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Use of a cysteine proteinase from *Carica candamarcensis* as a protective agent during DNA extraction

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Abstract

We describe the use of a plant cysteine proteinase isolated from latex of *Carica candamarcensis* as a protective agent during isolation of bacterial DNA following growth in culture of these cells. Between 100 to 720 units of proteinase (1 µg = 6 units) afforded good DNA protection when incubated with various kinds of microorganisms. Agarose gel electrophoresis showed that the resulting DNA was similar in size to DNA preparations obtained by treatment with proteinase K. The viability of the resulting material was checked by PCR amplification using species-specific primers. After standing at room temperature (25°C) for 35 days, the enzyme lost 10% of its initial activity. The enzyme stability and good yield of DNA suggest the use of this proteinase as an alternative to proteinase K.

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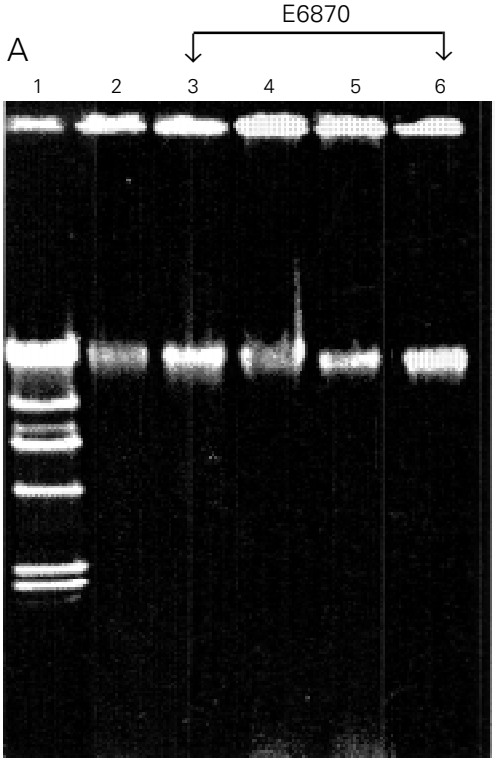
- Cysteine proteinase
- Proteinase K
- *C. candamarcensis*
- DNA extraction

Many procedures have been developed to facilitate the isolation of genetic material, by inactivating endogenous nucleases (1-3). These protocols must be gentle enough to permit later use of the DNA in techniques such as molecular cloning, enzyme digestion or PCR. One of the most ubiquitous procedures involves the use of proteinase K (4), a Ca^{2+}-dependent enzyme from *Tritirachium album* that remains substantially active following incubation at temperatures higher than 50°C in buffer systems containing SDS (5,6). In a search for alternative sources of proteolytic enzymes, we isolated a plant cysteine proteinase from *Carica candamarcensis*. The enzyme has been purified by chromatography on Sephadex G-10, CM-Sephadex, and MonoS Sepharose (7). The isolated enzyme, named E6870, was characterized as a single chain protein with an estimated Mr of 22-24 kDa, and behaves like a classic cysteine proteinase (Salas CE, García O and Mouhm V, unpublished data). The active fractions obtained from the last purification step display proteolytic activity in the pH range 5.5 to 8.3. They were concentrated by ultrafiltration through a PM 10 membrane and stored in 0.1 M sodium acetate, pH 5.0, at 4° or -20°C in the presence of 25% glycerol. The activity of this concentrated stock remained unchanged for one year at 4°C.

The amidolytic activity was assayed us-



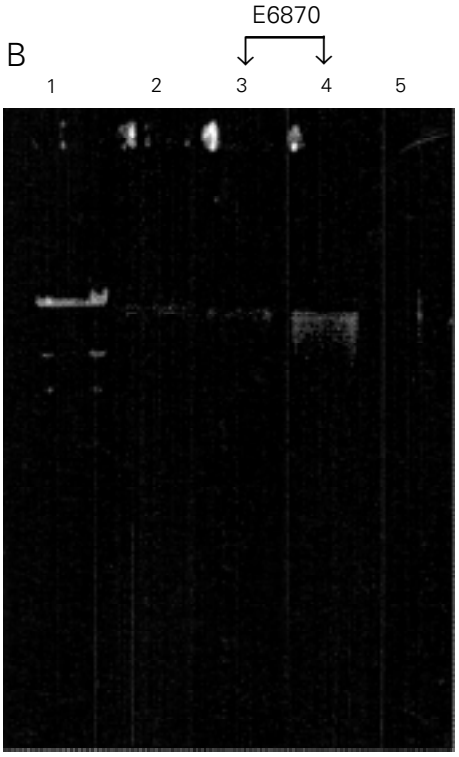


Figure 1 - Isolation of DNA from *Lepstospira* and *Mycobacterium* with E6870.

A, Leptospira tarasovi (10^8 cells) was sedimented at 14,000 *g* for 20 min. The sedimented cells were resuspended in 50 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS and 120 U of proteinase K or 100-400 U of E6870 and the mixture was incubated at 50ºC for 2 h. The solution was then centrifuged at 10,000 *g* for 10 min and the supernatant deproteinized by phenol extraction (phenol:chloroform:isoamylalcohol = 25:24:1). Before precipitation, 1 µg tRNA was added as carrier. After ethanol precipitation and washing with 70% ethanol the dried pellet was dissolved in 20 µl TE (Tris-EDTA) and 10 µl of this solution was electrophoresed on a 1.5% agarose gel along with molecular weight markers. Lanes: 1, λ-*Hin*dIII; 2, 120 U proteinase K; 3, 100 U; 4, 200 U; 5, 300 U and 6, 400 U of E6870.

B, Mycobacterium bovis previously inoculated into milk was isolated as follows: a milk sample (1 ml) containing 10^8 CFU/ml of *M. bovis* was incubated overnight with 720 U proteinase K (Boehringer, Mannheim, Germany), 360 U (lane 3), or 720 U (lane 4) of E6870 at 56ºC in buffer 10 mM Tris, 5 mM EDTA, pH 8.0, 1.5% SDS. The suspension was acidified with 200 µl 10% acetic acid and proteins were removed with 1 ml of a mixture containing phenol (3x) as shown in Figure 1A. The remaining procedure was as described in 1A. An aliquot (20% of total volume) was electrophoresed on agarose gel (1.5%) followed by ethidium bromide staining. Lanes: 1, λ-*Hin*dIII; 2, 720 U proteinase K; 3, 360 U; 4, 720 U E6870; 5, no proteinase added.

ing benzoyl-arginyl-p-nitroanilide (BAPNA) as substrate (7) and the proteolytic activity using casein as substrate (8). E6870 showed no signs of DNAase or RNAase activity, as indicated by incubation of 106 U of E6870 with λDNA (0.5 µg) or *E. coli* tRNA (5 µg) at 37ºC for 24 h, followed by agarose electrophoresis (data not shown).

In this study, we evaluated the performance of E6870 in protocols used to extract DNA from *Leptospira* and *Mycobacterium bovis*. The protective effect of E6870 in DNA extracting protocols was compared with that of proteinase K. We used the caseinolytic assay to standardize the amounts of both enzymes used (8). Figure 1A,B shows the data obtained after extraction of *Leptospira* and *Mycobacterium bovis* DNA with E6870 or proteinase K. The data show that both E6870 and proteinase K release *Leptospira* DNA of similar size and amounts (compare intensity of bands in lane 2 and 3 in Figure 1A). Also, the amounts of DNA recovered with E6870 were similar in experiments carried out to isolate mycobacterial DNA (Figure 1B). The effects of E6870 and proteinase K were also similar.

However, since we were uncertain about whether the use of E6870 could have an adverse effect, we further checked the intactness of DNA resulting from this extraction by applying the PCR technique. Primers were chosen to amplify specific targets of DNA from the genera *Leptospira* (9) and *Mycobacterium* (10).

These experiments are summarized in Figure 2A,B. The amplification profiles of different serovars from *Leptospira* showed an 849-bp band present in each serovar at 2 mM Mg^{2+}, although the band intensity varied from isolate to isolate (Figure 2A, lanes 3-8). In addition, a 370-bp band was evident in serovars *L. bratislava*, *L. hardjo*, *L. norma* and *L. hardjobovis*. The different intensity observed in the 849-bp band from various serovars can be explained by differences in Mg^{2+} requirement during PCR for each

serovar. This can be corrected by adjusting the Mg^{2+} concentration in the PCR of each serovar to its optimal value (data not shown).

In a second group of experiments, we applied the PCR technique to identify *M. bovis* in milk samples from animals suspected of being infected. The screening revealed that PCR obtained from milk samples from two animals (see Figure 2B, lanes 3 and 7) were positive (383 bp), while those from the remaining animals were negative. The negative results in Figure 2B are not caused by a selective loss of bacterial DNA during DNA extraction since each sample was shown to contain similar amounts of total DNA by agarose electrophoresis before PCR (data not shown). To our knowledge, this is the first time that *M. bovis* was identified directly in milk samples from cattle by PCR. A full report on this technique is being published elsewhere (11).

The present data show that E6870 from *C. candamarcensis* represents a good option during isolation of bacterial genomic DNA. One of the advantages is the stability of E6870 since thermal inactivation of the enzyme was less than 10% following a 35-day incubation period at room temperature (data not shown). The lack of divalent cation requirements in E6870 is another advantage compared to proteinase K, the calcium protein most widely used in nucleic acid extractive protocols. Therefore, E6870 performs well in mixtures containing 5-15 mM EDTA, thus improving the inhibition of nucleases that otherwise could degrade their target.

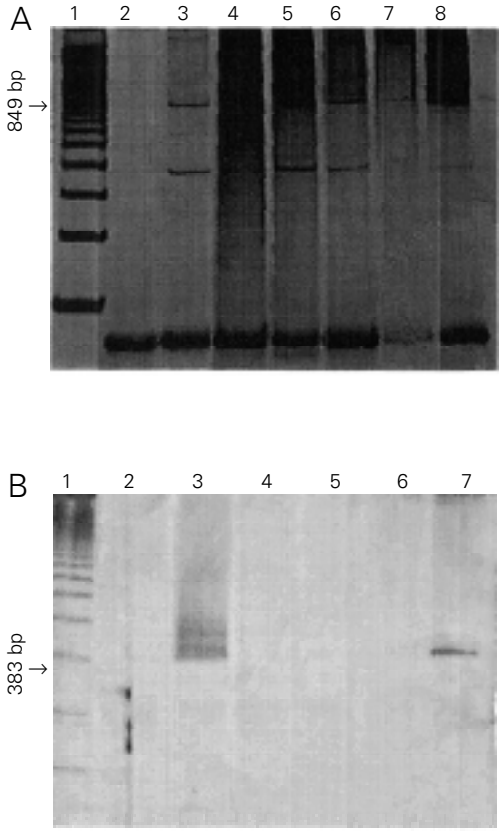


Figure 2 - PCR amplification of DNA from *Leptospira* and *Mycobacterium*.

A, DNA (10 ng) from *Leptospira* was subjected to 30 amplification cycles consisting of denaturation at 94ºC for 90 s, annealing at 58ºC for 90 s and extension at 72ºC for 2 min. The last extension step lasted 10 min. The other components of the mix were: 2 mM $MgCl_2$, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 10 U Taq polymerase and 800 nM each of primers Lep13/Lep14 (9). Lane 1, 100 bp; lane 2, negative control; lanes 3-8 are *L. bratislava*, *L. hardjo*, *L. norma*, *L. hardjobovis*, *L. mini*, and *L. neguita,* respectively.

B, Mycobacterial DNA (0.001-500 ng) was subjected to 42 cycles of amplification consisting of denaturation at 95ºC for 30 s, annealing at 68ºC for 60 s and extension at 72ºC for 30 s. The final extension step was carried out at 72ºC for 30 min. The other components of the mixture were: 50 mM KCl, 10 mM Tris, pH 8.3, 2.0 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and 0.4 mM dUTP or dTTP, 0.5 µmol each of primers BW6/BW7 (10,12), 0.5 U of uracil DNA glycosylase (Gibco BRL, Gaithersburg, MD), and 2.5 U Taq DNA polymerase. Before PCR the mixture was preincubated at 37ºC for 10 min. The glycosylase was then heat inactivated by incubation at 95ºC for 10 min. Lane 1, 100 bp; lanes 2-7 are DNA from cow's milk collected from different animals.

Finally, E6870 was isolated from the latex of *C. candamarcensis*, which is an abundant source of this enzyme, and therefore we anticipate that, if large-scale production is warranted, the supply of this enzyme would not be a problem. The thermal stability between 37º and 50ºC and pH range (5-8) in which the enzyme exhibits activity represent additional features of interest for those who intend to use E6870 in nucleic acid research.

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