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Evidence of metal binding activities of pentadecapeptide from *Panax ginseng*

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Abstract

A tetradecapeptide from ginseng (*Panax ginseng*) root showing anti-lipolytic activity in an isolated rat fat cell assay was chemically synthesized for analysis of metal binding activities in vitro. Binding activities against several metal ions were analysed by measuring mobility shifts during capillary zone electrophoresis experiments. The ginseng polypeptide (GPP) showed the greatest increase in effective molecular electrophoretic mobility in the presence of Mg^{2+}. Mobility was also affected in the presence of La^{3+}, Mn^{2+}, Ca^{2+} and Zn^{2+} ions. Analysis with the dye Stains-all revealed GPP to possess a cation binding site similar to those in Ca^{2+}-binding proteins. GPP thus appears to be a metal binding peptide. The results of this analysis suggested that GPP may perform its anti-lipolytic activities through an ability to modulate the level of free cellular Mg^{2+} and Mn^{2+} ions.

Keywords: *Panax ginseng*; Metal binding activity; Pentadecapeptide; Metal cations

1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer) is a traditional medicinal plant in Asia [1] and the activities of ginsenosides have long been studied for their pharmacological action. Ginseng roots also contains numerous regulatory chemicals and peptides [2]. One of these, an anti-lipolytic substance found in water extracts of ginseng roots, was reported to be an inhibitor of adrenaline-induced lipolysis in an isolated rat fat cell assay system [3]. The inhibitor was suggested to be a polypeptide on the basis of its inactivation following incubation with pronase. The anti-lipolytic activity was maintained even after heating. The amino acid sequence of this peptide was reported to be Glu-Thr-Val-Glu-Ile-Ile-Asp-Ser-Glu-Gly-Gly-Gly-Asp-Ala by Wang et al. [4]. In their report, the ginseng polypeptide (GPP) showed significant helical secondary structure and was stable in 1.0% sodium dodecyl sulphate (SDS).

From the available evidence, we considered that GPP may be a metal binding peptide because of its significant acidic amino acid content and its stability to heat and SDS treatments. We have already demonstrated an efficient metal binding assay system for proteins by capillary zone electrophoresis (CZE) [5]. To test this hypothesis, GPP was synthesized using a peptide synthesizer based on the reported amino acid sequence and its metal binding activities were tested in vitro by CZE. In addition, the conformational features of metal-binding by polypeptide were probed with the dye Stains-all which is known to bind to

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the anionic sites in Ca^{2+}-binding proteins [6]. The results are used to suggest a possible means by which GPP may effect its observed anti-lipolytic activity.

2. Experimental

2.1. Peptide synthesis

Peptide (Glu-Thr-Val-Glu-Ile-Ile-Asp-Ser-Glu-Gly-Gly-Gly-Asp-Ala, M_r=1392) was synthesized using a peptide synthesizer (Applied Biosystems, 431A) on the Fmoc-resin based on the FastMoc chemistry [8]. The peptide was de-protected and removed from the resin supports in a mixture of 0.75 g crystalline phenol, 0.25 ml 1,2-ethanedithiol, 0.5 ml thioanisole, 0.5 ml water and 10 ml trifluoroacetic acid. Resin supports were removed by filtration and the peptide was washed three times in diethyl ether.

2.2. Analysis of purity

The purification of synthetic GPP was completed by reversed-phase high-performance liquid chromatography (HPLC) (Gilson) using a C_{18} column (Wako, Wakosil 5C8). The amino acid sequence of purified GPP was determined by means of a protein sequencer (Applied Biosystems, 470A) [9]. The molecular mass of GPP was determined using a mass spectrometer (SX102A, JEOL).

2.3. Capillary zone electrophoresis

The analysis was performed by an Applied Biosystems Model 270HT analytical capillary electrophoresis system. The plain fused-silica capillary without coating had an I.D. of 50 μm, an O.D. of 192 μm, a total length of 72 cm and a length of 50 cm to the detector. Samples were introduced for 1 s by applied 16.8 kPa vacuum at the detector end of the capillary and then apparently eletrophoresed from the cathode end to the anode end. A 100 m*M* Tris–Tricine buffer (pH 8.3) containing 2 m*M* EDTA or 2 m*M* metal ion (Mg^{2+}, La^{3+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+} and Na^{+}) was used for CZE. A potential of 20 kV was applied and the detection was performed by measuring absorption at 200 nm [5]. The reference proteins, carbonic anhydrase and lactoglobulins, were purchased from Sigma.

2.4. Analysis by Stains-all

The dye, 0.035 m*M* Stains-all [1-ethyl-2-(3-(1-ethylnaphthol-([1,2-d]thiazoline-2-ylidine)-2-methylpropenyl)-naphtho[1,2-d]thiazolium bromide] was dissolved in 2 m*M* MOPS (pH 7.2) containing 30% ethylene glycol and 1 m*M* EDTA, 1 m*M* $MgCl_2$ or 1 m*M* $CaCl_2$. GPP (0.5 mg/ml) was incubated with Stains-all solution for 30 min at 37°C in the dark. Absorbance was observed between 400 and 700 nm using a Beckman DU-65 spectrophotometer [6].

2.5. Micellar electrokinetic capillary chromatography

The analysis was performed by the same CZE system with addition of SDS, methanol and Sudan III. The apparent capacity factors for toluene and GPP were calculated by the following formula [14]:

$$k' = (t_i - t_{eo})/t_{eo}(1 - t_i/t_{mc})$$

where k' is the capacity factor, t_i is the retention time of the analyte, t_{eo} is the retention time of the methanol and t_{mc} is the retention time of Sudan III. The mobility of GPP was ignored for the calculation.

3. Results and discussion

Synthesized GPP was soluble in 100 m*M* Tris–Tricine buffer (pH 8.3) but the solubility decreased in acidic solution (data not shown). Care was taken to avoid contamination of the synthetic material with metal ions during diethylether precipitation and HPLC purification. The purity of the synthetic GPP was tested by amino acid sequencing and mass spectrometry. The identities of the twelve N-terminal amino acids were confirmed by gas-phase amino acid sequencing. The remaining two amino acids were eluted from the reaction vessel of the protein sequencer and could not be identified. The molecular

mass of synthesized GPP was measured as 1392.2 by mass spectrometry. These analyses confirmed the primary structure and purity of synthetic GPP.

The metal binding activities of GPP and two control proteins, carbonic anhydrase and lactoglobulin, were tested against several metal ions. Carbonic anhydrase is a zinc binding protein [7] and served as a positive control, lactoglobulin has no metal-binding activity and served as a negative control. The electrophoretic mobilities of the controls were not shifted in the presence of any of the metal ions tested except for carbonic anhydrase in the presence of zinc [5]. Electro-osmotic flow (EOF) was measured by the addition of methanol: the methanol peak appeared close to the negative peak, as shown in Fig. 1 (data not shown). The velocities of EOF were not significantly changed by the addition of metals in the electrophoresis buffer. For GPP, migration times were significantly perturbed in the presence of various metal ions. The migration time of GPP was shifted from 15.140 to 19.646 min following addition of Mg^{2+} (Fig. 1). This was the largest shift recorded for any of the metal ions tested.

Grossman et al. [13] reported on the separation of peptides. They stated that the relationship of the charge and the number of amino acids. However, we had better discuss the peak shift of GPP by the following equation because we used a pentadecapeptide. For any metal-binding peptide or protein, the change in migration time during CZE is caused by a change in net charge and/or molecular conformation following metal ion binding. This may be approximated by the following formula [5]:

$$\mu = q/6\pi nr$$

where μ is the effective electrophoretic mobility, q is the net charge, n is the viscosity of the buffer and r is the Stokes radius. An increase in migration time suggests an increase in effective molecular electrophoretic mobility (decrease in apparent electrophoretic mobility) resulting from a decrease in molecular Stokes radius or increase in net negative molecular charge. Therefore, we infer that the Stokes radius of GPP was reduced by the binding of metal ions because the addition of cations to GPP decreased the two negative charges in 14 amino acids. Thus, a conformational change resulting in a smaller effective molecular radius of GPP occurred on addition of Mg^{2+}.

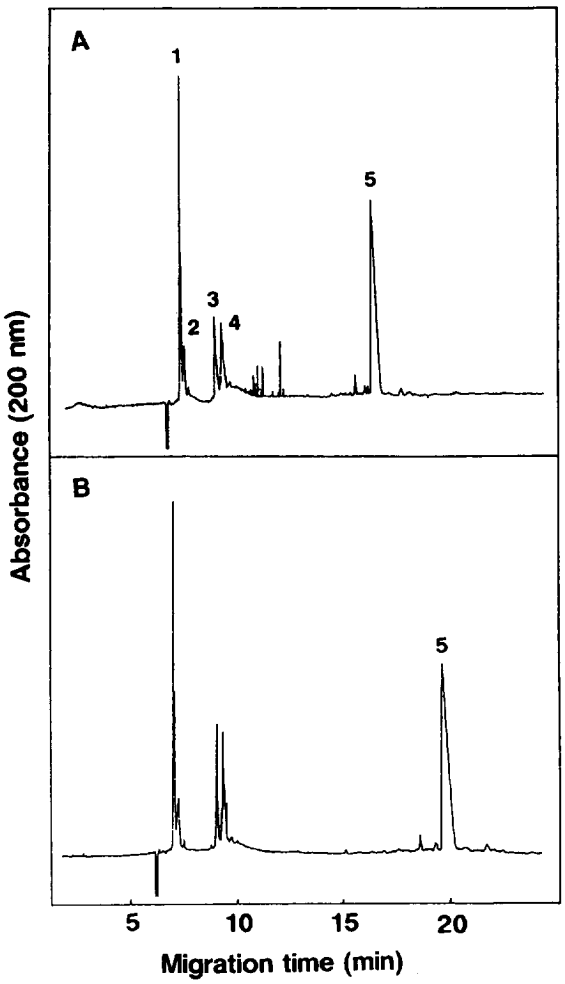

Fig. 1. CZE of GPP. (A) In the presence of 2 m*M* EDTA. (B) In the presence of 2 m*M* $MgCl_2$. Peaks 1 and 2: carbonic anhydrase; peaks 3 and 4: lactoglobulin; peak 5: GPP. The GPP peak was shifted following addition of Mg^{2+}.

The binding activities of metal ions to various proteins have been tested by CZE. For example, it was found that calmodulin bound not only to Ca^{2+} but also to Mg^{2+}, La^{3+} and Sr^{2+} [10]. In our experiments, GPP appears to bind Mg^{2+}, La^{3+} Mn^{2+}, Ca^{2+} and Zn^{2+}, indicated by the decrease in apparent migration velocity in the presence of these metal ions (Fig. 2). Therefore, it is possible to conclude that these metal ions caused a conformational change in GPP, at least, these metal ions

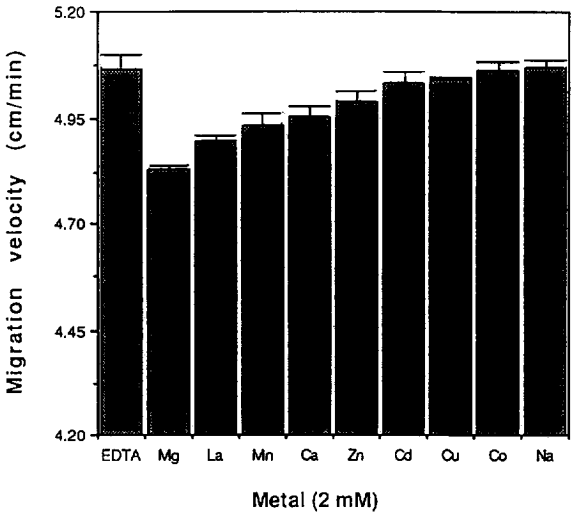

Fig. 2. Metal binding activities of GPP against metal ions. The apparent migration velocity of GPP was deceased by the addition of metal ions. Bars show the standard error, $n=5$.

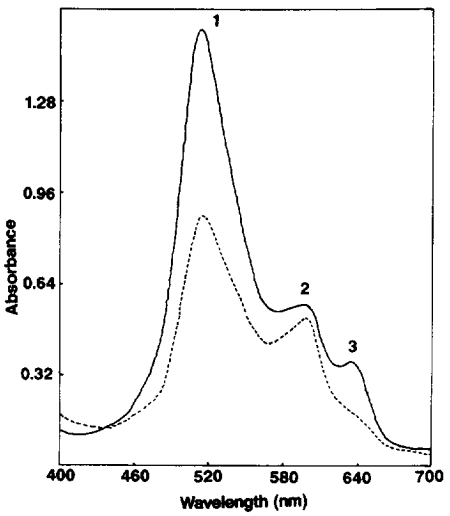

Fig. 3. Absorption spectra of the complex between GPP and Stains-all. 1 = the r band; 2 = the unknown band; 3 = the J band. The solid line shows the spectrum measured in the presence of EDTA. The broken line shows the spectrum measured in the presence of 2 m*M* $MgCl_2$.

changed GPP in some manner. On the other hand, GPP in the presence of Cd^{2+}, Cu^{2+} Co^{2+} and Na^{+} did not show a significant change in apparent migration velocity. However, it is not possible to unambiguously determine whether or not these ions bound to GPP because, for example, the opposing influences of changes in Stokes radius and in net charge may conceivably have resulted in minimal change in the apparent migration velocity. In conclusion, we can say that the electrophoretic velocity of GPP was influenced by the divalent metal cations Mg^{2+}, La^{3+}, Mn^{2+}, Ca^{2+} and Zn^{2+} as would be consistent with specific binding of these ions by the polypeptide.

It is necessary to prove that the binding of metal ions to GPP was not a simple ion binding process. The dye Stains-all was applied in order to probe the conformational features of GPP (Fig. 3). According to [6], Stains-all binds to the anionic sites in Ca^{2+}-binding proteins and generates a J band at 600–650 nm. The dye also generates an r band at 500–510 nm by binding to helical regions in proteins. In the case of GPP, the J band was detected and the r band decreased in the presence of Mg^{2+}. The decease in the r band in the presence of metal ions suggests that the extent of helical secondary structure was decreased by a conformational change. Also, the appearance of the J band by the chelation suggested that the metal ions significantly bound to the negatively charged amino acids. Although an additional unidentified band appeared around 600 nm, the data obtained by Stains-all analysis demonstrated a conformational change in GPP by a decrease in the r band in the presence of Mg^{2+}.

Micellar electrokinetic capillary chromatography (MECC) was used to investigate the interaction between GPP and SDS. Essentially, SDS bound the molecule and the interaction was observed by capillary electrophoresis. In this analysis, GPP is a highly acidic peptide and it was not thought that GPP and SDS did not make micelles. Normally, molecules in SDS solution make micelles and they behave like toluene (Fig. 4). Other organic molecules showed linear relationships between SDS concentration and the capacity factor [11]. In the case of GPP, there was no linear relationship between SDS concentration and the capacity factor. This evidence showed that GPP did not make micelles in less than 60 m*M* SDS. Although Wand et al. [4] reported that GPP was stable in 1% SDS, the MECC analysis gave the

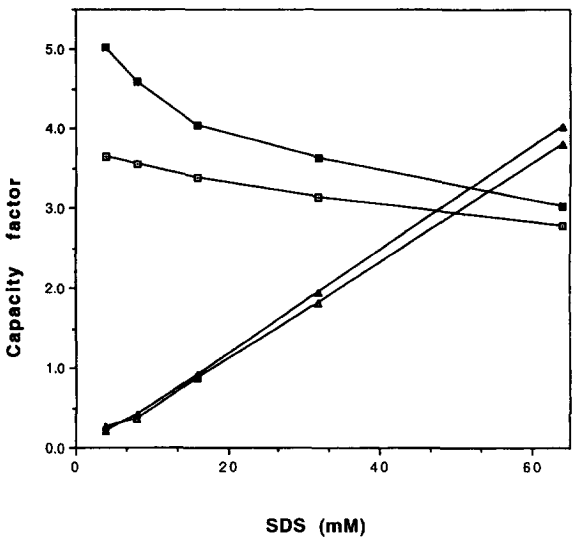

Fig. 4. MECC of GPP and toluene. (□) GPP with EDTA, (■) GPP with Mg^{2+}, (△) toluene with EDTA and (▲) toluene with Mg^{2+}.

evidence that GPP and SDS did not make complete micelles. However, significant differences were obsered between the curve in the presence of Mg^{2+} and that in the absence of Mg^{2+}. The differences were high in low SDS concentrations and low in high SDS concentrations. Addition of Mg^{2+} to GPP resulted in an increase of capacity factors. This change means the GPP became hydrophobic by binding of Mg^{2+}. We speculate that SDS interferes with the binding of Mg^{2+} to GPP in high concentration.

There have been no studies of the function of GPP in ginseng root. Although we do not have any information concerning the expression and function of this polypeptide in plants, the results of this study indicate that one role of GPP may be to chelate essential divalent cations from the soil.

GPP has been reported to act as an inhibitor of adrenaline-induced lipolysis in rat fat cells [3]. Although the mechanism of this inhibition has not yet been reported, the mechanism of induction of lipolysis by adrenaline has been well studied [12]. In brief, adrenaline binds to the β-adrenergic receptor and activates G-protein by the exchange of GTP for GDP. G-protein then stimulates membrane-bound adenylate cyclase thereby increasing the level of cAMP in the cytoplasm. cAMP acts as a second messenger to activate kinases which phosphorylate (and thus activate) the lipases necessary to effect lipolysis. Almost all nucleoside triphosphate-hydrolyzing enzymes require Mg^{2+} or Mn^{2+} for activity, as does adenylate cyclase. Therefore a number of the enzymes in the signal transduction cascade of adrenaline-induced lipolysis may be sensitive to cellular Mg^{2+} and/or Mn^{2+} level. Our CZE results showed that Mg^{2+} and Mn^{2+} reduced the apparent migration velocity of GPP to a greater extent than any other cations, a result consistent with specific binding of these ions by the polypeptide. Therefore, we suggest that GPP may effect its anti-lipolytic activity by chelating Mg^{2+} and Mn^{2+} and thus modulating the free cellular concentrations of these ions.

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