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Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation

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The loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that uses only one type of enzyme. One of the characteristics of the LAMP method is its ability to synthesize extremely large amount of DNA. Accordingly, a large amount of by-product, pyrophosphate ion, is produced, yielding white precipitate of magnesium pyrophosphate in the reaction mixture. Judging the presence or absence of this white precipitate allows easy distinction of whether nucleic acid was amplified by the LAMP method. Since an increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction was achieved by real-time measurement of turbidity.

Key Words: **nucleic acid detection; loop-mediated isothermal amplification; LAMP, turbidity; magnesium pyrophosphate.**

Although several nucleic acid amplification methods have been developed so far (1–4). Polymerase chain reaction (PCR) is the most widely used (1). Because of its apparent high simplicity and probability, routine use of PCR as a standard approach in biotechnology and medical diagnostic laboratories has been extensively practiced. However, the PCR method has several intrinsic disadvantages, such as the requirement of rapid thermal cycling, insufficient specificity, and rather low amplification efficiency. Taking such disadvantages into account, we have developed a new nucleic acid amplification method, loop-mediated isothermal amplification (LAMP) (5, 6), to establish a specific nucleic acid amplification method with ease of operation. The primary characteristic of the LAMP method is its ability to amplify nucleic acid under isothermal conditions in the range of 65°C. As a result, it allows the use of simple and cost effective reaction equipment. The second characteristic of the LAMP method is that it has both high specificity and high amplification efficiency. As the LAMP method uses 4 primers recognizing 6 distinct regions on the template DNA, its specificity is extremely high. For example, the LAMP method can specifically amplify a specific gene from a human genomic specimen discriminating a single nucleotide difference (H. Kanda, *et al.*, in preparation). Amplification efficiency of the LAMP method is extremely high because there is no time loss of thermal change due to its isothermal reaction, the reaction can be conducted under optimal temperature of enzyme, and the inhibition reaction at the later stage of amplification (7) is less likely to occur compared with the PCR. The LAMP method synthesizes 10–20 μg of specific DNA for 25 μl of reaction mixture in 30–60 min.

It was observed that when nucleic acid is amplified by the LAMP method, the turbidity derived from precipitate is produced according to progress of the reaction. Chemical and spectroscopic analyses identified the produced precipitate as magnesium pyrophosphate. The purpose of this study is to clarify the cause of the production of precipitate of magnesium pyrophosphate by the LAMP reaction, and establish a new detection method of the LAMP reaction based on such turbidity.

MATERIALS AND METHODS

Template DNA. A 569-bp fragment of prostate-specific antigen (PSA) cDNA was amplified with PCR using primers of 5′-CGGGATCCAGCTGTGTCACCATGTGGGT and 5′-CGGGATCCTG-CGCACACACGTCATTGGAA, and then the products were digested with *Bam*HI. The *Bam*HI-digested PCR products were inserted in *Bam*HI-digested pBluescript II before being used as the template DNA. Commercially available λ DNA (Takara Shuzo) was also used as the template DNA.

LAMP reaction. The LAMP reaction was conducted according to the reference (5) using the following primers: For PSA amplification—5′-TGTTCCTGATGCAGTGGGCAGCTTTAGTCTG-

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CGGCGGTGTTCTG (PSA-FIP); 5′-TGCTTGTGGCCTCTCGTG (PSA-F3), 5′-TGCTGGGTCGGCACAGCCTGAAGCTGA-CCTGAAATACCTGGCCTG (PSA-BIP), and 5′-GGGTGTGT-GAAGCTGTG (PSA-B); and for λ DNA amplification—5′-AGGCCAAGCTGCTTGCGGTAGCCGGACGCTACCAGCTTCT (lambda-FIP), 5′-AAAACTCAAATCAACAGGCG (lambda-F3), 5′-CAGGACGCTGTGGCATTGCAGATCATAGGTAAAGCGCCACGC (lambda-BIP), and 5′-GACGGATATCACCACGATCA (lambda-B3).

Amplification reaction was conducted at 65°C by mixing 1.6 μM each of FIP and BIP primer, 0.2 μM each of F3 and B3 primer, 8 U of *Bst* DNA polymerase (New England Biolabs) with attached buffer (Thermopol buffer including 2 mM of $MgSO_4$), 0.8 M Betaine (Aldrich Chemical), 2 mM $MgSO_4$ (Nakalai Tesque), and a specified amount of the template DNA. The same reaction mixture without template DNA was used as negative control. Ten milliunits of Tth-pyrophosphatase, thermostable (PPase; Roche Diagnostics), was added when necessary. The amplified regions including primer-binding site were 136 and 132 bp for PSA and λ DNA, respectively.

PCR. PCR was conducted with the Expand Long Template PCR System (Roche Diagnostics), under the conditions described in its protocol, using pBluescript II plasmid DNA containing the inserted PSA cDNA fragment as template and PSA-F3 and PSA-B3 LAMP primers as primer. After the initial denaturation step at 96°C for 3 min, thermal cycling at 96°C for 20 s, 62°C for 40 s and 68°C for 40 s was repeated 35 times.

Measurement of turbidity. For endpoint turbidity measurement, absorbance at 400 nm was measured with a Ultrospec 2000 spectrophotometer (Pharmacia Biotech) transferring 75 μl of the LAMP reaction mixture into a microvolume spectrophotometry cell (light pass: 1 cm). As a reference, absorbance of the reaction buffer of the same concentration was measured. Turbidity was determined by taking the difference between the absorbance of the sample solution and a reference. By measuring the absorption spectra of the reaction mixture before amplification, almost no absorbance around 400 nm was confirmed. The change in turbidity with reaction time was monitored by conducting the LAMP reaction in a cell, which was placed in a sample-folder attached to a circulation jacket maintained with isothermally circulating water (65°C).

Isolation and analysis of precipitate. To isolate precipitate, the LAMP reaction of 50-ml scale was conducted in a glass vial, using PSA as template DNA. After the LAMP reaction, the reaction mixture was centrifuged (5000*g*, 10 min) and then the supernatant was removed. The precipitate was resuspended by adding 50 ml of distilled water. These operations were repeated 3 times, and then the precipitate was isolated by centrifuge (5000*g*, 10 min). Isolated precipitate was dried in a silica gel desiccator at room temperature for 1 week. The amplified DNA by 50-ml scale LAMP was subjected to 2% agarose gel electrophoresis, and was confirmed to show the same pattern as that for normal scale (25 μl) LAMP reaction for PSA (5) (data not shown).

The FT-IR spectrum of precipitate was measured with a FTIR-8100 (Shimadzu Scientific Instruments) using standard KBr method at room temperature. The IR spectrum of commercially available magnesium pyrophosphate (Aldrich Chemical) was similarly measured as reference.

Qualitative analysis of magnesium ion in the precipitate was performed by Titan Yellow method (8, 9). Two milligrams each of isolated precipitate and commercially available magnesium pyrophosphate was dissolved with 10 ml of 0.01 N hydrochloric acid. By adding 1 μl of 0.1% Titan Yellow to 20 μl each of the suspension, the color of each solution was determined.

To trace the reaction to produce magnesium pyrophosphate by the LAMP method, the reaction between magnesium sulfate and potassium pyrophosphate or potassium phosphate (Aldrich Chemical) was observed by the turbidimetric method. $MgSO_4$ at 2 mM was dissolved with Thermopol buffer (final magnesium ion concentration of 4 mM) and incubated for 60 min at 65°C by adding certain concentration of potassium pyrophosphate or potassium phosphate. The turbidity of such solutions was measured with the same procedure as that for the LAMP reaction solution.

TABLE 1

Turbidity and DNA Yield in the LAMP Reaction Mixture after 60 min Reaction at 65°C

Amplification method	Template DNA	Starting amount of template DNA (copies)	Turbidity	[DNA] (μg/25 μl)
LAMP	PSA	6000	1.256	11.2
		0	0.001	0.0
	λDNA	6000	1.245	10.9
		0	0.000	0.0
PCR	PSA	6000	0.000	0.2
		0	0.002	0.0

Quantification of synthesized DNA. Concentration of double-stranded DNA in the DNA amplification reaction mixture was measured with a PicoGreen dsDNA Quantitation Kit (Molecular Probes) according to protocol with an RF-5000 spectrofluorophotometer (Shimadzu Scientific Instruments).

RESULTS

Turbidity of LAMP Reaction Mixture

Table 1 shows the results of turbidity measurements for the LAMP reaction mixture conducted for 60 minutes at 65°C. Increases in turbidity were observed for specimens containing template DNA independent of the type of template DNA in the reaction mixture. However, no increase in turbidity was observed for specimens that did not contain template DNA. These results suggest that the presence or absence of template DNA in testing specimen can be judged with the amplification by the LAMP method followed by the measurement of turbidity for the reaction mixture. When a reaction mixture in which DNA is amplified by the LAMP method is centrifuged (6000 rpm) for several seconds on a small desktop centrifuge, precipitate accumulates at the bottom of the tube. As this accumulated precipitate can easily be confirmed with a naked eye, detection of the LAMP reaction can be done by judging the presence of accumulated precipitate after centrifugation as well as visual judgment of turbidity. When amplification of almost the same target region was conducted by PCR under the normal condition, no increase of turbidity was observed (Table 1). Usually, in detection of template DNA with the PCR method, the progress of the amplification reaction was confirmed by complementary procedures such as gel electrophoresis.

Analysis of Precipitate

Figure 1 shows IR spectra of isolated precipitate and commercially available magnesium pyrophosphate.

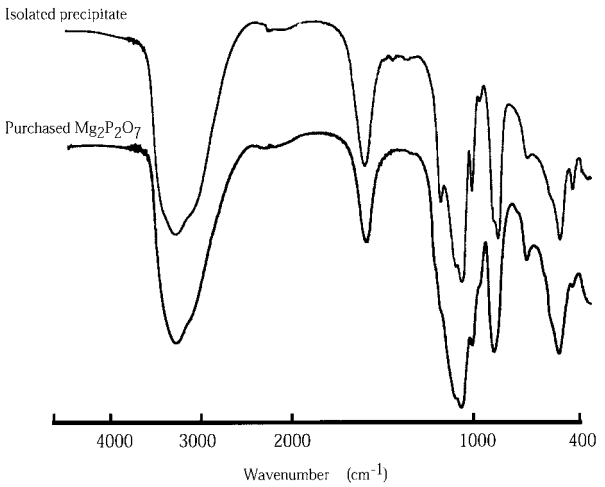

FIG. 1. IR spectra of precipitate produced by the LAMP reaction (top) and commercially available magnesium pyrophosphate (bottom).

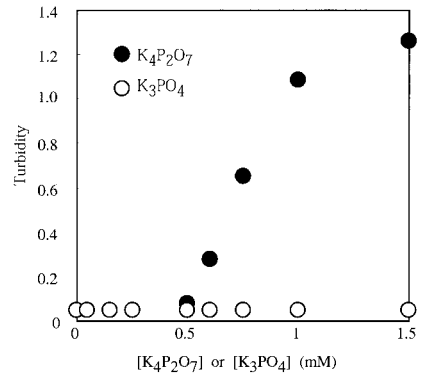

FIG. 2. Turbidity of reaction mixture containing Mg^{2+} ion and $P_2O_7^{4-}$ (filled circle) or PO_4^{3-} (open circle) ion in Thermopol buffer after reaction for 60 min at 65°C.

The IR spectrum of isolated precipitate coincided well in almost all peaks with that of commercially available magnesium pyrophosphate. From this result, the major component in the precipitate was found to be magnesium pyrophosphate. Furthermore, the finding that the peak in the vicinity of 2900 cm^{-1}, which is typical for CH stretching vibration of organic compounds, was not portrayed in the IR spectra of the precipitate indicates the absence of organic compound. This also indicates that the presence of DNA or organic compounds in reaction buffer (e.g., surfactant) hardly affects the generation reaction of precipitate. When the precipitate dissolved with hydrochloric acid was treated with Titan Yellow, the color of solution changed from yellow to red (data not shown). Moreover, this red color was similar to that of commercially available magnesium pyrophosphate with the same weight when compared with qualitative analysis. This result qualitatively indicates that the main cationic component in the precipitate was composed of magnesium ion. Furthermore, as shown in Table 2, when the LAMP reaction was conducted with the presence of Tth-pyrophosphatase, enzyme that hydrolyzes pyrophosphate ion to orthophosphate ion, no precipitate was produced while DNA amplification reaction occurred. This result suggests that pyrophosphate ion is essential for generation of precipitate.

TABLE 2

Effect of PPase on the Turbidity of the LAMP Reaction Mixture after 60 min Reaction at 65°C

Template DNA	Starting amount of template DNA (copies)	PPase (mU)	Turbidity	[DNA] (μg/25 μl)
PSA	6000	10	0.001	11.1
	6000	0	1.269	11.5
	0	10	0.000	0.0

Tracing of Precipitating Reaction

According to the above results, production of precipitate based on the LAMP reaction can be represented by the following reactions:

$$(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-} \quad [1]$$

$$P_2O_7^{4-} + 2Mg^{2+} \rightarrow Mg_2P_2O_7 \quad [2]$$

In DNA polymerization by DNA polymerase (1), pyrophosphate ion is released from dNTP as a by-product. When a large amount of this pyrophosphate ion is produced, it reacts with magnesium ion in the LAMP reaction buffer yielding a precipitate (2). The concentration dependency of pyrophosphate ion affecting the reaction between magnesium ion and pyrophosphate ion was investigated to ascertain reaction (Fig 2). When potassium pyrophosphate was added to the LAMP reaction buffer containing 4 mM magnesium sulfate and allowed to react for 60 min at 65°C, turbidity increases were observed when the concentration of added pyrophosphate ion exceeded 0.5 mM. Consequently, it was calculated that the solubility of magnesium pyrophosphate ($K_{sp} = [Mg]^2[P_2O_7]$) in the LAMP reaction buffer was about 8×10^{-9} mol^3 (dm^{-3}). According to the reaction (1), on a 25-μl scale reaction, a DNA yield of more than 4 μg is required to elevate the pyrophosphate ion concentration to more than 0.5 mM. As shown in Tables 1 and 2, the LAMP reaction synthesizes 10 μg/25 μl or more DNA, and therefore produced pyrophosphate ion react with magnesium ion to induce the precipitate. However, DNA yield by PCR is about 0.2 μg/25 μg and the resulting pyrophosphate

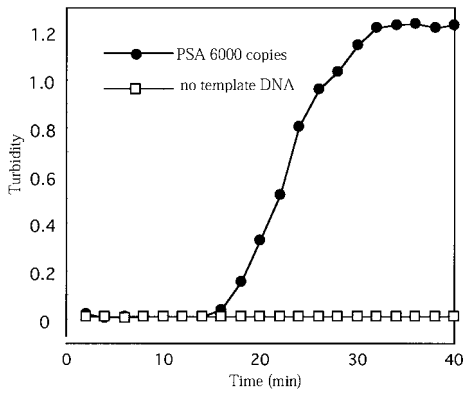

FIG. 3. Time-related change of turbidity in the course of LAMP reaction. LAMP reaction was conducted with 6000 copies of template DNA (PSA) at 65°C.

ion approximates 0.02 mM. Therefore, precipitate of magnesium pyrophosphate cannot be produced by the DNA amplification by the PCR. In addition, as shown in Fig. 2, magnesium phosphate was not produced within this concentration range. This also accounts for nonprecipitation when pyrophosphate ion was hydrolyzed to phosphate ion by PPase. While it is known that pyrophosphate ion is hydrolyzed to phosphate ion by heating (10), this probably occurs under the high temperature conditions typical of the denaturation step of the PCR, and it would be unlikely to occur under the isothermal condition at 65°C used in the LAMP reaction.

Correlation between Precipitate Yield and DNA Yield

If the precipitate is produced according to the above mentioned reaction equation, turbidity would increase in correlation with the DNA yield. The time-related change of turbidity in the LAMP reaction was therefore determined accordingly (Fig. 3). The turbidity of the reaction mixture containing template DNA began to increase at 15 min after initiation of the reaction and showed continuous gradual increase until about 30 min. To judge whether or not these turbidity increases reflected the progress of DNA synthesis by the LAMP reaction, the following investigation was carried out. In brief, the amounts of synthesized double-stranded DNA in the reaction mixture were successively monitored from 15 to 30 min after initiating the reaction with monitoring of the turbidity of the solution. From these data, the yields of DNA and the turbidity were found to be in a linear relationship as shown in Fig. 4. Accordingly, this proves that turbidity reflects DNA yield in the LAMP reaction, namely progress of the LAMP reaction. These finding indicated that time-related turbidity determination could furnish persistent monitoring of accumulative DNA synthesis in the LAMP reaction.

DISCUSSION

So far, several detection methods for amplified DNA have been developed (10–12). Among those, methods to detect pyrophosphate ion derived from nucleic acid amplification (or phosphate ion hydrolyzed from pyrophosphate ion) are included (10, 12). However, when amplification reaction is conducted with PCR, pyrophosphate ion or phosphate ion is produced only to orders of within a few tenths of μM. For this reason, special coloration reagents or enzymes are required to detect produced pyrophosphate ion. In contrast, the LAMP reaction enables visual judgment for the presence of pyrophosphate as turbidity or precipitate. Therefore, no special reagent to detect pyrophosphate ion is needed. By enlarging the target region to be amplified or by changing the reaction conditions of number of cycles or magnesium ion, etc., PCR may be able to synthesize large amount of DNA from which precipitate of pyrophosphate is produced. In such cases, however, it is highly possible that nonspecific amplification could arise (13). In such ambiguous amplifications, it would be of no significance to perform such a sequence nonspecific detection as turbidity measurements. Production of magnesium pyrophosphate along with amplification may occur even in amplification methods other than PCR (2–4). However, specificities of those amplification methods are definitely not of high levels, and it is difficult to judge if the specific amplification had been established merely based on the generation of magnesium pyrophosphate. The LAMP method has advantageously favorable characteristics of high specificity and high amplification efficiency not found in other methods, and therefore the absence or presence of precipitate can respectively demonstrate the absence or presence of template DNA in testing specimens without reservations. In other

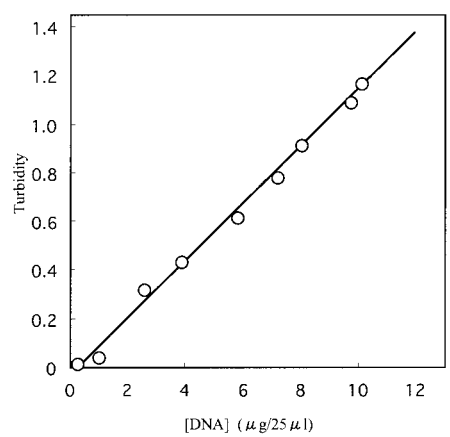

FIG. 4. Relation between turbidity of the LAMP reaction mixture and its DNA yield during continuous amplification reaction process. LAMP reaction was conducted with 6000 copies of template DNA (PSA) at 65°C.

words, the LAMP method is a superior nucleic acid amplification method, which conducts amplification and detection in one-step without the use of any detection reagents.

In cases such as quantitative diagnosis of infectious deceases, it is important to examine the presence of nucleic acid in the samples not only qualitatively, but also quantitatively. As a quantitative determination method for the amount of template DNA in the original specimen, kinetic analysis with PCR has been widely spread (14, 15). As mentioned above, the time-related changes in turbidity realize the application of kinetic analysis by the LAMP reaction. If template DNA amount dependence on the LAMP reaction rate is able to be monitored with turbidity measurement, it is expected that a novel quantitative determination method of nucleic acid utilizing high specificity and high amplification efficiency of the LAMP method could be established.

Former nucleic acid amplification techniques including PCR require expensive reaction equipment for rapid thermal cycling as well as time, labor, and cost-consuming detection tests. As indicated above, execution of the LAMP reaction and measurement of its turbidity is an extremely simple nucleic acid detection method. As turbidity can be confirmed visually, the only device required is a water-bath that furnishes a constant temperature of 65°C. If natural energy could be used as a power source to furnish an isothermal environment, even electricity (electrical connections) is not required. For instance, we have confirmed the progress of the LAMP reaction with heat generated from oxidation of iron micro-particles. Furthermore, in cases where detection of amplification is based on changes of turbidity, there is no need to use any detection reagent such as fluorescent intercalating dyes, which are commonly employed in DNA detection but environmentally unfriendly and highly toxic. By the use of turbidity measurement of the LAMP method, a small, simple, economically feasible, and safe DNA detection device could be established. The development of such device could realize the genetic point-of-care (gPOC) testing at the bedside or in underdeveloped nations in the near future.

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