Availability of Nagarse for DNA Analysis as a Substitute for Proteinase K

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The availability of Nagarse, a protease, as a substitute for proteinase K for digestion of leukocytic or bacterial DNAs was studied. The amount and purity of DNAs extracted from leukocytes and several bacterial strains with Nagarse were compared with those of DNAs treated with proteinase. Nagarse exhibited the same behavior as proteinase K in digesting leukocytes, and it could also be used for bacterial digestion

for physical mapping of genomic DNA by biased sinusoid field gel electrophoresis. Nagarse was thus comparable to proteinase K for use in biochemical experiments. The principal advantage of Nagarse is that it is inexpensive, unlike proteinase K, and our findings indicated that Nagarse is very useful as a substitute for proteinase K for the DNA study. *J. Clin. Lab. Anal.* 14:97–100, 2000. © 2000 Wiley-Liss, Inc.

Key words: Nagarse; subtilisin BPN⁺; DNA extraction; biased sinusoid-field gel electrophoresis; pulsed-field gel electrophoresis

INTRODUCTION

The clinical importance of DNA analysis is widely recognized, and this method of analysis is routinely performed in many laboratories. White cells and bacteria are commonly used as materials for DNA analysis. When leukocytes or bacteria are digested for analysis, proteinase K is used in combination with EDTA and sodium dodecyl sulfate (SDS) in the lysing solution. Cell lysis using this solution enables steady extraction of DNA from the cells, but proteinase K is relatively expensive as a reagent for routine use. We therefore examined the usefulness of nagarse for digestion of these cells in place of proteinase K.

Nagarse (subtilisin BPN⁺), a serine proteinase with a single peptide chain of 275 amino acids (1), is known generally as a meat tenderizer. It exhibits proteolytic activity in alkaline conditions, like proteinase K, and its activity is not suppressed by EDTA (2). We therefore compared the usefulness of Nagarse during digestion for white cells and bacteria with that of proteinase K.

MATERIALS AND METHODS

Blood Specimens

Specimen collection

Venous blood anticoagulated with K2-EDTA was obtained from healthy donors (aged 25 to 35 years). Total white cell counts were performed using an automated blood cell counter (NE-8000, Sysmex Corp., Kobe, Japan).

Sample preparation and DNA extraction from EDTA blood

Blood containing 1×10^6 leukocytes was collected into a polypropylene tube (1.5 mL) and centrifuged at 2,000g for 1

min. Supernatant was removed with a Pasteur pipette and resuspended in 500 μ L of 0.2% saline solution to hemolyse it. About 30 sec later, 500 μ L of 1.6% saline solution was added, and then mixed and centrifuged at 2,000g for 5 min. The pellet was resuspended in 100 μ L of TNE buffer (10 mM of Tris-HCl at pH 8.0, 1 mM of Na₂-EDTA, and 100 mM of NaCl) and treated with 400 μ L of a lysing solution of TNE buffer (TNE buffer containing 1.25% SDS and 125 μ g/mL proteinase K, Wako Pure Chemicals Co., Osaka) or a solution of Nagarse-TNE buffer containing 1.25% SDS and 25 proteolytic U/mL Nagarse (Nagase Co, Tokyo).

The mixture was incubated for 4 hr at 45°C. Extraction of DNA was performed as described in our previous report (3), and the DNA extracted was suspended in 1 mL of TE buffer at 55°C for 4 hr, and stored at 4°C before use.

DNA quantitation

Extracted DNA was quantified by measurement of optical density at 260 nm (OD260) using a spectrophotometer (U2000; Hitachi, Tokyo). To determine the purity of extracted DNA, the optical density at 280 nm (OD280) was measured, and the OD260/OD280 ratio was determined.

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Polymerase chain reaction

To determine the feasibility of amplification, the HLA-DQA1 locus was studied with GH26 and GH27 (4) primers. The PCR method and digestion of PCR products with restriction endonucleases for HLA-DQA1 genotyping were performed as described in our previous report (3).

Study With Bacteria

Bacterial strains and culture conditions

Isolates of *Escherichia coli* O157 (eight strains) and *Salmonella enteritica* serovar Enteritidis (five strains) were obtained from clinical specimens. The isolates were subcultured on Trypticase soy agar (Becton Dickinson and Co., Cockeysville, MD) and incubated at 37°C for 18 to 20 hr.

Biased sinusoid-field gel electrophoresis (BSFGE)

The general procedures for BSFGE (5) analysis were the same as reported for pulsed-field gel electrophoresis (PFGE) (6) with minor modifications. Bacteria were treated as described by Izumiya (7). Briefly, the solidified plugs containing each bacterial strain were transferred to 1 mL of lysis solution (1 mg/mL of lysozyme, 0.5 M Na₂-EDTA [pH 8.0]), and were incubated for 4 to 24 hr in a 37°C water bath with gentle shaking. Then, the plugs were transferred to 1 mL of lysis buffer (0.5 M Na₂-EDTA [pH 8.0] and 1% sodiumlauroyl sarcosin) containing either 1 mg/mL of proteinase K or 200 proteolytic U/mL of Nagarse. The floated plugs were incubated overnight at 45°C with gentle shaking. After incubation the plugs were cooled to room temperature, placed in 1 mL of 1 mM phenyl-methyl-sulfonyl fluoride (PMSF, Wako Pure Chemical Co. Ltd. Osaka) to block proteinase activity, and incubated for 2 hr in a 45°C water bath with gentle shaking. After incubation, the PMSF solution was replaced with a new PMSF solution and the plug was incubated for 2 hr in a 45°C water bath with gentle shaking. The plug was transferred to 1 mL of TE buffer (pH 8.0), and incubated for 2 hr at room temperature. Then the TE buffer was replaced with 1 mL of new TE buffer for storage of the plugs at 4°C until restriction digestion was performed.

Restriction digestion and BSFGE of bacterial DNA

A plug was cut into a slice about 4 mm wide using a glass-made cover glass, and the slice was placed into 200 µL of the manufacturer's recommended restriction buffer solution for 1 hr at 4°C. After incubation, the buffer was replaced with 100 µL of fresh buffer containing 20 U of *Xba*I (Nippon Gene Co. Ltd., Tokyo) for *E. coli* O157:H7 or 20 U of *Bln*I (Takara) for *S. serovar* Enteritidis. The plugs in the digestion mixture were incubated at 37°C overnight. BSFGE was performed with a 1.5% agarose gel (Pulsed Field Certified agarose, Bio-Rad Laboratories) using a Genofield® (ATTO Co. Ltd., To-

TABLE 1. Amounts of extracted DNA from 1×10^6 leukocytes¹

	Digestion with proteinase K	Digestion with Nagarse
Mean $\pm$ SD (μ g)	7.35 $\pm$ 0.81	7.35 $\pm$ 0.15
Minimum (μ g)	6.64	7.16
Maximum (μ g)	8.16	7.52

¹n = 6.

kyo) in 0.5 $\times$ TBE (1 $\times$ TBE is 89 mM Tris, 89 mM boric acid, and 25 mM Na₂-EDTA [pH 8.3]) buffer at 24°C at DC 48 V and AC 288 V. For separation of genomes, a log-ramped cycle alteration (0.005 Hertz/Start and 0.3 Hertz/End) was added to the electrophoresis for hr. Following the electrophoresis, the gel was stained with ethidium bromide (0.2 µg/mL) and photographed under ultraviolet light.

Statistics

Student's *t*-test was used to determine the significance of differences between groups.

RESULTS

Amounts and Purity of DNA Extracted From White Cells

The amount and purity of leukocytic DNAs digested with proteinase K and Nagarse were 7.35 $\pm$ 0.81 µg and 7.35 $\pm$ 0.15 µg, respectively (Table 1). No significant differences were noted in the amount of DNA extracted between testing with proteinase K and Nagarse in the lysing solution.

Comparing the two proteinase-treated DNAs, the OD260/OD280 ratios of both distributed between 1.82 and 1.89, and

Fig. 1. Electrophoretic patterns of extracted DNAs. Approximately 100 ng of DNAs were applied. 1, λ DNA/*Hind*III; 2–7, extracted DNAs obtained by digestions with proteinase K; 8–13, extracted DNAs obtained by digestions with Nagarse; 14, control DNA (Germline Human Placenta Control DNA, Uncut; Oncor®, Gaithersburg, MD).

Fig. 2. Amplification of the HLA-DQA1 gene. **Left:** 1 and 15, ϕ X174/*Hae*III; 2–7, results of amplification with DNAs obtained by digestions with proteinase K; 8, negative control; 9–14, results of amplification with DNAs obtained by digestions with Nagarse. **Right:** Polymorphic restriction fragment patterns of PCR products. 1, 8, and 15, ϕ X174/*Hae*III; 2, 5, 9, and 12,

digested with *Hae*III; 3, 6, 10, and 13, digested with *Dde*I; 4, 7, 11, and 14, digested with *Rsa*I. Samples 2–7, 9–14 are from the same subjects; 2–4 and 9–11 are from DNAs obtained by digestions with proteinase K, and 5–7 and 12–14, from DNAs obtained by digestions with Nagarse.

the purity of the extracted DNAs was equivalent. The electrophoretic patterns of extracted DNAs with approximately 100 ng of DNAs applied were the same, as shown in Figure 1.

Amplification and HLA-DQA1 Genotyping Using Extracted DNA

The suitability of extracted DNA for polymerase chain reaction was tested using the HLA-DQA1 gene, which could be amplified from DNA extracted from all specimens (Fig. 2). Amplified DNA of 242 bp was digested with three restriction endonucleases, *Hae*III, *Dde*I, and *Rsa*I, for HLA-DQA1 genotyping (Fig. 2). Of these specimens, genotypes of the HLA-DQA1 gene were identical to those in DNAs obtained by digestions with proteinase K and Nagarse.

BSFGE Analysis of *E. coli* O157:H7 and *Salmonella* Serovar Enteritidis Lysed With Proteinase K and Nagarse

The patterns of BSFGE-separated DNA fragments of bacterial strains with proteinase K treatment were consistent with those of corresponding strains treated with Nagarse (Fig. 3).

Stability Test of Nagarse Solution

Nagarse (2,000 proteolytic units) was dissolved in 1 mL of 30% glycerol solution and the solution was stocked at -20°C . After 2 months of storage, it was used for DNA analysis of

Fig. 3. BSFGE of restriction enzyme-digested total genomic DNA obtained by digestions with proteinase K and Nagarse from bacterial strains of *E. coli* O157:H7 (**left**) and *Salmonella* serovar Enteritidis (**right**). DNA from *E. coli* O157 was digested with *Xba*I, and DNA from *S. serovar enteritidis*, with *Bln*I. Lanes with the same number are paired samples of

bacteria. Lanes: M1, molecular weight marker (lambda ladder); M2, molecular weight marker (*Saccharomyces cerevisiae* chromosomal DNA); 1 to 7 (left) and 1 to 5 (right), with proteinase K treatment; 1 to 7 (left) and 1 to 5 (right), with Nagarse treatment.

leukocytes and bacteria. Yield of DNA from leukocytes and electrophoretic patterns of genomic DNA from bacteria were the same results obtained using a freshly-prepared Nagarse solution. That is, Nagarse was stable in this storage condition for at least more than two months.

DISCUSSION

The outstanding points of our study were as follows: (1) Nagarse can be used as a substitute of proteinase K for digestion of leukocytes to extract DNA, since it yielded sufficient amount and purity of DNAs from leukocytes, equal to those with proteinase K; (2) extracted DNA could be used for PCR and enzymatic analyses after Nagarse treatment; and (3) Nagarse could also be used for bacterial digestion in physical mapping of genomic DNA with BSFGE. These findings demonstrate that Nagarse is comparable to proteinase K for use in biochemical experiments. Another advantage of Nagarse is that it is less expensive than other proteinases we have used for a similar purpose.

Nagarse could digest leukocytes or bacteria in the presence of SDS and EDTA without difficulty. Five hundred μL of a solution containing 20 proteolytic units/mL of Nagarse was sufficient for digestion of 1×10^6 leukocytes. Nagarse is free of DNase and RNase (8). Nagarse that we used was in crystallized enzyme form and had an activity of $>1,000,000$ PU/g. Nagarse dissolved in 30% glycerol solution could be stored for at least 2 months at -20°C and was easy to handle; these are advantages for routine laboratory use.

Recently, physical mapping of genomic DNA of bacterial strains has often been performed using a PFGE method (7, 9–12), but we have used a BSFGE as a substitute of PFGE and performed the present study using BSFGE and obtained well-defined electrophoretic patterns. Our findings indicated

that Nagarse is useful for digestion of bacteria for physical mapping of genomic DNA of bacteria.

In conclusion, Nagarse is very useful as a substitute for proteinase K in the study of DNA leukocytes and bacteria.

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