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TECHNICAL NOTE

Isolation of high quality DNA: a protocol combining “rennet” and glass milk

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High quality DNA is essential for many molecular biology techniques. However, the reagents used for that purpose usually are expensive and/or cause a high environmental impact. Here, we describe two alternative protocols that use inexpensive reagents and are not hazardous to the environment. The first protocol utilizes the enzyme chymosin, normally used as “rennet” in cheese production and which is easily obtained on the commercial market. The second protocol uses “rennet DNA extraction protocol” combined with the DNA binding capacity of glass powder (glass milk), which can easily be “home made”. The first protocol is used when a high yield of DNA is needed, whereas the second protocol is used for production of a higher quality DNA, being able to work with sparse samples.

There are many protocols for DNA extraction and most use reagents, such as proteinase K or phenol, for DNA deproteinization (Chan et al. 2001; Niemi et al. 2001; Sato et al. 2001; Biase et al. 2002; Grachev et al. 2006). Reagents such as proteinase K normally come with an elevated cost or, such as phenol, are hazardous and require special discard procedures to minimize environmental impacts.

Here, we describe two protocols that can be used separately. However, the combination of both protocols is particularly useful to solve problems related to the quality of DNA extracted from some plants, since this is generally associated with secondary metabolites or latex. The first protocol uses the enzyme chymosin (rennin) present in “rennet”, which is used in making cheese. This enzyme has proteolytic activities and is widely used for protein coagulation of milk in the production of cheese (Bansal et al. 2007; Choi et al. 2007; Sandra et al. 2007).

According to UniProt (2008), the chymosin enzyme (CYM - P00794/A8RRP5) is an aspartic endopeptidase that belongs to the peptidase A1 family. This protein presents three molecular functions: aspartyl protease, hydrolase and protease. Its function in the biological process is defined as a protein whereby nutrients are rendered soluble and capable of being absorbed by the organism or cell and the activity specific is defined as catalysis of the lysis of peptide bonds with broad specificity similar to that of pepsin A (Mohanty et al. 2003; Rampilli et al. 2005).

The second protocol is also based on the capacity of chymosin proteolysis, but now the obtained DNA is additionally purified through its ability of binding positive electrical charged silica particles, also known as “glass milk”. The binding of DNA in the presence of chaotropic agents, such as NaI or $NaClO_4$, to silica or glass particles is well known (Boom et al. 1990). Melzak et al. (1996) describes some features that control the absorption of DNA by silica particles such as: (i) weak electrostatic repulsion forces, (ii) dehydration, and (iii) hydrogen bond formation. Glass milk, having these well-known characteristics, has been used in other methodologies of purification and DNA capture (Haugland et al. 1999; Huijun et al. 2000; Backer et al. 2001; England et al. 2001; Haugland et al. 2002; Nakama and Morishita 2004; Rohland et al. 2004; Zhang et al. 2004; Ros-Chumillas et al. 2007).

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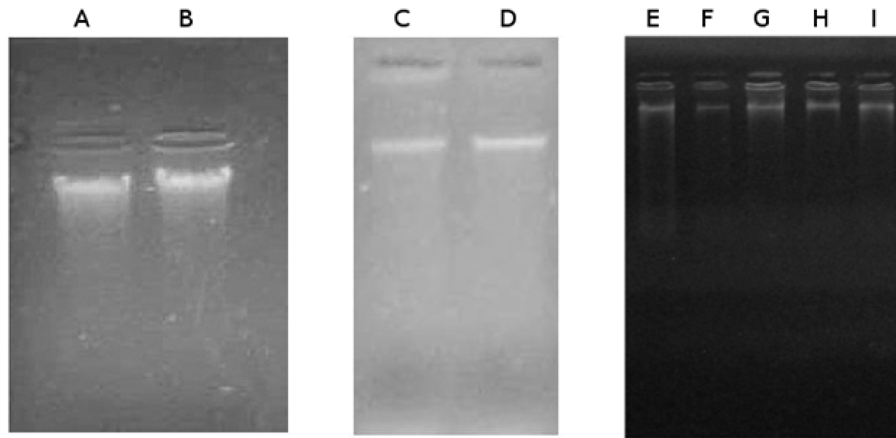

Figure 1. Comparison between two protocols: (A) *Drosophila* DNA extraction chymosin-based and (B) *Drosophila* DNA extraction phenol-chloroform; (C) *Oryza sativa* DNA extraction combined protocols (rennet/glass milk) and (D) *Oryza sativa* DNA extraction phenol-chloroform; (E) to (I): diverse biological materials; (E) Cicada exuviae with the "rennet/glass milk" protocol; (F) *Saintpaulia ionantha* with the "rennet/glass milk" protocol; (G) *Saccharomyces cerevisae* with the "rennet/glass milk" protocol; (H) *Escherichia coli* with the "rennet/glass milk" protocol; (I) *Ipomoea batatas* with the "rennet/glass milk" protocol.

Both protocols have low cost and small or no environmental impact and produced satisfactory results in extraction of genomic DNA. We have applied the DNA obtained through these protocols for different purposes, as PCR, Dot and Southern Blot, and to construction of partial genomic libraries.

MATERIALS AND METHODS

Diverse biological materials have been tested through the developed protocols, including insects of order Diptera (*Drosophila*), Hemiptera (family Cicadidae) cicada exuviae, yeast (*Saccharomyces cerevisae*), bacteria (*Escherichia coli*) and plants (*Oryza sativa*, *Ipomoea batatas*, *Saintpaulia ionantha*).

In the first protocol, which is chymosin-based, a work solution of calf rennet is prepared at 0.25 g/ml and maintained at -20ºC. We normally use the rennet "Coalho em pó HA-LA" (CHR HANSEN IND.COM.LTDA, Valinhos, SP, Brazil) purchased in supermarkets or in farm stores.

Roughly 100 mg of biological material, equivalent to a drop of blood, is homogenized in 600 µl of lysis buffer (0.1 M of Tris/HCl pH 8, 0.1 M of EDTA, 0.06 M of NaCl). Usually we perform this homogenization directly in a 1.5 ml microtube using a pistol homogenizer. After homogenization, 60 µl of 10% SDS is added and the tube is maintained in a water bath at 60ºC for one hour. After this, 60 µl of the rennet work solution is added and the tube is maintained in a water bath at 37ºC for an additional hour. Following this, 30 µl of potassium acetate (3 M) is added, and the tube is maintained for 15 min in an ice bath (0ºC). Next, 300 µl of chloroform are added to the tube and mixed gently for 10 min. The tube is centrifuged using a benchtop microcentrifuge (8.000 to 12.000 rpm) for 10 min and the supernatant is transferred to a fresh microtube. The chloroform tube is then discarded into an appropriate container. Two volumes of ethanol are then added to the supernatant, mixed gently, centrifuged for one minute and the pellet is left to dry. The pellet is then resuspended in 50 µl of ultrapure water or TE (0.01 M Tris/HCl, pH 8.0; 0.05 M EDTA, pH 8.0).

The second protocol uses glass milk, which is prepared using glass (we normally use broken test tubes). The glass initially is cleaned with hydrogen peroxide (20 volumes), rinsed two times with distillated water and then powdered into fine particles using a mortar and pestle. This procedure, nevertheless, needs some precautions, since the glass powder may be hazardous if breathed or swallowed. Thus, during the glass pulverization protective goggles and

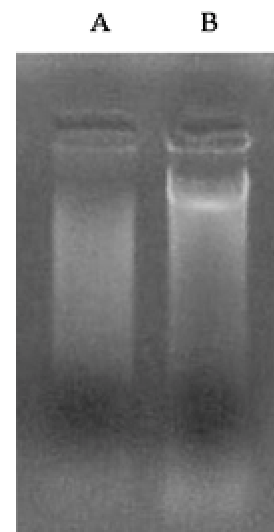

Figure 2. Comparative *Hind III* digestion profiles exhibited by genomic DNA using *Hind III* enzymes and DNA obtained with isolated through different protocols. (A) chymosinbased, and (B) phenol-chloroform.

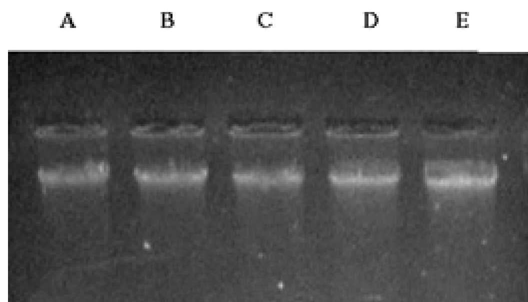

Figure 3. Test of DNA degradation. DNAs were incubated overnight with chymosin (rennet solution) with different final concentrations. (A) 0.071 g/µl; (B) 0.057 g/µl; (C) 0.042 g/µl; (D) 0.023 g/µl; and (E) 0.0 g/µl.

masks must be used. The use of a fume hood is also recommended. After pulverization the glass powder is dissolved in water and decanted over-night. The supernatant is centrifuged and the glass pellet is resuspended in two volumes of distilled water (pH 2.0) and stored at -20ºC. Additional details of the glass milk preparation can be obtained at http://www.ufsm.br/labdros/links/glassmilk.pdf.

Next, 20 to 30 mg of biological material are homogenized in 400 µl of buffer solution (0.1 M of Tris/HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.06 M NaCl) in an 1.5 ml microcentrifuge tube. After homogenization, 50 µl of 10% SDS are added and the tube is maintained in a 60ºC water bath for one hour. Following this, 50 µl of the "rennet work solution" are added to the tube, which is maintained in a 37ºC water bath for one additional hour, after which 30 µl of potassium acetate (3 M) are added. The solution is then mixed gently and maintained for 15 min in an ice bath. Next, 300 µl of chloroform are added and the solution is mixed for 10 min, being then centrifuged for 10 min in a benchtop microcentrifuge (8.000 to 12.000 rpm). To the supernatant so obtained, 600 µl of 6 M NaI and 80 µl of the glass milk solution are subsequently added. This solution is maintained on the benchtop for 5 min, inverting the tube every 30 sec. Next, it is centrifuged for 30 sec, the supernatant is removed, and 1 ml of 70% ethanol is added to completely wash the "glass milk pellet". This last step is repeated two times. Finally, the solution is centrifuged for 30 sec, the supernatant is removed, and the pellet is left to dry at room temperature, after which it is resuspended in 20 µl of ultra pure water or TE (0.01 M Tris/HCl, pH 8.0; 0.05 M EDTA, pH 8.0).

For the cleavage of genomic DNA, approximately 3 µg were digested over-night at 37ºC with *Hind III* restriction endonuclease (Invitrogen) following the manufacturer's instructions. Cleaved DNA was fractioned on a 0.8% agarose gel and visualized under a UV transiluminator.

To determine whether the rennet solution contained cow DNA, primers specific to the bovine gene *IGF-IR* (insulin-like growth factor-1 receptor) were used: IGF1-F= 5'-ACCCGCCAAGAAATTGTTTC-3' and IGF1-R 5'-GGCTCCTCCATACTTCCTGTA-3' (Schoenau et al. 2005). The PCR reactions we performed in a final volume of 25 µl, using approximately 20 ng of DNA, 0.4 µM of each primer, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 1.25 units of Taq DNA polymerase (Invitrogen) and 1 x PCR buffer. After an initial denaturation step of 5 min at 94ºC, 30 cycles consisting of 1 min at 94ºC, 30 sec at 55ºC and 1 min at 72ºC were carried out, followed by a final extension step of 4 min at 72ºC.

Additional PCR amplifications were performed to show the efficiency of the chymosin/glass milk-based protocol to obtain DNA pure enough for PCR using specific primers. In this case, the primers used were specific to Tip 100, that correspond to a *hAT* transposable element from *Ipomoea* and to the mitochondrial ITS region. The Tip 100 primers sequences were 5'-GCTTCTCAATGGGGCACTTC-3' and 5'-CGTTCTCCTTTTGTTGGTGT-3' (designed by authors), whereas the primers to ITS were 5'-AAGGTTTCCGTAGGTGAAC-3' and 5'-TATGCTTAAACTCAGCGGG-3' (Desfeux and Lejeune, 1996). The PCR conditions and parameters were the same as above, except that the annealing temperatures corresponded to 50ºC and 58ºC for Tip 100 and ITS, respectively.

RESULTS AND DISCUSSION

The rennet chymosin showed excellent activity as a proteolytic agent for DNA isolation. As can be seen in Figure 1A, the full amount of DNA obtained using this protocol is comparable to those using phenol-chloroform (Sambrook and Russel, 2001). However, it is important to note that rennet is much cheaper than proteinase K and, in contrast to phenol, has no environmental impact. Digestion assay with restriction enzymes have shown that the DNA obtained is completely digested (Figure 2), being suitable to be further applied in different techniques, including Dot and Southern blotting, PCR and to partial genomic libraries

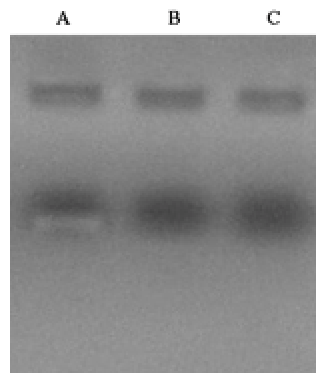

Figure 4. PCR to test the possibility of contamination of rennet with cow DNA. (A) bovine DNA, (B) negative control (without DNA), and (C) *Drosophila* DNA extracted with the chymosin-based protocol.

construction (data not shown).

The rennet is a commercial product for domestic or industrial use and not an enzyme isolated for molecular biology purposes. For this reason, the presence of other enzymes as DNAses, or even residual cow DNAs, could jeopardize the use of this product as a proteolytic agent. However, no DNAse activity was detected, since DNA samples exposed overnight to different concentrations of rennet solution did not show any signs of degradation (Figure 3). In addition, contaminant DNA was also not detected. The PCR performed using primers specific to the bovine *IGF-IR* gene showed no amplification signal in DNA preparations from different biological materials (Figure 4).

Some biological materials, mainly from plants, are sometimes problematic when trying to obtain high quality DNA able to be cleaved or used in PCR amplification. This is due to the presence of secondary metabolites and/or latex in these species. The major components of latex were shown to be conjugates of guaianolide sesquiterpene lactose and lactusin, in others words, polyphenolic conjugates which are produced constitutively as secondary metabolites and phytoalexins. The presence of polyphenolic content makes the isolation of high-quality nucleic acids problematic; in addition, residual polyphenolics interfere in enzymatic reactions such as PCR and endonuclease restriction digestion (Michiels et al. 2003).

We have solved the problem described above for some plants that we have tested, nominally *Oriza sativa, Saintpaulia ionantha* and many *Ipomoea* species (Figure 1B; Figure 1C), by adding to the “rennet DNA extraction protocol” a further step using glass milk. The high affinity of DNA to silica in the presence of a chaotropic salt permits the isolation of high quality DNA, free of polyphenolic contaminants. For the plants mentioned prior, the separate use of only the "rennet extraction DNA protocol" or the described "glass milk protocol" (Boom et al. 1990) does not produce DNA able to be amplified by PCR when using different sets of primers. However, the use of the combined protocol (rennet/glass milk) produced DNA that was capable of being amplified (Figure 5).

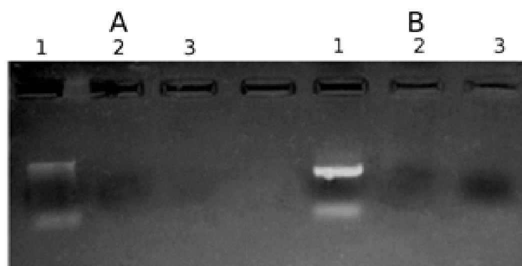

Figure 5. Comparison of PCR results obtained with the use of DNA from *Ipomoea purpurea* as template, and the Tip 100 (A) or the ITS (B) set of primers. (1) DNA extracted with the protocol rennet/glass milk based; (2) DNA extracted with rennet protocol, and (3) Negative control (without DNA).

Finally, these combined protocols possess the advantage of obtaining DNA from sparse biological materials. For example, we were able to obtain around 1 µg of DNA from a single *Drosophila* fly using this methodology (data not shown). Additionally, the combined protocol was also successfully applied in the extraction of DNA from cicada *exuviae* (Figure 1E). Feinstein (2004) and Su et al. (2007) have emphasized that DNA extraction protocol to insect *exuviae* are important to perform population analyses once do not need collect living wild animal. In fact, the combined use of these protocols increases the possibility of obtaining high quality DNA from diverse biological materials by using safe and inexpensive reagents.

From our knowledge, it is the first description of rennet use as a deproteinization agent for DNA isolation. The major advantage that can be attributed to these protocols refers to costs. The inexpressive price of rennet and the "home made" silica put these protocols among the cheaper ways to obtain DNA with quality to perform PCR, Southern Blot and other procedures. These characteristics make these protocols very useful in laboratories in developing countries, in which the resources to buy commercial kits is, sometimes, sparse. The major problem associated to these protocols is related to time and handwork involved in the glass milk preparation, but this cost is compensated for if a great quantity is made each time and stocked in the freezer (-20ºC). Other characteristic of this protocol is that it is easy and fast to be performed. Usually, in 4-5 work hrs, high quality DNA is isolated and available to be used for many different proposes.

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