Thermal-denatured PDRN exhibits increased *in vitro* pharmacological activities: A functional role of single-stranded DNA random coils

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Abstract. Polydeoxyribonucleotide (PDRN) is a DNA-based remedy that is broadly utilized for the treatment of wounds, inflammatory diseases and other diverse disorders. The present study aimed to clarify which conformational state of PDRNs, single-stranded DNA (ssDNA) random coil or double-stranded DNA (dsDNA) helical form, was responsible for its *in vitro* pharmacological properties, such as antioxidant, anti-inflammatory, skin whitening and anti-wrinkling properties. A PDRN solution was subjected to thermal denaturation at a high temperature, which generated heated PDRN (PDRN-H) with augmented ssDNA random coils. Skin *in vitro* beneficial properties of PDRN and PDRN-H were analyzed and compared using the non-celled *in vitro* models. PDRN itself was shown to exhibit positive effects, such as a scavenging or inhibitory effect, in all tests done, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS), superoxide and nitrite scavenging assays, and tyrosinase, elastase and collagenase inhibition assays. However, PDRN-H exhibited 10.3- and 1.6-fold higher activities in the DPPH and ABTS scavenging assays compared with PDRN, respectively. PDRN-H showed 2.2- and 4.7-fold higher activities in the superoxide and nitrite scavenging assays compared with PDRN, respectively. PDRN-H also displayed 2.3- and 1.8-fold higher activities compared with PDRN in the inhibition assays of L-tyrosine hydroxylase and L-DOPA oxidase activities of tyrosinase, respectively. PDRN-H was able to suppress elastase and collagenase activities and the degrees of their inhibition activities were 3.9- and 2.2-fold higher than those of PDRN, respectively. Although both PDRN and PDRN-H demonstrate *in vitro* beneficial properties for the skin, such as antioxidant, anti-inflammatory, skin whitening and antiwrinkle properties, PDRN-H exerts remarkably higher skin beneficial properties than PDRN due to its augmented single-stranded DNA random coils. Overall, these findings suggest that the single-stranded DNA random coils of PDRNs serve as an active player for the *in vitro* pharmacological properties.

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Introduction

PDRN, an abbreviation of polydeoxyribonucleotide, denotes a family of DNA fragments with low molecular weights or otherwise a mixture of polydisperse deoxyribonucleotide heteropolymers with different chain lengths and diverse nucleotide sequences. PDRNs, originally isolated from human placentas through a proprietary extraction protocol, were found to exhibit therapeutic roles against various diseases, particularly tissue wounding (1,2). As a result of cost limitation and ethical reason, its source was switched to the sperm cells of some salmon fishes, *Oncorhynchus mykiss* (salmon trout) and *Oncorhynchus keta* (chum salmon), which could provide relatively pure genomic DNA with less impurities such as lipids, peptides and proteins, and have been further extending to other marine organisms, such as laver (3), sea cucumber sperm (4), and starfish (5). In recent years, PDRNs have been prepared and characterized from botanic sources, including aloe (6), ginseng (7) and roselle (8).

PDRNs possess different pharmacological properties such as wound healing (9,10), anti-inflammatory (11,12), skin whitening (13), anti-apoptotic (14), anti-ischemic (15), anti-ulcerative (16), anti-osteoporotic (16), anti-allodynic (16), anti-osteonecrotic (16), and bone regenerative activities (16). They were also identified to have protective effects against cadmium-induced toxicity (17) and carbon tetrachloride-induced acute liver injury (18). PDRN, lately prepared from sea cucumber, was found to attenuate H_2O_2-induced

oxidative stress through multiple pathways, which suggests its plausible application in the prevention and treatment of diverse oxidative stress-induced diseases (4).

The two action mechanisms of PDRNs have been known for last a few decades. The first one is that PDRN, acting as an agonist, selectively stimulates adenosine A2A receptor, which leads to the regulation of various pharmacological properties (19). PDRN was basically recognized to have wound healing and anti-inflammatory properties through the activation of adenosine A2A receptor (19). PDRN plays a mesenchymal stem cell-based therapeutic role against osteoarthritis through the activation of adenosine A2A receptor (20). PDRNs alleviate inflammatory response by the inhibition of Janus kinase/signal transducer and activator of transcription pathway via the mediation of adenosine A2A receptor expression and by the inhibition of neuronal cell death in the model of ischemia/reperfusion injury (19). A second relevant action mechanism of PDRNs is that their pharmacological properties are mediated by nucleotide salvage pathways. PDRNs are likely cleaved by cell membrane enzymes, supply a source for purines and pyrimidines to different tissues, and are then converted to deoxyribonucleotides which incorporate into DNA, then activating the proliferation and growth of various cells, including fibroblasts, preadipocytes, osteoblasts and chondrocytes (21). The wound healing and anti-inflammatory properties of PDRNs have been estimated to be mediated by both the activation of adenosine A2A receptor and the nucleotide salvage pathways (21).

Regardless of biological sources, the most current protocols utilized for the preparation of PDRNs include high temperature treatment, for the purpose of proteins removal, DNA fragmentation and/or sterilization, which, however, can disrupt the double-helical DNA conformation then forming the single-stranded shapes. It is considered that the most of the final PDRN products usually contain ssDNA forms at the different ratios between dsDNA and ssDNA, depending on the detailed processes used. But it remains to clarify whether of the two DNA forms, dsDNA and ssDNA forms, is directly responsible for the pharmacological efficacies of PDRNs.

In this work, by thermal denaturation, a commercial PDRN, named PDRN, was transformed to PDRN-H with substantially higher ssDNA forms. When skin *in vitro* beneficial properties of PDRN and PDRN-H were comparatively analyzed, PDRN-H showed much higher skin beneficial properties than PDRN, which was supposedly based on the increased ssDNA proportion in PDRN-H. Collectively, the ssDNA random coils of PDRNs, as an active player, are responsible for their *in vitro* pharmacological efficacies.

Materials and methods

Chemicals. Ethidium bromide (EtBr), ascorbic acid (AA), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], ammonium persulfate, sodium nitrite, NADH, nitroblue tetrazolium, phenazine methosulfate, L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), mushroom tyrosinase type I, kojic acid (KA), Griess reagent, porcine pancreatic elastase, collagenase type I, *N*-succinyl-(L-Ala)$_3$-*p*-nitroanilide (STANA), epigallocatechin gallate (*EGCG)*, and *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) were from Sigma-Aldrich Chemical Co.. QuantiFluor® dsDNA System was obtained from Promega Corporation. A PDRN product, denoted as PDRN throughout this work, was obtained from Mastelli s.r.l. Officina Bio-Farmaceutica (Placentex, biological source/salmon sperm). Additional chemicals used were purchased from global commercial chemical companies.

Preparation of thermal-treated PDRN (PDRN-H). PDRN solution was subjected to 94˚C for 30 min, appropriately diluted with phosphate-buffered saline, and kept on ice-water bath. If necessary, the thermal-treated PDRN solution, named PDRN-H, was frozen in the refrigerator.

Characterization of thermal-treated PDRN solution (PDRN-H). Whether PDRN-H contained increased ssDNA portions than PDRN or not was determined through the comparisons of the absorbance at 260 nm, dsDNA levels and EtBr-stained DNA portions after agarose gel electrophoresis. The dsDNA levels in PDRN and PDRN-H were quantified using QuantiFluor® dsDNA System. EtBr-stained portions of PDRN and PDRN-H were compared through 2% agarose electophoresis, EtBr staining and the image analysis with ImageJ version 1.54h (NIH).

DPPH radical scavenging assay. The scavenging activities of PDRN and PDRN-H against stable DPPH radical were determined as earlier described (22). In a whole volume of 250 μl, the reaction mixture containing 120 μl of 1.8 mg/ml PDRN or PDRN-H and 130 μl of 0.1 mM DPPH was kept for 30 min at room temperature (RT) under the darkness. Ascorbic acid (AA, 1 μg/ml) was employed as a positive control. The absorbance at 517 nm was detected using a microplate reader and the percent scavenging was calculated using the formula, Percent Scavenging (%)=[(Control-Test)/Control] x100.

ABTS radical scavenging activity assay. The ABTS radical scavenging activities of PDRN and PDRN-H were determined as previously described (23) with a slight modification. ABTS radical cations ($ABTS^{\bullet+}$), generated by reacting ABTS stock solution (0.07 mM) with 0.12 mM ammonium persulfate, were kept standing for 16 h in the dark at RT before use. In a whole volume of 300, 100 μl of 1.8 mg/ml PDRN or PDRN-H was mixed with 200 μl of $ABTS^{\bullet+}$ solution, and then the reaction mixture was incubated for 15 min at RT under the darkness. AA (1 μg/ml) was used as a positive control. The absorbance was measured at 745 nm and the percent scavenging was calculated as described above.

Superoxide radical scavenging assay. As previously described (24), the *in vitro* superoxide radical scavenging activities of PDRN and PDRN-H were determined. In a whole volume of 200, 20 μl of 1.2 mg/ml PDRN or PDRN-H was mixed with 180 μl of 1 mM Tris buffer (pH 8.0) with 100 μM nitroblue tetrazolium, 156 μM NADH, and 20 μM phenazine methosulfate. The reaction mixture was stood for 5 min at RT. AA (20 μg/ml) was used as a positive control. Absorbance at 560 nm was measured at a microplate reader. The percent scavenging was calculated as described above.

Nitrite scavenging assay. The *in vitro* nitrite scavenging activities of PDRN and PDRN-H were determined as previously described (25). In a whole volume of 150, 60 μl of 1.2 mg/ml PDRN or PDRN-H was mixed with 30 μl of 0.1 mM citrate buffer (pH 3.0), 6 μl of 50 μg/ml sodium nitrite and 54 μl of distilled water. The reaction mixture was stored for 60 min at 37˚C, and mixed with the equal volume of Griess reagent. After incubation for 10 min, absorbance at 538 nm was measured at a microplate reader. AA (20 μg/ml) was used as a positive control. The percent scavenging was calculated as described above.

Tyrosinase inhibition activity assay. As previously described (26), the inhibitory activities of PDRN and PDRN-H on both L-tyrosine hydroxylase and L-DOPA oxidase activities of tyrosinase were determined. For the measurement of the hydroxylase activity, each reaction mixture (200 μl) contained 80 μl of 0.04 mM phosphate buffer (pH 6.8), 40 μl of 0.16 mg/ml PDRN or PDRN-H, 40 μl of 0.5 mM L-tyrosine in 0.04 mM phosphate buffer (pH 6.8), and 40 μl of 6 units/ml mushroom tyrosinase type I, and was then stored for 10 min at 37˚C. The amount of L-DOPA generated was quantitated by absorbance at 475 nm using a microplate reader. Kojic acid (KA, 2 μg/ml) was employed a positive control. For the L-DOPA oxidase activity, 0.5 mM L-DOPA was used, instead of L-tyrosine, as a substrate, and dopaquinone generated was quantified by absorbance at 450 nm. Kojic acid (KA, 10 μg/ml) was used a positive control. The percent inhibition was calculated using the formula, Percent Inhibition (%)=[(Control-Test)/Control] x100.

Elastase inhibition activity assay. Elastase inhibition activity was determined by measuring an attenuation in elastase activity in the presence of PDRN or PDRN-H. Elastase activity was quantified based upon the generation of *p*-nitroaniline from STANA utilized as a chromogenic substrate (27). The reaction mixture consisting of 100 μl of 0.2 M Tris buffer (pH 8.0) and 50 μl of 1.2 mg/ml PDRN or PDRN-H was pre-incubated with 100 μl STANA (0.8 mM) for 20 min at 37˚C, and the enzymatic reaction was started by adding 50 μl porcine pancreatic elastase (0.1 U/ml) in 0.2 M Tris buffer (pH 8.0). Absorbance at 410 nm was measured at a microplate reader. EGCG (20 μg/ml) was used as a positive control, and the percent inhibition was calculated as described above.

Collagenase inhibition activity assay. Collagenase inhibition activities of PDRN and PDRN-H were determined by measuring a diminishment in collagenase activity, based on previously described spectrophotometric assay (28) with a slight modification. *Clostridium histolyticum* collagenase type I (1 mg/ml, 20 μl) was allowed to react with 1.8 mg/ml PDRN or PDRN-H (20 μl) in a 96-well microtiter plate containing 20 μl of 50 mM Tris buffer (pH 7.4) with 0.36 mM $CaCl_2$ for 20 min in the dark at 37˚C. After the pre-incubation, 40 μl FALGPA (2.4 mM) was added to each well and the mixture was further incubated for 30 min in the dark at 37˚C. Absorbance at 335 nm was measured at a microplate reader. EGCG (20 μg/ml) was used as a positive control, and the percent inhibition was calculated as described above.

Statistical analysis. Experiments, in this work, were repeated at least three times. The data were presented as mean ± SD. The differences between experimental groups were analyzed using one-way ANOVA subsequently with post hoc Tukey HSD test for multiple comparisons. A $P<0.05$ was recognized to be statistically significant.

Results

The increase of ssDNA portion in PDRN-H. The UV light absorbances of PDRN and PDRN-H at 260 nm, shown in Fig. 1A, indicated that PDRN-H exerted ~10% higher absorbance than PDRN. If PDRN was presumed to be full of dsDNA, the treatment was thought to induce ~26.3% denaturation, since the complete denaturation of dsDNA causes ~38% increase in absorbance at 260 nm. However, since commercial PDRNs are believed to already have certain degrees of ssDNA portions due to their manufacturing protocols, the percentage of new thermal denaturation from the dsDNA portion might be >26.3%. As shown in Fig. 1B, dsDNA content was reduced to 71.6% in PDRN-H, compared to that of PDRN, suggesting that ~28.4% of the existing dsDNA in PDRN would be transformed to ssDNA in PDRN-H.

The transformation of dsDNA to ssDNA due to the thermal denaturation was further examined by EtBr staining after agarose gel electrophoresis. As shown in Fig. 2A, the EtBr-stained area of PDRN-H became smaller and migrated faster than that of PDRN, suggesting the increased portion of ssDNA in PDRN-H. Using ImageJ software, the intensity profiles as a function of gel distance were drawn as shown in Fig. 2B. When profile areas of the lanes of PDRN and PDRN-H were calculated by comparative densitometric quantification, the profile area of the PDRN-H lane appeared to be 76.4% of that of the PDRN lane (Fig. 2C). This diminishment in PDRN-H proposes that 24.6% of dsDNA in PDRN were transformed to ssDNA in PDRN-H, which was not sufficiently stained with EtBr. Based upon the mean of the three estimated values, the about 26.4% of the existing dsDNA in PDRN was determined to denature to ssDNA in PDRN-H.

Total antioxidant capacity. ROS, regarded as chemically reactive chemical species containing oxygen, present in the body are mostly of endogenous origin, and can also be produced in response to external stimuli, such as ionizing radiation, UV light, pollution, alcohol and tobacco consumption, toxic agents, and drugs (29). Although ROS play essential biological roles, such as cell survival, proliferation, growth, signaling and differentiation and immune response, oxidative stress is induced from an imbalance between production and elimination of ROS in cells and tissues. Oxidative stress is closely linked with many disorders, such as chronic obstructive pulmonary disease, cancer, atherosclerosis, Alzheimer's disease, inflammatory diseases, and others (30). Scavenging activities against free radicals have been thought as an antioxidant strategy in the prevention of oxidative stress-related disorders.

Total antioxidant activities of PDRN and PDRN-H were evaluated using DPPH and ABTS scavenging assays. As shown in Fig. 3A, PDRN and PDRN-H exhibited 1.8 and 18.8% scavenging activities in DPPH scavenging assay,

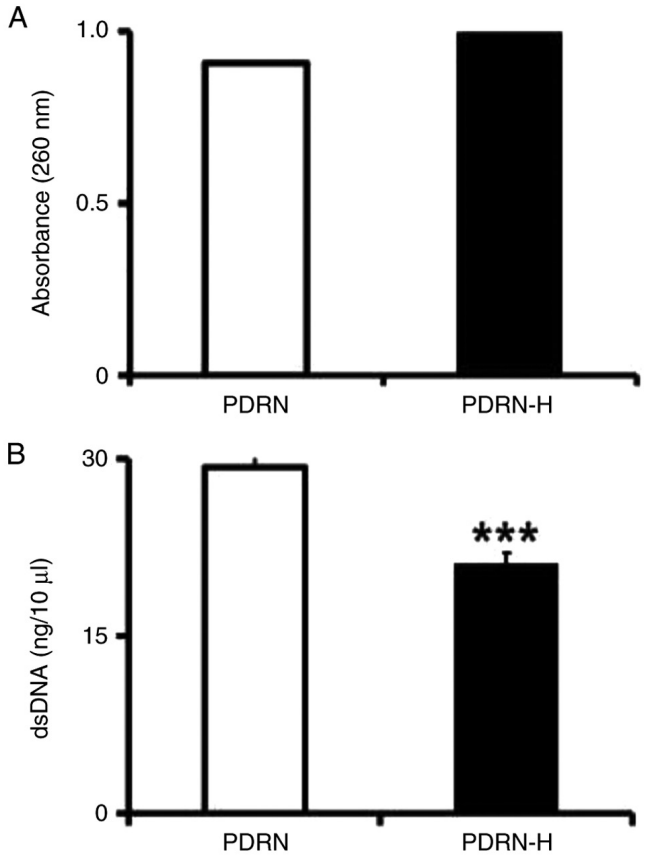

Figure 1. The light absorbances at (A) 260 nm and (B) dsDNA levels of PDRN and PDRN-H. They were measured using a (A) spectrophotometer and (B) a specific dsDNA detection kit. ***P<0.001 vs. the non-thermal control (PDRN). dsDNA, double-stranded DNA; PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide.

respectively. In ABTS scavenging assay, PDRN and PDRN-H gave rise to 8.7 and 14.2% scavenging activities, respectively (Fig. 3B). The results show that PDRN-H contains 10.4- and 1.6-fold higher scavenging activities over PDRN in DPPH and ABTS scavenging assays, respectively. AA, used as a positive control, exhibited 36.1 and 10.6% scavenging activities in DPPH (Fig. 3A) and ABTS (Fig. 3B) scavenging assays, respectively. In brief, PDRN-H possesses significantly higher total antioxidant activities than PDRN, due to the increased ssDNA random coils.

Superoxide scavenging activity. Superoxide radical anion ($O_2^{\bullet -}$) is a primary ROS which is the first species formed in the enzymatic respiratory chain by the reduction of oxygen by the transfer of an electron. It is converted to hydrogen peroxide by superoxide dismutase and further to hydroxyl radical. Although its cellular level is *in vivo* controlled by superoxide dismutase and superoxide reductase, their insufficiency becomes responsible for oxidative stress in organisms (29). In the scavenging assay against superoxide radical, PDRN and PDRN-H displayed 23.0 and 51.5% scavenging activities, respectively (Fig. 4A). It suggests that PDRN-H has 2.2-fold higher scavenging activity over PDRN, which is based upon the enhanced ssDNA portion of PDRN-H. AA, used as a positive control, showed 69.2% scavenging activity in superoxide scavenging assay (Fig. 4A).

Nitrite scavenging activity. Although nitric oxide (NO) displays an anti-inflammatory activity under normal physiological situations, it also acts as *a key mediator in the* induction of inflammation based upon its inappropriate or excessive production in diverse cells (31). Low-level constitutive generation of NO is involved in in the maintenance of barrier function, whereas NO synthetase activity, stimulated by skin wounding or UV light, results in high-level NO through complex reactions among diverse skin cells, and causes serious cutaneous diseases such as atopic dermatitis and psoriasis (32). The generated NO undergoes the oxidative breakdown metabolism to nitrite, a stable NO storage form, and nitrite is reversely recycled to NO through nitrate-nitrite-NO axis under inflamed skin conditions, such as UV exposure and other inflammation-related diseases (33). Accordingly, anti-inflammatory capacities of certain substances have been widely evaluated using *in vitro* nitrite scavenging activity assay.

When the nitrite scavenging activities of PDRN and PDRN-H were analyzed using the *in vitro* assay, they brought about 11.1 and 52.3% scavenging activities, respectively (Fig. 4B). The result indicates that PDRN-H has 4.7-fold higher scavenging activity on nitrite than PDRN. This increment is thought to arise from the enhanced ssDNA random coils in PDRN-H. Accordingly, PDRN-H was presumed to have significantly higher anti-inflammatory property over PDRN. AA, used as a positive control, showed 58.1% scavenging activity in nitrite scavenging assay (Fig. 4B).

Tyrosinase inhibition activity. Although melanin protects the skin against the harmful effects of UV irradiation, DNA damage and oxidative stress, its overproduction leads to severe physical and psychological distress, including skin darkening and aging, requiring the approaches to preserve skin homeostasis (34). Tyrosinase, a copper-containing rate-limiting enzyme located in the membrane of melanocytes, catalyzes the rate limiting reactions of melanin synthesis: the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and the oxidation of L-DOPA to *o*-dopaquinone. The inhibition of tyrosinase activity diminishes melanin synthesis, then whitening the skin.

The inhibition activities of both PDRN and PDRN-H were analyzed separately on the tyrosine hydroxylase and L-DOPA oxidase activities of tyrosinase (Fig. 5). As shown in Fig. 5A, PDRN and PDRN-H exhibited 11.5 and 26.2% inhibition activities on the tyrosine hydroxylase activity, implying the 2.2-fold increased inhibition activity of PDRN-H. In the L-DOPA oxidase inhibition assay (Fig. 5B), they gave rise to 11.0 and 19.6% inhibition activity, respectively. This results in the 1.8-fold higher inhibition activity of PDRN-H. Kojic acid (KA), used as a positive control, displayed 19.8 and 20.4% inhibition activities on the tyrosine hydroxylase (Fig. 5A) and the L-DOPA oxidase (Fig. 5B) activities of tyrosinase, respectively. Collectively, PDRN-H has the significantly increased inhibition activities against both tyrosine hydroxylase and L-DOPA oxidase activities of tyrosinase and is subsequently estimated to have much improved whitening activity.

Elastase and collagenase inhibition activities. Elastin and collagen, produced by fibroblasts, are predominantly rich components in skin dermis extracellular matrix (ECM).

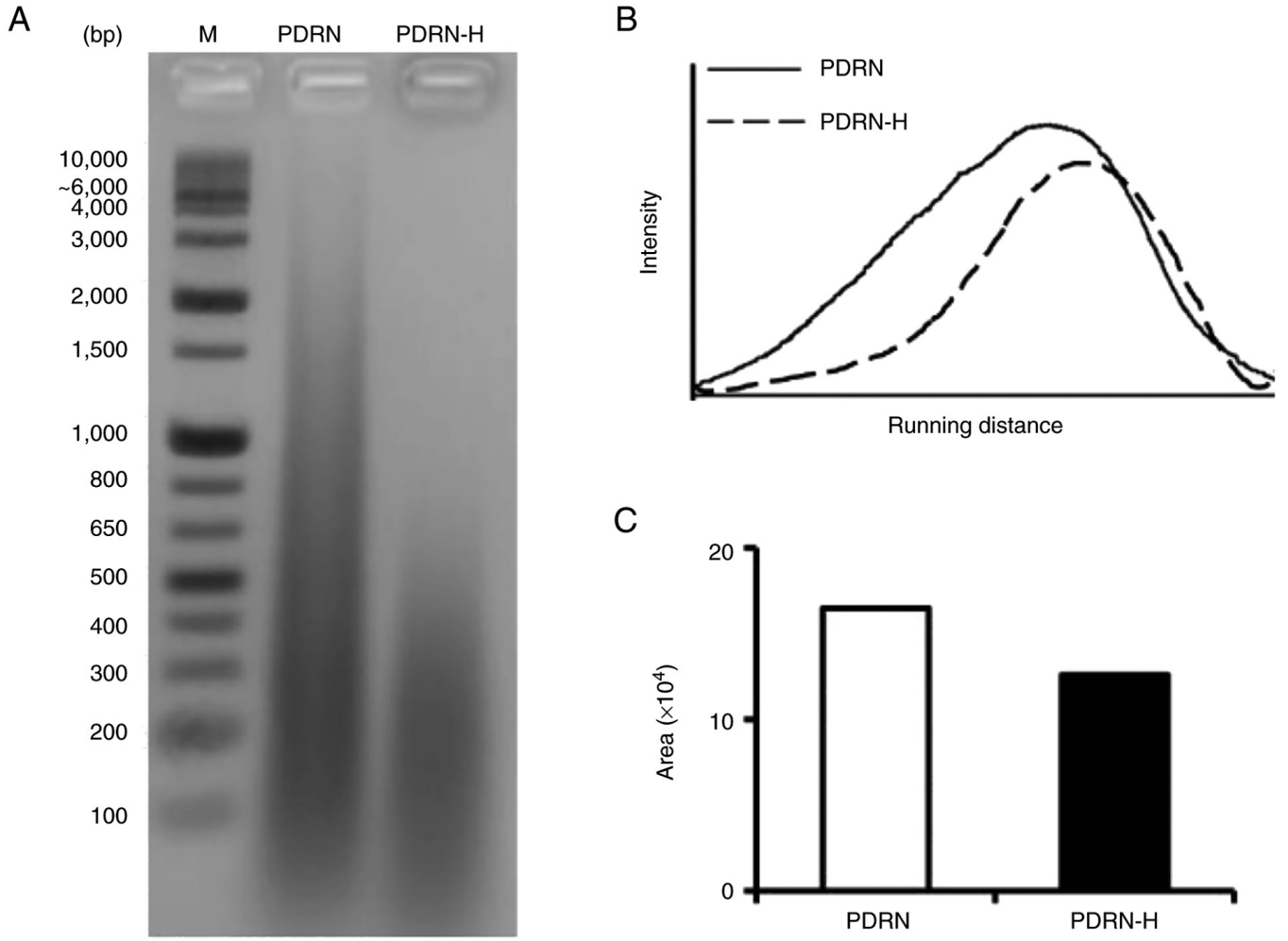

Figure 2. Electrophoretic evidence for the enhanced single-stranded DNA forms in PDRN-H, in comparison to that in PDRN. (A) 2% agarose gel electrophoretogram stained with ethidium bromide; (B) the scanning densitometric patterns of PDRN and PDRN-H lanes in (A) using Image J; (C) area comparison under the profiles of PDRN and PDRN-H obtained in (B). bp, base pairs; dsDNA, doublestranded DNA; PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide.

Breakdown and disorganization of elastin and collagen, two main ECM proteins, bring about major characteristics of skin aging, such as wrinkles, sagging, pigmentation and skin thinning, due to the enhanced activation of elastase and collagenases (27). If the substances of certain origin inhibit elastase and collagenase activities, they are presumed to delay skin aging.

The inhibition activities of both PDRN and PDRN-H were evaluated on elastase and collagenase activities. As shown in Fig. 6A, PDRN and PDRN-H showed 9.0 and 35.1% inhibition activities against elastase activity, respectively. As shown in Fig. 6B, PDRN and PDRN-H showed 10.2 and 22.4% inhibition activities against collagenase activity, respectively. From the results, PDRN-H was found to have the 3.9- and 2.2-fold higher inhibition activities against elastase and collagenase activities over PDRN, respectively. EGCG, used as a positive control, displayed 50.5 and 20.4% inhibition activities on the elastase (Fig. 6A) and collagenase (Fig. 6B) activities, respectively. Based upon the increased level of ssDNA portion in PDRN-H, it retains significantly high inhibition activities against the two wrinkling enzymes, which further suggests its strong skin anti-aging properties.

Discussion

PDRNs have been proved to possess skin beneficial properties using various experimental techniques. PDRN exhibits antioxidant activities which suppress oxidative stress in skin cells (35). It directly inhibits mushroom and cellular tyrosinase activities, then lowering the cellular melanin content in the melanocytes, and has attenuating ability on the gene expression of tyrosinase-related protein 1, another enzyme involved in the synthesis of melanin (35). It inhibits *in vitro* elastase activity and suppresses matrix metalloproteinase-1 gene expression in human skin fibroblast cells (35). In human dermal fibroblasts under ultraviolet-B radiation, PDRN was identified to bring about the attenuation of cyclobutane pyrimidine dimers, the enhancement of DNA repair and the activation of p53 protein, suggesting its protective effect against UV-induced DNA damage (36,37). It has a treatment effect against psoriasis associated with chronic inflammation, which is linked to its dual mode of action (38). Taken together, PDRNs retain potential roles in preserving healthy skin homeostasis and protecting against skin photoaging.

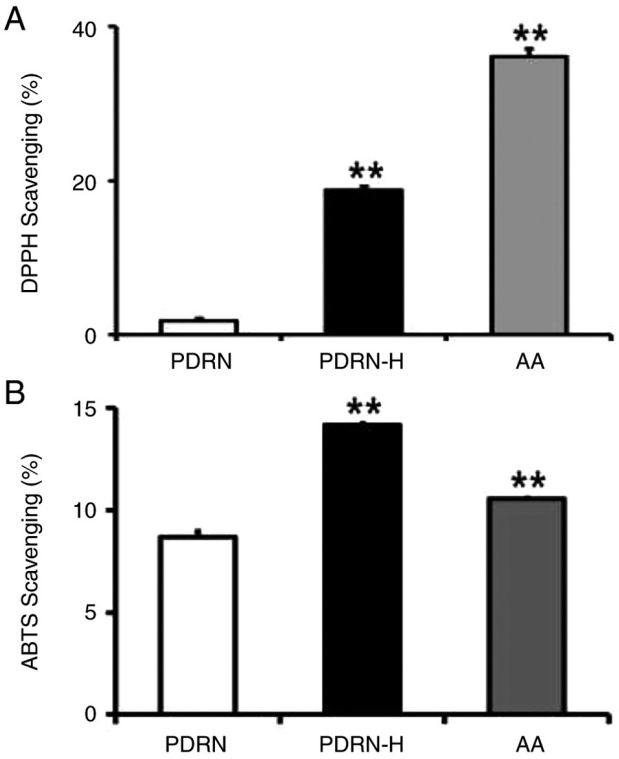

Figure 3. *In vitro* scavenging activities of PDRN and PDRN-H against (A) DPPH radical and (B) ABTS radical cations ($ABTS^{\bullet+}$). AA at 1 μg/ml was used as a positive control. $^{**}P<0.01$ vs. the non-thermal control (PDRN). AA, ascorbic acid; PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

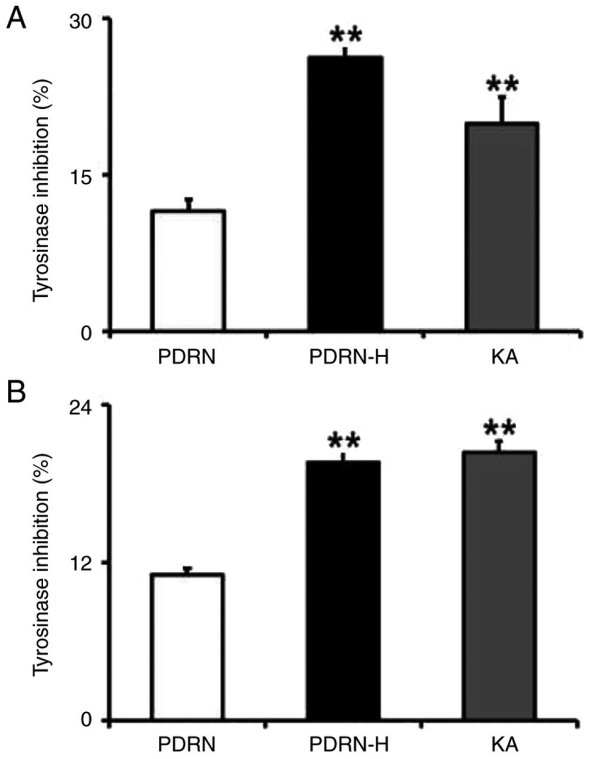

Figure 5. Inhibitory activities of PDRN and PDRN-H on the activities of tyrosinase. Their inhibitory activities on tyrosine hydroxylase and L-DOPA oxidase activities of tyrosinase were determined using (A) L-tyrosine or (B) L-DOPA as its substrate, respectively. KA at 2 and 10 μg/ml was used as a positive control in panels A and B, respectively. $^{**}P<0.01$ vs. the non-thermal control (PDRN). KA, Kojic acid; PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide; L-DOPA, L-3,4-dihydroxyphenylalanine.

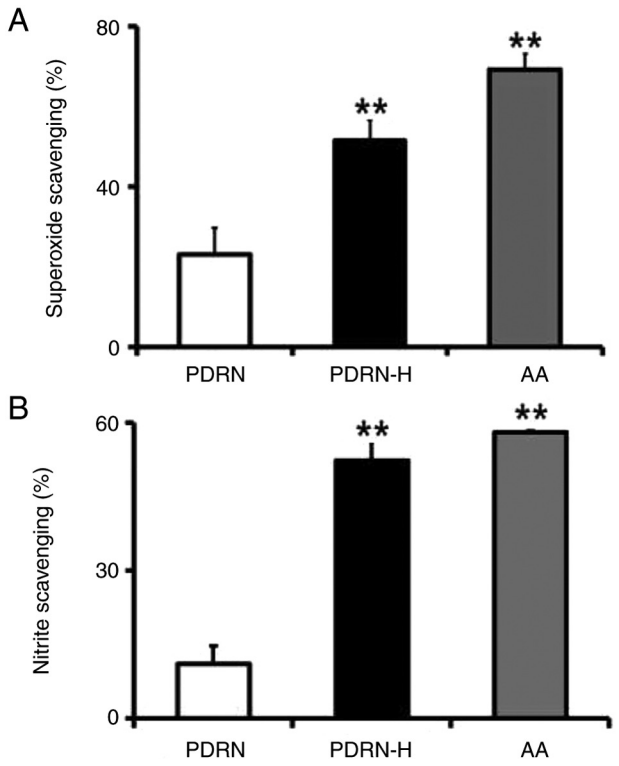

Figure 4. *In vitro* scavenging activities of PDRN and PDRN-H against (A) superoxide radical anions and (B) nitrite ions. AA at 20 μg/ml was used as a positive control. $^{**}P<0.01$ vs. the non-thermal control (PDRN). AA, ascorbic acid; PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide.

When thermal denaturation was carried out to augment the ssDNA content in a commercial PDRN, the thermal-denatured PDRN (PDRN-H) was determined to have the significantly enhanced ssDNA content, as a result of the denaturation from about 26.4% of the existing dsDNA in the non-thermal PDRN. When several skin *in vitro* pharmacological properties of PDRN-H and PDRN were comparatively measured, PDRN-H was found to have considerably higher activities than PDRN, within the increasing range of 1.6- to 10.3-fold (Table I). This finding strongly assures that the ssDNA random coils, markedly enriched in PDRN-H, but not the dsDNA helical forms, relatively rich in the non-thermal PDRN, are actually responsible for the pharmacological activities tested. This work also demonstrates that non-thermal PDRN contains the tested activities to a certain degree. It could be thought that PDRN itself contains some degree of the ssDNA portions which are presumed to be formed during the preparation processes. The notion that ssDNA random coil acts as an active and functional player of PDRNs is assisted indirectly by defibrotide which is a polydisperse mixture of single-stranded oligodeoxyribonucleotides prepared by controlled depolymerization of purified porcine intestinal mucosa genomic DNA (39). Defibrotide was originally begun to use to cure veno-occlusive disease, and has been found to contain plasmin activating, anti-atherosclerotic, anti-inflammatory, anti-ischemic and anti-thrombotic properties (39,40). The pharmacological properties of defibrotide,

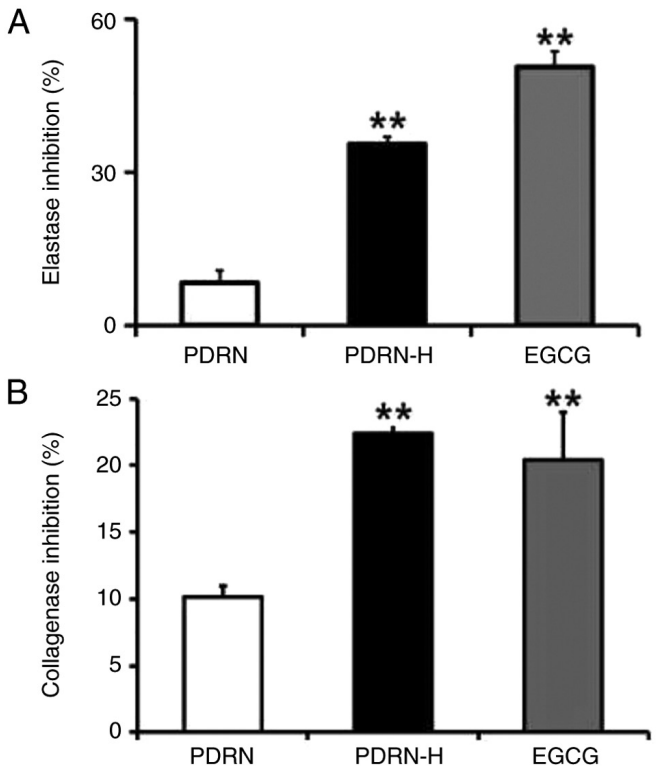

Figure 6. Inhibitory activities of PDRN and PDRN-H on the activities of (A) elastase and (B) collagenase. EGCG at 20 μg/ml was used as a positive control. **P<0.01 vs. the non-thermal control (PDRN). PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide; EGCG, epigallocatechin gallate.

Table I. A summary on the thermal-induced enhancements in *in vitro* skin beneficial properties of a commercial PDRN product.

Beneficial properties	Enhancement (PDRN-H/PDRN)[a]
Antioxidant properties	
DPPH scavenging activity	10.3
ABTS scavenging activity	1.6
Superoxide scavenging activity	2.2
Anti-inflammatory properties	
Nitrite scavenging activity	4.7
Skin whitening properties	
Tyrosinase inhibition activity[b]	2.3
Tyrosinase inhibition activity[c]	1.8
Antiwrinkle properties	
Elastase inhibition activity	3.9
Collagenase inhibition activity	2.2

[a]Units for the enhacement were given as unitless quantities. [b]L-tyrosine was used as a substrate to detect the inhibitory activity on tyrosine hydroxylase of tyrosinase. [c]L-DOPA was used as a substrate to detect the inhibitory activity on L-DOPA oxidase activities of tyrosinase. DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); PDRN, polydeoxyribonucleotide; PDRN-H, heated polydeoxyribonucleotide.

chemically as single-stranded oligodeoxyribonucleotides, have been naturally attributed by the ssDNA forms. However, diverse PDRNs and PDRN-based products, in the absence of the detailed information on the proportions of dsDNA and ssDNA, have been utilized for the purposes of academic research and medical and industrial application. In the advanced research, the various types of next-phase works, such as the validation in cellular *in vitro* models (for example, keratinocytes, skin fibroblasts, etc.), large-scale preparation of PDRNs in ssDNA forms, optimization of size and composition of single-stranded PDRNs, elucidation of mechanistic mechanism, application to animal and human skin, and so on, can be accomplished.

The insight, obtained from the non-celled *in vitro* assays used, strongly implies that the ssDNA forms of PDRNs, without metabolic alteration, can interact with their reacting molecules, such as protein molecules, such as tyrosinase, elastase and collagenase, and small molecules such as DPPH and ABTS radicals, superoxide radical ion and nitrite ion. That is, the primary hydrolytic metabolites of PDRNs inside and/or outside cells, such as deoxyribonucleotides, deoxyribonucleosides and free bases, are not directly involved in their skin *in vitro* properties. Direct contact between the ssDNA forms of PDRNs and corresponding protein molecules can be more convinced by binding studies, such as molecular docking and modified mobility shift assay.

Based on this work, it is thought that the ssDNA form, but not the dsDNA form, of PDRN acts as an active player in performing its various pharmacological properties, including skin *in vitro* properties. Also based on the use of the non-celled *in vitro* assays, it is presumed that the skin *in vitro* properties of PDRNs are displayed in a manner which is independent on adenosine A2A receptor activation and nucleotide salvage pathway, the two current mechanisms of PDRNs. Through the coming studies, it can be decided whether the functional role of the ssDNA forms of PDRNs is linked with their known mechanisms or not. Considering that the free bases of PDRNs are necessarily required for their action as an agonist of adenosine A2A receptor, the ssDNA forms of PDRNs, based upon the fact that ssDNA only retains unpaired, exposed bases, are thought to be much more adequate than the dsDNA forms.

In conclusion, the ssDNA forms of PDRNs act as an active and functional player in the performance of their *in vitro* pharmacological properties, including skin *in vitro* beneficial properties. Non-celled *in vitro* assay system used implies that PDRNs are effective without metabolic alteration and that their ssDNA random coils make a direct contact with the appropriate reacting molecules. It seems that the *in vitro* pharmacological properties of PDRNs are not mediated through adenosine A2A receptor activation and nucleotide salvage pathways. This is the novel finding on the functional action mechanism of PDRNs, which greatly influence their academic research and production. In the future, PDRNs in single-stranded DNA forms but not double-stranded DNA forms are expected to be more desirable in the generation of PDRNs and PDRN-based products for cosmetic and pharmaceutical uses.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HL and CL conceptualized this study. HL, KK and CL designed the experiments of this study. YH, HB and HK performed the experiments. HK analyzed the data. KK and CL wrote the manuscript. All authors have read and approved the final version of manuscript. HK and CL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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