Natural Product Sciences
4(2) : 62 ~ 67 (1998)

The Glycopeptide, a Promoter of Thymidine Uptake, from *Aloe Vera*

Mi-Rim Yang[1], Chun Geun Kang[1], Yeon Suk Roh[1], Byeng Wha Son[1]*, Hong Dae Choi[2], Young In Park[3], Seung Ki Lee[4], Sung Won Choi[5], and Myung Hee Chung[5]

[1]Department of Chemistry, Pukyong National University, Pusan 608-737
[2]Department of Chemistry, Dongeui University, Pusan 614-714
[3]Graduate School of Biotechnology, Korea University, Seoul 136-701
[4]College of Pharmacy, Seoul National University, Seoul 151-742
[5]Department of Pharmacology, Seoul National University, College of Medicine, Seoul 110-799, Korea

Abstract – As a part of search for new biologically active constituents from aloe, we have isolated a glycopeptide, called G1G1M1DI2, from the gel(G1) of *Aloe vera*. Chemical and spectroscopic evidence indicated that G1G1M1DI2 is a glycopeptide. The molecular weight of G1G1M1DI2 was about 5,500 daltons, and the carbohydrate and protein contents were 20.9% and 32.6%, respectively. Periodate oxidation and enzymic degradation gave peptide moiety and carbohydrate moiety, respectively. Carbohydrate moiety is composed of fucose, galactose, glucose and mannose in a molar ratio of 0.5 : 2.4 : 48.8 : 48.3. Peptide moiety is composed of fifteen amino acids, and glutamic acid and glycine were the major componants. The glycopeptide, G1G1M1DI2, stimulated thymidine uptake of SCC 13 cells about 6.5 times the control. This result suggests that this glycopeptide has a skin cell proliferating activity.

Key words – Glycopeptide, *Aloe vera*, Thymidine uptake assay, Squamous cell carcinoma 13 (SCC 13), Cell proliferation.

Introduction

Aloe is known as an important traditional medicine in many countries and has been commonly used for health, medical and cosmetic purposes (Blitz *et al.*, 1963; Lee *et al.*, 1980; Norton, 1961). Some people keep one or a few plants at home to provide a readily available gel source for treatment of burns or other wounds. The chemical constituents of *Aloe* species have been investigated by several groups (Park *et al.*, 1997; Reynolds, 1985a; Reynolds, 1985b), and the aromatic derivatives of the constituents have been reported to be effective in wound healing, antitumor, antimicrobial, immune modulating, peptic, laxative, and gastric juice controling activities (Hirata and Suga, 1977; Speranza *et al.*, 1993).

As a part of chemical studies on biologically active metabolites from aloe, we have investigated an activity-guided fractionation and an isolation of bioactive compounds from a freeze-dried aloe gel.

This paper deals with details of the isolation and the structural characterization of a bioactive glycopeptide on the basis of chemical and physicochemical evidence.

Experimental

General – IR spectra were obtained by us-

*Author for correspondence.

ing the Bruker FT-IR IFS48 spectrometer. NMR spectra were measured with Bruker AM-400 spectrometer with TSP[3-(trimethylsilyl)propinoic-2,2,3,3-d4 acid, sodium salt] as an internal standard. GC-MS was taken on Hewlett Packed 5890 HP5970 MSD spectrometer.

UV charts were recorded on Hitachi V-3210 spectrometer. The amino acid analysis was performed with Hitachi amino acid analyzer model L-8500A (with ninhydrin method). The carbohydrate analysis was taken with Glyco System Diones DX 300.

The following experimental conditions were used for chromatography: HPLC, Spectra system P2000; column chromatography, Amberlite XAD-2 (Organo Co.), DEAE-Toyopearl 650M (Tosoh Co.) and silica gel 60 (Merck, 60~230 mesh); TLC, silica gel 60 F_{254} (Merck, pre-coated TLC plate)(detection by spraying with 1% $Ce(SO_4)_2$/10% aq. H_2SO_4 followed by heating and with Dragendorff reagent) and cellulose (Merck, pre-coated TLC plate)(detection by spraying with 5% aniline phthalate).

Materials – The freeze-dried aloe gel used as materials was obtained from Nam Yang Aloe Co. and made as follows. Mature *Aloe vera* leaves were cut from the plant and the gel of leaves was mechanically isolated. Cellulase was added to the crude gel and blended. The digested gel was filtered through Filter. The filtrate was concentrated and then lyophilized to give freeze-dried aloe gel.

Isolation of glycopeptide (G1G1M1DI2) – The aloe gel (25 g, designated as G1) was suspended in 200 ml of distilled water and 4 volumes of 95% EtOH were added. This solution was allowed to stand for 12 hr at 4℃. The clear yellowish supernatant was decanted off from the white residue and the precipitate was centrifuged for 30 min at 4℃ (13,000 g).

The supernatants were combined, evaporated and lyophilized to give a mixture of small and medium size molecules (G1G1, 14.5 g). G 1G1 (1 g) was applied to Amberlite XAD-2 column (250 ml of resin, 2.5×25 cm). The column was eluted with distilled water (500 ml), 50% aq. MeOH (200 ml), MeOH-acetone (1:1, 400 ml), successively. The eluates with 50% aq. MeOH were concentrated and lyophilized to afford dark-gray powder (G1G1M 1, 53 mg), which was active on the thymidine uptake assay.

G1G1M1 (53 mg) was dissolved in deionized water, then dialyzed for 3 days (m.w. 5,000 cut), and lyophilized (G1G1M1D, 23 mg). This fraction (G1G1M1D, 23 mg) was dissolved in 0.02 M NH_4HCO_3 and applied to an ion-exchange column (2.5×20 cm) of diethylaminoethyl (DEAE)-Toyopearl 650M.

The column was equilibrated and eluted with 0.02 M NH_4HCO_3 (600 ml), and then eluted with 0.3 M NaCl (600 ml). Fractions of 15 ml were collected and analyzed by the UV (210 and 280 nm) and by phenol-sulfuric acid method to give two fractions, G1G1M 1DI1 and G1G1M1DI2, according to the elution pattern (Fig. 1). Each fraction was concentrated, dialyzed and lyophilized. The yields of G1G1M1DI1 and G1G1M1DI2 were 7.8 mg and 2.7 mg, respectively.

TLC of glycopeptide(G1G1M1DI2) – TLC

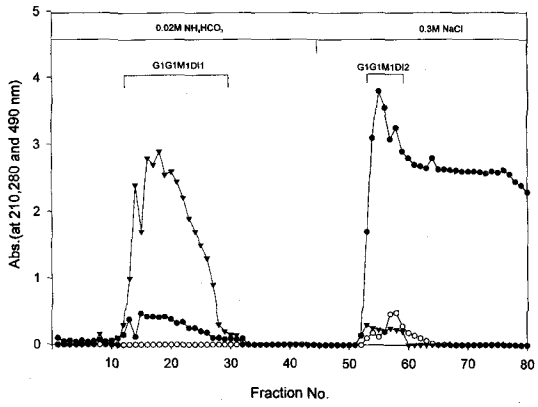

Fig. 1. Elution profile of G1G1M1D by DEAE-Toyopearl 650 M CC. The sample was dissolved in 0.02 M NH_4HCO_3, and the colunm was *eluted with 0.02 M NH_4HCO_3 and then* 0.3 M NaCl at 4℃. Each fraction was 15 ml. (●): absorbance at 210 nm; (○): absorbance at 280 nm; (▼): absorbance at 490 nm (phenol-H_2SO_4 method).

was performed on Merck precoated Kieselgel $60F_{254}$ plates and cellulose plates using *n*-butanol-ethanol-water (5 : 5 : 7) as a developing solvent. Detection was done by spraying 1% $Ce(SO_4)_2$ in 10% aq. H_2SO_4 followed by heating at 110℃ and by 5% aniline phthalate. Rf values of G1G1M1DI2 were 0.67 in the silica gel plate and 0.35 in the cellulose plate.

Electrophoresis – The purity and molecular weight of bioactive G1G1M1DI2 were determined by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970) using 20% separation gel with standard protein markers. Carbohydrate and protein bands which are the same distance from the origin were stained by the periodate-Schiff procedure (Dubray and Bezard, 1982; Kinchel and Bryan, 1990) and with Coomassie blue reagent (Bollag annd Edelstein, 1991).

Measurement of total carbohydrate and protein – Total carbohydrate was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) using D-mannose as standard. Total protein was also determined by the Lowry method (Lowry *et al.*, 1951) with egg albumin as standard.

Analysis of component sugar – G1G1M1DI2 (10 mg) was dissolved in 2 N H_2SO_4 (10 ml) and hydrolyzed at 100℃ for 6 hr in a sealed tube. The hydrolysate was neutralized with $BaCO_3$, and then filtered. The filtrate was passed through a column of Dowex 50W-X8 resin (acidic form) with deionized water. Component sugars were eluted and analyzed by HPLC.

Analysis of amino acid – G1G1M1DI2 (10 mg) was hydrolyzed with 6 N HCl (10 ml) for 24 hr at 110℃ in a sealed tube. After evaporation to dryness, the hydrolysate dissolved in 1 ml of citrate buffer (pH 2.2) were analyzed by an automatic amino acid analyzer (Hitachi L-8500 A).

Periodate oxidation – G1G1M1DI2 (10 mg) was dissolved in distilled water (2 ml), then sodium metaperiodate (5 mg) was added. The reaction mixture was stirred at 20℃ for 20 hr in the dark. The excess of sodium metaperiodate was decomposed by the addition of ethylene glycol (1 ml) with stirring for 1hr. The solution was dialyzed overnight and lyophilized. The product was dissolved in distilled water (1 ml) and $NaBH_4$ (5 mg) was slowly added. Then the solution was stirred at r.t. for 7 hr in the dark. The excess of $NaBH_4$ was decomposed by the addition of 0.1 M acetic acid. After dialysis and lyophilization, small amounts of 0.05 M sulfuric acid were added and the mixture was stored overnight at r.t. The solution was neutralized with 0.05 M NaOH. Dialysis and lyophilization gave carbohydrate-degraded product (3 mg).

Enzymic degradation – To a solution of G1G1M1DI2 (10 mg) in 0.01 M phosphate buffer (pH 7.0)(0.5 ml), pronase E (Sigma Chemical Co.) (0.1 mg) was added, and incubated at 37℃ for 24 hr. Dialysis for 24 hr and lyophilization gave protein-degraded product (2 mg).

Thymidine uptake assay (Kopan, 1989) – SCC 13 (human squamous cell carcinoma 13) Cells were suspended in the culture medium (a mixture of 3 parts of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and one part of Ham's nutrient mixture F12) and aliquots (0.2 ml) were put into the wells of a 96 well-plate (5,000 cells/well). The cells were cultured until 80% confluency was reached. The culture medium was replaced with 0.2 ml of the culture medium containing G1G1M1DI2 (1 mg/ml) (for the control, saline was added instead of G1G1M1DI2) and the cells were cultured for 24 hr. The culture medium was replaced again with the same medium containing G1G1M1DI2 and the cells were cultured further for 24 hr. At this time, [^{3}H]thymidine was added into each well (5 μCi/ml) and the cells were incubated for 3 hr. The cells were washed twice with phosphate buffered saline, treated with 10% trichloroacetic

acid for 1 hr and dried out. The dried cells were mixed with 100 μl of 0.5 M NaOH for 3 hr and then mixed with 0.5 M HCl for neutralization of the alkali. Aliquots were used for thymidine uptake by a scintillation counter (Hewlett Packard).

Results and Discussion

A crude glycopeptide fraction (G1G1) obtained from the gel (G1) of *Aloe vera* was applied to a column chromatography of Amberlite XAD-2. The eluate with 50% aq. MeOH which showed activity in the thymidine uptake assay was subjected to ion-exchange chromatography on DEAE-Toyopearl 650 M to afford a non-binding fraction (G1G1M1DI1) and a binding fraction (G1G1M1DI2) (Fig. 1). Among them, G1G1M1DI1 did not show any activity in thymidine uptake assay, but the main activity was found in the binding fraction G1G1M1DI2 (Table 1). G1G1M1DI2, dark gray amorphous solid, was freely soluble in water, but slightly soluble in alcohol and insoluble in organic solvents. It was found to be homogenous in the electrophoresis.

Elementary analysis (%) of G1G1M1DI2 indicated that it was: C, 35.8; H, 5.2; N, 2.5; and O, 56.5.

G1G1M1DI2 showed the positive reactions with 1% $Ce(SO_4)_2$ in 10 % aq. H_2SO_4 in TLC (silica gel) and with aniline phthalate in TLC (cellulose). It also revealed a band with periodic acid-Schiff's reagent and with Coomassi reagent in electrophoresis (20% SDS-PAGE), which are specific reagents to detect carbohydrate and protien, respectively.

The IR spectrum of the G1G1M1DI2 also showed absorption bands at 3359, 1601, 1382, 1077 cm^{-1} indicating the presence of carbohydrate. Although the NMR spectra (^1H and ^{13}C) were poorly resolved, the ^{13}C NMR spectrum (100 MHz, D_2O) showed signals ascribable to alipathic carbons (δ_c 12.7, 12.9, 16.2, 21.4, 24.0, 32.8), carbonyl carbons (δ_c 160.5, 171.8, 186.5), geminal carbons of oxygen function (δ_c 61.2, 61.6, 62.0, 69.6, 70.6, 72.1, 75.7, 76.5), and anomeric carbons (δ_c 100.9, 101.1), and ^1H NMR spectrum (400 MHz, D_2O) also showed signals assignable carbohydrate moiety (δ3.4 ~4.5, a mass of signals).

Evidence above has led us to assume a glycopeptide for G1G1M1DI2. Glycopeptide G1G1M1DI2 contained 32.6% of the total protein as egg albumin and 20.9% of the total carbohydrate as mannose (Table 1). G1G1M1DI2 had a molecular weight of 5,500 daltons, estimated by SDS gel electrophoresis. A peptide moity of G1G1M1DI2 was obtained by degradation with $NaIO_4$, and the carbohydrate moiety by digestion with pronase E. The presense of each moiety was confirmed by electrophoresis. Carbohydrate moiety was suggested to contain fucose, galactose, glucose and mannose (Fig. 2) and the molar

Table 1. Thymidine Uptake, and Contents of Carbohydrate and Protein in Active Fractions Purified from the Gel (G1) of *Aloe vera*

Sample	Thymidine Uptake[a)]	Carbohydrate[b)]	Protein[b)]
G1	210	47.9	2.6
G1G1	270	50.7	3.9
G1G1M1	320	62.6	11.8
G1G1M1DI2	645	20.9	32.6

[a)] Relative activity(%) to the control, taken as 100%, in [^3H]thymidine uptake assay.

[b)] Presented as % and determined by the phenol-sulfuric acid and Lowry's methods, respectively.

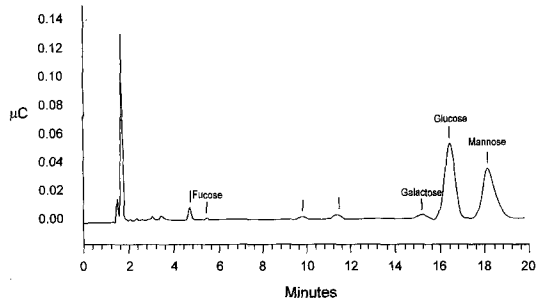

Fig. 2. HPLC Chromatogram of acid hydrolysate obtained from carbohydrate moiety of G1G1M1DI2. The colunm (CarboPak PA1, 4×250 mm) was eluted with H_2O-0.2 M NaOH (92:8) at r.t. and flow rate of 1.0 ml/min.

Table 2. Monosaccharide Composition of G1G1M1DI2

Monosaccharide	Composition (molar ratio, %)
fucose	0.5
galactose	2.4
glucose	48.8
mannose	48.3

Table 3. Amino Acid Composition of G1G1M1DI2

Amino acid	Composition (%)
Asp	12.68
Thr	0.72
Ser	0.94
Glu	22.70
Gly	18.47
Ala	10.96
Val	8.75
Met	0.62
Ile	2.98
Leu	8.50
Tyr	1.70
Phe	2.47
Lys	5.24
Arg	2.16
His	1.18

ratio (%) of these component sugars was 0.5, 2.4, 48.8 and 48.3 (Table 2). To determine amino acid composition and contents, the HCl-hydrolysate of peptide moiety of G1G1M1DI2 was analyzed by amino acid analyzer. Fifteen kinds of amino acid were detected, and glutamic acid and glycine comprised 41.2 molar % of the total detected amino acids (Table 3). From these observation, G1G1M1DI2 was characterized as a glycopeptide. The relative activity of glycopeptide G1G1M1DI2 was 645% of the control, taken as 100%, in thymidine uptake assay (Table 1), and also showed significant activities of wound healing in experiments using SCC 13 (Squamous cell carcinoma 13) cells. The detailed result and the molecular mechanism of wound healing will be reported elsewhere.

Acknowledgement

This research was financially supported by CAP project program from Namyang Aloe Co., and we greatfully acknowledge.

References

Blitz, J. J., Smith, J. W. and Gerard, J. R., *Aloe vera* gel in peptic ulcer therapy: preliminary report. *J. Amer. Osteop. Asso.* **62**, 731-735 (1963).

Bollag, D. M. and Edelstein, S. J., Gel electrophoresis under denaturing condition. Protein Methods, Wiley-Liss. Inc., New York, 1991.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., Colorimetric method for determination of sugars and related substance. *Anal. Chem.* **28**, 350-356 (1956).

Dubray, G. and Bezard, G., A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**, 325-329 (1982).

Hirata, T. and Suga, T., Biologically active constituents of leaves and roots of *Aloe arborescens*. *Z. Naturforsch* **32c**, 731-734 (1977), and references cited therein.

Kinchel, C. O. and Bryan, A., Detection of glycoproteins separated by nondenaturing polyacrylamide gel electrophoresis using the periodic acid-Schiff stain. *Anal. Biochem.* **187**, 147-150 (1990).

Kopan, R. E., The use of retinoic acid to probe the relation between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. *J. Cell. Biol.* **109**, 295-307 (1989).

Laemmli, U. K., *Nature* **227**, 680 (1970).

Lee, L. M., Haggers, J. P., Robson, M. C. and Hagstrom, W. J., The therapeutic efficacy of *Aloe vera* cream (Dermaide Aloe) in thermal injuries: two case reports. *J. Amer. Animal Hosp. Asso.* **16**, 768-772 (1980).

Lowry, O. H., Rosebrough, N. L., Farr, A. L. and Randall, R. J., Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951).

Norton, J. F., Folk uses and commercial exploitation of aloe leaf pulp. *Economic Bot.* **15**(4), 311-319 (1961).

Park, M. K., Park, J. H., Shin, Y. G., Choi, Y. S., Kim, K. H., Cho, T. H. and Lee, S, K., Chemical constituent of *Aloe capensis*. *Arch. Pharm. Res.* **20**, 194-196 (1997).

Reynolds, T., The compounds in aloe leaf exudates: a review. *Botanical J. Linnean Soc.* **90**, 157-177 (1985a), and references cited therein.

Reynolds, T. Observations on the phytochemistry of the aloe leaf-exudate compounds. *Botanical J. Linnean Soc.* **90**, 179-199 (1985b).

Speranza, G., Manitto, P., Cassara, P. and Monti, D., Studies on aloe, 12. Furoaloesone, a new 5-methylchromone from Cape aloe. *J. Nat. Prod.* **56**(7), 1089-1094 (1993).

(Accepted March 5, 1998)