Production and Evaluation of *Taq* DNA Polymerase

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Astract

Taq DNA polymerase is an enzyme essential in performing Polymerase Chain Reaction (PCR) which has recently become a basic technology in research and diagnostic laboratories. In order to reduce the cost of research work in Thailand, recombinant *Taq* DNA polymerase was locally produced from pTaq cloned in *E. coli.* The enzyme was characterized and evaluated in comparison with the commercial *Taq* DNA polymerase produced by Perkin Elmer Cetus, U.S.A. The yield of enzyme was 6.72 mg/ml and the activity of 9,524 units/mg protien with the total of 448,000 units/litre of the bacterial culture. The preparation was free of DNase based upon its ability to degrade Lambda DNA evaluated by gel electrophoresis. Although the enzyme produced gave a high DNA polymerase activity, the preparation was not as pure as the enzyme produced by Perkin Elmer Cetus. Immunoblot analysis indicated that the enzyme preparation contained the products of enzyme degradation obtained during preparation and bacterial protein contaminations. In spite of the existence of bacterial proteins in the preparation, the *Taq* enzyme produced was proved to be applicable in performing PCR such as the PCR-SSP (Sequence Specific Primers) typing for HLA-DR. The cost of enzyme preparation was about 256 times less than that of the commercial enzyme. Economically, the locally produced *Taq* DNA polymerase can be used efficiently in the research laboratories performing PCR based typing of the HLA genes.

Thermus aquaticus DNA polymerase or *Taq* DNA polymerase is a heat stable enzyme used to amplify specific DNA sequences *in vitro*. It is an essential enzyme in Polymerase Chain Reaction (PCR) which is widely used in research and diagnostic laboratories[1,2]. The enzyme was originally extracted from thermophilic bacteria. The process of production and purification was quite complicated using special equipment and skill[3]. Recent advances in molecular technology make it possible

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to clone and highly express the Taq gene in other fast growing bacteria such as *E. coli*[4,5]. New methods have been developed to make the preparation of enzyme quick and simple with high yield and good quality of recombinant *Taq* enzyme[6,7]. Local production of *Taq* enzyme for research purposes will reduce the cost, especially in the field of molecular biology in Thailand.

In this report, we adopted the clone and method used by F.G Pluthero[6] to produce the recombinant *Taq* enzyme. The enzyme preparation was evaluated for the yield, activity, purity and stability as well as its application to PCR based typings of the HLA genes.

MATERIAL AND METHOD

Preparation of recombinant *Taq* DNA polymerase

The *pTaq* clone[5], containing the *Taq* gene under control of *tac* promoter kindly provided by J Corbett (Corbett Research Laboratory, NSW, Australia), was introduced into *E. coli* strain INV1αF' (Invitrogen Corp, CA, U.S.A.) using the standard heat shock method[8]. The recombinant *Taq* DNA polymerase was produced according to the rapid method of F.G. Pluthero[6]. Briefly, one colony of the transformant was regrown in 2 ml of LB medium with 80 mg/l of ampicillin at 37°C for overnight. To prepare one litre of bacterial culture, 500 µl of the overnight growth bacterial culture was transferred to 1 litre of LB with 80 mg/l of ampicillin and regrown at 37°C until Optical Density (OD) at 600 nm of the culture reached 0.8 which would take approximately 11 hours. The bacteria was then induced for high expression of the *Taq* gene by isopropyl-1-thio-ß-D-galactopyranoside (IPTG; Boehringer-Mannheim, Germany) at a final concentration of 125 mg/l for 12 hours. The bacterial pellet was then washed in 100 ml of prelysis buffer (50 mM Tris-HCl *p*H 7.9, 50 mM dextrose, 1 mM EDTA) and resuspended in 50 ml of prelysis buffer with 4 mg/ml of lysozyme (Boehringer-Mannheim, Germany) for 15 minutes at room temperature. To release the recombinant *Taq* enzyme from the cells, the bacteria were lysed using an equal volume of lysis buffer (10 mM Tris-HCl *p*H 7.9, 50 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl, 0.5% tween 20 and 0.5% nonidet-P 40) at 75°C for 1 hour. The bacterial debris was separated by centrifugation at 15,000 rpm, 4°C for 10 minutes. The supernatant was transferred to a new flask and 30 g of $(NH_4)_2SO_4$/100 ml of lysate was slowly added with stirring at room temperature. The protein precipitation was collected by centrifugation at 15,000 rpm, 4°C for 10 minutes. The protein pellet was then resuspended in 20 ml of prelysis buffer and dialysed two times, 12 hours each in storage buffer (50 mM Tris-HCl *p*H 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl and 50% gylcerol) at 4°C. The final enzyme preparation was tested for purity using SDS-PAGE and immunoblot analysis as well as separately stored in storage buffer at 4°C, -20°C, -70°C and weekly tested for the enzyme activity in PCR.

Determination of yield and purity of the enzyme preparation

Total protein concentration of the enzyme preparation was determined using the Lowry method[9]. The *Taq* enzyme produced was analysed for purity compared to the commercial *Taq* enzyme (Perkin Elmer Cetus, U.S.A.) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli's method[10]. The protein preparation was separated through 5 per cent and 12 per cent stacking and separating gels under 60 and 150 volts, respectively. The protein bands were revealed by staining with 0.1 per cent of Coomassie brilliant blue R-250. Immunoblot analysis was performed according to the standard method[10] using mouse monoclonal antibody against *Taq* (Clonetech, U.S.A.) and anti-mouse IgG conjugated with alkaline phosphatase (Sigma, U.S.A.) using ß-naphthyl phosphate as well as O-dianisidine as substrates (Sigma, U.S.A.). The protein bands revealed by the Coomassie blue and alkaline phosphatase/substrate stainings were analysed quantitatively by densitometry (LDS-200, Beckman, U.S.A.).

Determination of enzyme activity

The activity of the enzyme preparation was determined by amplifying the PCR product of the PERB11.1 gene. The product is approximately 600 bp covering exon 2, intron 2 and part of exon 3 flanked by the JA3 and JB3 primers[11]. The DNA template used for PCR testing was a plasmid clone, p7J-9-3, carrying PERB11.1 derived from the 7.1 ancestral haplotype[11]. The PCR procedure for amplification of this gene was described previously[11].

To determine the activity of enzyme, the locally produced *Taq* DNA polymerase was titrated

against the *Taq* enzyme from Perkin Elmer Cetus, U.S.A. Two-folded dilutions of Taq from the two sources were used in the PCR reactions. The intensities of PCR products were compared from different dilutions of the two sources revealed by agarose gel electrophoresis. Accordingly, the activity of the locally produced *Taq* enzyme was roughly calculated.

Determination of DNase contamination

The locally produced *Taq* enzyme was incubated with 2.4 μg of lambda DNA (Gibco, U.S.A.) in a 50 μl reaction at 37°C overnight. Positive controls consisted of lambda DNA and DNase (Boehringer Mannheim, Germany) at 1, 0.1, 0.01, 0.001 and 0.0001 μg. The contamination of DNase activity in the preparation was evaluated by the degree of integrity of the lambda DNA by 0.8 per cent agarose gel electrophoresis.

Determination of enzyme stability

The enzyme preparations kept at 4°C, -20°C and -70°C were tested weekly for 13 weeks to determine the stability of the enzyme produced. Dilutions of each aliqout of enzyme preparations were used in PCR reactions as described above. The PCR products obtained from different dilutions of each aliqout were blindly scored according to their intensities by two readers using the criteria as follows: 0 = no PCR product, 1 = probably negative; positive cannot be excluded, 2 = weakly positive, 3-10 = positive 1+ to 8+. This semi-quantitative detection system was adopted from Abraham et al (12). The average scores were presented in this report. F-test (ANOVA) and pair *t*-test were used to evaluate the differences of enzyme activities amongst the preparations kept at three different temperatures.

Feasibility of the recombinant *Taq* DNA polymerase in HLA typings

The locally produced enzyme was used to perform the HLA-DR, HLA-DQ, HLA-A, HLA-B and HLA-C typings using PCR-SSP for HLA class II[13,14] and ARMS-PCR (Amplification Refractory Mutation System) for HLA class I (12th International Histocompatibility Workshop).

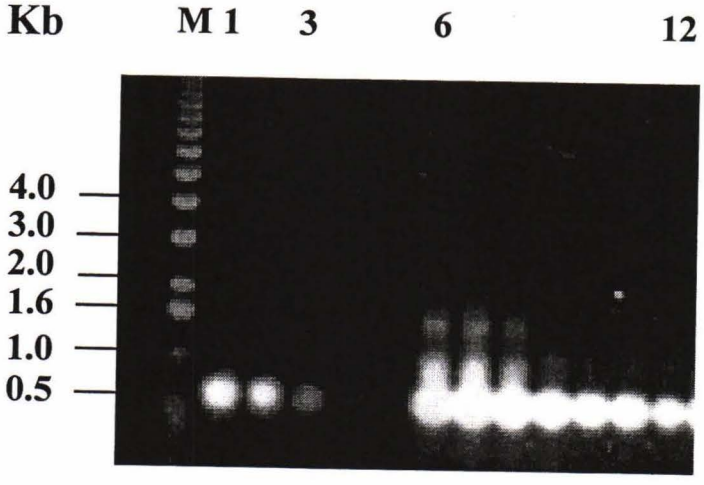

Fig. 1. The activity of locally produced *Taq* DNA polymerase is approximately 64 units/μl. The enzyme preparation was titrated against the Perkin Elmer's *Taq* DNA polymerase. The PCR product is a 600 bp genomic fragment carrying part of the PERB11.1 gene. M = 1 kb lambda ladder. Lanes 1-5 are dilutions of the commercial *Taq* equivalent to 1, 0.5, 0.25, 0.125 and 0.0625 units added to the PCR reactions. Lanes 6-12 are dilutions of our enzyme preparation added to the reactions as follows : undiluted, 1:2, 1:4. 1:8, 1:16, 1:32 and 1:64, respectively. It can be seen that the 1:64 dilution of the enzyme preparation produced the PCR band with an intensity equivalent to that of 1 unit of the commercial *Taq* DNA polymerase. Consequently, the activity of the locally produced enzyme is approximately 64 units/μl.

RESULTS

Production of recombinant *Taq* DNA polymerase

A total of 7 ml of the recombinant *Taq* DNA polymerase was obtained from one litre of the bacterial culture. The protein concentration of the enzyme preparation was 6.72 mg/ml. Thus, the total protein isolated from the preparation was 47.04 mg. As shown in Fig. 1, the 1:64 dilution of the locally produced enzyme had the activity equivalent to 1 unit of the commercial *Taq* enzyme. This is indicated by the same intensity of the generated PCR product. Accordingly, the enzyme preparation possesses 64 units/μl. Totally, the activity of the enzyme preparation is 448,000 units with 9,524 units/mg protein.

Evaluation of the recombinant Taq DNA polymerase

To determine the purity of the locally produced *Taq* DNA polymerase, the enzyme preparation was analysed by SDS-PAGE compared to the *Taq* enzyme from Perkin Elmer Cetus. Only one major band of 97 kD was found in the commercial *Taq* (Fig. 2a). In contrast, multiple protein species were found in our enzyme preparations kept for 13 weeks at 4°C, -20°C and -70°C. The 97 kD protein represented an intact *Taq* DNA polymerase which was the major protein component found in the enzyme preparations kept at -20°C and -70°C but not at 4°C. Interestingly, the enzyme kept at 4°C revealed an additional protein band of 69 kD absent from those kept at -20°C and -70°C (Fig. 2a).

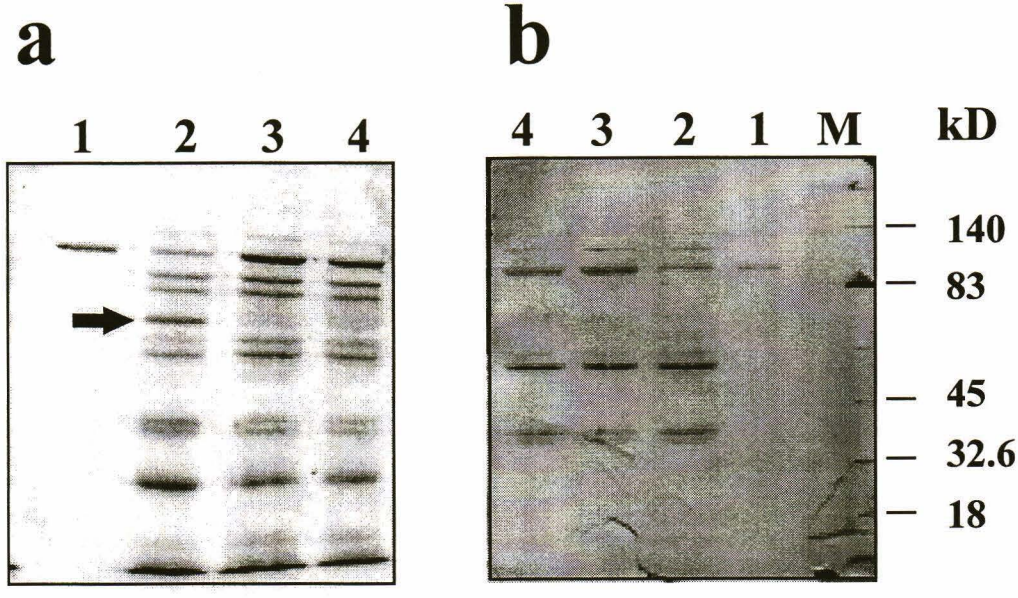

Fig. 2. The locally produced enzyme preparation contains multiple protein species including the *Taq* degradation products. The enzyme preparation was analysed for purity using SDS-PAGE (a) and immunoblot with monoclonal anti-*Taq* (b). Lane 1 represents the commercial *Taq* polymerase. Lanes 2-4 represent the locally produced *Taq* kept at 4°C, -20°C and -70°C, respectively, for 13 weeks. M = size standard markers. It can be seen that only a protein band of 97 kD is found in Perkin Elmer's *Taq* but mulitple protein species are revealed in the locally produced enzyme. Interestingly, an additional protein band of 69 kD is present (arrow pointed) with less intensed 97 kD band in the enzyme preparation kept at 4°C. It is anticipated that the 69 kD protein is a degradation product of the *Taq* 97 kD. Surprisingly, this protein component is not reactive to the monoclonal *Taq* antibody. Immunoblot analysis shown in (b) indicates that at least three protein components, 97, 59 and 41 kD, are strongly reactive to the *Taq* antibody.

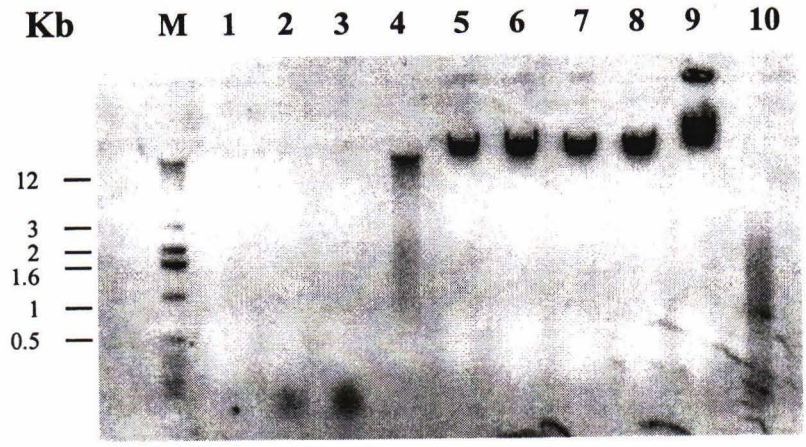

Fig. 3. The enzyme preparation is free of DNase contamination. The enzyme preparation was tested for DNase contamination by incubating the praparation with lambda DNA. The mixtures were tested for DNA degradation by electrophoresis through 0.8% agarose gel. M = 1 kb lambda markers. Lanes 1-3, 10 and 4 are lambda DNA and DNase mixtures at 1. 0.1, 0.01, 0.001 and 0.0001 μg., respectively. Lanes 5-9 are the mixtures of lambda DNA and our enzyme preparation at undilution, 1:2, 1:4, 1:8 and 1:16 dilutions, respectively. At a high concentration of DNase, the lambda DNA is completely degraded (lanes 1-3). The smearing of DNA appears at 0.001 and 0.0001 μg of Dnase (lanes 10 and 4). Apparently, there is no DNA degradation detected in the mixtures of lambda DNA and our enzyme preparation.

Table 1. The highest activity per mg protein was obtained from the intact *Taq* enzyme.

Enzyme	% curve area of 97 kD	Units/mg protein of 97 kD	% curve area of immunoblot positive bands	Units/mg protein of immunoblot positive
Perkin Elmer Cetus	46.4	5986.6	46.4	5986.6
Locally produced 4°C	8.9	6689.0	58.4	1019.4
Locally produced -20°C	20.6	11560.7	62.7	3797.8
Locally produced -70°C	21.1	22567.0	66.8	7128.5

Immunoblot analysis was performed to determine which protein species were reactive to monoclonal anti-*Taq* antibody (Clonetech, U.S.A.). It can be seen in Fig. 2b that three protein bands, 97, 59 and 41 kD, were reactive. Surprisingly, the 69 kD found in the enzyme kept at 4°C was not reactive to the anti-*Taq*. The enzyme activities according to SDS-PAGE and immunoblot analysis are shown in Table 1. It is clear that the enzyme activity kept at -70°C has the highest enzyme activity/mg protein especially when the calculation is based upon the quantity of intact enzyme (97 kD).

The enzyme preparation was examined for DNase contamination by incubating the enzyme preparation with lambda DNA at 37°C overnight. It is clear that the recombinant *Taq* preparation is free of DNase (Fig. 3) based upon its capability of DNA degradation examined by agarose gel electrophoresis.

Stability of the recombinant *Taq* DNA polymerase

The activities of three aliqouts of the enzyme preparation kept at 4°C, -20°C and -70°C

Table 2. The stability of the locally produced *Taq* DNA polymerase kept at three different temperatures.

week	Score of PCR products amplified by the recombinant Taq DNA polymerase kept at																									
	4 °C								-20 °C								-70 °C							
	undilute	1:2	1:4	1:8	1:16	1:32	1:64	total	undilute	1:2	1:4	1:8	1:16	1:32	1:64	total	undilute	1:2	1:4	1:8	1:16	1:32	1:64	total
1	10	9	8	7	7	6	5	52	10	9	10	9	8	7	5	58	10	10	9	8	8	8	7	60
2	10	9	7	6	6	6	5	49	10	10	9	8	8	6	5	56	10	10	9	8	8	8	7	60
3	10	9	8	7	6	5	4	49	10	10	9	8	7	6	5	55	10	10	9	8	8	6	5	56
4	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
5	10	8	7	6	5	5	4	45	10	10	9	8	7	6	5	55	10	10	9	8	8	6	4	55
6	10	8	7	6	4	3	0	38	10	10	8	7	6	5	5	51	10	10	9	8	7	6	3	53
7	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
8	10	8	7	5	5	3	0	38	10	10	8	8	7	6	5	54	10	10	9	8	7	6	5	55
9	9	7	6	4	0	0	0	26	10	10	8	7	6	5	4	50	10	9	8	8	6	5	4	50
10	8	5	4	3	0	0	0	20	10	9	6	5	4	3	2	39	10	9	8	6	4	2	2	41
11	8	6	5	4	3	0	0	26	10	10	7	6	5	0	0	38	10	10	9	9	8	7	3	56
12	6	5	4	0	0	0	0	15	10	8	6	5	3	0	0	32	10	9	8	7	4	3	2	43
13	6	5	3	0	0	0	0	14	10	8	7	6	6	0	0	37	10	10	9	8	7	5	0	49
total								372								525								578

0 = no PCR product 2 = Weekly positive PCR product 3-10 = PCR product positive 1+ to 8+

NT = not tested

were determined weekly for 13 weeks. The result is presented in Table 2. The total scores of the enzyme activities were markedly decreased after the eighth week with rapid decline for the enzyme kept at 4°C. The activities were significantly decreased when compared between the enzyme preparations kept at 4°C *vs* -20°C and 4°C *vs* -70°C ($p<0.05$) but not between -20°C and -70°C. However, there was a significant decrease when vertical comparison was made between the first *vs* thirteenth weeks for each aliqout of enzyme. The activities were 8 times, 4 times and 2 times less in the thirteenth week than those of the first week for the enzyme kept at 4°C, -20°C and -70°C, respectively.

HLA typings using the recombinant *Taq* DNA polymerase

The locally produced *Taq* DNA polymerase were used in PCR-SSP typings for the HLA-DR/DQ genes and ARMS-PCR typings for the HLA-A/B/C genes. The enzyme preparation was feasible to perform the HLA typings of the genes as mentioned. Examples of the PCR-SSP typing for HLA-DR are shown in Fig. 4.

DISCUSSION

We have successfully expressed and produced *Taq* DNA polymerase from the *pTaq* clone which was introduced into *E. coli*[5]. We adopted the simple isolation step[6] without utilization of any chromatographic purification. The yield of enzyme is high with 9,524 units/mg protein and even higher (up to 22,567 units/mg protein) when the activity is calculated based upon the amount of intact enzyme contained in the preparation. In previous reports, the activity per mg protein of *Taq* enzyme was varied depending on the methods of preparation ranging from 2,000-300,000 units/mg protein[5-7].

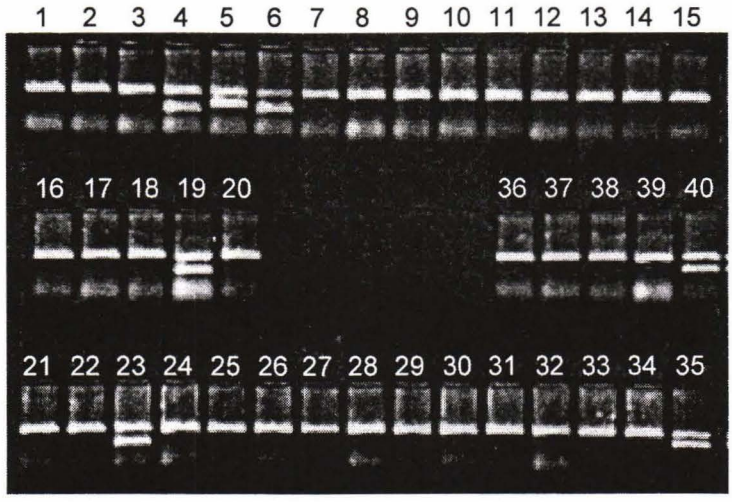

Fig. 4. The locally produced *Taq* DNA polymerase can be used successfully in the PCR-SSP typing of HLA-DR. Lanes 1-20 and 21-40 represent two samples of HLA-DR typing using 20 pairs of primers. The larger band in each lane is an internal control representing a genomic fragment amplified from the human growth hormone gene. The lower bands in lanes 4, 5, 6 and 19 in the first sample are specific PCR products of HLA-DRB1*1601/2/3 (lane 4), 03011/03012 (lanes 5 and 6) and HLA-DRB3 (lane 19) which is associated with HLA-DR3. Accordingly, this individual carries HLA-DRB1* 1601/2/3 and HLA-DRB1*03011/03012. Typing of the second sample reveals specific PCR products of HLA-DRB1*1501/2/3 (lane 23), 0701/2 (lane 35) and HLA-DRB4 (lane 40) which is associated with HLA-DR7. Thus, this individual carries HLA-DRB1*1501/2/3 and HLA-DRB1*0701/2.

Our enzyme preparation is free of DNase as evaluated by its ability to degrade DNA examined by gel electrophoresis. Although the evaluation method is insensitive, it is applicable and suitable for our purpose. We attempt to use this Taq enzyme for amplification of small target sequences (< 1 kb) and detect the presence or absence of products. We have not tested whether the enzyme preparation would be suitable for amplification of a large genomic sequence, direct sequencing or cloning of PCR products.

In spite of high activity and DNase free, the enzyme preparation was not homogeneous. It contained multiple protein species in addition to the 97 kD intact enzyme. The preparation method included a heating step at 75°C during isolation of *Taq*. In theory, other bacterial proteins should be denatured and would be removed from the preparation[7]. However, when we probed these protein species contained in the preparation with monoclonal antibody against *Taq*, only three protein components were reactive to the antibody suggesting that they are the proteolytic products of *Taq*. It is possible that the immunoblot negative protein species are also the products of *Taq* degradations which have lost the epitope reactive to the monoclonal antibody. Indeed, this was the case for the *Taq* enzyme kept at 4°C for 13 weeks. The percentage of 97 kD intact enzyme was much less in this aliqout than those of the enzyme kept at -20°C and -70°C, with an additional protein band of 69 kD. Apparently, this is a truncated form of *Taq* which lacks the epitope and may also have lost the activity. Alternatively, the additional proteins contained in the preparation are bacterial protein contaminations. These two views are not mutually exclusive.

It is quite clear that the activity of enzyme preparation kept at 4°C declined quicker than those kept at -20°C and -70°C especially after the eighth week. Significant loss of the enzyme activity was also found after the 13th week even though the enzyme was kept at -70°C.

We have proved that our enzyme preparation could be used successfully for HLA typings using PCR-SSP and ARMS-PCR for class II and

class I, respectively. The total cost of enzyme preparation was about 14,000 baht excluding the cost of labour and equipment. Considering the cost of commercial *Taq* which is approximately 8 baht per unit, the cost of our enzyme preparation is 256 times less than that of the commercial enzyme.

In conclusion, we have locally produced recombinant *Taq* DNA polymerase which can be used economically and efficiently in research laboratories.

ACKNOWLEDGEMENTS

The authors wish to thank Assistant Professor Ganjana Lertmemongkolchai, Department of Clinical Immunology, Faculty of Associated Medical Sciences, and Associate Professor Dr Sopit Wongkum, Department of Biochemistry, Faculty of Medicine, Khon Kaen University for their advice and the use of equipments. We are appreciative in the support and technical advice of David Townend, Brian Brestovac and Professor Roger Dawkins from the Departments of Clinical Immunology, Royal Perth Hospital and Sir Charles Gairdner Hospital and the Centre for Molecular Immunology and Instrumentation (CMII), The university of Western Australia. This work was supported by CKICC, Faculty of Associated Medical Sciences, Faculty of Medicine and Khon Kaen University.

(Received for publication on June 24, 1997)

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การผลิตและตรวจสอบคุณสมบัติของเอนไซม์ *แทค* ดีเอ็นเอ โพลีเมอเรส

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Thermus aquaticus DNA polymerase (*Taq* DNA polymerase) เป็นเอนไซม์ที่มีความจำเป็นในการใช้เพิ่มจำนวน ดีเอ็นเอ ในปฏิกิริยาลูกโซ่โพลีเมอเรส (Polymerase chain reaction) แต่ปัจจุบันเอนไซม์นี้ยังมีราคาแพง เพื่อเป็นการลดต้นทุนในการศึกษาวิจัยงานทางด้านชีวโมเลกุล จึงได้ผลิตเอนไซม์จาก Clone *pTaq* ซึ่งอยู่ในแบคทีเรีย *E. coli* และตรวจสอบคุณสมบัติของเอนไซม์ที่ผลิตได้ เปรียบเทียบกับเอนไซม์จากบริษัท Perkin Elmer Cetus ในเรื่องของความคงตัว ความสามารถในการทำปฏิกิริยา การปนเปื้อนของเอนไซม์ดีเอ็นเอส และความบริสุทธิ์ของเอนไซม์ โดยพบว่าปริมาณโปรตีน และความสามารถในการทำงานของเอนไซม์ที่ผลิตได้ มีค่าเท่ากับ 6,720 ไมโครกรัมต่อมิลลิลิตร และ 9,524 ยูนิตต่อมิลลิกรัมโปรตีน โดยได้ปริมาณเอนไซม์ทั้งหมด 448,000 ยูนิต จากการเพาะเลี้ยงแบคทีเรีย 1 ลิตร เอนไซม์ที่ผลิตได้ไม่มีการปนเปื้อนของเอนไซม์ดีเอ็นเอส ถึงแม้เอนไซม์ที่ผลิตได้จะสามารถทำปฏิกิริยาได้ดีแต่ยังมีความบริสุทธิ์ต่ำหากเปรียบเทียบกับเอนไซม์จากบริษัท เนื่องจากมีการปนเปื้อนของโปรตีนจากแบคทีเรีย และโปรตีนจากการสลายตัวของเอนไซม์ในระหว่างการเตรียม อย่างไรก็ตามเมื่อนำเอนไซม์ที่ผลิตได้มาใช้ในการตรวจหาอัลลีลของจีนเอชแอลเอ พบว่าได้ผลดี เนื่องจากค่าใช้จ่ายในการผลิตเอนไซม์นี้จะน้อยกว่าการซื้อเอนไซม์ที่ผลิตจากต่างประเทศประมาณ 256 เท่า ดังนั้นการใช้เอนไซม์ที่ผลิตได้จะสามารถประหยัดค่าใช้จ่ายในการทำวิจัยซึ่งต้องอาศัยเอนไซม์ *แทค* โพลีเมอเรส ในประเทศไทย

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