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Short communication

A new method for random mutagenesis by error-prone polymerase chain reaction using heavy water

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ABSTRACT

Error-prone polymerase chain reactions (epPCRs) are often used to introduce mutations in random mutagenesis, which has been used as a tool in protein engineering. Here, we developed a new method of epPCR using heavy water as a solvent instead of normal water (H_2O). Rhodopsin cDNA of the Ayu fish (*Plecoglossus altivelis*) was used as a template and was amplified using five different conditions: (A) 100% H_2O with no Mn^{2+}, (B) 100% H_2O/0.6 mM Mn^{2+}, (C) 99% D_2O with no Mn^{2+}, (D) 99% D_2O/0.6 mM Mn^{2+} and (E) 99% $H_2{}^{18}O$ with no Mn^{2+}. The 13,960 (for each of the conditions A to D) and 33,504 (for condition E) base pairs were sequenced. A maximum error rate of 1.8×10^{-3} errors/bp was detected in condition D, without any particular hot-spot mutations. A high preference for AT → GC transitions was observed in condition D, whereas a high preference for transitions over transversions was observed in condition C. All of the mutations observed in condition E were transversions. When conditions A and C were applied to another template, the honeybee actin gene, the results were comparable to those for Ayu rhodopsin. Based on these results, the use of heavy water, instead of H_2O, as a solvent for epPCR can introduce random mutations without positional bias, template dependency or decreased yield. Our new epPCR method, and possibly combining the use of D_2O and $H_2{}^{18}O$, may be a powerful random mutagenesis technique.

1. Introduction

Random mutagenesis is used as a tool for generating more optimized enzymes or altered proteins that have specific functions. The strategy involves altering DNA that encodes different proteins, expressing the mutated proteins, and screening of these proteins based on the desired properties. This approach needs no prior estimation or knowledge of the functional importance of each residue, and is thus widely used for purposes associated with protein engineering (Labrou, 2010). In addition to biological applications, random mutagenesis can also be used for more fundamental research, such as the functional analysis of each residue in a protein (Krebs and Fierke, 1993).

Several methods for random mutagenesis are currently established, such as error-prone PCR (epPCR) (Cadwell and Joyce, 1994), UV irradiation or chemical mutagenesis (Kadonaga and Knowles, 1985; Witkin, 1976), and saturation mutagenesis (Kotzia and Labrou, 2009). Of these, epPCR is the most widely used for *in vitro* mutagenesis. epPCR is generally performed using DNA polymerases, such as the native DNA polymerase of *Thermus aquaticus* (*Taq*) or the artificially mutated polymerase of *Pyrococcus furiosus* (*Pfu*), without 3′ to 5′ exonuclease activity (also known as proof-reading activity) with error-prone conditions using Mn^{2+}, nucleotide analogs, or biased nucleotide concentrations (Biles and Connolly, 2004; Cadwell and Joyce, 1994). However, epPCR using *Taq* polymerase has a major drawback in that it creates biased patterns of substitution in which AT → GC transitions and AT → TA transversions appear more frequently than other substitutions (Keohavong and Thilly, 1989; Lin-Goerke et al., 1997). To compensate for this, some procedures combine different epPCR methods having opposite tendencies for substitutions (Vanhercke et al., 2005).

In this study, a new method for epPCR was developed using heavy water (D_2O), which consists of one oxygen and two deuterium (D, also depicted as ^2H), a stable isotope of hydrogen. Deuterium contains one proton and one neutron in its nucleus, whereas hydrogen contains only one proton. Deuterium exists in natural environments, and, on average, 0.015% of H is D. Therefore 0.03% of water molecules contain D. D_2O can be created by a simple electrolysis reaction, making it relatively inexpensive to produce. The chemical characteristics of solvents are of great importance in enzymatic reactions; the replacement of H by D causes significant

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changes because of the two-fold increase in molecular weight of D versus H. Thus, it was expected that replacing H_2O by D_2O would cause an increase in the error rate of the PCR as a result of decreased substrate specificity. We also describe the results of epPCR with another kind of heavy water, $H_2{}^{18}O$, as the solvent. Here, we propose a new epPCR method and report the change in error rate in PCR when heavy water was used as a solvent.

2. Materials and methods

The complete open reading frame of rhodopsin (Rh) cDNA (DDBJ accession number AB086225) from Ayu fish (*Plecoglossus altivelis*) was cloned into the pGEM easy-T vector and was used as a template for subsequent PCR experiments. The length of the cloned fragment was 1,436 base pairs (bp) and the GC content of the analyzed region (1396 bp) was 53%. All PCRs were performed using Gene *Taq* polymerase (Nippon Gene; Tokyo, Japan), 10× Gene *Taq* universal buffer (Nippon Gene), dNTPs (Nippon Gene), $MnCl_2$ (Nacalai Tesque, Kyoto, Japan), and gene-specific primers Ayu-Rh-ALL-F: 5′-GCCAGACAAAACAACACC-3′ and Ayu-Rh-ALL-R: 5′-TACGTTGCCTTGATTACATGCC-3′ that were designed previously (Minamoto and Shimizu, 2003). Initially, all PCR reagents, excluding *Taq* polymerase, were mixed and dried using a centrifugal evaporator, and re-dissolved in H_2O or D_2O (Sigma–Aldrich, St. Louis, MO, USA). *Taq* polymerase was then added to the mixture. Given that the polymerase was dissolved in H_2O, a maximum D_2O concentration of 99% was used in these experiments. Based on reports that the addition of Mn^{2+} reduces base pair specificity in DNA replication (Beckman et al., 1985), four PCR conditions were tested: (A) 100% H_2O with no Mn^{2+}, (B) 100% H_2O/0.6 mM Mn^{2+}, (C) 99% D_2O with no Mn^{2+}, and (D) 99% D_2O/0.6 mM Mn^{2+}. In addition, we tested the solvent effect of another type of heavy water, $H_2{}^{18}O$, in the absence of Mn^{2+}: condition (E) 99% $H_2{}^{18}O$ (SI Science, Saitama, Japan) with no Mn^{2+}. The reaction mixture (50 μl) contained 1× Gene *Taq* Universal Buffer, 0.2 mM each of dNTPs, 0.4 μM each primer, 100 pg template DNA, 0 or 0.6 mM $MnCl_2$, and 0.05 units/μl *Taq* polymerase. The thermal cycling conditions consisted of 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min using a GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA). Amplified fragments were cloned into the pGEM easy-T vector and sequenced with DYEnamic ET Terminator Sequencing Kit (GE Healthcare, Piscataway, NJ, USA) with M13 universal primers. The sequence was determined in both directions using the 373S automatic sequencer (Applied Biosystems). The complete open reading frames of 10 different Ayu-Rh clones amplified using each of the conditions A–D were sequenced. For condition E, 24 amplified Ayu-Rh clones were sequenced, giving a total of 33,504 sequenced bp (24 × 1396 bp).

To confirm that the introduction of mutations was not specific to Ayu-Rh, the PCR experiment was also performed using the actin gene of the honeybee *Apis cerana* (DDBJ accession number AB072495) (Shimizu et al., 2001) as a template. The length and GC content of the analyzed region were 311 bp and 42%, respectively. PCR conditions A and C were used, as described above, and 37 and 35 clones, respectively, were sequenced.

3. Results and discussion

In this study, we established a new epPCR method using heavy water instead of normal water. Based on the difference in the solvent's chemical characteristics, DNA polymerase function could be affected with regard to the substrate specificity of the reaction and, consequently, more mutations could be introduced. The occurrences of each type of substitution, the error rate and the bias indicators under each PCR condition with each template are listed in Table 1.

Table 1
Occurrence of each type of substitution for each PCR condition.

Template	PCR condition	Total analyzed sequence (bp)	AT → GC	GC → AT	AT → TA	AT → CG	GC → CG	GC → TA	Total Ts	Total Tv	Deletion	Total	Error rate	Ts/Tv	AT → GC/ GC → AT	AT changes (%)	GC changes (%)
Ayu-Rh	(A) 100% H_2O/with no Mn^{2+}	13,960	1	1	0	0	0	0	2	0	0	2	1.4×10^{-4}	–	1.0	50	50
	(B) 100% H_2O/0.6 mM Mn^{2+}	13,960	1	0	0	1	2	0	1	3	0	4	2.9×10^{-4}	0.3	–	50	50
	(C) 99% D_2O/with no Mn^{2+}	13,960	8	6	2	0	0	0	14	2	0	16	1.1×10^{-3}	7.0	1.3	63	38
	(D) 99% D_2O/0.6 mM Mn^{2+}	13,960	15	3	2	2	1	2	18	7	0	25	1.8×10^{-3}	2.6	5.0	76	24
	(E) 99% $H_2{}^{18}O$/with no Mn^{2+}	33,504	0	0	0	3	2	4	0	9	0	9	2.7×10^{-4}	–	–	33	67
Honeybee actin	(A) 100% H_2O/with no Mn^{2+}	11,581	0	0	0	0	0	0	0	0	0	0	0	–	–		
	(C) 99% D_2O/with no Mn^{2+}	11,305	9	2	0	1	1	0	11	2	1	14	1.2×10^{-3}	5.5	4.5	71	21