



Effects of Peanut-Skin Procyanidin A1 on Degranulation of RBL-2H3 Cells

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Peanut skin contains large amounts of polyphenols having antiallergic effects. We found that a peanut-skin extract (PSE) inhibits the degranulation induced by antigen stimulation of rat basophilic leukemia (RBL-2H3) cells. A low-molecular-weight fraction from PSE, PSEL, also had inhibitory activity against allergic degranulation. A main polyphenol in PSEL was purified by gel chromatography and fractionated by YMC-gel ODS-AQ 120S50 column. Electrospray ionization mass spectrometry (ESI-MS) analysis of the purified polyphenol gave m/z 599 $[M + Na]^+$. Based on the results of ^1H -NMR, ^{13}C -NMR spectra, and optical rotation analysis, the polyphenol was identified as procyanidin A1. It inhibited the degranulation caused by antigen stimulation at the IC_{50} of 20.3 μM . Phorbol-12-myristate-13-acetate (PMA) and 2,5-di(*tert*-butyl)-1,4-hydroquinone (DTBHQ)-induced processes of degranulation were also inhibited by procyanidin A1. These results indicate that peanut-skin procyanidin A1 inhibits degranulation downstream of protein kinase C activation or Ca^{2+} influx from an internal store in RBL-2H3 cells.

Key words: peanut skin; polyphenol; procyanidin; rat basophilic leukemia (RBL)-2H3; degranulation

Polyphenols are ubiquitous in plant species. Recent studies have revealed their antioxidative, anti-carcinogenic, and anti-hyperglycemic effects and anti-allergic activities as well as their suppression of fat accumulation.¹⁻³⁾

The skin of the peanut, *Arachis hypogaea* L., contains high concentrations of polyphenols, about 90–125 mg/g in total.⁴⁾ It also contains many procyanidins, as in the case of grape, litchi, cacao, quince, and apple.⁵⁻¹⁰⁾ Notably, peanut skin contains A-type procyanidin dimers,⁵⁾ which consist of monomeric catechin units linked with a C2–O–C7 or C2–O–C5 linkage in addition to a C4–C8 or C4–C6 linkage, called B-type (Fig. 1). In the food industry, peanut skin is generally discarded as waste during the production of peanut-based foods. However, the skin is used to prevent and to treat chronic hemorrhage and bronchitis in Chinese traditional medication.¹¹⁾ Our group also found a hypocholesterolemic effect of PSE in rats fed a high-cholesterol diet.¹²⁾

Recent studies have indicated that oral injection of procyanidin A1 lowers serum IgE and IgG1 levels in mice, suggesting that it has an anti-allergic effect *in vivo*,¹³⁾ but no effect of peanut-skin procyanidins has been reported at the cellular level.

Type-1 allergy is defined as a hypersensitivity reaction. Its frequency is increasing worldwide.¹⁴⁾ This type of allergy is known to be evoked by antigen-induced activation of high-affinity IgE receptors (Fc ϵ RI) expressed in mast cells and basophils. Crosslinking of IgEs is essential, and is the first step triggering the signaling cascades that lead to degranulation. The signaling pathways are involved in the activation of protein kinase C induced by 1,2-diacylglycerol, or Ca^{2+} influx into the intracellular induced by diacylglycerol.¹⁵⁻¹⁷⁾ Activation of mast cells triggers allergic and inflammatory responses through the release of mediators, such as histamine, arachidonic acid metabolites, and neutral proteases, and modulates immune responses through the production of cytokines and chemokines.

In this study, we investigated the anti-allergic activity of peanut-skin polyphenols at the cellular level. First we divided an aqueous extract of peanut skin (PSE) into a low-molecular-weight fraction, PSEL, and a high-molecular-weight fraction, PSEH, and then isolated a procyanidin from PSEL to examine its anti-allergic activity by quantification of β -hexosaminidase release from RBC-2H3 cells as a marker of degranulation.¹⁸⁾

Materials and Methods

Preparation of PSEL and PSEH from PSE. PSE was prepared as described previously.¹²⁾ In brief, peanut skins were boiled in purified water, and the residue was separated from the supernatant and boiled again in purified water. This was done 5 times, and then all the supernatants were concentrated *in vacuo* to produce PSE. This was ultrafiltered by centrifugation (MWCO 10000; Advantec, Tokyo). The resulting ultrafiltrate, which contained low-molecular-weight materials, was designated PSEL, and the residual non-filtered fraction, which contained high-molecular-weight materials, was designated PSEH. The amounts of polyphenols in the two fractions were determined by Folin–Ciocalteu assay, and were expressed as (+)-catechin equivalents.

Isolation of a major component of PSEL. Twenty mL of PSEL was fractionated by column chromatography on 26 $\times$ 800 mm TSK-gel Toyopearl HW-40 F (Tosoh Bioscience, Tokyo). MeOH was used as the mobile phase, and the polyphenols in the eluates were detected by

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Abbreviations: BSA, bovine serum albumin; COSY, correlation spectroscopy; DMEM, Dulbecco's Modified Eagle's Medium; DNP, dinitrophenyl; DTBHQ, 2,5-di-*tert*-butylhydroquinone; ESI-MS, electrospray ionization mass spectrometry; FBS, fetal bovine serum; HMBC, heteronuclear multiple-bond coherence; IgE, immunoglobulin E; PMA, phorbol 12-myristate 13-acetate; PSE, peanut-skin extract; PSEH, high-molecular-weight fraction of PSE; PSEL, low-molecular-weight fraction of PSE; RBL, rat basophilic leukemia

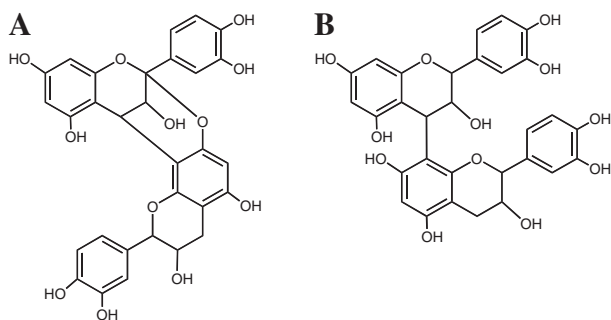


Fig. 1. Structures of A-Type Procyanidin (A) and B-Type Procyanidin (B).

monitoring the absorbance at 280 nm. The main fraction (Peak IV), 150 mL, was obtained and concentrated to 10 mL. Subsequently, the concentrate was further treated on a YMC-gel ODS-AQ 120S50 column (YMC, Kyoto, Japan), which was pre-equilibrated with solvent A (0.1% formic acid v/v), and then with solvent B (MeOH), and the eluted at a flow rate of 5 mL/min in a linear gradient from 20% to 34% solvent B over 180 min. The polyphenolic compounds in the eluate were monitored by the absorbance at 280 nm. This was done 5 times to collect all the eluates. The purity of peak IV-1, 50 mL $\times$ 5, was confirmed with a Shimadzu LC-20AD HPLC system using two columns, with solvent A (0.1% formic acid v/v) and solvent B (MeOH). A Capcell Pak C18 MG (150 mm $\times$ 4.6 mm, Shiseido, Tokyo) column was used for chromatography at a flow rate of 1 mL/min. The column was equilibrated with 5% solvent B, and then elution was performed with a 5–40% gradient. The isolated compound was lyophilized *in vacuo* to obtain 7.55 mg from 1 g of peanut skin, and the isolate was stored at -80°C in the dark until use.

Chemical analyses. NMR spectra were recorded using a JNM-ECX 400 (JEOL, Tokyo) at 400 MHz (^1H) and at 100 MHz (^{13}C), with acetone- d_6 as solvent and tetramethylsilane (TMS) as internal standard. ^1H - ^1H COSY was used to assign the ^1H chemical shifts and couplings. HMBC was employed to assign the correlations between ^1H and ^{13}C signals. Mass spectrometric analyses were performed using a JMS-T700LCK (JEOL) equipped with an electrospray ionization (ESI) interface. Specific rotation was measured at 19°C using a Jasco Digital Polarimeter DIP-370 (Jasco, Tokyo).

Characterization of Peak IV-1 (procyanidin A1). The brown amorphous powder was characterized as follows: $[\alpha]_D^{19} = +45.8^{\circ}$ (c 0.55, MeOH); ESI-MS m/z : 599 $[\text{M} + \text{Na}]^+$; HRESI-MS m/z : 99.11433 $[\text{M} + \text{Na}]^+$, 599.11654 calcd. for $\text{C}_{24}\text{H}_{30}\text{NaO}_{12}$. The ^1H - and ^{13}C -NMR spectral data are shown in Table 1.

Measurement of β -hexosaminidase release from cultured cells. RBL-2H3 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), 250 mg/mL of kanamycin (Meiji Seika, Tokyo), and 7.5% NaHCO_3 . The cells were incubated at 37°C in a 5% CO_2 incubator. Degranulation of RBL-2H3 cells was monitored by measuring released β -hexosaminidase activity, as described by Kawasaki *et al.*,¹⁹⁾ with minor modifications. Anti-DNP (dinitrophenyl)-IgE, DTBHQ, and PMA were purchased from Sigma-Aldrich (St. Louis, MO), DMEM was from Nissui Pharmaceuticals (Tokyo), DNP-BSA (bovine serum albumin) was from Cosmo Bio (Tokyo), and *p*-nitrophenyl-2-acetoamide- β -D-glucopyranoside was from Wako Pure Chemicals (Osaka, Japan). The RBL-2H3 cells were diluted with 10% FBS in DMEM to 2×10^5 cells/mL. Anti-DNP-IgE was added to them at a final concentration of 50 ng/mL, and a 1-mL aliquot of the cell suspension was dispensed into a 24-well plate (Corning, Oneonta, NY), which was then incubated at 37°C for 16 h. After the cells were washed twice with DMEM containing 2% FBS to remove free IgE, various concentrations of PSE, PSEH, or PSEL were added to the wells. A control buffer solution was added to the wells instead of PSE or its fractions. After 15 min of incubation, 100 μL of 100 $\mu\text{g}/\text{mL}$ DNP-BSA was added and the mixture was incubated at 37°C for 1 h. We also prepared plates in which the cells were stimulated with 50 μL of 100 μM DTBHQ and 50 μL of

100 $\mu\text{g}/\text{mL}$ PMA without sensitization with anti-DNP-IgE. Fifty μL of the supernatant of the cells was mixed with 200 μL of 3.8 mM *p*-nitrophenyl-2-acetoamide- β -D-glucopyranoside in 0.05 M citrate buffer (pH 4.5), and the mixture was incubated at 37°C for 1 h. After incubation, 500 μL of 0.2 M glycine buffer (pH 10.0) was added, and the consequent absorbance at 405 nm was measured to analyze free β -hexosaminidase activity. To quantify the enzyme activity in the cells, 500 μL of 0.2% Triton X-100 was added and the cell lysate was analyzed as described above. Total β -hexosaminidase activities were calculated by adding the free β -hexosaminidase activities of the cell supernatant as the absorbance at 405 nm to the enzyme activities in the cells as the absorbance at 405 nm. β -Hexosaminidase releases (%) were calculated as follows:

$$\frac{\text{free } \beta\text{-hexosaminidase activity}}{\text{total } \beta\text{-hexosaminidase activity}} \times 100$$

Statistical analysis. Each data point was expressed as the mean $\pm$ standard error of three independent determinations for each experiment. The effects of treatment, concentration, and treatment $\times$ concentration were evaluated by two-way reiterated measures ANOVA (Figs. 2, 5A). One-way ANOVA and Fisher's PLSD test were used for group-to-group comparison at given concentrations (Figs. 2, 5A). Differences in the percentage values of inhibitory activities between procyanidin A1 and procyanidin A2 were analyzed by Student's *t* test (Fig. 5B). A probability level of <0.05 was considered to indicate significance.

Results and Discussion

Inhibitory activities of PSE and its ultrafiltrated fractions against IgE-mediated β -hexosaminidase release

The total polyphenol in PSE amounted to 140.9 mg of (+)-catechin equivalent/g of dry skin. PSE contained 32.2% high-molecular-weight polyphenols (45.4 mg/g, dry skin) and 56.6% low-molecular-weight polyphenols (79.7 mg/g, dry skin).

We measured the inhibitory activities of PSE, PSEH, and PSEL against IgE-mediated β -hexosaminidase release from RBL-2H3 cells. After the data were analyzed by two-way ANOVA, both treatment and concentration effects were observed. There were significant differences among PSE, PSEL and PSEH at a concentration of 0.15 mg/mL. PSE inhibited β -hexosaminidase release in a polyphenol concentration-dependent manner ($\text{IC}_{50} = 0.16$ mg/mL, Fig. 2). PSEH and PSEL also showed inhibitory activity when the polyphenol concentrations were higher than 0.05 and 0.15 mg/mL respectively. The IC_{50} value of PSEH was 0.10 mg/mL, lower than those of PSE (0.16 mg/mL) and PSEL (N.D.). The average β -hexosaminidase release amounted to 31.9% of total β -hexosaminidase in the absence of PSE, PSEH, and PSEL activity. Incidentally, it is known that high-molecular-weight procyanidins isolated from grape seed can inhibit the degranulation of cells by binding to the mast-cell surface.²⁰⁾ DNP-BSA does not bind to the IgE antibody because the cell surface must be covered with PSEH polyphenols. PSEL also inhibited the degranulation of RBL-2H3 cells. Hence, the high-molecular-weight polyphenols in PSEH probably inhibit degranulation in a similar manner. Fujimura *et al.*²¹⁾ reported that (–)-epigallocatechin-3-*O*-gallate (EGCG)-induced inhibition of degranulation actin remodeling is caused by decreased myosin regulatory light chain phosphorylation mediated through the binding of EGCG to the 67-kDa laminin receptor in KU812 cells. It is thus likely that high-molecular-weight

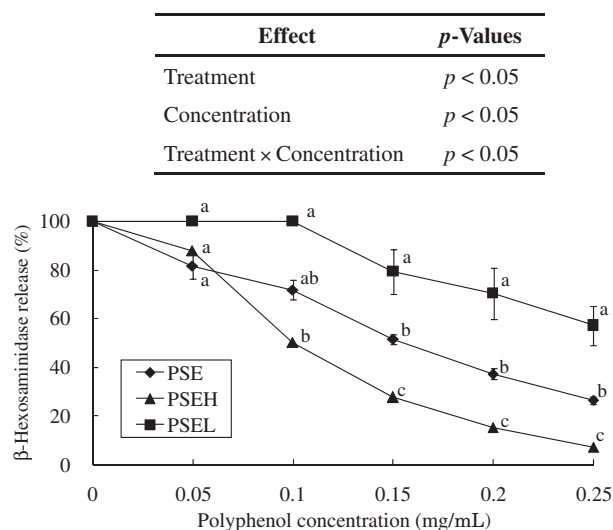


Fig. 2. Inhibition of the β -Hexosaminidase Release from RBL-2H3 Cells in the Presence of PSE.

β -Hexosaminidase release at the concentrations, of the various polyphenols is represented as a relative value (%) obtained with β -hexosaminidase release in the absence of polyphenol taken as unity. Points with bars represent means $\pm$ standard errors ($n = 3$). Different alphabetical superscripts indicate significant difference at a given concentration at $p < 0.05$.

and low-molecular-weight polyphenols inhibit cell degranulation by different mechanisms. Since it has been reported that only low-molecular-weight polyphenols, epicatechin, procyanidin A1, A2, and B2, can be absorbed in the digestive tract,²²⁾ and only partially, our study focused on the PSEL fraction to clarify its inhibitory mechanism against degranulation.

Isolation and identification of the main polyphenol in PSEL

PSEL was further separated into four fractions by purification with a Toyopearl HW-40F column. The main fraction, Peak IV, was subjected to YMC-gel ODS-AQ 120S50 column chromatography (Fig. 3A) and the main peak, Peak IV-1, was fractionated. Purification of Peak IV-1 was confirmed by the Shimadzu LC-20AD HPLC system. Only one sharp peak was obtained (Fig. 3B).

The compound from Peak IV-1 was analyzed by ESI-MS and NMR spectroscopy. The cluster ion peak at m/z 599 $[M + Na]^+$ in the ESI-MS spectrum and the molecular formula was found to be $C_{24}H_{30}O_{12}$ based on the HRESI-MS data (m/z 599.11433 $[M + Na]^+$, 599.11654 calcd. for $C_{24}H_{30}NaO_{12}$). Hence it was suggested that Peak IV-1 was an A-type dimer (M.W. 576), with two-bond linkage between the upper and lower units. All the ^1H -NMR and ^{13}C -NMR spectroscopic data are shown in Table 1. The ^1H - and ^{13}C -NMR data, including the ^1H - ^1H COSY data, indicated that the lower unit was catechin, which had a coupling constant of 8 Hz between H-2F and H-3F, whereas that of epicatechin was 5 Hz (Table 1). The two-bond linkage was confirmed by an HMBC experiment. For the upper unit, the ^{13}C -NMR chemical shift at C-2C (δ_C 100.0) indicated the absence of the H-2 proton of catechin or epicatechin, and indicated the presence of a C-2C $\rightarrow$ O $\rightarrow$ C-7D bond. In addition, the cross peaks between H-4C and C-7D, C-8D, and C-8aD and between

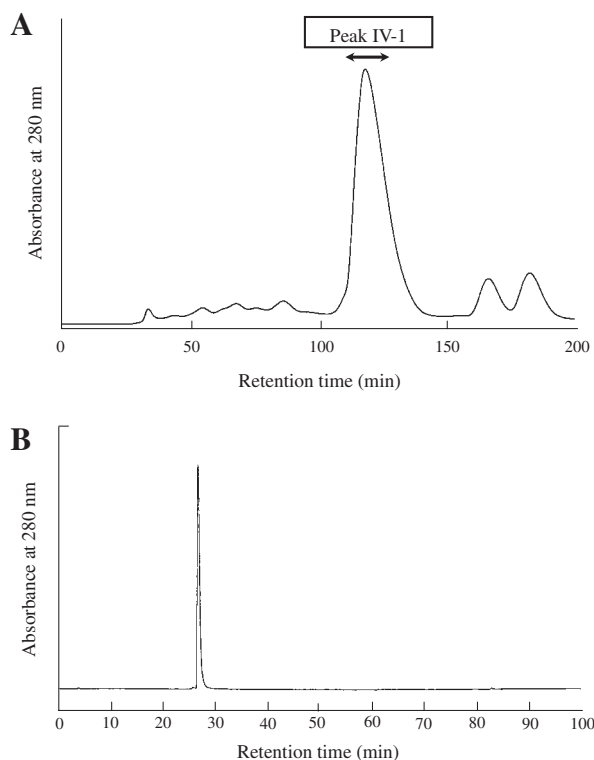


Fig. 3. Chromatographic Profiles of PSEL and Peak IV-1.

A, Gel chromatography of PSEL with YMC-gel ODS-AQ 120S50 column; detection at 280 nm. B, HPLC profile of Peak IV-1 with a Capcell Pak C18 MG column detected at 280 nm.

H-3D and C-8D suggested the presence of another C-4C $\rightarrow$ C-8D bond (Fig. 4). The upper unit was predicted to be an epicatechin unit, because Lou *et al.*²³⁾ reported that most A-type procyanidin dimers in peanut skin have the epicatechin unit as the upper unit. Furthermore, the optical rotation of Peak IV-1 was $[\alpha]_D^{19} = +45.8^\circ$ (c 0.55, MeOH). Taken together, these spectral data indicate that this compound from Peak IV-1 was procyanidin A1. Approximately 7.55 mg of Peak IV-1 was obtained from 1 g of dry peanut skin. Yu *et al.*⁴⁾ reported that 1.27 mg of A-type procyanidin dimers was extracted from 1 g of roasted peanut skin with 80% MeOH. In the present study, we extracted polyphenols with boiling water, and the amount of procyanidin A1 obtained was 6 times that extracted with 80% MeOH. This indicates that the extraction with boiling water is more effective than with aqueous alcohol.

Inhibitory activities of procyanidin A1 against β -hexosaminidase release

Procyanidin A1 inhibited β -hexosaminidase release from RBL-2H3 cells in a dose-dependent manner (Fig. 5A). After the data were analyzed by two-way ANOVA, both treatment and concentration effects were observed. There was a significant difference between the two procyanidins and (+)-catechin. The IC_{50} value of 20.3 μM for procyanidin A1 was similar to that of epigallocatechin gallate, 22.0 μM ,²⁴⁾ indicating that its inhibitory activity against β -hexosaminidase release is stronger (Table 2). The average β -hexosaminidase release amounted to 33.2% of total β -hexosaminidase in the absence of procyanidin A1, procyanidin A2, and (+)-catechin. Procyanidin A1 was contained by 6.6% of

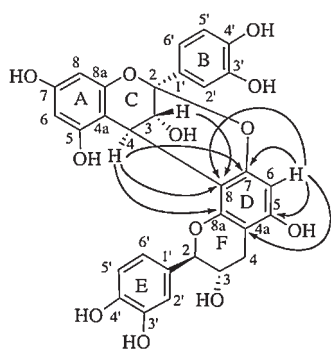
Table 1. ^1H -(400 MHz) and ^{13}C -(100 MHz) NMR Spectral Data for Procyanidin A1 in Acetone- d_6

Position	δ_{H}^*	δ_{C}^*
2C	—	100.0
3C	4.14 (d ^a , $J^{**} = 4$)	67.5
4C	4.23 (d, $J = 4$)	28.8
4aC	—	103.8
5A	—	154.0
6A	5.99 (d, $J = 2$)	96.2
7A	—	157.9
8A	6.06 (d, $J = 2$)	97.9
8aA	—	156.6
1'B	—	132.2
2'B	7.19 (d, $J = 2$)	115.6
3'B	—	145.0
4'B	—	145.9
5'B	6.84 (d, $J = 8$)	116.0
6'B	7.07 (d, $J = 2$)	119.8
2F	4.70 (d, $J = 8$)	84.7
3F	4.19 (m)	67.4
4F	2.60 (dd, $J = 9, 16$)	29.9
4F'	3.05 (dd, $J = 6, 16$)	—
4aF	—	103.2
5D	—	155.6
6D	6.15 (s)	96.6
7D	—	151.9
8D	—	106.7
8aD	—	151.2
1'E	—	130.1
2'E	7.05 (d, $J = 2$)	116.0
3'E	—	146.4
4'E	—	146.2
5'E	6.85 (d, $J = 8$)	115.3
6'E	6.87 (dd, $J = 2, 8$)	120.9

*Chemical shifts (δ) are given in ppm relative to tetramethyl silane (TMS) as internal standard.

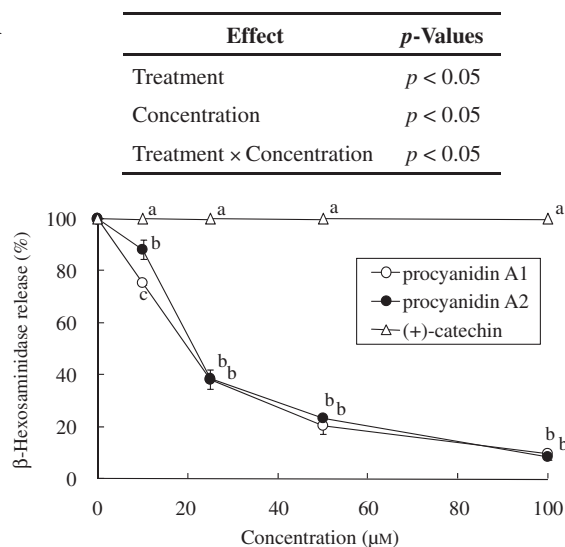
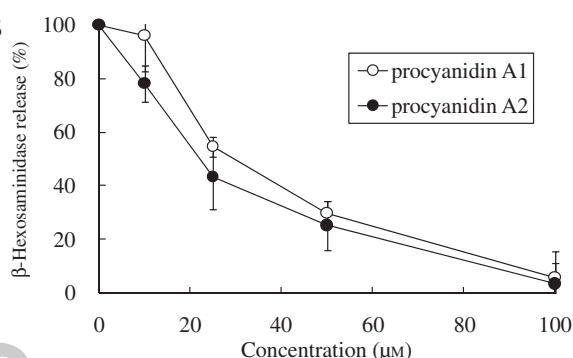
**Coupling constant (J) values are given in Hz.

^aMultiplicity

**Fig. 4.** HMBC Correlations for the Determination of (2 → O → 7, 4 → 8) Double Linkage of Peak IV-1.

Arrows correspond to HMBC correlations.

the PSEL polyphenol. The inhibitory activity of this polyphenol was greater than that of procyanidin A1 at the point of concentration, and thus PSEL might contain many other polyphenols that have inhibitory activity on degranulation. We also compared the inhibitory activity of procyanidin A1 to those of procyanidin A2 and (+)-catechin. Procyanidin A2 is a stereoisomer of procyanidin A1, and (+)-catechin is the element of procyanidin A1. Procyanidin A2 presented inhibitory activity as procyanidin A1 ($\text{IC}_{50} = 21.5 \mu\text{M}$), while (+)-catechin was not involved in inhibiting β -hexosaminidase release

A**B****Fig. 5.** Inhibition of β -Hexosaminidase Release from RBL-2H3 Cells in the Presence of Procyanidins.

β -Hexosaminidase release at the concentrations of the various polyphenols, is represented as a relative value (%) obtained with β -hexosaminidase release in the absence of polyphenol taken as unity. A, Inhibition by procyanidin A1, procyanidin A2, and (+)-catechin of antigen-stimulated β -hexosaminidase release from RBL-2H3 cells. B, Inhibition by procyanidins of PMA and DTBHQ induced β -hexosaminidase release. Points with bars represent means $\pm$ standard errors ($n = 3$). There was no significant difference between procyanidin A1 and procyanidin A2 ($p < 0.05$).

Table 2. IC_{50} Values for Procyanidin A1, Procyanidin A2, and Authentic (+)-Catechin

Sample	IC_{50} (μM)	
	Antigen	DTBHQ + PMA
Procyanidin A1	20.3	29.7
Procyanidin A2	21.5	22.1
(+)-Catechin	N.D.	N.D.

IC_{50} values were determined graphically.

(Table 2). These results indicate that there is no difference in inhibitory activity between these two stereoisomeric forms. The dimer formation of the catechin structure might thus be essential for inhibition of β -hexosaminidase release from cells.

To determine which step is involved in A-type procyanidin inhibition of the signaling pathway, we measured the activities against DTBHQ- and PMA-induced β -hexosaminidase release. PMA is a protein kinase C activator that induces activation of protein kinase C. DTBHQ is a Ca^{2+} -ATPase inhibitor. It

releases Ca^{2+} from intracellular stores. Thus, PKC activation and Ca^{2+} release induce degranulation of cells. A-type procyanidins inhibited DTBHQ- and PMA-induced β -hexosaminidase release, with inhibitory activity comparable to that against IgE-mediated β -hexosaminidase release (Fig. 5B). There was no significant difference between procyanidin A1 and procyanidin A2 (IC_{50} values of $29.7\ \mu\text{M}$ and $22.1\ \mu\text{M}$ respectively, Table 2). The average β -hexosaminidase release amounted to 45.7% of total β -hexosaminidase in the absence of procyanidin A1 and procyanidin A2. Kim *et al.*²⁵⁾ reported that *Vitis amurensis* Rupr. (Vitaceae), which has been used for centuries as a traditional oriental medicine, inhibited degranulation from mast cells by inhibition of DNA binding of NF- κ B as an important transcriptional regulator of inflammatory cytokines. The crude material of this drug contains polyphenols, mainly catechin, procyanidin, and amurensin. A detailed mechanistic understanding of degranulation is lacking though procyanidin A1 might attenuate the activation of NF- κ B. Consequently, our results indicate that peanut-skin procyanidin A1 inhibits degranulation downstream of protein kinase C activation or Ca^{2+} influx from internal stores in RBL-2H3 cells.

In conclusion, we isolated 7.55 mg of procyanidin A1 from 1 g of peanut-skin, and found that it can inhibit degranulation of RBL-2H3 cells, mainly by inhibition of signal transduction leading to secretion. This suggests that peanut-skin polyphenolic compounds, including procyanidin A1, might serve as effective therapeutic food components against allergic diseases.

Acknowledgments

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