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# A novel nuclear DNA helicase with high specific activity from *Pisum sativum* catalytically translocates in the 3′→5′ direction

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A novel ATP-dependent nuclear DNA unwinding enzyme from pea has been purified to apparent homogeneity and characterized. This enzyme is present at extremely low abundance and has the highest specific activity among plant helicases. It is a heterodimer of 54 and 66 kDa polypeptides as determined by SDS/PAGE. On gel filtration chromatography and glycerol gradient centrifugation it gives a native molecular mass of 120 kDa and is named as pea DNA helicase 120 (PDH120). The enzyme can unwind 17-bp partial duplex substrates with equal efficiency whether or not they contain a fork. It translocates unidirectionally along the bound strand in the 3′→5′ direction. The enzyme also exhibits intrinsic single-stranded DNA- and $Mg^{2+}$-dependent ATPase activity. ATP is the most favoured cofactor but other NTPs and dNTPs can also support the helicase activity with lower efficiency (ATP > GTP = dCTP > UTP > dTTP > CTP > dATP > dGTP) for which divalent cation ($Mg^{2+}$ > $Mn^{2+}$) is required. The DNA intercalating agents actinomycin $C_1$, ethidium bromide, daunorubicin and nogalamycin inhibit the DNA unwinding activity of PDH120 with $K_i$ values of 5.6, 5.2, 4.0 and 0.71 μM, respectively. This inhibition might be due to the intercalation of the inhibitors into duplex DNA, which results in the formation of DNA–inhibitor complexes that impede the translocation of PDH120. Isolation of this new DNA helicase should make an important contribution to our better understanding of DNA transaction in plants.

*Keywords*: DNA-dependent ATPase; helicase inhibitors; plant DNA helicase; unwinding enzyme.

Despite the energetically stable genomes of all living organisms including plants, they have to partially unwind for a very short time to create a single-stranded (ss) DNA template, which is required for most of their important cellular functions, including replication, repair, recombination and transcription [1]. The ssDNA template is provided by a group of enzymes called DNA helicases, which catalyse the DNA unwinding in an ATP-dependent manner and thereby act as an essential molecular tool for cellular machinery [2–6]. All helicases exhibit intrinsic DNA-dependent ATPase activity, which provides energy for the reaction [1]. Mechanistically, there are two classes of DNA helicases, those that can translocate in the 3′→5′ direction and the others in the 5′→3′ direction with respect to the strand on which they initially bind. Most organisms encode multiple DNA helicases because of their involvement in numerous biological processes at different stages of cell metabolism [3,6–8]. All the helicases share at least three common biochemical properties: (a) nucleic acid binding; (b) NTP/dNTP binding and hydrolysis; and (c) NTP/dNTP hydrolysis-dependent unwinding of duplex nucleic acids [9].

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*Abbreviations*: ss, single stranded; ds, double stranded; DP, degradation product.

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In plants, multiple DNA helicases must be present in three different organelles of the cell – nucleus, mitochondrion and chloroplast – where DNA transactions takes place independently of each other [4,5]. In plants, helicases play an important role in growth and development, which are the result of controlled cell proliferation that is cell division, elongation and arrest of the cell cycle [5]. Although the existence of first eukaryotic DNA helicase was reported from a plant in 1978 [10], but not much progress has been made on helicases in plant systems. In order to study the function of various helicases from a plant system, we have initiated a systematic study which involves purification and characterization of some of them. In this context we have previously reported four DNA helicases from plants: two from pea chloroplast, CDH I and CDH II [11,12] and two from pea nuclei, PDH45 and PDH65 [13,14]. We now report the purification and characterization of another novel DNA helicase from pea nuclei, which is the fifth candidate from pea whose properties have been characterized at the protein level. This enzyme is a heterodimer of 54 and 66 kDa subunits with a native molecular mass of 120 kDa and is designated *p*ea *D*NA *h*elicase *120* (PDH120). We have also tested the effect of different DNA intercalating agents on the unwinding activity of PDH120.

## Experimental procedures

### Materials, DNA polymers, compounds and buffers

Seeds of *Pisum sativum* L were imbibed in aerated water for 12 h and then germinated at 18 °C for 7–8 days. M13

ss- and double-stranded (ds) DNA and total RNA from pea leaves were prepared by standard methods. NTPs/dNTPs, ATPγS, poly(A), poly(U), poly(C), poly(G) and yeast tRNA were from Boehringer-Mannheim. [γ-$^{32}$P]ATP (185 TBq·nmol$^{-1}$) and [α-$^{32}$P]dCTP (≈ 110 TBq·mmol$^{-1}$) were from Amersham. The DNA oligonucleotides were synthesized chemically and purified electrophoretically. A total of 10 different oligonucleotides (ranging in length from 17 to 101 nucleotides) have been used in this study for constructing various DNA substrates with tail(s), no tails and small linear synthetic substrates as well as direction-specific substrates (see Fig. 5A–J). The sequences and details of these oligonucleotides have been described previously [11,15]. All of the electrophoresis reagents, protein markers, silver stain kit and BioRex 70 resin were from Bio-Rad. Miracloth was from Cal Biochem; column chromatography resins DE-52 cellulose, phosphocellulose, dsDNA cellulose and ssDNA cellulose were from Whatman and Pharmacia; T4 polynucleotide kinase and DNA polymerase I were from New England Biolabs; trypsin was from Serva (Heidelberg, Germany); the DNA-intercalating compounds daunorubicin, camptothecin, VP-16 and m-AMSA were from Topogene Inc. (Ohio, USA); novobiocin, and nogalamycin were from Sigma; ethidium bromide was from BDH and actinomycin $C_1$ was from Boehringer Mannheim. Most of these compounds were dissolved in dimethyl sulfoxide and stored at 4 °C in the dark; dimethyl sulfoxide has no effect on the enzyme activity of the helicase. Buffers were: NaCl/Pi, 10 mM sodium phosphate pH 7.4, 140 mM NaCl, 3 mM KCl; NE-1 buffer, 0.55 M sucrose, 50 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 25 mM KCl, 10 mM $Na_2S_2O_3$, 7 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride; NE-2 buffer, 600 mM KCl, 50 mM Tris/HCl pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, 0.5 μM leupeptin, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM pepstatin; Buffer A, 50 mM Tris/HCl pH 8.0, 0.1 M KCl, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium bisulfite, 1 μM pepstatin, 1 μM leupeptin, 20% glycerol. Buffer B is buffer A plus 1 mM ATP and 1 mM $MgCl_2$.

## Preparation of nuclear extract

The pea nuclear extract was prepared from 12 kg of pea leaves (top three to four leaves of 7-to 8-day-old pea seedlings) as described below. The leaves were washed with ice-cold NaCl/$P_i$, submerged in ice-cold NE-1 buffer and homogenized with kitchen mixer. The homogenate was then passed through two layers of cheesecloth and two layers of Miracloth. The filtrate was then centrifuged at 1000 ***g*** for 10 min at 4 °C in a Sorvall RC 5B centrifuge. The pellet was slowly resuspended in NE-1 buffer containing 2.5% Triton × 100, and incubated at 4 °C with slow shaking (to lyse the chloroplast) and followed by centrifugation at 2000 ***g*** for 30 min at 4 °C. If the pellet was still green in colour the above step could be repeated until all the chloroplast is removed. The resulting nuclear pellet was then resuspended in NE-2 buffer and homogenized in a Potter-Elvehjem glass homogenizer (Kimble/Kontes, Kimble Glass Inc. and Kontes Glass Co., Vineland, NJ, USA). Then the homogenate was centrifuged at 12 000 ***g*** for 30 min at 4 °C and the clear supernatant (nuclear extract) was dialysed against buffer containing 50 mM KCl, 50 mM Tris/HCl pH 8, 20% glycerol and protease inhibitors and stored at −80 °C.

## Preparation of DNA helicase substrates

The DNA substrate used in the helicase assay consisted of $^{32}$P-labelled complementary oligonucleotides hybridized to M13mp19 phage ssDNA or synthetic oligonucleotides to create a partial duplex. A substrate with 5′ and 3′ hanging tails (Fig. 5D) was used for purification and for most of the characterization unless stated otherwise. The structures of the various DNA substrates used in this study are shown in Fig. 5A–J. All the M13 substrates (Fig. 5A–F) including direction specific substrates (Fig. 5I and J) and small synthetic oligonucleotide substrates (Fig. 5G and H) were prepared as described previously [11,15].

## ATP-dependent DNA helicase and DNA-dependent ATPase assays

The standard DNA helicase reaction was performed in a 10-μL reaction mixture consisting of 20 mM Tris/HCl pH 8.0, 1 mM ATP, 2 mM $MgCl_2$, 250 mM KCl or NaCl, 8 mM DTT, 4% (w/v) sucrose, 80 μg·mL$^{-1}$ BSA, 40 pmol $^{32}$P-labelled substrate (approximately 1000–2000 c.p.m.) and the helicase fraction. The reaction mixture was incubated for 30 min at 37 °C and the reaction was terminated by addition of 1.5 μL 75 mM EDTA, 2.25% SDS, 37.5% (by vol.) glycerol and 0.3% Bromophenol blue. The reaction products were separated by 12% native PAGE and analysed as described previously [11]. The percentage unwinding was quantitated and calculated as described [11]. One unit of DNA helicase activity is defined as the amount of enzyme that unwinds 30% of the DNA helicase substrate at 37 °C in 30 min (1% in one min). For examining the effect of DNA-interacting compounds on DNA unwinding activity of PDH120, the compounds were added at 50 μM final concentrations in the helicase reaction mixture prior to the addition of enzyme. For determining the $K_i$, a concentration curve of the inhibitor was performed. The $K_i$ values here signify the inhibitor concentration necessary to inhibit enzyme activity by 50%. The ATPase reaction condition was identical to that described above for the helicase reaction, except that the $^{32}$P-labelled helicase substrate was replaced by 1665 Bq [γ-$^{32}$P]ATP and the reaction was performed for 30 min, 60 min and 2 h at 37 °C and analysed as described [11].

## Other methods

The DNA topoisomerase, polymerase, ligase, nicking and nuclease activities were performed as described earlier [11,12]. Glycerol gradient centrifugation and gel filtration chromatography were performed as described earlier [11,15]. Protein concentration was determined using the protein assay kit of Bio-Rad. SDS/PAGE was performed by a standard method, followed by silver staining of the gel with Bio-Rad kit.

**Table 1. Purification of pea nuclear DNA helicase 120.** Twelve kilograms of pea leaves were used as the starting material. ND not determined, due to the presence of nucleases.

| Fraction | Step | Total volume (mL) | Total protein (mg) | DNA helicase activity | |
|---|---|---|---|---|---|
| | | | | Total units (U) | Specific activity ($U \cdot mg^{-1}$) |
| I | Nuclear extract (after dialysis) | 140 | 201 | ND | |
| II | DE-52 cellulose | 156 | 12.6 | ND | |
| III | Bio-Rex70 | 124 | 4.72 | ND | |
| IV | Phosphocellulose | 22 | 0.283 | 29 333 | 103 650 |
| V | ds-DNA cellulose | 6 | 0.053 | 24 800 | 467 924 |
| VI | ss-DNA cellulose | 5 | 0.006 | 11 390 | 1 898 333 |

## Results

### Purification of PDH120

The results of purification are summarized in Table 1. The elution profiles of each chromatographic step along with the helicase gel pictures and the SDS/PAGE of pure protein are shown in Fig. 1. All purification steps were performed at 4 °C. Nuclear extract (fraction I, 140 mL) was prepared from 12 kg pea leaves and dialysed against buffer A. Fraction I was loaded on to a DE-52 cellulose column equilibrated with buffer A. After washing the column with buffer A, the bound proteins were eluted by linear gradient of 0.1–0.8 M KCl in buffer A. Fractions eluting at ≈ 0.3 M KCl contained helicase activity. These fractions also contained the nuclease activity as shown in Fig. 1A as degradation product (DP). The active fractions were pooled and diluted with buffer A to obtain a 0.1 M final concentration of KCl (fraction II, 156 mL) and loaded onto a Bio-Rex70 column equilibrated with buffer A. After thorough washing, bound proteins were eluted with linear gradient of 0.1–0.6 M KCl in buffer A. Fractions eluting at ≈ 0.4 M KCl contained helicase activity. These fractions still contained nuclease activity as shown in Fig. 1B as DP. The active fractions were pooled and diluted with buffer A without KCl (fraction III, 124 mL). Up to this step the activity was not quantified due to the contamination with nuclease activity.

Fraction III was applied to a phosphocellulose column equilibrated with buffer A. Following washing with buffer A, the bound proteins were eluted with a linear gradient of 0.1–1 M KCl in buffer A. The active fractions eluting at ≈ 0.7 M KCl (Fig. 1C) were pooled and dialysed against buffer B (fraction IV, 22 mL, 29 333 units). Fraction IV was loaded onto a dsDNA-cellulose column equilibrated with buffer B. The column was washed thoroughly and bound proteins were eluted with a linear gradient of 0.1–1 M KCl in buffer B. The activity eluted from the column at ≈ 0.65 M KCl (Fig. 1D) (fraction V, 6 mL, 24 800 units). After adjusting the KCl concentration to 0.1 M with buffer B, fraction V was loaded onto a ssDNA-cellulose column equilibrated with buffer B. After washing the column excessively with buffer B the bound proteins were eluted in steps with 0.2, 0.4, 0.6, 0.8 and 1 M KCl in buffer B. The helicase activity was detected in the fraction eluting with 0.6 M KCl (Fig. 1E) (fraction VI, 5 mL, 11 390 units).

SDS/PAGE followed by silver staining revealed the presence of two polypeptides of 54 and 66 kDa in fraction VI (Fig. 1F, lane 1), which showed that nuclear PDH120 was purified to apparent homogeneity with specific activity of $1.89 \times 10^6$ $U \cdot mg^{-1}$ (Table 1). The enzyme preparation did not contain any detectable DNA polymerase, ligase, topoisomerase, nicking or nuclease activity (data not shown). PDH120 did not cross-react with antibodies against plant helicases including PDH45 and PDH65 and also against human DNA helicases I, II, III and IV (data not shown). ssDNA-dependent ATPase activity was present at a level of $0.6 \times 10^3$ pmol ATP hydrolysed at 37 °C in 30 min by 3 ng pure PDH120 enzyme (fraction VI) in the presence of 100 ng M13 ssDNA. This activity increases up to 60 min ($1.15 \times 10^3$ pmol), saturates at 2 h and shows maximum activity of $1.5 \times 10^3$ pmol. There was no ATP hydrolysis without ssDNA and $Mg^{2+}$ (data not shown).

### Native molecular mass of PDH120

The native molecular mass of PDH120 was determined by its hydrodynamic properties, i.e. by glycerol gradient centrifugation (Fig. 2A) and gel filtration chromatography (Fig. 2B) by using 200 U concentrated fraction VI. Purified PDH120 (fraction VI, 85 μL, 105 ng, 200 U) was mixed with markers (catalase, alcohol dehydrogenase, BSA and ovalbumin) and centrifuged on a glycerol gradient (15–40%) in buffer A containing 0.5 M KCl. The autoradiogram of helicase gel and activity profile representing only fractions 9–16 are shown in Fig. 2A. The peak active fraction number 11 contains both the polypeptides of 54 and 66 kDa on SDS/PAGE as shown in Fig. 2A (right side of the graph). The DNA helicase activity (Fig. 2A, lane 4) and ssDNA-dependent ATPase activity (data not shown) sedimented together between alcohol dehydrogenase and BSA (fraction 11) and gave a molecular mass of 120 kDa with a sedimentation coefficient of 6.0. For gel filtration chromatography the concentrated fraction VI (50 μL, 105 ng, 200 U) was used. The autoradiogram of helicase gel and the activity profile representing only active fractions 18–24 of gel filtration chromatography are shown in Fig. 2B. The same fractions were also active for ssDNA-dependent ATPase activity (data not shown). The peak active fraction number 22 contains both the polypeptides of 54 and 66 kDa on SDS/PAGE as shown in Fig. 2B (right

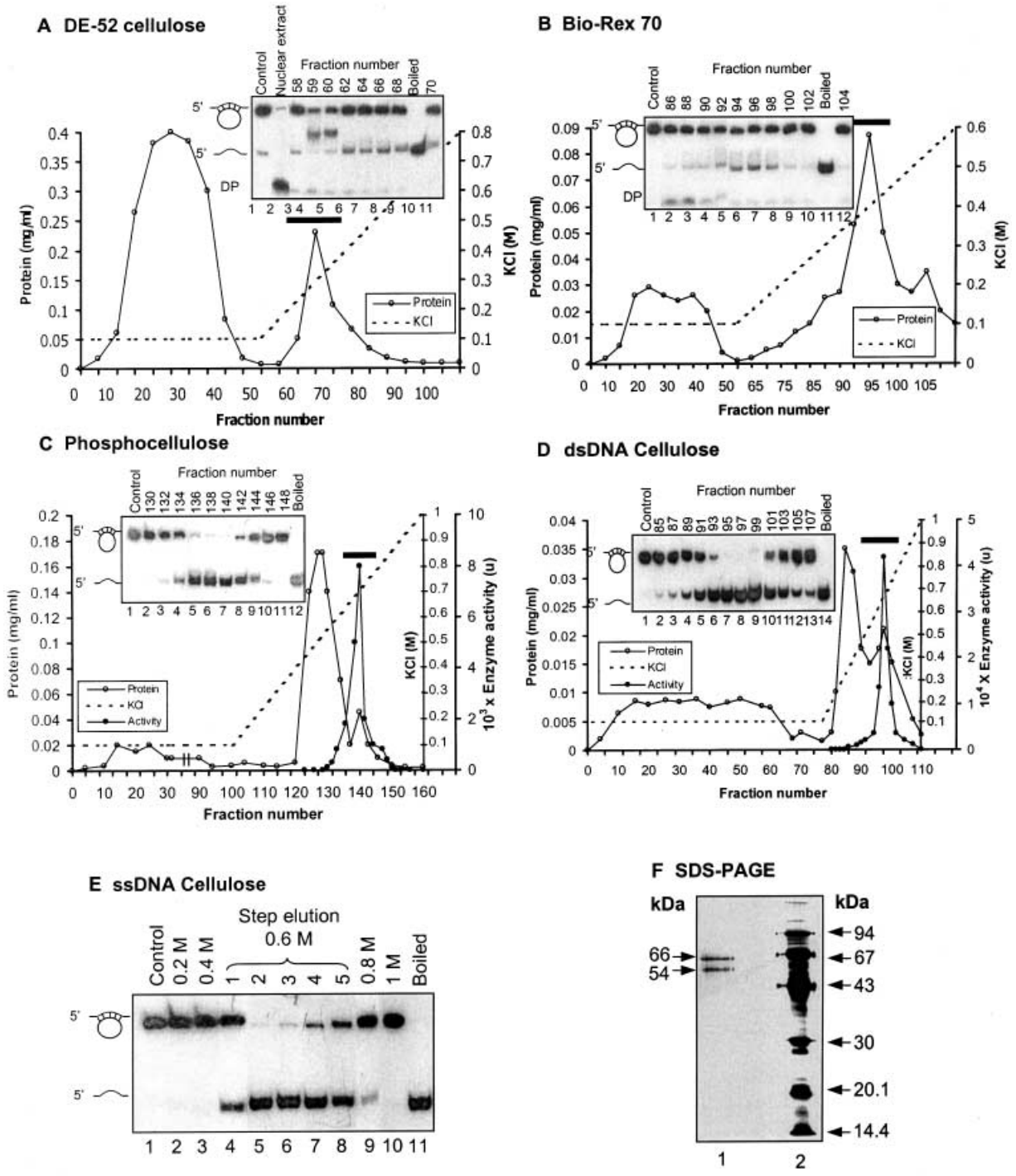


**Fig. 1. The protein elution and helicase activity profiles and SDS/PAGE of PDH120.** (A–E) The purification of PDH120 through chromatography on (A) DE-52 cellulose, (B) Bio-Rex70, (C) phosphocellulose, (D) dsDNA-cellulose, and (E) and ssDNA-cellulose columns. The detailed description of each chromatographic procedure is given in the text. The dotted line indicates the KCl gradient. The active fractions, which were pooled, are indicated by a horizontal bar on the top of the active peak. The autoradiogram of helicase gel representing only active fractions is also shown in corresponding panels. The structure of the hanging tail-bearing substrate (also shown in Fig. 5D) used for the helicase assay is shown on the left side of each gel. On each helicase gel, control and boiled lanes represent reactions without enzyme and with heat-denatured substrate, respectively. The rest of the lanes represent active fractions. The smears at the bottom of the gel in panels (A) and (B) are due to the action of nucleases on the substrate and are represented as DP (degradation products). The species that migrates intermediate to the released oligonucleotide and substrate in lanes 4 and 5 of panel (A) is the band of slower mobility (band shift) which is due to the binding of released oligonucleotide to the ssDNA binding protein present in the particular fraction of nuclear extract. (F) The silver stained SDS/PAGE of purified PDH120 (lane 1, fraction VI, 45 ng) and molecular-mass markers (lane 2). Arrows show the size in kDa.

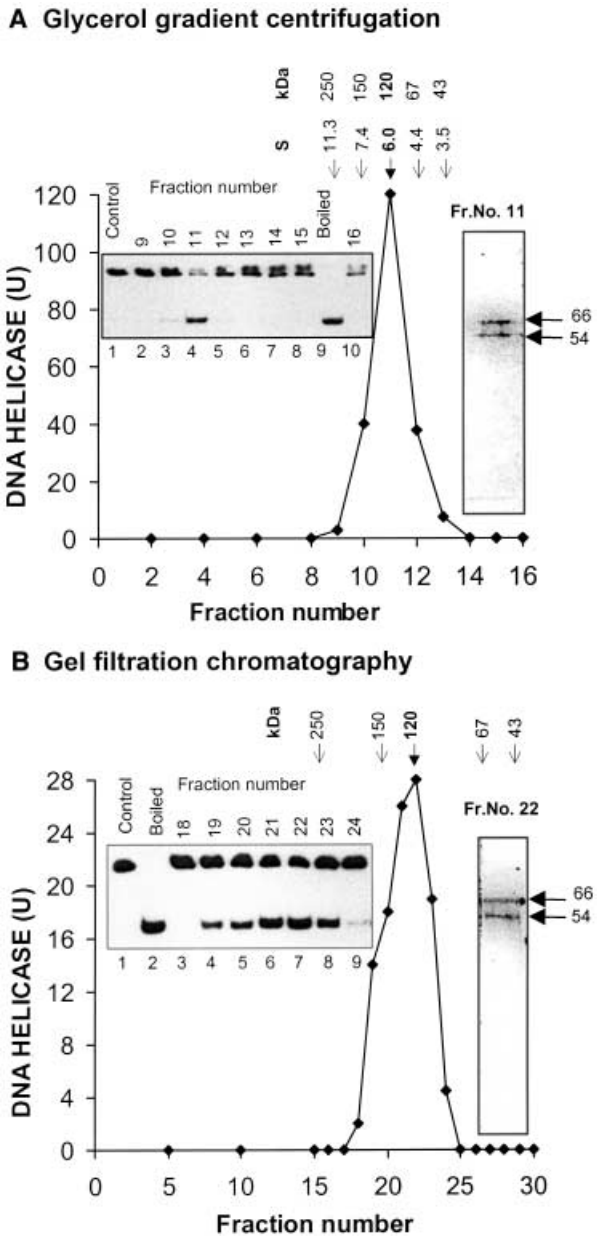


**Fig. 2. Glycerol gradient centrifugation and gel filtration chromatography of PDH120.** The pure PDH120 (fraction VI) was first concentrated before use. (A) Glycerol gradient (15–40%) centrifugation of 50 μL (105 ng, 200 U) purified PDH120 (fraction VI) was performed at 48 000 r.p.m. for 18 h at 4 °C in SW 60 rotor. Fractions of 0.2 mL were collected from the bottom of the tube and assayed for DNA helicase activity. The distribution of helicase activity, position of the sedimentation coefficient and molecular mass markers are shown. The markers were catalase (250 kDa, 11.3S), alcohol dehydrogenase (150 kDa, 7.4S), BSA (67 kDa, 4.4S), and ovalbumin (45 kDa, 3.5S). An autoradiogram of helicase gel of some fractions is shown on the left side of the active peak. The hanging tail-bearing substrate (as shown in Fig. 5D) was used for helicase assay. The silver stained SDS/PAGE of active peak fraction number 11 (30 ng) is shown on the right side of the graph. (B) Gel filtration chromatography of 50 μL of concentrated PDH120 (fraction VI, 105 ng, 200 U) was performed on a Sephadex G-150 column (240 × 4 mm). The column was run at 4 °C with buffer A containing 0.5 M KCl. Fractions of 0.2 mL were collected and assayed for helicase activity. Markers were same as above. An autoradiogram showing helicase activity of the active fractions is shown on the left side of the graph. In both the gels (A and B) the control and the boiled lanes are reactions without enzyme and heat-denatured substrate, respectively. The hanging tail-bearing substrate (as shown in Fig. 5D) was used in a standard helicase assay. The silver stained SDS/PAGE of concentrated fraction 22 (25 ng) is shown on the right side of the graph.

side of the graph). The native molecular mass of PDH120 on gel filtration was also 120 kDa (Fig. 2B). The glycerol gradient and gel filtration data collectively suggest that PDH120 is a heterodimer of 54 and 66 kDa polypeptides.

### Reaction requirements and characterization of DNA unwinding activity of PDH120

The enzyme is heat labile and loses its activity upon heating at 56 °C for 1 min (data not shown). Significant unwinding activity was observed in the broad pH range (pH 7.5–9.0) with an optimum near pH 8.0 (data not shown). The activity was completely inhibited by trypsin (1 U), EDTA (5 mM), potassium phosphate (100 mM), ammonium sulfate (45 mM), M13 ssDNA (30 μM as P, phosphate), M13 dsDNA (30 μM as P), pea leaf total RNA (30 μM as P), *E. coli* t-RNA (30 μM as P) and histone (1 mg·mL$^{-1}$) (data not shown). Probably helicase is binding to these DNA and RNA molecules nonspecifically and acting as trap. The enzyme showed an absolute requirement for divalent cations. Magnesium at 2.0 mM concentration optimally fulfilled this requirement (Fig. 3A). However, at 8.0 mM $MgCl_2$ the activity was totally inhibited (Fig. 3A, lane 9). Manganese at equivalent concentration supported 80% of the activity while other divalent cations such as $Ca^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Ag^{2+}$ and $Co^{2+}$ were unable to support the activity (data not shown). The optimum concentration of KCl required for the helicase reaction was 250 mM (Fig. 3B, lane 6). At a higher concentration of KCl (400 mM) the activity was totally inhibited (Fig. 3B, lane 9).

The optimum concentration of ATP for DNA helicase activity was 1.0 mM (Fig. 3C, lane 6). At higher concentration (8 mM ATP) the DNA unwinding activity of PDH120 was inhibited (Fig. 3C, lane 9). All of the other NTPs or dNTPs also supported the unwinding activity but with lower efficiency (ATP > GTP = dCTP > UTP > dTTP > CTP > dATP > dGTP) (Fig. 3D). ADP, AMP and the poorly hydrolysable ATP analogue ATPγS were inactive as a cofactor for DNA unwinding activity of PDH120 (data not shown).

The kinetics of the helicase reaction under standard assay condition with 3 ng purified enzyme (fraction VI) showed a linear rate up to 30 min (Fig. 4A). After further incubation it deviated from the linearity and became saturated at ≈ 60 min. Titration of helicase activity with increasing amounts of the pure enzyme showed an approximately linear response; up to 82% unwinding

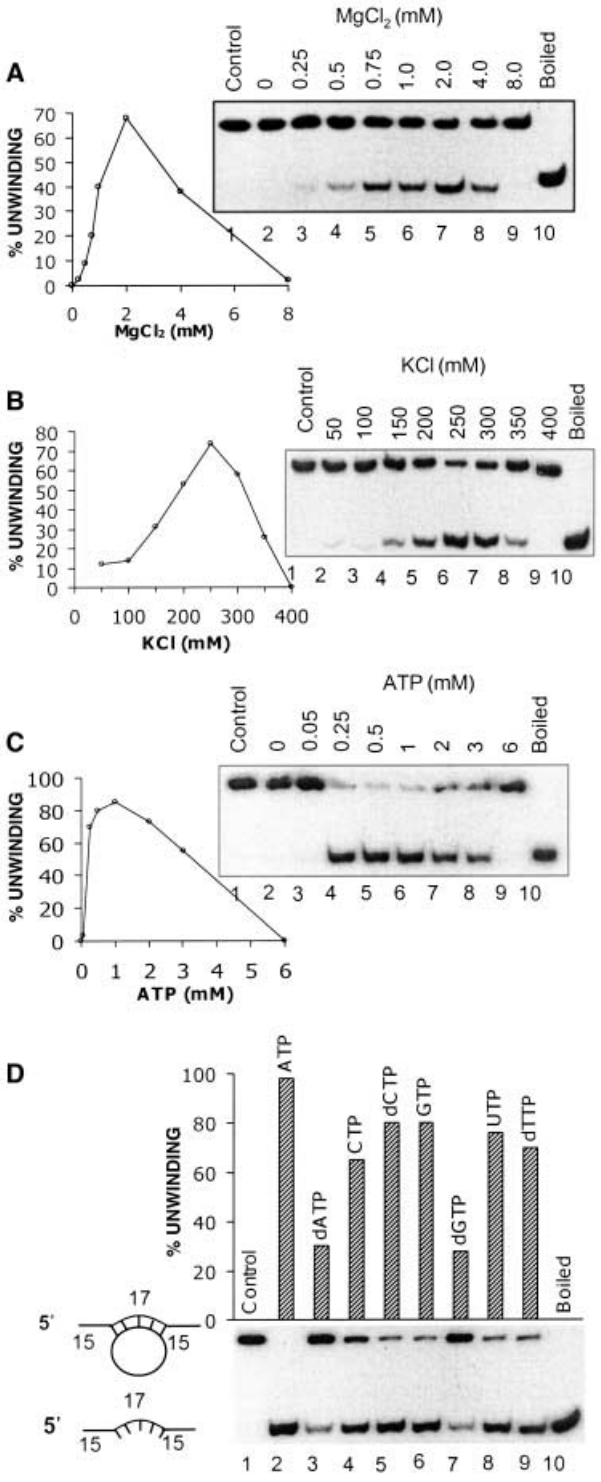


**Fig. 3. Requirement of $MgCl_2$ (A), KCl (B) ATP (C) and NTPs/dNTPs (D) for PDH120 activity.** (A–C) In each reaction 3 ng of fraction VI with 40 pmol of 5′ and 3′ hanging tail-bearing substrate (as shown in Fig. 5D) was used with varying concentration of $MgCl_2$ (A), KCl (B), or ATP (C). The concentrations used are given at the top of each lane of each gel. The quantitative data are displayed on the left side of each autoradiogram. In all gels, lane 1 (control) is the reaction without enzyme and lane 10 (boiled) is heat-denatured substrate. The activity is shown as percentage unwinding. (D) The standard helicase reactions were performed with 3 ng fraction VI, 40 pmol substrate and 1 mM NTP or dNTP. The amount of unwound DNA was quantified and plotted as a histogram above the autoradiogram of the gel. Lanes 2–9 are reactions in the presence of ATP, dATP, CTP, dCTP, GTP, dGTP, UTP, and dTTP, respectively. The structure of the hanging tail-bearing substrate is shown on the left side of the autoradiogram.

with 3 ng of the protein and 40 pmol of the substrate (Fig. 4B).

## Fork structures have no influence on DNA unwinding activity of PDH120

The unwinding activity of PDH120 was examined by using four different substrates (forked or nonforked) in standard assay conditions. All four of the substrates had the same duplex length (17 base pairs) with identical sequence but they differed in the presence of noncomplementary tails at the 5′ end (Fig. 5B), the 3′ end (Fig. 5C), both the 5′ and 3′ ends (Fig. 5D) or at neither end (Fig. 5A). The results showed that there was no significant difference in the DNA unwinding activity of PDH120 with forked or nonforked substrates. Almost the same activity was seen with all four of the above substrates (Fig. 5A–D). However, the enzyme was unable to unwind longer duplex even if it contained tails (Fig. 5E) or no tail (Fig. 5F). The use of synthetic oligonucleotide partial duplex containing the same duplex length (17 base pairs) as substrate showed almost the same activity (Fig. 5G). However, the enzyme failed to unwind synthetic blunt-ended duplex DNA (Fig. 5H) suggesting that PDH120 requires ssDNA adjacent to the duplex as a loading zone.

## Direction of DNA unwinding by PDH120

The strand to which the enzyme binds and moves defines the direction of unwinding. In order to determine the direction of unwinding, two different substrates were prepared with long ssDNA bearing short stretches of duplex DNA at the ends. The models of the direction-specific substrates are shown above each autoradiogram in Figs 5I and J. The results show that PDH120 moves unidirectionally in the 3′→5′ direction (Fig. 5I, lanes 2 and 3) and not in the 5′→3′ direction (Fig. 5J, lanes 2 and 3). The 5′→3′ directional activity was not detected even at higher concentration of the PDH120 protein (data not shown).

## Effect of DNA-interacting compounds on DNA unwinding activity of PDH120

The chemical structures of the compounds used have been described previously [16]. Initially, each compound was used at a final concentration of 50 μM. The results are shown in Fig. 6A. Camptothecin, VP-16, novobiocin and m-AMSA did not show any significant effect on DNA helicase activity (Fig. 6A, lanes 3, 8, 9 and 10). However, ethidium bromide, daunorubicin, nogalamycin,

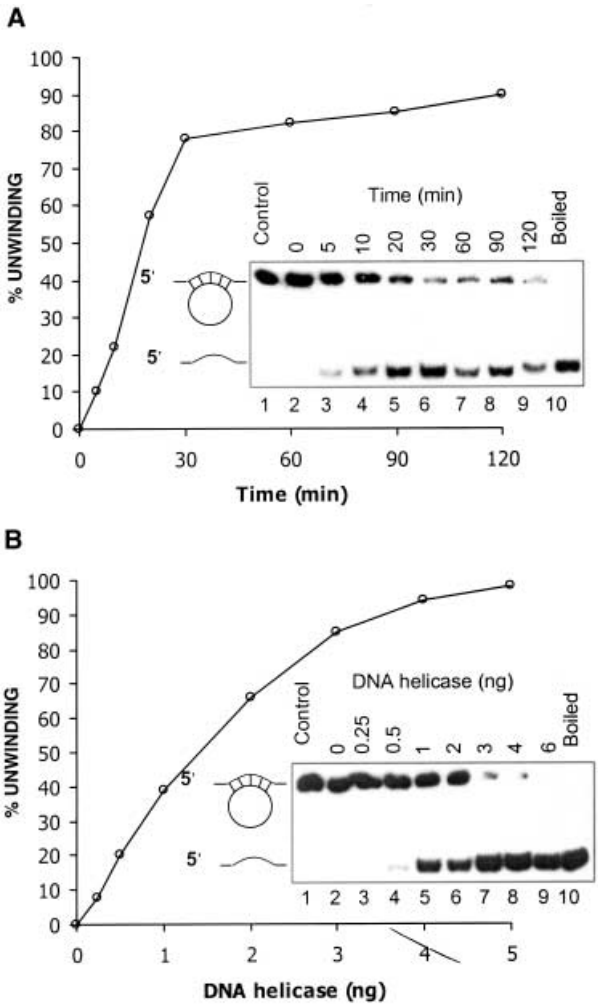


**Fig. 4. Kinetics and concentration dependence of unwinding activity of PDH120.** The enzyme activity data from the autoradiograms were quantified and plotted. The structure of the hanging tail-bearing substrate (also shown in Fig. 5D) used is shown on the left side of the gel. (A) The standard helicase reaction was carried out with 3 ng of fraction VI for the time indicated on the top of each lane (2–9). Lanes 1 (control) and 10 (boiled) are the reactions without enzyme and heat-denatured substrate, respectively. (B) An increasing amount of fraction VI was used in the standard helicase assay. The concentrations used are indicated on the top of each lane. Lanes 1 (control) and 10 (boiled) are the reactions without enzyme and heat-denatured substrate, respectively.

and actinomycin $C_1$ were inhibitory to the enzyme activity (Fig. 6A, lanes 4–7). The kinetics of inhibition by these inhibitors was studied by including different concentrations of actinomycin $C_1$ (Fig. 6B), ethidium bromide (Fig. 6C), daunorubicin (Fig. 6D), and nogalamycin (Fig. 6E) in the standard helicase reactions. The titration curve is plotted as a graph and shown on the left side of the autoradiogram of the gel in Fig. 6B–E. The apparent $K_i$ values for inhibition by intercalating agents actinomycin $C_1$, ethidium bromide, daunorubicin and nogalamycin were 5.6, 5.2, 4.0 and 0.71 μM, respectively (Fig. 6B–E).

## Discussion

In this study we have described the isolation and properties of a novel plant DNA helicase (PDH120), which exists in extremely low abundance in plants, has a high specific activity and is inhibited by DNA major groove binding agents. It did not cross-react with antibodies against various DNA helicases from human [6], pea chloroplast [11,12] and PDH45 and PDH65 from pea nuclei [13,14], suggesting that it is a new enzyme. The comparison of various properties of PDH120 with PDH45 and PDH65 as shown in Table 2 further strengthen the fact that PDH120 is different from other plant nuclear helicases.

The PDH120 was fractionated from pea nuclear extract on the basis of its behaviour on DE-52 cellulose, Bio-Rex70, phosphocellulose, dsDNA and ssDNA chromatography. It binds more strongly to ssDNA column and elutes at 0.6 M salt as compared to previously described pea nuclear helicases PDH45 [13] and PDH65 [14], which eluted from the same column at 0.2 M and 0.4 M salts, respectively (Table 2). PDH120 is a heterodimer of 54 and 66 kDa subunits with a native molecular mass of 120 kDa. Human DNA helicase II was also reported to be a heterodimer [15] while PDH45 [13] and PDH65 [14] were monomers. The PDH120 contains an ATP- and $Mg^{2+}$-dependent DNA unwinding activity and it catalytically translocates on ssDNA in the 3′→5′ direction similar to PDH45 [13], PDH65 [14], pea chloroplast DNA helicases I and II [11,12], human DNA helicases I, II, III, V and VI [6], simian virus-40 large tumour antigen [17] and nuclear DNA helicases from calf thymus [18].

The enzyme does not require a fork-like structure for its optimum activity as it has similar activity whether the substrate contains tail(s) or not. This property is similar to human DNA helicases I, IV and V [6], pea chloroplast DNA helicase I [11], PDH45 [13], and soybean helicase [19]. In contrast, the pea chloroplast DNA helicase II [12] and human DNA helicases II, III and VI [6] showed maximum activity with forked substrates. Furthermore, the enzyme acts catalytically in displacing short duplex regions and is unable to unwind 32-bp duplex. This kind of limited unwinding activity was also reported for *E. coli* Rep helicase [3] and human MCM-4, -6 and -7 protein complexes [20]. If PDH120 plays a role in replication, additional proteins would be required for its ability to unwind long stretches of duplex DNA, as reported for *E. coli* Rep helicase [3]. PDH120 requires ATP as cofactor for optimal activity and all the other NTPs or dNTPs supported the activity but with lower efficiency. This property is similar to the pea chloroplast DNA helicase I [11] and human DNA helicase II [15].

The hydrolysis of ATP is an absolute requirement for the unwinding reaction of PDH120, as a poorly hydrolysable analogue of ATP, ATPγS, was unable to be utilized by the enzyme. PDH120 contains ssDNA-dependant ATPase activity, which has been reported to be the intrinsic activity of all the helicases [1]. The ssDNA-dependent ATPase activity is also known to be required for translocation of the helicase protein on the DNA [1].

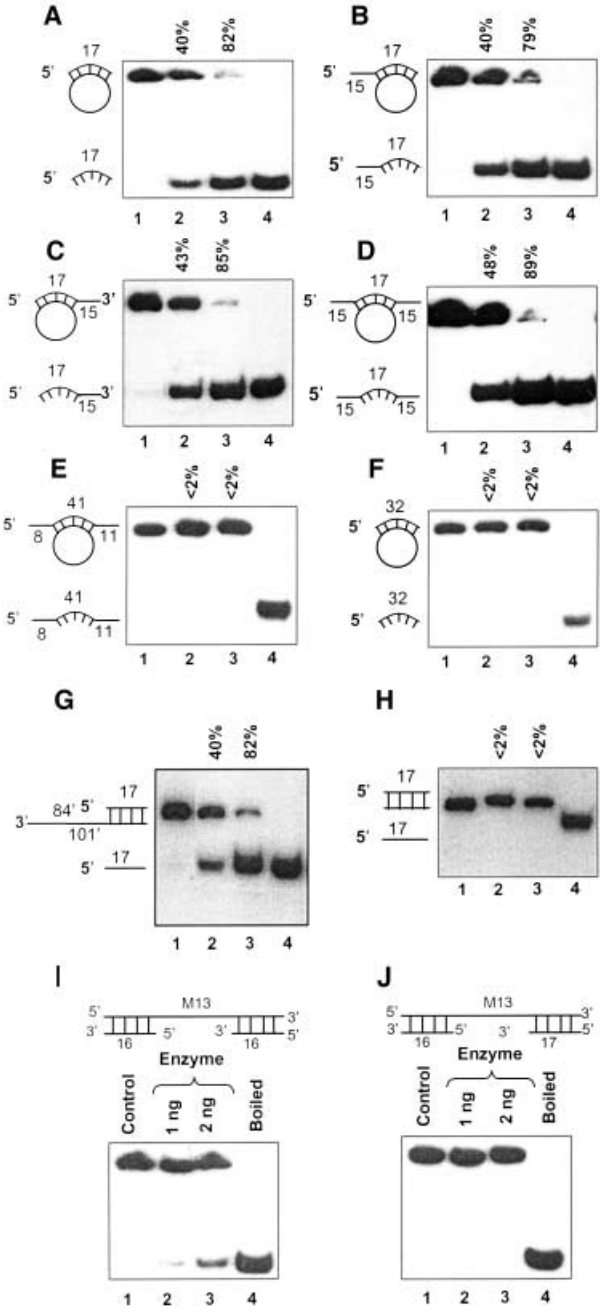


**Fig. 5. DNA helicase activity with various substrates and direction of unwinding by PDH120.** (A–H) The DNA helicase reactions were performed under standard conditions using 1.5 and 3 ng of purified PDH120 with different DNA substrates that contained either no tail (A) and (F), a 5′ tail (B), a 3′ tail (C) or both 3′ and 5′ tails (D) and (E). The substrates in panels (E) and (F) contained longer duplex annealed to M13 ssDNA as compared to (D) and (A). The substrate in panel (G) is a linear synthetic oligonucleotide partial duplex containing the same duplex length (17 base pairs). The substrate in panel (H) is a synthetic blunt-ended duplex DNA of 17 base pairs. The schematic structure of each substrate is shown on the left side of the autoradiogram of the gel. The percentage unwinding is shown on the top of each panel. In each panel, lane 1 is the reaction without enzyme, lane 2 is the reaction with 1.5 ng of enzyme, lane 3 is the reaction with 3 ng of enzyme and lane 4 is the heat-denatured substrate. (G,H) The structure of the direction-specific linear substrates for the 3′→5′ direction (G) and 5′→3′ direction (H) is shown on the top of the autoradiogram. In each gel, lane 1 is the reaction without enzyme, lane 2 is the reaction with 1 ng of fraction VI, lane 3 is the reaction with 2 ng of fraction VI, and lane 4 is the heat-denatured substrate.

In order to understand the mechanism of DNA unwinding we tested the effect of different compounds and found that actinomycin $C_1$, ethidium bromide, daunorubicin and nogalamycin inhibited the DNA unwinding activity of PDH120. All four of these compounds were also reported to inhibit the human DNA helicase II [16] and pea chloroplast DNA helicase I [21]. However, PDH45, Werner's helicase, Bloom's helicase and *E. coli* helicase II were not inhibited by actinomycin $C_1$ [22]. Actinomycin $C_1$, a polypeptide containing the properties of an antibiotic, intercalates into dsDNA and thereby inhibits nucleic acid synthesis [23]. Nogalamycin and daunorubicin are anthracycline antibiotics and are considered to be universal inhibitors of all the helicases tested so far [22]. Daunorubicin intercalates into the major groove of DNA while nogalamycin intercalates into both major and minor grooves of DNA. Ethidium bromide, a potent inhibitor of DNA synthesis, is a phenathridium compound, which intercalates into DNA [22].

The mechanism by which these compounds inhibit the unwinding reaction of PDH120 might be through intercalation into the duplex DNA substrate. This probably provides a physical block to continued translocation by the helicase, causing the unwinding reaction to be inhibited. Yet another possibility could be that these inhibitors bind directly to the PDH120 protein and negatively impact upon the catalytic function of the enzyme and/or prevent the protein from binding to the partial duplex DNA substrate. However, this possibility was ruled out by preincubating PDH120 with the inhibitory concentration of all these inhibitors prior to dilution in the unwinding reaction. Under these conditions, there was no inhibition of the unwinding reaction (data not shown). This further confirmed that the inhibition was due to the formation of an inhibitor–DNA complex that impeded the translocation of the protein. The exact mechanism of DNA unwinding by the helicase is not yet fully understood. Therefore, these findings should make an important contribution to our better understanding of the mechanism by which the plant nuclear duplex DNA is unwound by a helicase and also, more generally, the mechanism by which these agents act to inhibit cellular function.

Although many helicases have been characterized biochemically, it is often difficult to determine the *in vivo* role of a specific helicase. However, the biological roles of only a few DNA helicases have been determined. For example, the DnaB, PriA protein, Rep protein and helicase II from *E. coli* and the SV-40 large T antigen helicase have been shown to play a role in DNA

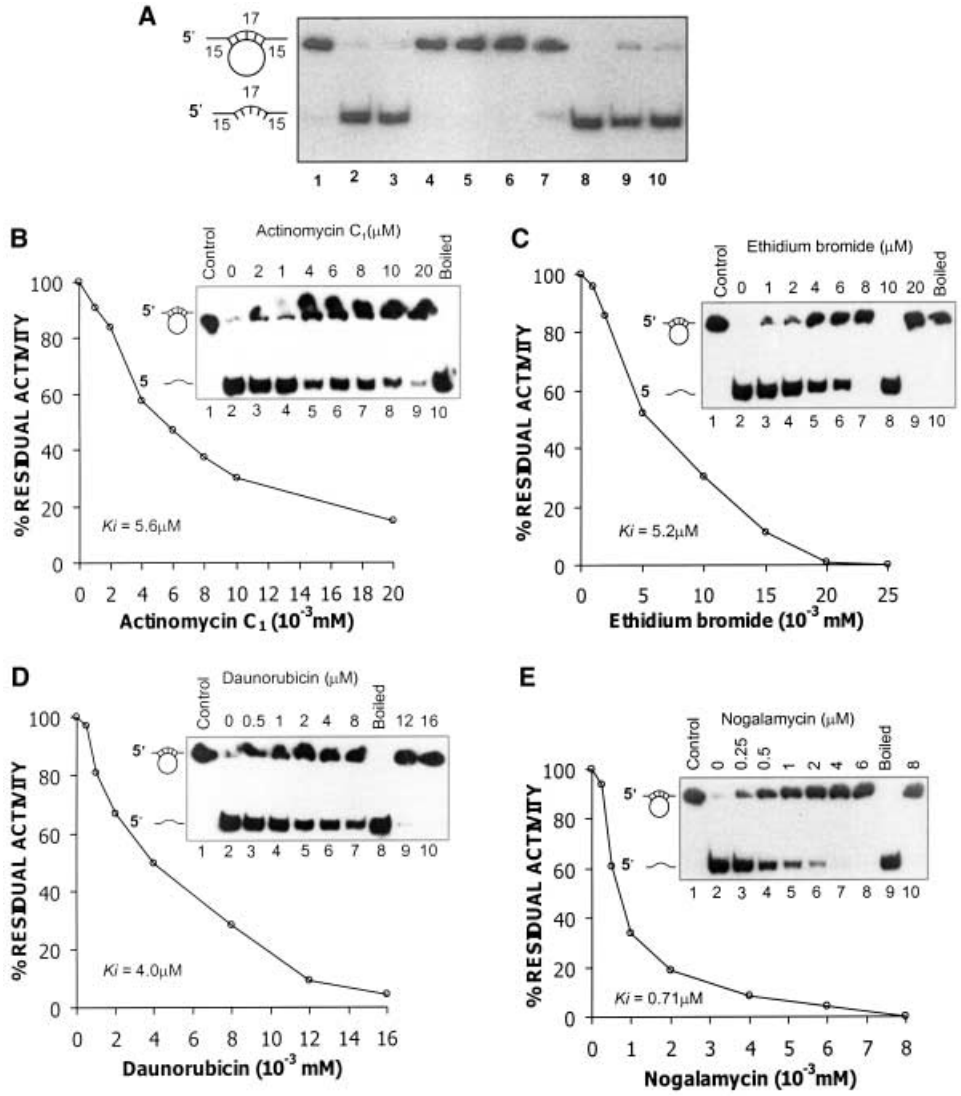


**Fig. 6. Effect of DNA interacting agents on DNA unwinding activity of PDH120 and the kinetics of inhibition.** (A) The standard helicase reaction was performed with 3 ng fraction VI, ≈ 40 pmol of the substrate having hanging tails of 15 nucleotides on the 3′ and 5′ ends (see Fig. 5D) and 50 μM of the compound. Lane 1 is the reaction without enzyme, lane 2 is the reaction with enzyme in the presence of 1 μL of the solvent (dimethyl sulfoxide), and lanes 3–10 are reactions in the presence of camptothecin, ethidium bromide, daunorubicin, nogalamycin, actinomycin $C_1$, VP-16, novobiocin and m-AMSA. The structure of the substrate used is shown on the left side of the autoradiogram. (B–E) Titration of inhibition of unwinding activity of PDH120 by actinomycin $C_1$ (B), ethidium bromide (C), daunorubicin (D) and nogalamycin (E). The DNA helicase reactions were performed in the presence of increasing concentrations of the compound using 40 pmol of $^{32}$P-labelled substrate with hanging tails (see Fig. 5D) and 3 ng of the pure enzyme. The quantitative curve is shown on the left side of each autoradiogram of the gel. The concentrations of each compound used are given on the top of each lane. The structure of the hanging tail-bearing substrate used is shown on the left side of each gel. The $K_i$ value is also given. The 100% relative activity in panels (B) to (E) is ≈ 90%, ≈ 98%, ≈ 91% and ≈ 94%, respectively.

replication [1–3,7]. A DNA repair helicase has been shown to be a component of basic transcription factor 2 (TFIIH) [24]. Recently we have reported the first biochemically active malarial DNA helicase and shown that it is homologous to eIF-4A [25] similar to previously reported PDH45 [13] and hepatitis C virus NS3 helicase [26]. These helicases may also have a role in translation initiation. Isolation of DNA helicase is the first step towards elucidating the DNA transaction mechanism in plants. Therefore, the discovery of this novel helicase should make an important contribution to our better understanding of: (a) DNA transactions in plants; (b) mechanism by which the plant nuclear duplex DNA is unwound by helicase; and (c) in general the mechanism by which these compounds act to inhibit cellular function.

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**Table 2. Differences between pea nuclear DNA helicase I (PDH45), II (PDH65) and III (PDH120).** P, phosphate; ND, not determined; HDH, human DNA helicase; eIF-4 A, eukaryotic translation initiation factor 4A.

| Property | PDH45[a] | PDH65[b] | PDH120 |
|---|---|---|---|
| Molecular mass: SDS/PAGE | 45.5 kDa | 65 kDa | 54 and 66 kDa |
| Native | 45.5 kDa | 65 kDa | 120 kDa |
| Oligomeric nature | Monomer | Monomer | Heterodimer |
| Behaviour on ssDNA-column | Eluted at 0.2 M salt | Eluted at 0.4 M salt | Eluted at 0.6 M salt |
| Optimum concentration ATP (mM) | 0.6 | 3.0 | 1.0 |
| $MgCl_2$ (mM) | 0.6 | 3.0 | 2.0 |
| KCl (mM) | 150 | 10.0 | 250 |
| Divalent cation requirement | $Mg^{2+} \geq Mn^{2+} \gg Ca^{2+}$ | $Mg^{2+} > Mn^{2+} > Ca^{2+}$ | $Mg^{2+} > Mn^{2+}$ |
| Nucleotide requirement | ATP > dATP > dCTP > CTP, GTP > UTP > dTTP | ATP > dATP | ATP > GTP = dCTP > UTP > dTTP > CTP > dATP > dGTP |
| Inhibition by: | | | |
| $(NH_4)_2SO_4$ (45 mM) | Yes | No | Yes |
| M13RFI DNA (30 μM as P) | No | Yes | Yes |
| Pea total RNA (30 μM as P) | No | Yes | Yes |
| Unwinding longer duplex (> 17 bp) | No | Yes | No |
| Enzyme concentration curve | Not sigmoidal | Sigmoidal | Not sigmoidal |
| Reaction with anti-PDH45 Ig | Yes | No | No |
| Reaction with anti-PDH65 Ig | No | Yes | No |
| Stimulation of topoisomerase I | Yes | No | n.d. |
| *In vitro* translation inhibition by the respective antibodies | Yes | No | n.d. |
| Substrate for CK2 protein kinase | No | Yes | n.d. |
| Substrate for cdc2 protein kinase | No | Yes | n.d. |
| Localization | Nucleus and cytosol | Nucleolus | Nucleus[c] |

[a] Pea DNA helicase 45 kDa in size [13]. [b] Pea DNA helicase 65 kDa in size [14]. [c] Isolated from highly purified pea nuclei.

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