. It was found that a low concentration of pAsp (0.01%) provides successful amplification of specific DNA targets. The inhibitory effect of pAsp is due to its polymeric structure since aspartic acid did affect neither specific nor nonspecific amplification. Strongly acidic polyelectrolyte heparin does not possess the same selectivity since it suppresses any DNA synthesis. The applicability of pAsp to prevent nonspecific reactions and reliable detection of the specific target has been demonstrated on the genetic material of SARS-CoV-2 coronavirus using Loop-mediated isothermal amplification.

1. Introduction

DNA and RNA polymerases are molecular machines that provide the synthesis of nucleic acids (NA) by catalysis of the nucleotidyl transfer reaction. The main functions of polymerases are replication, repair and recombination in living organisms [1], but some of them are used for *in vitro* NA synthesis as well, e.g., in amplification reactions and other biotechnological applications [2–4]. NA amplification has become one of the most important methods for molecular genetic studies, DNA diagnostics, forensics, food control, analysis of environmental samples, etc. [5]. Many genetically engineered DNA polymerases have been obtained to achieve high efficiency and specificity of amplification [6].

Although polymerase chain reaction (PCR) [7] remains the most widely used amplification technique, new isothermal reactions have been developed as well [8]. Isothermal amplification requires polymerases with strand-displacement activity, which provide denaturation of double-stranded NA at a constant temperature. Among these, Bst exo- (large fragment of DNA polymerase I from *Geobacillus stearothermophilus*) and Vent exo- (large fragment of DNA polymerase from *Thermococcus litoralis*) polymerases are the most commonly used. Bst exo-has strong strand-displacement activity, moderate thermal stability and high processivity, and Vent exo-has moderate strand-displacement activity and processivity but high thermal stability. However, both these polymerases cause nonspecific amplification (i.e., in the absence of a specific target), which courses through multimerization [9] or *ab initio* DNA synthesis [10]. Multimerization is the most studied type of nonspecific reaction that readily occurs for short linear DNA templates or primer dimers under isothermal conditions and leads to multimeric products [11]. The products of multimerization represent tandem nucleotide repeats that correlate with the sequence of the initial template and appear as a ladder on electrophoretic gels. The highest efficiency of multimerization is observed for Bst 2.0 DNA polymerase in buffers with high salt content or reducing agents (e.g., 2-mercaptoethanol) for short linear single-stranded DNA templates or primer dimers (of about 50 nucleotides in length), and at

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55–60°C. Side reactions decrease the efficiency of amplification and make it impossible to obtain reliable results. Previously, methods were proposed to exclude the results of nonspecific amplification [12], or to prevent multimerization by varying the reaction conditions and/or the composition of the reaction mixture [13] or by modified primers [14]. However, more convenient approaches to achieve highly specific isothermal amplification are still demanded.

Specificity and efficiency of PCR-based assays can be increased using low molecular-weight additives such as sulfoxides [15], tetraalkylammonium salts [16], carbohydrates [17], etc. The effect of these small organic molecules is mediated by the protection of polymerase from stress factors due to the changes in their molecular environment and hydration state [18,19]. Although isothermal amplification provides the fast detection of viral infections that was become even more relevant over the last year on the background of the Covid-19 pandemic [20,21], there are only a few reports on the improvement of isothermal methods [22,23]. This study aims to develop an approach to suppress nonspecific reactions and increase the specificity and reliability of isothermal amplification using poly (aspartic) acid.

2. Materials and methods

2.1. Reagents

The following reagents were used: Bst 2.0, Bst 3.0 and Vent exo- DNA polymerases, Thermopol and Isothermal buffers (pH 8.8), exonuclease I (Exo I), 50 bp DNA ladder (New England Biolabs); T4 DNA ligase, T4 polynucleotide kinase, dNTP (Thermofisher Scientific); Taq-ME buffer (Sibenzyme, Russia); SYBR Green I (Lumiprobe); sodium heparin, acrylamide, N,N′-methylenbisacrylamide, Tris, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, N,N,N′,N′-ethylenediaminetetraacetic acid disodium salt; o-phosphoric acid (Sigma); L-aspartic acid (L-Asp), organic solvents (Acros Organics). Isothermal buffers with pH 7.2, 7.6, 8.0, 8.4 and 9.2 were prepared as well. All solutions were prepared with highly purified water (>18 MOm) (Millipore).

2.2. Synthesis of poly(aspartic) acid (pAsp)

The polysuccinimides (pSIs) were synthesized via thermal polycondensation of L-aspartic acid as described previously [24,25]. Briefly, the synthesis was carried out in the presence of o-phosphoric acid (2 : 1) under reduced pressure at 185°C for variable time to achieve different molecular weight (MW) of produced polymers, i.e. polysuccinimides (pSIs). pSIs were dissolved in N,N-dimethylformamide (DMF), precipitated by the addition of water, dried, and milled. Then, pSIs were subjected to alkaline hydrolysis in aqueous solution to produce poly (aspartic acid)s, sodium salts. The average MW of three resultant pAsps (pAspI, pAspII and pAspIII) determined by viscosimetry of pSI precursors [24] were 0.9, 3.9 and 6.8 kDa, respectively.

The polypeptide structure of pAsps was verified by FTIR spectroscopy in attenuated total internal reflection mode on a Frontier spectrometer (PerkinElmer) in the range of 400–4000 cm^{-1} (the resolution was 1 cm^{-1}, and the number of scans was 10). The chemical structure of pAsps was analyzed by ^{1}H NMR spectroscopy on an Avance-400 NMR spectrometer (Bruker) (400 MHz, ^{1}H; 100.6 MHz, ^{13}C).

In FTIR spectrum of pAsps the bands at 1600 cm^{-1} (C = O stretching vibrations of amide link and amide I) and 1520 cm^{-1} (joint N–H deformation vibrations and stretching vibrations of C–N and amide II) typical of polypeptides were detected.

^{1}H NMR (400 MHz, DMSO-d_6), 4.70 br.s; 4.54 br.s; 4.35 br. s; 4.30 br. s; 2.63 br. s; 2.57 br. s; 2.44 br. s. pAsp, 13C–{^{1}H} NMR (100.6 MHz, DMSO-d_6), 177.56 br.s (C(O)O); 172.66 br.s (C(O)NH); 171.95 br.s (C(O)NH); 51.3–51.8 br.s (CH); 38.97 br.s (CH_2); 37.31 br.s (CH_2).

2.3. Oligonucleotide primers, DNA templates and RNA material

Artificial DNA template LT, primers F and R, and splint probe S were designed using OligoAnalyzer 3.1 (Integrated DNA Technologies). Primers for LAMP reaction (srs-F3, srs-B3, srs-FIP and srs-BIP) were designed using NEB LAMP Primer Design Tool (New England Biolabs); the nucleotide sequence of coronavirus S-protein gene was chosen as an amplification target in this case. All oligonucleotides were purchased from Syntol (Russia) and are listed in Table 1. The circular DNA template CT was prepared as described in Ref. [14].

The genetic material of SARS-CoV-2 coronavirus was obtained from nasopharyngeal swab samples of the patients affected by COVID-19 (N = 100 patients) using M-Sorb-OOM-96 extraction kit (Syntol, Russia). The COVID-19 diagnosis was confirmed by RT-PCR assay using RT-PCR-SARS-CoV-2 detection kit (Syntol, Russia). LAMP assay was performed directly with RNA-containing material without conversion to cDNA.

2.4. Isothermal amplification

All amplification samples were prepared in UVC/T-M-AR PCR box (Biosan). The working space, dispensers, and plasticware were preliminarily irradiated with ultraviolet for 20 min**.** **A**mplification was carried out in iQ5 thermal cycler (Bio-Rad Laboratories). Reaction mixtures for model experiments with a volume of 10 μl contained 10^{7} copies of linear or circular DNA targets per reaction, 5 pmol of each primer, 1 μl of 2.5 mM dNTP, 1× buffer, 0.1× SYBR Green I intercalating dye, 1.0 U of DNA polymerase (Bst 2.0 or Vent exo-) and 0.01, 0.05 or 0.1% of pAspI, pAspII, pAspIII or sodium aspartate, respectively. The program of amplification consisted of the following steps: 1) 70°C – 30 s, and 2) 60°C – 4 h for Bst 2.0 polymerase; or 1) 95°C – 30 s, 2) 60°C – 1 min, and 3) 68°C – 4 h for Vent exo-polymerase.

LAMP was performed as described in Ref. [26] with little variations. Samples for LAMP with a volume of 20 μL contained 2 μL 10× Thermopol buffer, 2 μl of 2.5 mM dNTP, 3.0 U of Bst 3.0 DNA polymerase, 1.6 μM srs-FIP and srs-BIP primers, 0.2 μM srs-F3 and srs-B3 primers, and 0.5× SYBR Green I intercalating dye. Bst 3.0 DNA polymerase possessing reverse transcriptase activity and providing reverse transcription LAMP was used. The test samples contained 1 μl of nasopharyngeal swab extracts from COVID-19 patients; nontemplate controls contained no NA material. In some cases, the amplification results were additionally analyzed by electrophoresis in 10% polyacrylamide gels (PAGE) followed by ethidium bromide staining and visualization in the Gel Camera System (UVP Inc.).

3. Results and discussion

Isothermal amplification techniques are an excellent alternative to PCR. However, strand-displacement DNA polymerases are very prone to undesirable reactions that drastically reduce the specificity and sensitivity of the assays and efficiency of amplification due to the competition for reagents between specific and nonspecific DNA syntheses. In particular, multimerization can proceed along with the amplification of a specific DNA target in the same reaction tube [11] that provides close threshold reaction times for test samples and negative controls and makes it impossible to distinguish the results of specific and nonspecific amplification. We noticed that besides Bst exo-, other commercial DNA polymerases with strand-displacement activity (Vent exo-, 9°Nm, KlenTaq, etc.) cause an appreciable multimerization (unpublished data).

According to Ref. [27], multimerization is initiated by the formation of cycle-like DNA structures due to the partial denaturation of amplicons termini followed by the bending of free 3′-ends and their annealing at the opposite part of the DNA duplex. The cause of DNA termini bending remains unclear. We hypothesized that this phenomenon occurs due to the ionic interaction between the phosphate backbone of synthesized DNA strands with surface amino groups (from lysine and arginine) of the polymerase (Fig. 1A).

Table 1
Oligonucleotide primers and templates.

Name	Sequence, 5'→3′	Size, nt
F	CCTCTTGCTTTCGCTCTCGTTCTTT	25
R	TGGTCTTCTTCTCGTCTGTGTTCTGT	26
S	AGCAAGAGGTGGTCTTC	18
LT	CCTCTTGCTTTCGCTCTCGTTCTTTACAGAACACAGACGAGAAGAAGACCA	51
srs-F3	GCATGTGACTTATGTCCCTG	20
srs-B3	TGACAATTCCTATTACAACATCA	23
srs-FIP	AGGAAAGTGTGCTTTTCCATCACACAAGAAAAGAACTTCACAAC	44
srs-BIP	CACACACTGGTTTGTAACACAAAGCAGTTACCAGACACAAATGTG	45

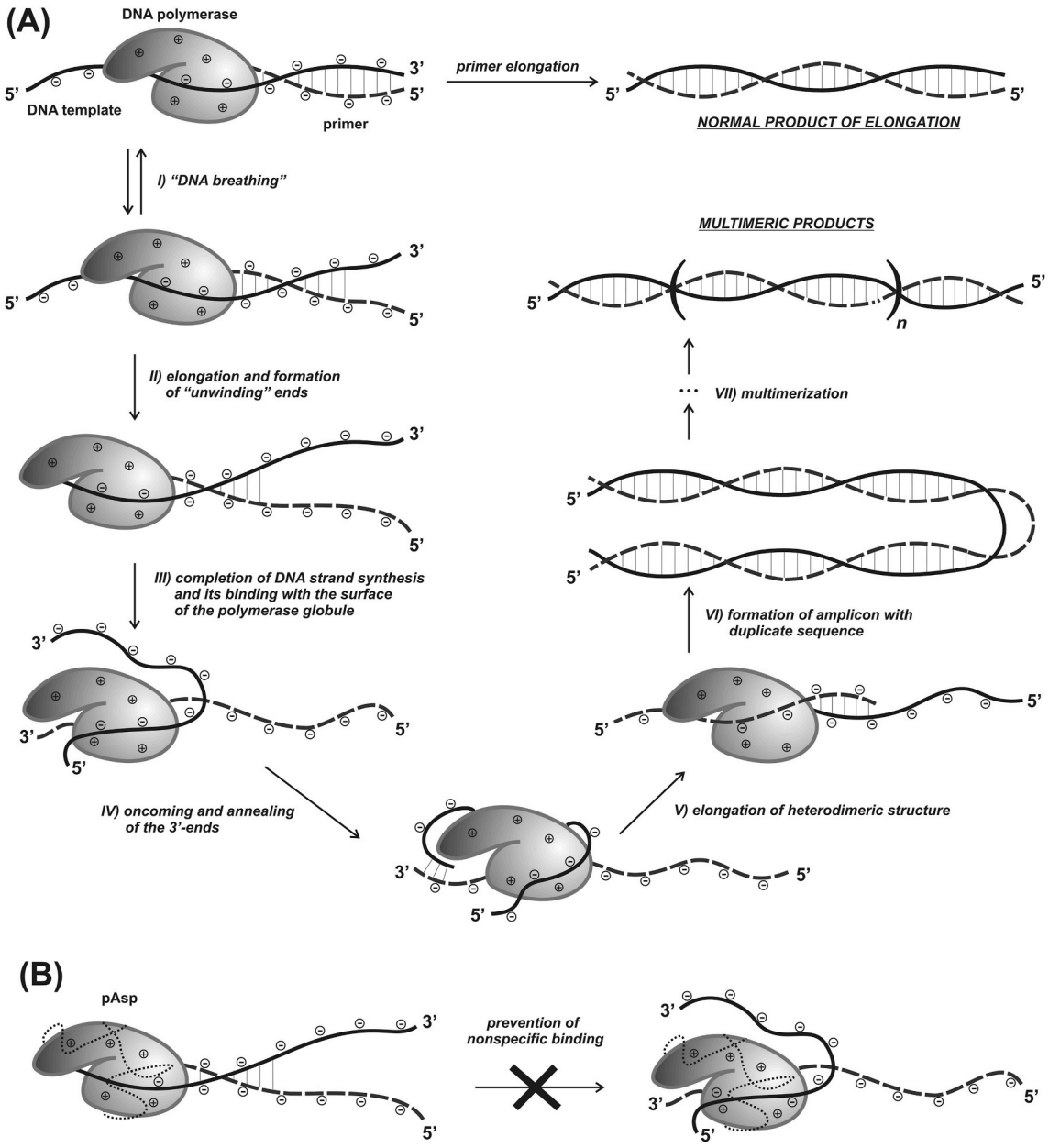

Fig. 1. The scheme of polymerase-DNA interaction and cycle-like DNA structure formation (A), and shielding polymerase molecule by pAsp (B).

Extension of the primer on the template strand should result in the formation of a normal double-stranded amplicon. However, a partial unwinding of the free end of the duplex is possible due to "DNA breathing" [28,29] (Fig. 1, stages I and II). In our opinion, for short amplicons, such a chain untwisting can lead to ionic interaction between positively charged amino groups on the surface of the enzyme globule and negatively charged phosphate groups of DNA (stage III). As a result, DNA envelops the enzyme molecule, and it becomes possible to converge the 3′-ends of the strands to form a heterodimer (stage IV). The heterodimeric structure is elongated by polymerase followed by the formation of an amplification product with duplicate nucleotide sequence (stages V and VI), which facilitates further multimerization

(stage VII). It should be taken into account that the probability of the reaction occurs through the described pathway is extremely low, and the start of multimerization is a sporadic event.

The abovementioned assumption is confirmed by two of our observations obtained for Bst exo- and Vent exo-polymerases: 1) multimerization occurs in the temperature range just below the maximum activity of the enzymes (55-60 °C and 65-70 °C, respectively), apparently, due to efficient unwinding of the amplicons termini, and 2) multimerization is most efficient for DNA of about 50 nucleotides in length, which correlates with the diameter of the enzyme molecules, and does not occur for more extended templates (>60 nucleotides). If the hypothesis is valid, multimerization could be prevented by anionic polyelectrolytes due to their binding with polymerase and shielding polymerase molecules from the interaction with DNA (Fig. 1B). It is known that anionic polyelectrolyte heparin effectively inhibits polymerases even at low concentrations, apparently, due to tightly binding with polymerase through strongly acidic sulfate groups [30].

The model experiments with single-stranded linear DNA template LT and two primers (F and R) that are not homologous to any known genomic sequences were performed to confirm the assumption. A similar molecular set (linear DNA template and two primers) is common for most DNA or RNA amplification reactions, for example, in PCR after cDNA synthesis or in primer dimers formation, in LAMP, or during microRNA amplification. We have shown that efficient multimerization occurs for relatively short (of about 50 nucleotides) DNA structures and for DNA templates with purine-rich 3′-ends [11], therefore, only one DNA template with AG-rich 3′-part was utilized. Since the rolling circle amplification (RCA) was used as an amplification control, the circular template CT was previously obtained from LT by its intramolecular ligation on the splint probe S [31]. Initially, the R primer is annealed to the 3′-part of the linear template or the circular template and is extended by DNA polymerase, resulting in a short double-stranded amplicon for LT amplification or long single-stranded DNA product for CT amplification at the first step of the reaction (Fig. 2).

Then, the F primer anneals to the new strand built by the polymerase after the extension of the R primer. Accumulation of the multimers (products of nonspecific reaction) is achieved through the unwanted amplification (multimerization) initiated by linear template LT, whereas concatamers (products of specific reaction) are formed on the CT template during RCA. The multimers and concatamers are visible on electrophoretic gels and represent the molecular ladders with bands that multiple to the length of the initial template (for example, Fig. 3C or 3D). Amplification was carried out with a program consisting of the following temperature steps: 1) 70°C – 30 s, and 2) 60°C – 4 h for Bst 2.0 polymerase; or 1) 95°C – 30 s, 2) 60°C – 1 min, and 3) 68°C – 4 h for Vent exo-polymerase. The first temperature depending on the polymerase used (70°C or 95°C, respectively) was included in order to dissociate the probable secondary structures. The elongation step was performed for 4 h at a constant temperature.

Three preparations of poly (aspartic) acids as sodium salts with different MW: pAspI (0.9 kDa), pAspII (3.9 kDa) and pAspIII (6.8 kDa) were used as model anionic polyelectrolytes (Fig. 3A). We supposed that since pAsp molecules contain many weakly acidic carboxyl groups, such a polymer would not hinder coordination of DNA with the active site of the polymerase, but would not allow it to bind with the surface of the enzyme molecules (Fig. 1B). We also assumed that a low concentration of pAsp would not significantly affect the specific polymerase activity but would decrease or eliminate the nonspecific one. Preliminary experiments showed that the optimal range of pAsp concentration is 0.1–0.01% (data not shown), so 0.1, 0.05 and 0.01% values were taken to evaluate the effect of pAsp on amplification.

We have recently shown that the most efficient multimerization observes for Bst exo- DNA polymerases in Isothermal and Taq-ME buffers and the weakest one in Thermopol buffer [11]. Here, amplification experiments were carried out with two different DNA polymerases (Bst 2.0 and Vent exo-) and three buffers: Thermopol (supplied with Bst Large Fragment and Vent exo- DNA polymerases), Isothermal (recommended for Bst 2.0 DNA polymerase), and Taq-ME (buffer for Taq DNA polymerase from SibEnzyme, Russia). The temperatures optimal for efficient multimerization were set depending on the DNA polymerase used (60 °C for Bst 2.0 and 68 °C for Vent exo-). Amplification efficiency was evaluated by Tt (time-to-threshold) values similar to the threshold cycle values (Ct) in real-time PCR. Lower Tt indicates a higher rate and efficiency of amplification.

In general, experiments showed the early rise of fluorescence (small Tt values) for samples with a circular template (Fig. 3B, curves marked by letter C), while for samples with a linear template, the curves had increased Tt (Fig. 3B, curves marked by letter L). Relatively high Tt for LT amplification is due to the late start of multimerization, which is a random and rare event that requires time to begin. High Tt values were also observed for amplification with Vent exo-because of its lower strand-displacement activity compared to Bst exo-. Electrophoresis showed the formation of typical ladder-like products of multimerization and RCA (Fig. 3C and D). It should be noted that the yield of the amplicons for Vent exo-is reduced compared to Bst exo-due to the lower strand-displacement activity and processivity of Vent exo-.

The influence of pAsp on amplification efficiency was assessed by the addition of pAspI, pAspII or pAspIII into reaction mixtures. L-aspartic acid was also used instead of pAsp to ensure that the ability of pAsp to bind with the protein and prevent nonspecific amplification relates to its polymeric structure. In addition, amplification with heparin as a control anionic polyelectrolyte was performed. It turned out that sodium aspartate at a concentration equivalent to pAsp monomers does not affect the rate of both specific and nonspecific reactions, while pAsp prevents or

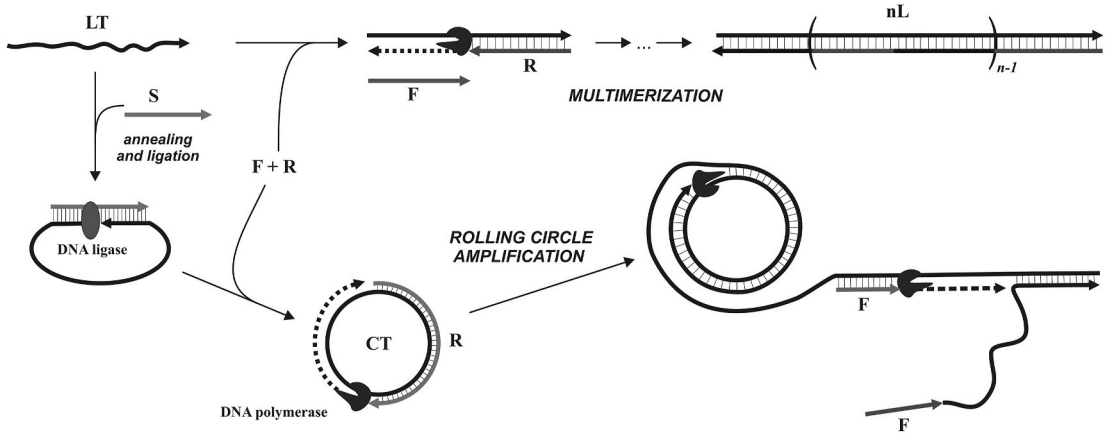

Fig. 2. Experimental design: LT - linear template, CT - circular template, S - splint probe, F and R - primers.

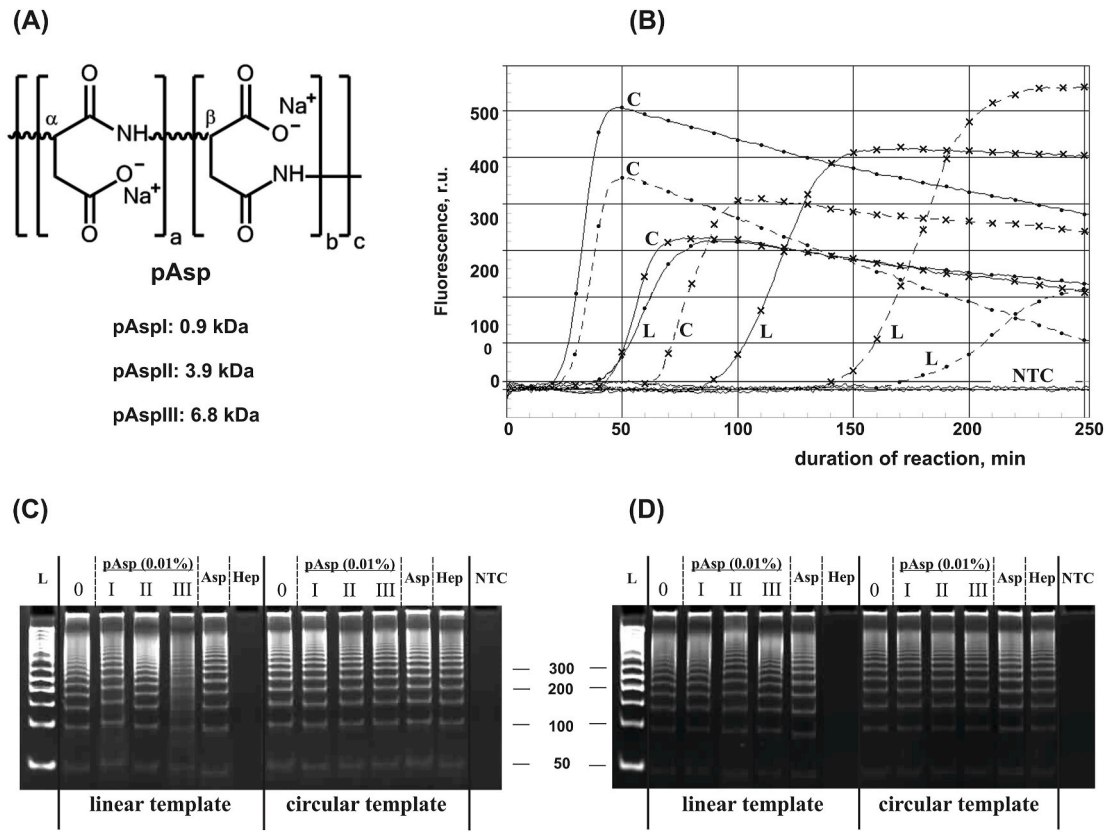

Fig. 3. (A) Structure of poly (aspartic) acid (pAsp, sodium salt). (B) Real-time amplification of linear (curves marked by L) and circular (curves marked by C) templates with Bst 2.0 (curves with dots) and Vent exo- (curves with crosses) DNA polymerases in Isothermal buffer without pAsp (solid curves) and in the presence of 0.01% pAspI (dashed curves). NTC - nontemplate control. Electrophoretic analysis of samples after amplification with Bst 2.0 (C) and Vent exo- (D) DNA polymerases. Lanes: L - 50 bp DNA ladder; 0 - samples without any additive; I, II, III - samples with pAsp I, pAsp II and pAsp III, respectively (0.01%); Asp - sodium aspartate (0,1%); Hep - sodium heparin (0,01 U/ml); NTC - nontemplate control.

significantly reduces the efficiency of nonspecific amplification (Table 2). For Bst 2.0 DNA polymerase, multimerization is prevented (for pAspI and pAspII) or significantly reduced (for pAspIII) at high concentrations of pAsp. As for Vent exo- DNA polymerase, all pAsp samples effectively prevented multimerization at ≥0.05% concentration. However, pAsp increased the duration of the specific reaction. For low pAsp concentrations, the increase was not considerable (less than 2-fold), but for higher concentrations, it was unacceptable (up to 4-fold). Heparin inhibited both Bst exo- and Vent exo-polymerases and blocked any amplification in all buffers except for Taq-ME, therefore, it cannot be considered a polyelectrolyte suitable to prevent nonspecific reactions. The activity of both polymerases in the used buffers was identical and did not depend on pAsp. The highest inhibition was observed in Thermopol buffer, which resulted in a significant increase in the reaction time (>1 h). The highest amplification rate and the highest specificity were achieved for pAspI in Isothermal and Taq-ME buffers, which allow to recommend this compound for the elimination of undesirable reactions.

The effect of ionic interaction between the phosphate backbone of synthesized DNA and surface amino groups of the polymerase should depend on pH due to the change of their protonation status. To verify this, amplification experiments were carried out at different pH in the presence of 0.01% pAsp I (Fig. 4).

For specific amplification, pH decrease led to the increase of Tt values, indicating a reduction in the polymerase activity of the enzyme, which lost at pH below 7.6. Under the same conditions, the decrease of nonspecific activity was more significant, and amplification ceased at pH below 8.0. The addition of pAspI resulted in an abrupt reduction in multimerization efficiency, which was prevented at pH below 8.4. Sodium aspartate did not affect polymerase activity.

To demonstrate the applicability of pAsp-mediated inhibition of nonspecific amplification, the experiments on the detection of RNA material of SARS-CoV-2 coronavirus were performed using the Loop-mediated amplification (LAMP) technique [32], which has become the most used method for detection of viral nucleic acids for the last decade [33]. LAMP-primers were designed using the NEB LAMP Primer Design Tool, and amplification conditions were preliminarily optimized (data not shown). Amplification was performed as a reverse transcription LAMP (RT-LAMP) variant directly with RNA-containing material isolated from nasopharyngeal swab samples of the patients, affected by COVID-19 and confirmed using reverse transcription PCR.

For samples without pAsp, detection of coronavirus RNA occurred within 15–20 min (Fig. 5A, curve 2), however, negative controls (NC) showed early rise as well (within 20–25 min) (Fig. 5A, curve 3). Such a small difference between the Tt values of the test samples and the controls cannot be satisfactory and can lead to a false-negative result when the amount of NA targets of interest is too small. The addition of pAspI slightly inhibited amplification of specific target (Fig. 5A, curves 4 and 5) but completely prevented nonspecific DNA synthesis (Fig. 5A, curve 6, and Fig. 5B, lane 6). Electrophoretic analysis showed the formation of amplification products typical for LAMP that appear on the gel as groups of bands with different lengths (Fig. 5B, lanes 1, 2, 4, 5). At the same time, ladder-like bands typical for multimerization were observed for NC in the absence of pAspI (Fig. 5B, lane 3), but no amplification occurred for NC in the presence of pAspI (Fig. 5B, lane 6). Although sets of amplicons are formed for a specific LAMP reaction and multimerization, they are not similar, namely, for LAMP, several groups of amplification products with close lengths are formed. The obtained results demonstrate the way to increase the reliability of the detection of

Table 2
The mean Tt (time-to-threshold) values for reactions of specific (with circular (CT) template) and nonspecific (with linear (LT) template) amplification (minutes).

Additive	Additive concentration, % (U[a])	Buffer	Bst 2.0 polymerase		Vent exo- polymerase	
			CT	LT	CT	LT
–	0	Thermopol	33 ± 2	47 ± 5	55 ± 2	103 ± 13
		Isothermal	20 ± 1	35 ± 5	40 ± 3	87 ± 8
		Taq-ME	15 ± 1	31 ± 6	37 ± 2	68 ± 12
Sodium aspartate	0.1	Thermopol	31 ± 3	48 ± 8	58 ± 2	107 ± 7
		Isothermal	21 ± 1	34 ± 7	42 ± 2	84 ± 10
		Taq-ME	17 ± 1	29 ± 5	39 ± 1	70 ± 9
pAspI	0.01	Thermopol	42 ± 4	180[b]	85 ± 4	183 ± 24
		Isothermal	26 ± 1	164 ± 15	63 ± 2	145 ± 10
		Taq-ME	18 ± 2	127 ± 5	57 ± 1	128 ± 16
	0.05	Thermopol	66 ± 2	–	117 ± 5	–
		Isothermal	50 ± 3	–	98 ± 5	–
		Taq-ME	41 ± 1	174 ± 11	86 ± 4	–
	0.1	Thermopol	81 ± 4	–	–	–
		Isothermal	65 ± 3	–	143 ± 5	–
		Taq-ME	60 ± 3	–	132 ± 4	–
pAspII	0.01	Thermopol	45 ± 2	87 ± 9	72 ± 2	174[b]
		Isothermal	23 ± 2	72 ± 5	52 ± 2	156 ± 21
		Taq-ME	20 ± 1	58 ± 7	48 ± 3	132 ± 13
	0.05	Thermopol	58 ± 2	165[b]	94 ± 3	–
		Isothermal	49 ± 3	170 ± 16	71 ± 2	–
		Taq-ME	37 ± 2	119 ± 7	69 ± 3	–
	0.1	Thermopol	79 ± 3	–	117 ± 4	–
		Isothermal	62 ± 2	–	93 ± 5	–
		Taq-ME	55 ± 3	–	87 ± 3	–
pAspIII	0.01	Thermopol	48 ± 4	85[b]	71 ± 2	–
		Isothermal	21 ± 2	77 ± 6	53 ± 1	167 ± 18
		Taq-ME	20 ± 3	73 ± 7	47 ± 3	152 ± 15
	0.05	Thermopol	53 ± 3	112[b]	89 ± 4	–
		Isothermal	47 ± 3	98 ± 11	77 ± 3	–
		Taq-ME	40 ± 2	81 ± 6	71 ± 3	–
	0.1	Thermopol	74 ± 3	172 ± 11	121 ± 3	–
		Isothermal	63 ± 4	117 ± 19	102 ± 2	–
		Taq-ME	62 ± 4	94 ± 12	93 ± 2	–
Sodium heparin	0.01	Thermopol	–	–	–	–
		Isothermal	–	–	–	–
		Taq-ME	154 ± 24	–	124 ± 18	–
	0.05	Thermopol	–	–	–	–
		Isothermal	–	–	–	–
		Taq-ME	–	–	142 ± 37	–
	0.1	Thermopol	–	–	–	–
		Isothermal	–	–	–	–
		Taq-ME	–	–	–	–

[a] Activity units (U/ml) for sodium heparin.

[b] Amplification for one sample of three repeats was occurred.

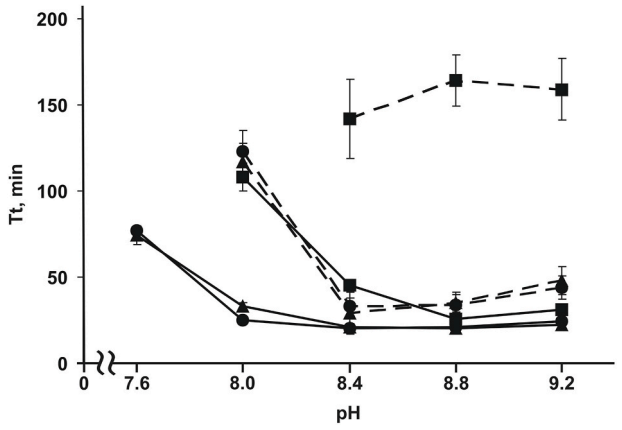

Fig. 4. Tt (time-to-threshold) values for amplification of circular (solid curves) and linear (dashed curves) templates depending on pH (Bst 2.0 DNA polymerase, Isothermal buffer). Tt values were obtained at the presence of 0.01% pAspI (squares), or 0.1% Asp (diamonds), or without additives (circles).

infectious agents using anionic polyelectrolytes as a reaction additive during amplification.

4. Conclusion

Strand-displacement DNA polymerases are extremely prone to catalyze nonspecific DNA synthesis, which complicates the interpretation of the amplification results. Fortunately, undesirable competing processes could be prevented by optimization of the reaction conditions. Using rolling circle amplification as a model specific reaction and multimerization as a nonspecific one, we have shown that weakly acidic anionic polyelectrolytes such as poly (aspartic) acid can selectively suppress nonspecific synthesis and thereby increase the reliability of the assays. It was found that poly (aspartic) acid at a concentration of 0.01% provides an admissible duration of the specific reaction and, at the same time, allow to discriminate specific and nonspecific amplification due to the significant increase of ΔTt values (the difference between Tt values for amplification of linear and circular templates, respectively). Aspartic acid did not affect the course of either specific or nonspecific amplification. Thus, the inhibitory effect of pAsp comes from its polymeric nature that probably allows pAsp to bind with the enzyme in a specific

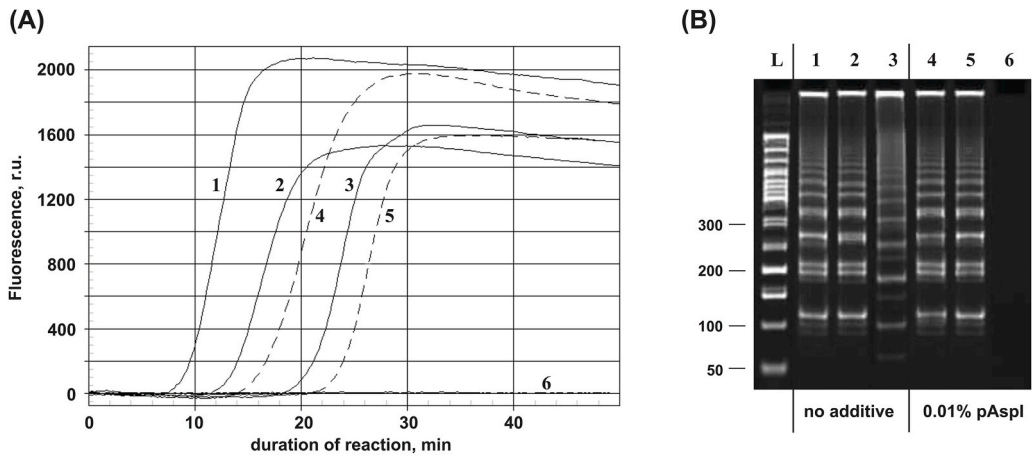

Fig. 5. Detection of the genetic material of SARS-CoV-2 using LAMP reaction. (A) Amplification curves for the positive control (curves 1 and 4), test sample (2 and 5), and negative control (curves 3 and 6). Solid curves represent samples without additive, dashed curves correspond to samples with 0.01% pAspI. (B) Electrophoretic analysis of amplification results. L - 50 bp ladder; lanes 1 and 4 - positive control, 2 and 5 - test samples, 3 and 6 - negative control.

manner due to the cooperative action of the polycarboxylic backbone. Nevertheless, the mechanism of binding of anionic polyelectrolytes with surface amino groups of the enzyme requires further detailed studies.

The inhibitory power of pAsp preparations differed depending on their MW. pAsp I was found to be the most effective inhibitor, probably, due to the smaller size of the polymer molecules that reduces steric hindrance when enveloping enzyme globule. Unlike pAsp, heparin that contains strongly acidic sulfate groups did not suppress nonspecific amplification indicating that selective inhibition of undesirable DNA synthesis requires less acidic polymers such as pAsp. The proposed mechanism of inhibition of multimerization using pAsp is confirmed by its dependence on the pH. Thus, with a decrease in pH, a more significant reduction or loss of nonspecific activity of the polymerase occurs compared to the specific one.

Although the possibility to avoid multimerization by varying combinations of DNA polymerases, buffers, and cofactors was previously demonstrated [13,23], these conditions only allow to reduce the probability of multimerization and increase the threshold amplification times for control samples, and they do not prevent the nonspecific interaction of DNA strands with the enzyme globule. Since the use of anionic polyelectrolytes addresses the crucial cause of nonspecific amplification, it should provide a more appropriate way to solve the problem. The applicability of pAsp for detection of a specific target has been demonstrated on the genetic material of SARS-CoV-2 coronavirus using LAMP assay. It was shown that although pAsp increases the duration of the reaction, it suppresses nonspecific amplification, which makes it possible to obtain reliable results.

CRediT author statement

Assol R. Sakhabutdinova: Methodology, Investigation, Writing - Original Draft. **Marat I. Kamalov:** Methodology, Resources. **Diana V. Salakhieva:** Formal analysis, Validation, Funding acquisition. **Ayrat R. Mavzyutov:** Resources. **Ravil R. Garafutdinov:** Conceptualization, Writing - Review & Editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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