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Motif 2 in adenosine kinase homologous ginseng polypeptide showed affinity to D-ribose by capillary zone electrophoresis and surface plasmon resonance

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Abstract

Affinity capillary electrophoresis (ACE) and surface plasmon resonance (SPR) were applied to the analysis of anti-lipolytic acidic tetradecapeptide from *Panax ginseng* roots. The ginseng polypeptide (GPP) and modified GPPs were chemically synthesized and their affinity to D-ribose and adenosine was examined by ACE and SPR. GPP had affinity to D-ribose and adenosine and the binding constants (K_b) to GPP were calculated by both methods ($K_b = 1.04 \cdot 10^4$ mol^{-1} to D-ribose by ACE and $K_b = 1.91 \cdot 10^4$ mol^{-1} to adenosine by SPR). Most of the modified GPPs lost their affinity to D-ribose and adenosine through substitution or rearrangement of the amino acids.

Keywords: Surface plasmon resonance; Affinity capillary electrophoresis; *Panax ginseng*; Ribose; Adenosine kinase; Enzymes; Peptides

1. Introduction

Roots of ginseng (*Panax ginseng* C.A. Meyer), a traditional medicinal plant, contain numerous regulatory chemicals and peptides [1]. One of these chemicals, an anti-lipolytic substance found in water extracts of ginseng roots, has been reported to be an inhibitor of adrenaline-induced lipolysis in an isolated fat cell assay system in the rat [2], in which the anti-lipolytic substance was an acidic tetradecapeptide [3]. We showed evidence of metal binding activities of ginseng polypeptide (GPP) [4] using affinity capillary electrophoresis (ACE) [5,6]. 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. This ACE system has become one of the most efficient tools in the analysis of affinity between biomolecules. Recently, Spychala et al. [7] reported that homologous amino acid sequences are commonly found in human adenosine kinase (AK) and microbial pentose and hexose kinases. One of the motifs, motif 2, was homologous with the amino acid sequence of GPP.

The report on GPP showed evidence of binding activities to several divalent metal ions [5]. A previous report had shown that the electrophoretic behavior was dependent on the pH using GPP and modified GPPs [8]. GPP and modified GPPs showed very complicated electrophoretic behavior on changing the pH. In this report, the affinity of GPP to D-ribose using the new ACE and surface plasmon resonance (SPR) systems is described.

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Peptides	Amino acid sequence
	1 5 10 14 (A A)
Consensus (7)	D T X G A G D X F X G G
AK (7)	D Q K E I I D T N G A G D A F V G G
Ribokinase (10)	F R V Q A V D T I A A G D T F N G A
Fructokinase (11)	Q V V S P I D T T G A G D A F V A G
Gluconate kinase (12)	P K E K V V D T T A A G D S F S A G
Glc dehydrogenase (13)	E T V E I I E G R G G R A F A F R
GPP (4)	E T V E I I D S E G G G D A
GPP	E T V E I I D S E G G G D A E G G G D A
Modified GPPs	
GPP	E T V E I I D S E G G G D A
Peptide-1	D - - - - - E - D - - - E -
Peptide-2	D - - D - - E - - - - - E -
E1Q	Q - - - - - - - - - - - - -
E4Q	- - - Q - - - - - - - - - -
D7N	- - - - - - N - - - - - - -
G11dA	- - - - - - - - - - dA - - -

Fig. 1. Alignments for maximal amino acid homology among GPP [4], human AK [7], ribokinase [10], fructokinase [11], gluconate kinase [12], glucose dehydrogenase [13], and the consensus sequence of the motif 2 [7] and modified GPPs. Homologous amino acids are shaded.

2. Experimental

2.1. Peptide synthesis and analysis of purity

GPP and modified GPPs (Fig. 1) were synthesized using a peptide synthesizer (431A, PE Applied Biosystems, San Jose, CA, USA) on 9-fluorenylmethoxycarbonyl (Fmoc) resin based on FastMoc chemistry (PE Applied Biosystems). The purification of synthetic GPP and modified GPPs was completed by reversed-phase HPLC system (Gilson, Villiers-le-Bel, France) using a C_{18} column (Wakosil 5C8, Wako, Tokyo, Japan). The amino acid sequences of purified GPP and modified GPPs were determined by means of a protein sequencer (470A, PE Applied Biosystems). The molecular masses of synthesized peptides were determined by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Voyager PerSeptive Biosystems Japan, Tokyo, Japan) [4,8].

2.2. Capillary electrophoresis

The analysis was performed using a PE Applied Biosystems model 270HT analytical CE system [4,8]. The fused-silica capillary used was 72 cm (50 cm to the detector)×50 μm I.D., 192 μm O.D. Samples were introduced for 1 s by applying a 16.8 kPa vacuum at the detector end of the capillary, and were then electrophoresed from the cathode to the anode at 30°C. A 50 m*M* sodium phosphate buffer was used for electrophoresis and 1.0 m*M* magnesium chloride, 0.1 m*M* EDTA, and/or 0.1 mg/ml D-ribose were added for the ACE analysis. Potentials of 15–25 kV were applied and the detection was performed by the absorption at 200 nm. All chemicals for CE were purchased from Wako.

2.3. Surface plasmon resonance

Buffers for SPR (BIAcore 1000, Pharmacia Biosensor, Uppsala, Sweden) were 50 m*M* sodium phosphate buffer containing 0.2 m*M* adenosine and 0.5 m*M* Mg^{2+}. Flow-rate was 5 μl/min and sensor chip SA (Pharmacia Biosensor) was used. Sensor chip SA was streptavidin covalently immobilized on a carboxymethylated dextran matrix. GPP was fixed on the sensor chip SA after the biotinylation of N-terminal amino acid. Other chemicals for SPR were purchased from Wako.

3. Results and discussion

3.1. Homology of GPP to motif 2 in AK and microbial pentose and hexose kinases

GPP is an acidic peptide which we considered to be a metal binding peptide. In previous studies using the ACE system, GPP significantly bound Mg^{2+} [4,8]. Recently, Spychala et al. [7] reported two kinds of motifs in AK and microbial pentose and hexose kinases (Fig. 1), though the function of the consensus sequence among them was not known.

GPP was homologous with the amino acid sequence of AK and microbial pentose and hexose kinases. GPP had four identical amino acids in the consensus sequence among those kinases. If the four C-terminal region of the consensus sequence is ignored, only the eighth (S to T) and the eleventh (G to A) amino acids in GPP were mismatched with the consensus sequence. Though these two amino acids were not identical with the consensus sequence, these two amino acids were replaced with the functionally similar amino acids.

3.2. ACE analysis of GPP

We considered that the consensus sequence of motif 2 has an affinity to D-ribose because AK and others were conserved in this region. The ACE system was used to search for evidence of the affinity between GPP and D-ribose or adenosine.

First, affinity between adenosine and GPP was analyzed by the ACE (data not shown). However, the peak of adenosine showed almost the same migration time with the peak of methanol for the endoosmotic flow marker. The peaks of methanol and adenosine were difficult to identify and it was impossible to calculate the change in electrophoretic mobility of GPP. Therefore, D-ribose was used for the ACE analysis (Fig. 2). D-Ribose did not have any charge and was not detected as a peak because it did not migrate into the capillary tube (data not shown).

The affinity between GPP and D-ribose was analyzed at pH 6.8 and pH 7.6 using a 50 m*M* sodium phosphate buffer. Two pH conditions were applied to the ACE system because the binding mechanisms of GPP to Mg^{2+} seemed to be different between pH 6.8 and pH 7.6 [8].

At pH 6.8, affinity between GPP and D-ribose was not observed in the absence of Mg^{2+} (Fig. 3), nor was a significant decrease in negative electrophoretic mobility observed. However, the negative electrophoretic mobility of GPP in the presence of D-ribose and Mg^{2+} was significantly slower than that without D-ribose. This delay was apparently caused by the affinity between GPP and D-ribose, which was independently observed.

At pH 7.6, affinity between GPP and D-ribose was observed in the presence and absence of Mg^{2+}. Binding shift of GPP to D-ribose at pH 7.6 was clearer than those at pH 6.8. This delay was not caused by the increase of viscosity by D-ribose in the CE buffer.

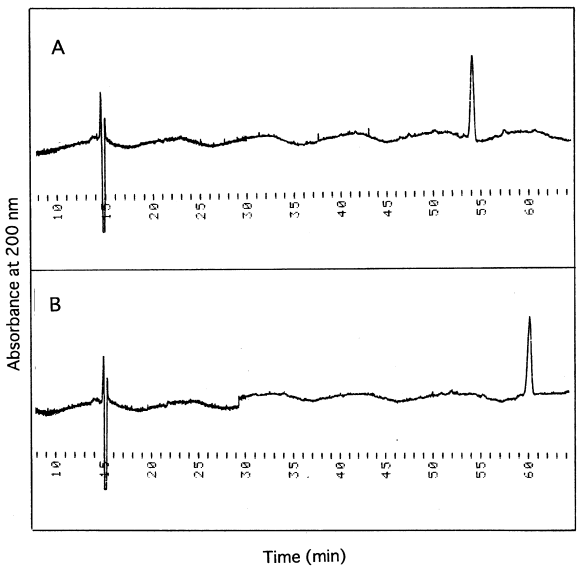

Fig. 2. ACE analysis of GPP. GPP was analyzed in 45 m*M* sodium phosphate buffer (pH 7.6) containing 10% glycerol and 1.0 m*M* magnesium chloride. A potential of 15 kV was applied. (A) No D-ribose (B) in the presence of 0.1 mg/ml D-ribose.

3.3. Binding constants of GPP to D-ribose

Scatchard plot was used to determine the binding constants (K_b) of GPP to D-ribose (Fig. 4). This calculation gave the binding constants $K_b = 1.04 \cdot 10^4$ mol^{-1}. Binding constants of lectins from plants were: *R. communis* agglutinin ($K_b = 3.3 \cdot 10^3$ mol^{-1} to lactobinate), peanut agglutinin ($K_b = 9.1 \cdot 10^2$ mol^{-1} to lactobinate) and soybean agglutinin ($K_b = 1.1 \cdot 10^2$ mol^{-1} to lactobinate) [15]. GPP had higher binding constants than these lectins. In this sense, GPP bound to D-ribose and adenosine. This result strongly suggested that the motif 2 consensus sequence in AK and microbial pentose and hexose kinase bound to monosaccharide.

3.4. ACE analysis of modified GPPs

Other modified GPPs, E1Q, E4Q, D7N, and G11dA, did not show clear affinity to D-ribose at pH 7.6 (Fig. 5). These three acidic amino acids at

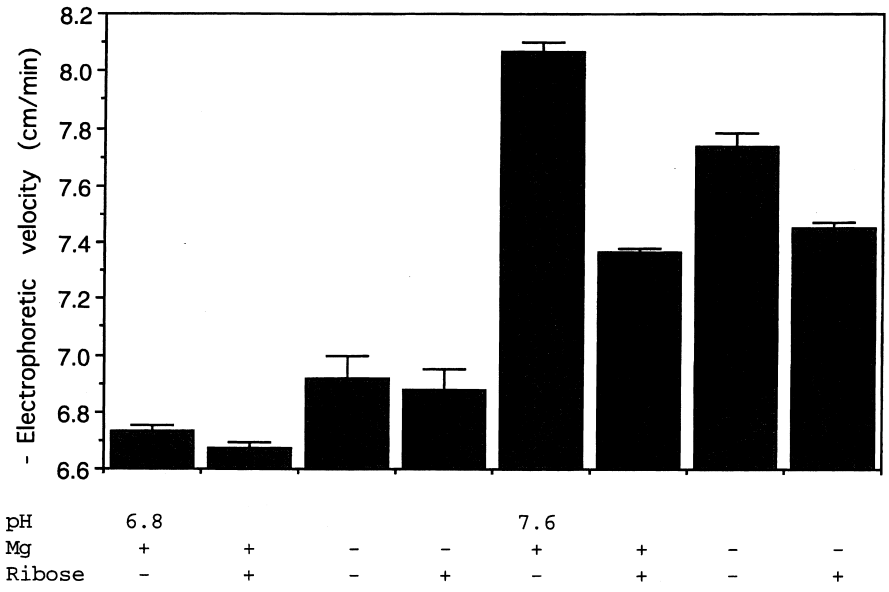

Fig. 3. ACE analysis of GPP against D-ribose in the presence and absence of Mg^{2+} at pH 6.8 and pH 7.6. A potential of 25 kV was applied. ACE was performed on 50 m*M* sodium phosphate buffer in the presence or absence of 0.1 m*M* D-ribose or 0.1 m*M* Mg^{2+}, $n=4$.

positions 1, 4, and 7 were apparently essential for the binding of D-ribose in GPP. These acidic amino acids were also essential for the binding of Mg^{2+} [4,8]. The eleventh amino acid in G11dA was not L-Ala, which was substituted from Gly to D-Ala at this position. This substitution of D-Ala also affected affinity to D-ribose. G11dA also lost its binding activities to Mg^{2+}. Therefore, these substituted amino acids, E1Q, E4Q, D7N, and G11dA, were essential for the affinity of GPP to D-ribose.

Two kinds of modified GPPs, peptide 1 and 2, were analyzed at pH 7.6 using the ACE system in

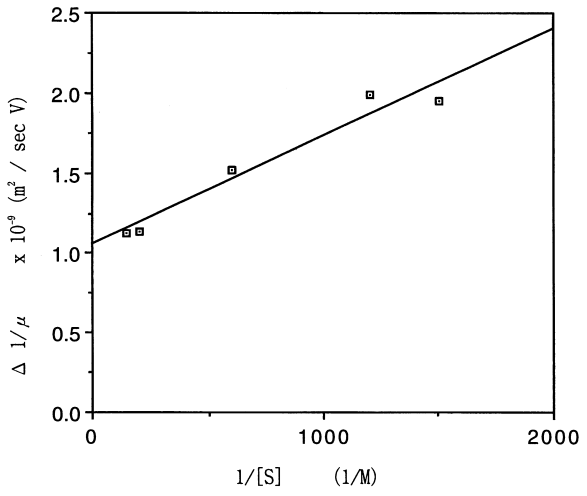

Fig. 4. Scatchard plot for the calculation of binding constants GPP against D-ribose. The data were obtained from binding shift assay by ACE. [s]=concentration of D-ribose, $\Delta 1/\mu=(1/\mu$ in the presence of D-ribose)$-(1/\mu$ in the absence of D-ribose).

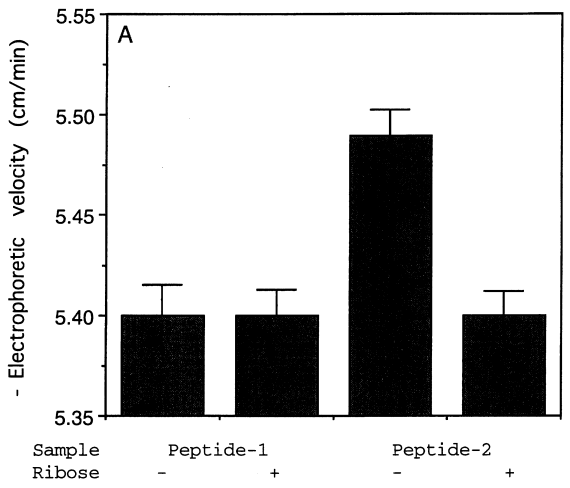

Fig. 5. ACE analysis of modified GPPs, peptide 1 and 2, in the presence of Mg^{2+} at pH 7.6. A potential of 20 kV was applied, $n=4$.

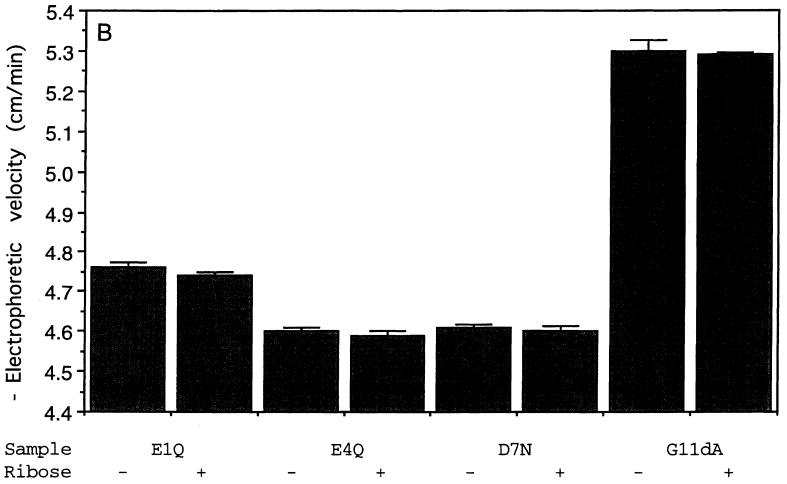

Fig. 6. ACE analysis of modified E1Q, E4Q, D7N, and G11dA in the presence of Mg^{2+} at pH 6.8 and 7.6, $n=4$.

the presence of Mg^{2+} because most of the cells contained a large concentration of Mg^{2+} in the cytosol (Fig. 6). Since GPP, peptide 1, and 2 had the same amino acid compositions, they also had the same molecular masses (1392.1) and same theoretical net charges (isoelectric point (pI)=2.38). Though affinity of peptide 1 to D-ribose was not observed, peptide-2 showed a delay of electrophoretic mobility in the presence of D-ribose. The reason for this lack of affinity to D-ribose in peptide 1 was not known. Though modified peptide 1 and 2 retained binding activities to Mg^{2+}, the change of length of side chains pf peptide 1 forced the loss of binding activity to D-ribose.

3.5. Analysis of GPP by SPR

In the SPR system, the increase of masses was detected on the sensor chip by a laser. SPR allows the observation of real-time kinetics for biomolecular interactions. This method has been applied to the study of antigen–antibody, protein–DNA and receptor–ligands interactions [16,17]. There is a lower limit of detection in the SPR system and large masses of ligands are expected. Adenosine was used as a ligand in SPR analysis because it has a much higher molecular mass than D-ribose and is also expected to bind to GPP. GPP was biotinylated at N-terminal amino acids and fixed on the sensor chip SA because amino groups in the N-terminal amino acids seemed to have no relationship to the binding of Mg^{2+} and D-ribose. Streptavidins were covalently immobilized on a carboxymethylated dextran matrix of the sensor chip SA and was found to bind strongly to biotin.

GPP showed increase of mass by SPR analysis (Fig. 7). The binding constants can be calculated by curve fitting, and the binding constant was $1.91\cdot10^4$ mol^{-1}. The binding constants obtained by SPR were of the same order as those obtained by ACE ($K_b=1.04\cdot10^4$ mol^{-1}). An experimental calculation of the binding constants using ACE and SPR between monoclonal antibody and antigen (DNA) complex concluded that both methods were reliable procedures for the analysis of binding constants [16].

G11dA did not show the increase of mass on the sensor chip SA. This modified GPP, G11dA, did not have Mg^{2+} binding abilities [8]. The flexibility of GPP was reduced by the substitution from Gly to D-Ala, and so the amino acid sequence of Gly–Gly–Gly seemed to be important for producing suitable conformation to bind Mg^{2+} and D-ribose.

3.6. Perspectives of inhibition mechanism of adrenaline-induced lipolysis by GPP

A signal by adrenaline was thought to be transduced into the cell through two ways, α-receptor and β-receptor [9]. Signal to the α-receptor increased Ca^{2+} in cells by intake from outside the cell. Signal

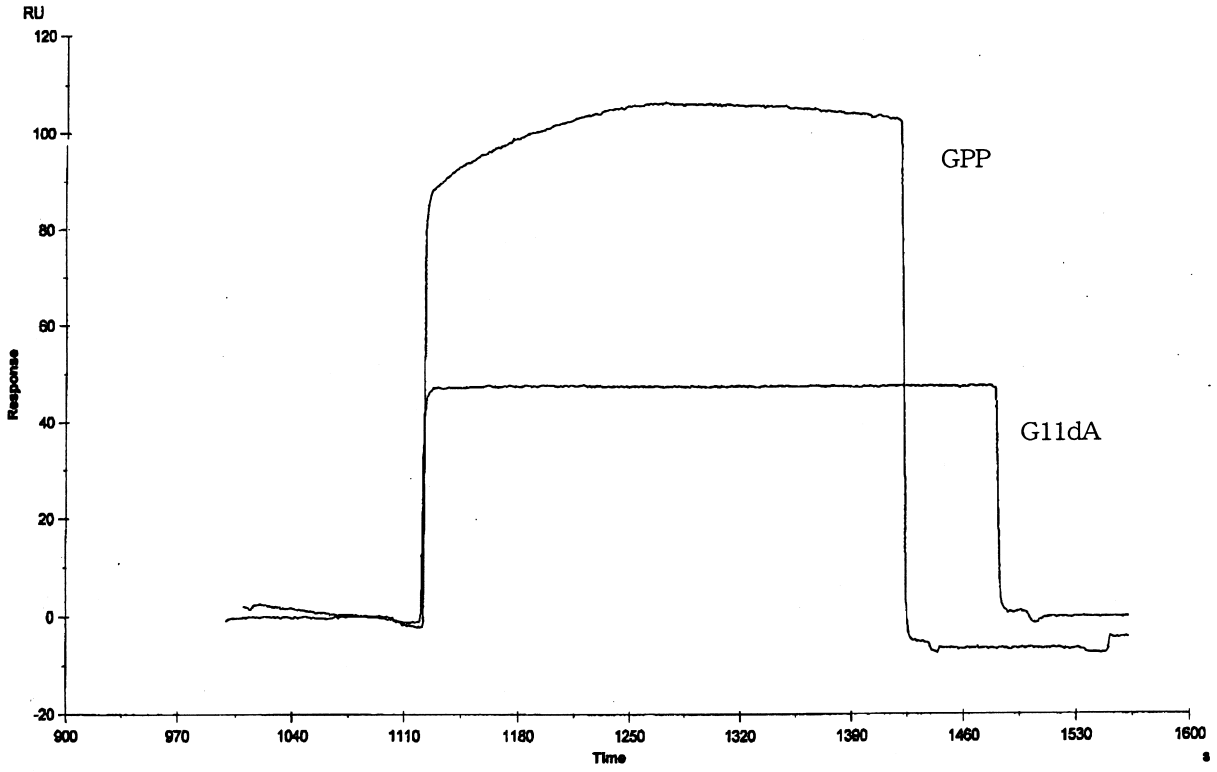

Fig. 7. SPR analysis of GPP: GPP and G11dA were fixed on the sensor chip SA. Sodium phosphate buffer containing adenosine flowed to the sensor chip SA.

to the β-receptor was transduced to G-protein and adenylate cyclase, then cAMP-dependent protein kinase was activated in cells. Previous reports have suggested that the binding activities of GPP to divalent cations interfered with the intake of Ca^{2+} into cells, otherwise GPP removed Mg^{2+} which was essential for the activities of adenylate cyclase. Based on the binding activity of GPP to D-ribose and adenosine, GPP inhibited the enzymatic activities of adenylate cyclase by binding of cAMP or ADP-ribose. To prove this possible inhibition mechanism of GPP, the translocation of GPP through the membrane and the inhibition of enzymatic activities of AK or adenylate cyclase was analyzed. Finally, Castan et al. [14] reported that neuropeptide Y and peptide YY were α-adrenergic agonists. Though the precise inhibition mechanism of these peptides is not known, GPP might inhibit adrenaline-induced lipolysis in the same way.

Therefore, there are three possible inhibition mechanisms of adrenaline-induced lipolysis. In vivo and in vitro biochemical assays and enzyme kinetic studies must be undertaken to clarify the inhibition mechanism of adrenaline-induced lipolysis by GPP.

4. Conclusions

ACE and SPR were not performed under the same experimental conditions. In SPR, GPP must be immobilized on a sensor chip. GPP was biotinylated on N-terminal amino acid and adenosine was used for the affinity experiments. On the other hand, adenosine could not be used in ACE analysis. Though there were several differences in experimental conditions, the binding constants of GPP obtained by ACE and SPR were of the same order. These binding constants were considered to be identical and both instruments can be used for the analysis of biomolecular affinities.

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