CHAPTER III

MATERIALS AND METHODS

Materials

1. Enzymes

Calf thymus topoisomerase I was purchased from Fermantas Inc. (Hanover, MD, USA) and Takara, Japan. Pronteinase K was purchased from USB (Cleveland, OH, USA).

2. Chemicals

Hoechst 33258 was perchased from Calbiochem (Darmstadt, Germany). Picogreen and Sybr Green I were perchased from Molecular Probes Inc. (Eugen, OR, USA). pBR322 was perchased Fermantas Inc. (Hanover, MD, USA). Hoechst 33342, camptothecin, menadione, heparin, quercetin dehydrate, etoposide, ellipticine, chelidonine, chelerythrine, sanguinarine, genistin, hesperidin, naringin were perchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was purchased from AJAX Chemical, Australia. 10X topoisomerase I reaction buffer and serum bovine albumin (BSA) were perchased from Fermantas Inc. (Hanover, MD, USA) and Takara, Japan. Agarose was purchased from BDH Chemical, England. Dimethyl sulfoxide (DMSO) and Tris-base were purchased from Fisher Scientific, UK. Boric acid and EDTA Triplex II® were purchased from Merck (Darmstadt, Germany). Triton-X was purchased from Fluka Chem, Switzerland.

3. Instruments

PCR tube 0.2 ml was purchased from Axygen Scientific (Union City, CA, USA). Black 96-well microplate was purchased from PerkinElmer Inc. (Netherland). Fluorescent microplate reader Victor^3 (PerkinElmer Inc, Waltham, MA, USA), PCR device Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany), EC370M Electrophoretic gel system with power supply model EC250-90 (EC Apparatus Corp., Holbrook, NY, USA). Gel Documentation (Bio-Rad, Hercules, CA, USA)

Methods

1. Preparation of topoisomerase I reaction

Reactions contained 35 mM Tris-HCl, pH 8.0, 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin, 250 ng of supercoiled pBR322 and 0.25 unit of calf thymus topoisomerase I in 20 µl and were incubated at 37°C for 45 min in PCR device. For substrate control, the addition of the enzyme was omitted. One unit of enzyme was the amount of the enzyme that completely relaxed

0.5 μg of supercoiled pBR322 in 50μl of reaction mixture that was defined by commercial product.

1.1 Agarose-gel electrophoresis

1% agarose gels were prepared in TBE buffer plus 0.1% SDS (pH 8.3) gel slab (10 X 10 X 0.5 cm). Samples were mixed with appropriate amount of loading buffer and run at 5 V/cm for 2 hr. Gels were stained for 15 min in 0.25 μg/ml ethidium bromide in distilled water and subsequently destained in distilled water over 1 hr.

2. Optimization of fluorescence microplate assay

2.1 Evaluation of fluorescence effect of dyes on supercoiled plasmid and relaxed plasmid

Enzyme reactions were prepared as described above. The volume of enzyme reaction was calculated to prepare adequately for 4 dyes. Samples were run in parallel by electrophoresis as described above to check a complete reaction. For fluorescence method, samples (25 μg/ml DNA) were diluted with 100 μl TE buffer or TEN buffer (pH 7.4) depend on type of dye buffer to obtain DNA concentration of 200 ng/ml in black 96-well microplate (Perkin Elmer) and then added equal volumes (100 μl) of 50 μg/ml H 33258 in TE Buffer, 50 μg/ml H 33342 in TE buffer, 50 μg/ml H 33258 in TEN Buffer, 50 μg/ml H 33342 in TEN buffer, 1 μg/ml H 33258 in TEN Buffer, 1 μg/ml H 33342 in TEN buffer, 1:200 Picogreen in TE buffer or 1:5000 Sybr Green I in TE buffer. After add each dye solutions, the final DNA concentration was 100 ng/ml. and total volume was 200 μl. The microplate was shaken orbitally for 10 s and the fluorescence intensity was read by fluorescence microplate reader Victor^3. The excitation and emission wavelength of each dyes were followed by Table 3.

Table 3 List of fluorescence dyes used in this study

Dye	Excitation wavelength	Emission wavelength
Hoechst 33258 Hoechst 33342	355	460
Picogreen Sybr Green I	485	535

The percent of coefficient of variation (% CV) and the Z′ value was calculated by following equations:

$$\% \text{ CV} = \frac{(\text{SD}/\sqrt{n})}{\text{average}}$$

$$Z' \text{ value} = \frac{(\text{average}_{max} - 3SD_{max}/\sqrt{n}) - (\text{average}_{min} + 3SD_{min}/\sqrt{n})}{(\text{average}_{max} - \text{average}_{min})}$$

2.2 dynamic range and limit of detection

Enzyme reactions were prepared as described above. Samples were run in parallel by electrophoresis as described above to check a complete reaction. Substrate control (only DNA) and reaction control (DNA + Enzyme) were diluted to obtained final DNA concentrations of 10, 50, 100, 500 and 1,000 ng/ml by 100 µl TE buffer in black 96-well microplate. And then equal volumes (100 µl) of 1:200 Picogreen were added. The microplate was shaken orbitally for 10 s and the fluorescence intensity was read by fluorescence microplate reader Victor[3]. The Z′ value was calculated by equation as described above.

2.3 Time-course study of topoisomerase I activity using Picogreen-based assay

Enzyme reactions were prepared as described above. For gel-based assay, Two microliter (50 ng DNA) aliquot of sample from enzyme reaction was mixed with 4 µl loading buffer and then loaded into well 1% agarose gel run 50 V (5 V/cm) for 2 hour. For microplate assay, Two microliter (50 ng DNA) aliquot of sample from enzyme reaction was diluted to obtain DNA concentration of 500 ng/ml in black 96-well microplate by 100 µl of TE buffer (pH 7.4) and then added 100 µl of 1:200 Picogreen,. The final DNA concentration was 250 ng/ml. Sample was collected from enzyme reaction tube at 0, 1, 2, 3, 4, 5, 10, 25 and 50 min.

The percentage of enzyme relaxation was calculated by the following equations:

$$\% \text{ relaxation} = \frac{\text{BI of sc DNA (substrate control} - \text{sample)} \times 100}{\text{BI of sc DNA (substrate control} - \text{reaction control)}}$$

BI = band intensity

$$\% \text{ relaxation} = \frac{\text{FI of sc DNA (substrate control} - \text{sample)} \times 100}{\text{FI of sc DNA (substrate control} - \text{reaction control)}}$$

FI = fluorescence intensity

3. Modification of fluorescence ratio assay

Enzyme reactions were prepared as described above. The method was modified from Rock *et al.* (2003). Two microlitre (50 ng DNA) aliquot of sample from enzyme reaction was diluted with 50 µl of TE buffer (pH 7.4) in black 96-well microplate and then 50 µl of Picogreen 1:200 was added. The microplate was shaken orbitally for 10 s and incubated for 5 min. After that the microplate was read for first fluorescence intensity value. One hundred µl of 0.1 M NaOH was added to obtain pH 12 in well, shaken for 10 s, incubated for 5 min and read secondly. The fluorescence intensities were calculated in ratio.

$$\text{Relative fluorescence} = \frac{\text{FI of pH 12}}{\text{FI of pH 7.4}}$$

FI = fluorescence intensity

3.1 Evaluation of candidate stop process

Enzyme reactions were prepared as described above. the experiment designed in three groups: stopped by cooling down at 4° C or freezing, stopped by heat at 95° C for 1 min and stopped by adding 10% SDS following 1 µg/ml proteinase K incubated at 37° C for 1 hour. Two microlitre (50 ng DNA) aliquot of each sample from enzyme reaction was diluted with 50 µl of TE buffer (pH 7.4) in black 96-well microplate and then 50 µl of Picogreen 1:200 was added. The microplate was shaken orbitally for 10 s and incubated for 5 min. After that the microplate was read for first fluorescence intensity value. One hundred µl of 0.1 M NaOH was added to obtain pH 12 in well, shaken for 10 s, incubated for 5 min and read secondly. The fluorescence intensities were obtained by following as modified Rock's method.

3.2 Evaluation of topoisomerase I amount on relative fluorescence.

Enzyme reactions were prepared as described above except enzyme amounts. The enzyme amounts were varied in 0.25, 1 and 5 unit and stopped reaction by cooling at 4°C. Two microlitre (50 ng DNA) aliquot of each sample from enzyme reaction was diluted with 50 µl of TE buffer (pH 7.4) in black 96-well microplate and then 50 µl of Picogreen 1:200 was added. The microplate was shaken orbitally for 10 s and incubated for 5 min. After that the microplate was read for first fluorescence intensity value. One hundred µl of 0.1 M NaOH was added to obtain pH 12 in well, shaken for 10 s, incubated for 5 min and read secondly. The fluorescence intensities were obtained by following as modified Rock's method.

3.3 Time-course analysis of topoisomerase I activity by using fluorescence ratio assay.

Enzyme reactions in time-course study using Picogreen-based assay were used to evaluate by using fluorescence ratio assay. Two microlitre (50 ng DNA) aliquot of each sample from enzyme reaction was diluted with 50 µl of TE buffer (pH 7.4) in black 96-well microplate and then 50 µl of Picogreen 1:200 was added. The microplate was shaken orbitally for 10 s and incubated for 5 min. After that the microplate was read for first fluorescence intensity value. One hundred µl of 0.1 M NaOH was added to obtain pH 12 in well, shaken for 10 s, incubated for 5 min and read secondly. The fluorescence intensities were obtained by following as modified Rock's method.

4. Inhibitor assays

Known topoisomerase inhibitors and unknown compounds were used to validate the assays. Non-intercalating topoisomerase I poisons, camptothecin and heparin, intercalating topoisomerase I poison, quercetin and catalytic inhibitor, menadione were used as positive control. Non-intercalating topoisomerase II poison, etoposide and intercalating topoisomerase II poison, ellipticine were used as negative controls. The enzyme reactions were prepared as above. Only menadione must be preicubated to obtain a good inhibitory effect. The samples were diluted following by Picogreen-based DNA relaxation assay or fluorescence ratio assay. The IC_{50} values were obtained from dose-response experiments and calculated by software, CurveExpert version 1.3. The percent of enzyme inhibition (% inhibition) was the inversion of % relaxation

Twelve unknown compounds such as glycosylated flavonoids (genistin, hesperidine, naringin and vitexin), isoquinoline alkaloids (chelidonine, chelerythrine, sanguinarine and tetrahydropalmatine) and miscellaneous compounds (oxostephanine, clausenidin, and γ-fagarine) were included for screening. All compounds were screened at concentration of 50 µM and 100 µM and calculated % inhibition compared with 50 µM camptothecin.

5. Statistical analysis

Data were expressed as mean ± S.D. or S.E.M. For differences between 2 mean values, independent t test was performed. Pearson correlation measures the strength and direction of a linear relationship between the X and Y variables that was used to compare relationship between two assays. Student's *t*-test was used for the comparison of two mean values, and statistical significance was taken as $p < 0.01$. All statistical analysis was performed according to the statistical program, SPSS.