RESEARCH/

EXTRAORDINARY STABILITY OF ENZYMES DRIED IN TREHALOSE: SIMPLIFIED MOLECULAR BIOLOGY

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We show that extremely fragile biomolecules such as DNA restriction and modifying enzymes can be dried *in vitro* in the presence of trehalose with no loss of activity, even after prolonged storage. A remarkable and unexpected property of the dried enzyme preparations is their ability to withstand prolonged exposure to temperatures as high as +70°C. This stability is unique to trehalose and is not found with other sugars irrespective of their physical or chemical properties. The immediate significance of these observations is the ability to convert enzymes used in molecular biology into stable reagents. The indefinite stability and high temperature tolerance of these dried enzymes should permit the design of convenient formats that may be of particular significance in the automation of genome mapping and sequencing projects. The stabilization of a wide range of biomolecules by trehalose also has practical implications for a number of areas ranging from basic science, through healthcare and agriculture, to bio-electronics.

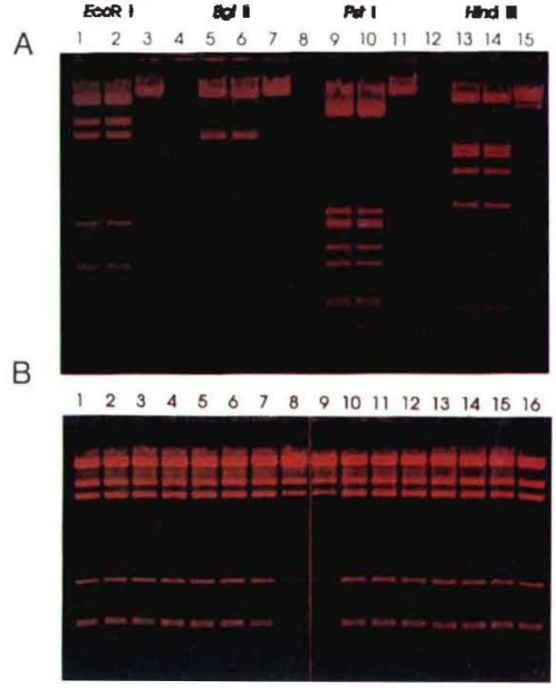
Cryptobiotic organisms, found in many phyla in both the plant and animal kingdoms[1–4], have evolved mechanisms to withstand repeated bouts of total desiccation during drought conditions and yet recover completely on rehydration. The better known examples include baker's yeast *Saccharomyces cerevisiae*, soil nematodes such as *Ditylenchus dipsaci*, tardigrades like *Adoribiotus coronifer*, the brine shrimp *Artemia salina* and resurrection plants such as *Selaginella lepidophylla* and *Myrothamnus flabellifolia*. Whilst the molecular mechanisms underlying cryptobiosis are unknown, a common theme linking these organisms is the presence of high concentrations of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) in their tissues when dry[5–8]. Although the *in vitro* effects of trehalose on the stabilization of cell membranes and enzymes during desiccation achieved by freeze-drying have been variable[9–11], dramatic effects are observed when desiccation is achieved by drying at ambient temperatures[12–13]. Anti-blood group antibodies air-dried at room temperature or 37°C in the presence of trehalose are undamaged and full biological activity can be recovered on rehydration, even after several years storage at ambient temperatures[14,15]. Similar results with a variety of antibodies and other proteins such as enzymes and blood coagulation factors[13–16] suggested that this process may be generally applicable.

As a stringent test of the capacity of trehalose to preserve labile biological molecules, the DNA restriction and modifying enzymes used in molecular biology were chosen for study. Though these enzymes, like other proteins, have a range of stabilities, many are notoriously fragile and at present are transported and stored at –20°C. We show that these endonucleases and DNA modifying enzymes can be dried from trehalose solutions at room temperature without loss of activity. Furthermore, the dried enzymes are stable for extended periods even when stored at elevated temperatures, a property uniquely conferred by trehalose.

RESULTS

Restriction enzyme stabilization. The results of drying four restriction enzymes (EcoRI, BglII, PstI and HindIII) with and without trehalose are shown in Figure

FIGURE 1 Stabilization of restriction enzymes by trehalose. (A) EcoRI (tracks 1–3) BglII (tracks 5–7), PstI (tracks 9–11) and HindIII (tracks 13–15) were dried either with 15% trehalose (tracks 2, 6, 10, 14) or without trehalose (tracks 3, 7, 11, 15) and then compared with five units of non-dried enzyme from the –20°C freezer (tracks 1, 5, 9, 13). (B) A titration of EcoRI (6, 4, 3, 2, 1, 0. 5 and 0.3 units) dried in 15% trehalose (tracks 1–8, decreasing titer) was compared with a titration of fresh non-dried enzyme from the –20°C freezer (tracks 9–16, increasing titer).



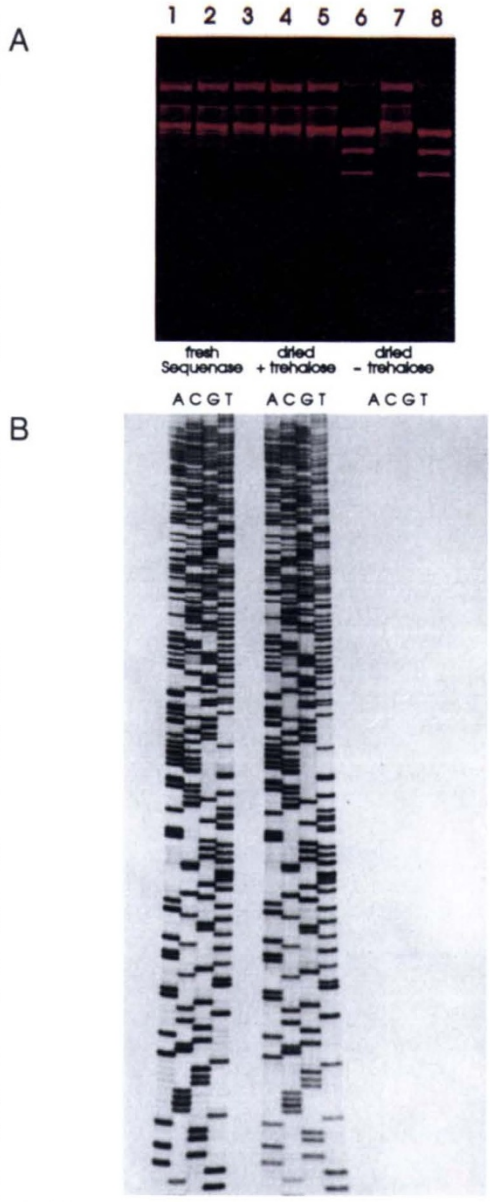


FIGURE 2 Stabilization of DNA modifying enzymes. (A) Stabilisation of T4 DNA ligase. T4 DNA ligase dried in the presence of 15% trehalose (6, 4, 3, 2 and 1 units, tracks 1–5) or without trehalose (3 units, track 6) was compared with 1 unit of fresh enzyme (track 7) and a control reaction without enzyme (track 8). (B) Stabilization of modified T7 DNA polymerase. Sequenase® (3 units per template) was dried with (tracks 5–8) and without (tracks 9–12) 15% trehalose, stored at room temperature for one week and compared with fresh enzyme (tracks 1–4) in a sequencing reaction using a ssDNA M13mp18 template with the –40 universal primer (reaction order ACGT).

1A. The enzymes dried without trehalose (tracks 3, 7, 11 and 15) lost all activity, whereas enzyme activity was preserved in all the trehalose-dried samples (tracks 2, 6, 10 and 14). The recovery of activity was quantitative as established by comparing the activities of serial dilutions of fresh enzyme with an identical series dried in the presence of trehalose (Fig. 1B). All other restriction enzymes so far tested also show a similar recovery of activity when dried in trehalose (these include AluI, BamHI, BssHII, BstXI, ClaI, DdeI, EcoRV, HaeIII, HincII, HinfI, HpaII, KpnI, MluI, MspI, NotI, NruI, SacII, SalI, SfiI, SmaI, TaqI, and XbaI, data not shown). Interestingly, trehalose did not show any improved stabilization of these enzymes in solution when compared to that observed with glycerol and showed some inhibition of enzymic activity at concentrations above 20% (data not shown).

Modifying enzyme stabilization. The universality of the stabilization conferred by trehalose is illustrated by the similar results obtained with DNA ligase and polymerases. T4 DNA ligase dried in the presence of trehalose retained its activity on drying and storage as assayed by ligation of HindIII fragments of lambda DNA (Fig. 2A). The modified T7 DNA polymerase, Sequenase®, also retained its activity when dried in the presence of trehalose as assayed by either the incorporation of labeled nucleotide in primer extension reactions (data not shown) or the much more stringent dideoxy chain-termination sequencing reaction, which is highly sensitive to enzyme denaturation (Fig. 2B). The addition of nucleotides and/or dideoxy-nucleotides to the polymerase before drying did not affect the results, and the dried enzyme gave identical results even after storage at room temperature for at least 20 weeks (data not shown). Similar results were obtained with both Taq polymerase and the Klenow fragment of *E. coli* DNA polymerase (data not shown).

High temperature stability. Even after storage at 70°C for 35 days, normally labile endonucleases could still cut DNA with fidelity, as illustrated for PstI in Figure 3C. Similar results were obtained for all other enzymes tested. Dry enzymes can also be stored at 37°C for at least 9 months or cycled repeatedly between their optimum activation temperature of 37°C and room temperature for at least 96 one-hour cycles with no loss of activity (data not shown). This stabilization property appears unique to trehalose as all other sugars tested failed to confer similar protection over long periods and to high temperatures, even though some prevented damage due to the drying process itself (Fig. 3A–C, Table 1). Reducing sugars, such as lactose and maltose, failed at 37°C within 35 days (the earliest time point at which they were tested) as did the non-reducing disaccharide sucrose, while the chemically more stable non-reducing sugars, the sugar alcohols, failed within 35 days at 55°C (Fig. 3A–C, Table 1). All monosaccharides tested, whether reducing or non-reducing, were ineffective as were polymers such as inulin, ficoll and dextran (Table 1).

DISCUSSION

The observations presented here demonstrate the ability of the simple disaccharide trehalose to convert normally labile molecular biology enzymes into stable dry reagents. This raises the possibility of developing simplified, pre-aliquotted kits for use in molecular biology research, as well as novel formats for applications such as the automation of techniques for genome mapping and sequencing. For example, a dry 96-well microtiter plate for DNA sequencing, consisting of a DNA polymerase dried in trehalose together with the deoxy and dideoxy nucleotides, provides the ability to perform twelve sequencing reactions simultaneously, either manually or robotically. The simplicity and speed of this process, together with the stability of the dry reagents, also lends itself to the application of molecular biology techniques in areas such as clinical diagnostics and education. For example, stabilization of a polymerase together with nucleotides in a pre-aliquotted dried PCR reaction mix provides a single-step reaction format that would reduce problems due to spurious contamination, and a multi-well format containing different primers could provide a versatile clinical diagnostic kit.

Though the precise molecular mechanism by which

trehalose stabilizes biological molecules is unknown, two hypotheses have been suggested. The water replacement hypothesis[18] states that, in common with other polyols, trehalose can make multiple external hydrogen bonds and could therefore replace the essential water molecules that are involved in maintenance of tertiary structure[20,21]. The glassy state theory[19] states that the tendency of trehalose solutions to undergo glass transformation results in an amorphous continuous phase, similar in structure to vitreous ice, in which molecular motion and thus degradative molecular reactions would be kinetically insignificant[22,23]. The results presented above are not entirely consistent with either theory. Although they do not eliminate the water replacement hypothesis, the strikingly different stabilities observed with trehalose and glucose are difficult to reconcile with this theory. Others have postulated that the glassy state is the sole important factor in

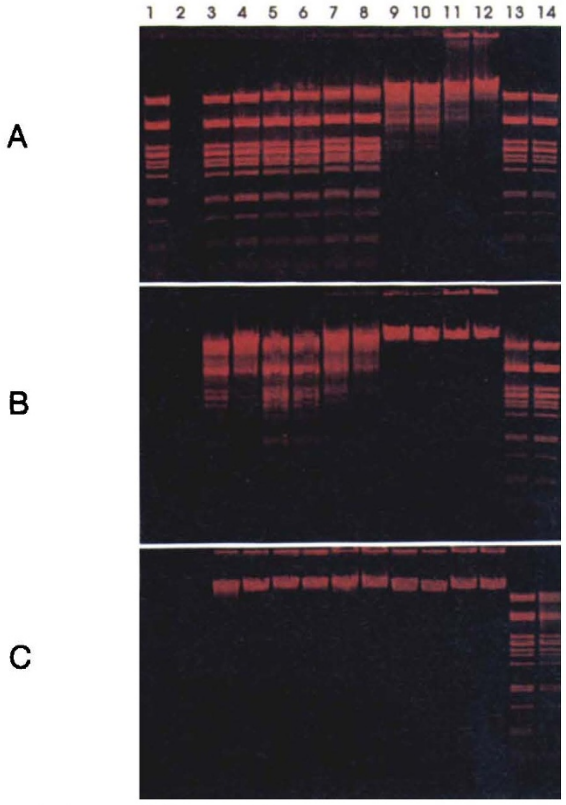


FIGURE 3 High temperature stability of dried enzymes. Five units of fresh PstI (track 1) was compared with 5 and 2.5 units of enzyme dried in 0.3M sugar solutions of either trehalose (tracks 13, 14), maltose (tracks 11, 12), the non-reducing disaccharide sucrose (tracks 9, 10) the disaccharide alcohol palatinit (the reduced sugar alcohol of isomaltulose consisting of a mixture of glucopyranosyl-sorbitol (GPS) and glucopyranosyl-maltitol (GPM) (tracks 7, 8) or its individual components GPM (tracks 3, 4) and GPS (tracks 5, 6). (A) Storage at 37°C for 35 days. (B) Storage at 55°C for 35 days. (C) Storage at 70°C for 35 days.

TABLE 1 Stability of restriction enzyme PstI dried in various sugars.

Carbohydrate	Chemical Name	Red. Prop.	Temp (°C)	Time (days)	Activity Recovered
Monosaccharides					
Threitol	(R*,R*)-1,2,3,4 butanetetrol	–	37°	14	–
Erythritol	(R*,S*)-1,2,3,4 butanetetrol	–	〃	14	–
Glucose	α-D-glucopyranose	+	〃	1	+
			〃	14	–
Sorbitol	sugar alchol of glucose	–	〃	14	+
			〃	35	+
			〃	70	–
Galactose	α-D-galactopyranose	+	〃	1	–
Galactitol	sugar alcohol of galactose	–	〃	1	–
Mannose	α-D-mannopyranose	+	〃	1	–
Mannitol	sugar alcohol of mannose	–	〃	1	–
Myo-inositol	(1α,2α,3α,4β,5α,6β)cyclo-hexanehexol	–	〃	14	–
Di- and Trisaccharides					
Trehalose	α-D-glucopyranosyl-α-D-glucopyranoside	–	〃	98	+ + +
			55°	70	+ + +
			70°	35	+ + +
Maltose	4-O-β-D-glucopyranosyl-D-glucopyranoside	+	37°	14	–
			〃	7	+
Maltotriose	O-α-D-glucopyranosyl(1→4)-O-α-D-glucopyranosyl(1→4)-D-glucopyranoside	+	〃	14	–
Lactose	4-O-β-D-galactopyranosyl-D-glucopyranose	+	〃	14	+
			〃	35	–
Lactulose	4-O-β-D-galactopyranosyl-D-fructose	+	〃	14	+
			〃	35	–
Palatinose	α-D-glucopranosyl-1,6-D-fructose	+	〃	14	–
Sucrose	β-D-fructofuranosyl-α-D-glucopyranoside	–	〃	14	+ +
			〃	35	–
Polymers					
Inulin	Polymer of 1-O-β-D-fructo-furanosyl-D-fructose	–	〃	7	–
Dextran	Polymer of α-(1,6)-D-gluco-pranose (1-3,1-4 branched)	+	〃	7	–
Ficoll	Polymer of β-D-fructofuranosyl-α-D-glucopyranose	–	〃	7	+

Quantitation of activity: (–) no detectable activity, (+) some activity (10–20% of titer), (+ +) partial activity (25–50% of titer), (+ + +) full activity (90–100% of titer). Reducing properties: (+) reducing sugar, (–) non-reducing sugar. PstI was dried in a universal cutting buffer containing the sugar or sugar alchol at 0.3M. After storage for the indicated time and temperature the activity recovered was titrated for its ability to cut 1μg of bacteriophage lambda DNA at 37°C for 1hr.

the stabilization of biological molecules in other systems[22,23] and argued, on a purely theoretical basis, that this is also the mechanism of action of trehalose[24]. However, this view is certainly not supported by the actual experimental data, as the high temperature stability results reported here are observed at temperatures well above the glass transition temperature of the samples as measured by differential scanning calorimetry (Y. Noel, S. Ring, BJR and CALSC manuscript in preparation). Furthermore, the comparative data presented here has been confirmed in other systems by other authors who also show the clear superiority of trehalose[11-13,25]. These results illustrate a basic deficiency of the glassy state theory, namely that it fails to consider any chemical reactions between the glass forming excipient matrix and the stabilized biomolecules embedded therein, both during drying and due to their juxtaposition. That these may be of primary importance, particularly when sugars are used as glass forming excipients, is suggested by results from studies on the storage of foods where deterioration is a result of the Maillard reaction between sugars and proteins[26]. As it is driven by the loss of water[27], the Maillard reaction is probably an even more important factor during stabilization achieved by desiccation. The relative chemical stability[28] and non-reducing nature of trehalose may thus be the most significant features in the results observed, especially in the long term stability at high temperatures.

Finally, the stability of the enzymes dried in trehalose reflects the hardiness of cryptobionts observed in nature. These organisms can remain apparently dead for many years and, when dry, are remarkably resistant to harsh environmental conditions such as extreme temperatures and megarads of ionizing radiation[1-4]. These data together with our earlier observations on trehalose-based drying of monoclonal antibodies[14,15] and previous work on other biological molecules and membranes[9-12], strongly suggest that the capacity of cryptobiotic organisms to accumulate high concentrations of trehalose is a sufficient explanation for their desiccation tolerance.

EXPERIMENTAL PROTOCOL

Drying experiments. Enzymes were made up to the required concentration in modified universal buffer[17] containing 1% BSA or manufacturer's recommended buffer and dried in 10–25 μl aliquots in microtiter 96-well plates. Trehalose concentrations of between 10–15% were routinely used or optimal concentrations determined by titration. Plates were dried at 37°C or ambient temperature overnight, under laminar airflow in a hood or under vacuum, or for 1–1.5 hrs under a captive dry atmosphere. Dried enzymes were stored in an anhydrous cabinet at various temperatures for various lengths of time before assay. In all experiments, the normal controls used were aliquots of the identical enzyme preparation that had been stored refrigerated as per the manufacturer's instructions.

Restriction enzyme assays. Enzymes were assayed for their ability to digest 1μg of non-methylated bacteriophage lambda DNA at 37°C for 1 hr. Dried enzyme samples were rehydrated directly in the test solution of lambda DNA in a volume equivalent to the volume initially dried and control digests of fresh or freezer stored enzymes also contained the same final concentrations of trehalose as in the dried enzyme assays. Reaction products were analysed on 1.2% agarose gels run in Tris-acetate and visualised by staining with ethidium bromide.

Other enzyme assays. T4 Ligase was assayed for the ability to ligate 1μg of HindIII fragments of non-methylated bacteriophage lambda DNA at 20°C for 30 min. Ligation products were analysed on 1.2% agarose gels run in Tris-acetate and visualised by staining with ethidium bromide. Dried enzyme samples were rehydrated directly in the test solution of lambda-HindIII fragments, in a volume equivalent to that initially dried and control reactions contained the same final concentrations of trehalose as in the dried ligase assays. Sequencing reactions were carried as in the USB Sequenase® sequencing booklet using a ssDNA M13mp18 template with the –40 univer-

sal primer, and run on buffer gradient sequencing gels. Dried polymerase samples were rehydrated directly in the test solution of primed-template DNA and control reactions again contained the same final concentration of trehalose as test samples.

Enzymes and chemicals. Restriction and modifying enzymes were purchased from various suppliers, and stored refrigerated as per the manufacturer's recommendations. For drying experiments, the enzymes were diluted in universal buffer to give the required concentration of enzyme just prior to aliquotting for drying. Trehalose was purchased from various suppliers and purified by repeated ion-exchange chromatography and crystallization.

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