Purification and identification of an IgE suppressor from strawberry in an in vitro immunization system

Akira Iwamoto · Kazuhiro Mitsuda ·
Aiko Inoue · Tamaki Kato · Yuichi Inoue ·
Hiroharu Kawahara

Received: 30 November 2011 / Accepted: 19 January 2012 / Published online: 11 February 2012
© Springer Science+Business Media B.V. 2012

Abstract We purified and identified an IgE suppressor from the strawberry ‘Toyonoka’, based on the decrease of IgE production in in vitro immunization (IVI). Gel filtration experiment indicated that fractions in a 15–48 kDa range and <10 kDa have an IgE suppressive activity. Furthermore, the fraction in 15–48 kDa was subjected to chromatofocusing and found to have activities at isoelectric points, pI 6.0, 7.0, and 8.0–9.2. We focused on the active fractions of pI 8.0–9.2 and the purified a large amount of strawberry extracts by cation exchange resins in batch. A purified 39 kDa protein showed homology to plant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in N-terminal amino acid sequence and had GAPDH enzymatic activity. Nucleotide sequence and deduced amino acid sequence of the obtained cDNA clone of the protein matched with the sequence of *Fragaria x ananassa* GAPDH in the GenBank with >98% identical nucleotides and >99% identical amino acids, respectively. The purified strawberry GAPDH suppressed total IgE

production in IVI in a dose-dependent manner. From these results, we identified GAPDH as IgE suppressor in the strawberry. Our study may be applicable to the development of new methods to relieve allergic conditions using GAPDH and the screening of other functional factors for human health.

Keywords Allergy · GAPDH · IgE · Strawberry

Introduction

IgE antibodies are well known as a trigger of acute hypersensitivity such as atopic dermatitis, asthma, food allergy and pollinosis, and their blood level is involved in the severity of these diseases (Holgate 1999; Corry and Kheradmand 1999). They enhance allergic reactions such as vasodilation, smooth muscle contraction, and mucosal edema through the release of chemical mediators by their binding to the FcεRI on mast cells and basophils, followed by their cross-link to the allergen (Metzger et al. 1986). These studies suggest that allergic symptom can be palliated by suppression of IgE antibodies.

There are some reports on IgE suppression by food components such as flavones (Yano et al. 2007; Hassanain et al. 2010). We also found that some species of strawberries (*Fragaria x ananassa*) suppressed total IgE production in in vitro immunization (IVI) where a population of human peripheral blood lymphocytes was cultured with the cedar pollen

A. Iwamoto · K. Mitsuda · T. Kato
Graduate School of Life Science and Systems
Engineering, Kyushu Institute of Technology,
2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196,
Fukuoka, Japan

A. Inoue · Y. Inoue (✉) · H. Kawahara
The Cell Engineering Center, Kitakyushu National
College of Technology, 5-20-1 Shii, Kokuraminami-ku,
Kitakyushu 802-0985, Fukuoka, Japan
e-mail: inoue@kct.ac.jp

antigen, Cry j 1, in a medium containing human plasma, fetal bovine serum (FBS), IL-2, IL-4, IL-6, and muramyl dipeptide (Mitsuda et al. 2010). But, we failed to determine the difference of the Cry j 1-specific IgE production between tested samples because their production level was very low. The IgE suppression by strawberry extract was accompanied a decreased expression of the suppressor of cytokine signaling-3 that evokes Th2 cytokines and IgE antibodies (Kubo and Inoue 2006). Strawberries have been studied for antioxidation and antitumor properties (Aaby et al. 2007; Zhang et al. 2008), but their anti-allergic effect was not fully understood yet. In this study, based on the results of IgE suppression in the IVI we purified an IgE suppressor from the strawberry ‘Toyonoka’, and identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the IgE suppressor.

Materials and methods

Preparation of strawberry extract

The strawberry ‘Toyonoka’ fruits were homogenized after the addition of equal weight volume of phosphate buffered saline (PBS). The homogenate was centrifuged at 9,400g for 60 min and the supernatant was sterilized by a 0.22 µm filter. The filtrate was used for the experiments.

Purification of an IgE suppressor in the strawberry

The strawberry extract was purified by the gel filtration column, HiLoad 26/60 Superdex 200 prep grade (Amersham Biosciences, UK) with 20 mM phosphate buffer (pH 7.0) and by the Chromatofocusing column, Mono P 5/200 GL (GE Healthcare, Sweden) with Polybuffer 96-acetic acid (pH 6.0), respectively, at a flow rate of 1.0 mL/min using the fast protein liquid chromatography (FPLC) system, ÄKTA™ explorer 10S (Amersham Biosciences, UK). Suppressive effect of each fraction on IgE production was examined by the IVI system.

To obtain an IgE suppressor in a large amount, the strawberry extract was newly prepared and applied to the cation exchange resins, TOYOPEAL SP-550C (TOSOH, Japan) with 100 mM acetate buffer (pH 5.0) in batch. The active fraction eluted with acetate buffer

containing 0.1 M NaCl was concentrated and subjected to SDS-polyacrylamide gel electrophoresis, followed by electroblotting to PVDF membrane and staining with Coomassie dye. A main band of 39 kDa in the membrane was cut out and subjected to the N-terminal protein sequence analysis.

The GAPDH enzymatic activity of the purified 39 kDa protein was examined according to the method described by Ferdinand (1964). The strawberry GAPDH concentration was determined by the Bio-Rad protein assay (Bio-Rad, Japan) with rabbit muscle GAPDH as a standard.

In vitro immunization (IVI) system

Peripheral blood lymphocytes (PBL) were separated from heparinized blood of healthy donors by using Ficoll-Paque Plus (GE Healthcare, Sweden). To induce IgE production of PBL, IVI was done as described by Kawahara et al. (2000). Briefly, 2.0×10^6 cells/mL of PBL were cultured in the 5% fetal bovine serum (FBS) and 10% human plasma containing ERDF medium (Kyokuto Pharmaceuticals, Japan) supplemented with 10 ng/mL of recombinant human IL-2, IL-4, and IL-6 (R&D systems, USA), 10 µg/mL of muramyl dipeptide (SIGMA, USA) and 100 ng/mL of the cedar pollen antigen Cry j 1 (HAYASHIBARA, Japan) with or without 5% (v/v) of strawberry extract. After 10 days, total IgE concentration in the supernatant was measured by an enzyme-linked immunosorbent assay (ELISA). Noteworthy, IgE production level was different between donors.

ELISA

Total IgE concentration in culture medium was measured by sandwich ELISA. 96 well microplates were coated with anti-human IgE (Biosource, USA) diluted with PBS. The antibody-coated wells were blocked with 1.0% BSA/PBS, and then, each sample was added to them. After washing with 0.05% Tween 20 containing PBS (TPBS) three times, biotin-conjugated anti-human IgE antibody (Biosource, USA) and horseradish peroxidase-conjugated streptavidin were added to them. After washing with TPBS three times, a substrate solution (0.1 M citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg/mL *p*-2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was added to them. After 15 min, the absorbance

was measured at 414 and 490 nm by a microplate reader.

cDNA cloning by PCR

Total RNA was extracted from ‘Toyonoka’ strawberry fruits using ISOGEN (NIPPON GENE, Japan). First strand cDNA was synthesized from 5 µg of total RNA using Thermoscript™ cDNA synthesis kit (Invitrogen, USA) according to the product instruction manual. For cloning the gene of the target protein, the cDNA fragment was amplified by polymerase chain reaction (PCR). A forward primer (5′-AAGAATCAGATCGG AATCAACGGATTC-3′) was designed from N-terminus protein sequence and optimized strawberry codon usage. A reverse primer (5′-TGACACAAGCTTGA CAAAGTTCT-3′) was designed from the mRNA partial sequence of strawberry GAPDH (GenBank, accession number: AF421145.1). PCR was performed with a 50 µL reaction mixture containing 1 µL of cDNA, 5 µL of 2 mM dNTPs, 3 µL of 25 mM MgSO₄, 10 pmol of each primer, KOD plus DNA polymerase, and 5 µL of 10 × KOD FX buffer (TOYOBO, Japan) using the following program: 94 °C for 2 min, then 40 cycles each with 98 °C for 10 s, 58 °C for 30 s, 68 °C for 2 min 30 s, and finally 68 °C for 10 min. The amplified product was analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The corresponding DNA band was recovered and cloned into the pTarget™ vector for sequencing (Promega, Japan).

Statistics

Data are means ± standard deviations (SD) from triplicate samples. Statistical significance was determined by the Student’s *t* test.

Results and discussion

Purification of an IgE suppressor in the strawberry

Among 18 species of strawberries tested, we selected the strawberry ‘Toyonoka’ as the material to purify an IgE suppressor because they showed relatively strong IgE suppression (Mitsuda et al. 2010). As shown in Fig. 1a and b, the separation of the strawberry extract by gel filtration indicated IgE suppressive activity in

Fig. 1 Effect of gel filtration fractions on IgE production in IVI. **a** Elution pattern and fractions of strawberry extract in gel filtration. MW numbers represent the molecular weight (kDa) of standard proteins. **b** IgE production in IVI. Data show the average values of two measurements

Fig. 2 Effect of chromatofocusing fractions on IgE production in IVI. Chromatofocusing was done in the pH gradient range between 9 and 6. The pI value shows isoelectric point of eluate. Data show the average values of two measurements

fraction number 4 (15–48 kDa) and 7 (<10 kDa). In this experiment, IgE suppression was weak, but it was confirmed that the position of the suppressive activity

50
AAG ATC AAG ATC GGA ATC AAC GGA TTC GGA AGA ATC GGA CGT TTG GTT GCT AGG GTC GCC CTA CAG AGG GAC GAT
 Lys Ile Lys Ile Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Leu Val Ala Arg Val Ala Leu Gln Arg Asp Asp

100
 GTT GAG CTC GTC GCT GTT AAC GAT CCA TTC ATC ACC ACC GAC TAC ATG ACC TAC ATG TTT AAG TAT GAC ACC GTT
 Val Glu Leu Val Ala Val Asn Asp Pro Phe Ile Thr Thr Asp Tyr Met Thr Tyr Met Phe Lys Tyr Asp Thr Val

200
 CAC GGA CCA TGG AAG CAC CAT GAG CTC AAG GTC AAG GAT GAG AAG ACC CTT CTC TTC GGT ACG AAG CCA GTT GCC
 His Gly Pro Trp Lys His His Glu Leu Lys Val Lys Asp Glu Lys Thr Leu Leu Phe Gly Thr Lys Pro Val Ala

250
 GTC TTC GGG CTC AGA AAC CCA GAG GAG ATC CCA TGG GGT TCA GTT GGT GCC GAT ATT GTT GTC GAG TCT ACT GGA
 Val Phe Gly Leu Arg Asn Pro Glu Glu Ile Pro Trp Gly Ser Val Gly Ala Asp Ile Val Val Glu Ser Thr Gly

350
 GTG TTC ACT GAC AAG GAC AAA GCC GCT GCT CAC TTG AAG GGA GGT GCC AAG AAG GTT GTC ATC TCT GCC CCA AGT
 Val Phe Thr Asp Lys Asp Lys Ala Ala Ala His Leu Lys Gly Gly Ala Lys Lys Val Val Ile Ser Ala Pro Ser

400
 AAG GAT GCC CCC ATG TTC GTT GTT GGT GTG AAT GAG CAC GAA TAC AAG TCC GAC CTT TGC ATT GIT TCC AAT GCT
 Lys Asp Ala Pro Met Phe Val Val Gly Val Asn Glu His Glu Tyr Lys Ser Asp Leu Cys Ile Val Ser Asn Ala

500
 AGT TGC ACT ACC AAC TGT CTT GCT CCC CTT GCC AAG GTT ATC AAT GAC AGG TTT GGA ATT GTT GAG GGT CTT ATG
 Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu Ala Lys Val Ile Asn Asp Arg Phe Gly Ile Val Glu Gly Leu Met

550
 ACC ACT GTG CAC TCC ATC ACT GCC ACC CAG AAG ACT GTT GAT GGG CCA TCA GCC AAG GAC TGG AGA GGT GGA CGT
 Thr Thr Val His Ser Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro Ser Ala Lys Asp Trp Arg Gly Gly Arg

650
 GCT GCC TCA TTC AAC ATC ATT CCT AGC AGC ACT GGA GCT GCC AAG GCT GTC GGA AAG GTT CTG CCT GCT CTC AAT
 Ala Ala Ser Phe Asn Ile Ile Pro Ser Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Leu Pro Ala Leu Asn

700
 GGC AAG TTG ACT GGA ATG GCC TTC CGT GTA CCC ACT GTT GAT GTT TCA GTT GTT GAC CTC ACT GTC AGA CTT GAG
 Gly Lys Leu Thr Gly Met Ala Phe Arg Val Pro Thr Val Asp Val Ser Val Val Asp Leu Thr Val Arg Leu Glu

800
 AAG AAG GCC ACC TAT GAC CAG ATC AAG GCT GCT ATC AAG GAG GAG TCT GAG GGA AAG ATG AAG GGT ATC TTG GGT
 Lys Lys Ala Thr Tyr Asp Gln Ile Lys Ala Ala Ile Lys Glu Glu Ser Glu Gly Lys Met Lys Gly Ile Leu Gly

850
 TAC ACC GAA GAT GAT GTT GTG TCA ACC GAC TTC ATT GGT GAC AAC AGG TCA AGC ATC TTT GAT GCC AAG GCT GGA
 Tyr Thr Glu Asp Asp Val Val Ser Thr Asp Phe Ile Gly Asp Asn Arg Ser Ser Ile Phe Asp Ala Lys Ala Gly

ATT GCA TTG AAC GAG AAC TTT GTC AAG CTT GTG TCA
 Ile Ala Leu Asn Glu Asn Phe Val Lys Leu Val Ser

Fig. 3 Nucleotide sequence and deduced amino acid sequence of the obtained cDNA clone. Numbers above refer to the nucleotide position. The primer sites for PCR are *underlined*

was almost the same between experiments. Low molecular weight constituents such as linocinnamarin and cinnamic acid in the strawberry 'Nohime' were reported to inhibit allergic reactions (Ninomiya et al. 2010). But, vitamin C, which is rich in strawberry, had little IgE suppressive activity in the IVI system (Mitsuda et al. 2010). This means that the IgE suppression is not necessarily caused by only antioxidation. Thus, the 15–48 kDa fraction was subjected to chromatofocusing and was found to have the following isoelectric point: pI 6.0, 7.0, and 8.0–9.2 (Fig. 2). These results suggest that the strawberry may include some IgE suppressors..

Identification of an IgE suppressor in the strawberry

We focused on the active fractions of pI 8.0–9.2 and newly purified a large amount of strawberry extract using cation exchange resins in batch. SDS-PAGE analysis of eluted active fraction showed a main band of 39 kDa in molecular size. The 39 kDa protein band was observed in the strawberry species with IgE suppressive activity at a relatively higher level than others (data not shown). Subsequently, the protein band was subjected to N-terminal protein sequence analysis. The resulting first N-terminal amino acid sequence, Ala Lys Ile Lys Ile Gly Ile Asn Gly Phe, showed homology to the sequence of rice and maize glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is a key glycolytic enzyme that plays a crucial role in energy production. In addition, the purified protein had GAPDH enzymatic activity. Based on the result of this protein sequence and the GAPDH mRNA sequence from the GenBank, a cDNA clone of the protein was obtained by RT-PCR cloning technique. Figure 3 shows its nucleotide sequence and deduced amino acid sequence. These sequences matched with that of strawberry GAPDH in the GenBank (accession number: AF421145.1 and AB363963.1) with >98% identical nucleotides and >99% identical amino acids, respectively. Strawberry GAPDH was purified by cation exchange resins and its purity was examined by SDS-PAGE. The protein bands of strawberry extract were almost invisible, but after purification, a single band of GAPDH was clearly confirmed (Fig. 4a). The purified strawberry GAPDH suppressed total IgE production in IVI in a dose-dependent manner (Fig. 4b). In addition, rabbit muscle

Fig. 4 Effect of purified strawberry GAPDH on IgE production in IVI. **a** SDS-PAGE of strawberry GAPDH purified by cation exchange resins. Lane 1: molecular weight marker, lane 2: rabbit muscle GAPDH, lane 3: strawberry extract, lane 4: purified strawberry GAPDH. **b** Effect of strawberry GAPDH on IgE production in IVI. Data are means $\pm$ SD ($n = 3$). * $p < 0.05$ versus control

GAPDH that exhibits about 67% similarity of strawberry GAPDH in amino acid sequence also showed a IgE suppressive activity (data not shown). Yamaji et al. (2005) reported that GAPDH could bind to mammalian cells in time- and dose-dependent manners and its binding was involved in the cysteine at position 151 of this enzyme. From these findings, we identified GAPDH as the IgE suppressor in the strawberries. Interestingly, Sugahara et al. (1995) reported that human and rabbit muscle GAPDH increased IgM production in human hybridomas and lymphocytes. IgM antibodies are known to be class-switched to IgE antibodies by IL-4 in activated B lymphocyte. Therefore, GAPDH may influence the process of the immunoglobulin class switching in addition to the production level. Recently, the multi-function of GAPDH in apoptosis and NO stress has

been reported (Sirover 1999; Hara et al. 2006). Our study indicates an additional new function of GAPDH.

Conclusions

We reported here that strawberry GAPDH suppressed IgE production in IVI. But, further study is needed to understand the mechanism underlying the IgE suppression by the GAPDH. At present, anti-allergic drugs are used to improve allergic symptoms. They inhibit the release and activity of chemical mediators from mast cells, but produce occasionally harmful side effects. Our study may lead to the development of new methods to relieve allergic conditions using GAPDH and the screening of other functional factors for human health.

Acknowledgments This work was supported in part by a Fukuoka prefectural fund for the promotion of agricultural research and development.

References

- Aaby K, Wrolstad RE, Ekeberg D, Skrede G (2007) Polyphenol composition and antioxidant activity in strawberry purees; impact of achene level and storage. *J Agric Food Chem* 55:5156–5166
- Corry DB, Kheradmand F (1999) Induction and regulation of the IgE response. *Nature* 402:B18–B23
- Ferdinand W (1964) The isolation and specific activity of rabbit muscle glyceraldehyde phosphate dehydrogenase. *Biochem J* 92:578
- Hara MR, Cascio MB, Sawa A (2006) GAPDH as a sensor of NO stress. *Biochim Biophys Acta* 1762:502–509
- Hassanain E, Silverberg JI, Norowitz KB, Chice S, Bluth MH, Brody N, Joks R, Durkin HG, Smith-Norowitz TA (2010) Green tea (*Camelia sinensis*) suppresses B cell production of IgE without inducing apoptosis. *Ann Clin Lab Sci* 40:135–143
- Holgate ST (1999) The epidemic of allergy and asthma. *Nature* 402:B2–B4
- Kawahara H, Maeda-Yamamoto M, Hakamata K (2000) Effective induction and acquisition of human monoclonal IgE antibodies reactive with house-dust mite extracts. *J Immunol Methods* 233:33–40
- Kubo M, Inoue H (2006) Suppressor of cytokine signaling 3 (SOCS3) in Th2 cells evokes Th2 cytokines, IgE, and eosinophilia. *Curr Allergy Asthma Rep* 6:32–39
- Metzger H, Alcaraz G, Hohman R, Kinet JP, Pribluda V, Quarto R (1986) The receptor with high affinity for immunoglobulin E. *Annu Rev Immunol* 4:419–470
- Mitsuda K, Inoue A, Nishino N, Inoue Y, Kawahara H (2010) Detection of anti-allergic effects in strawberry extracts. In: Kamihira M et al. (eds) Proceedings of the 21st annual and international meeting of the Japanese association for animal cell technology, Fukuoka, November 2008. *Animal cell technology: basic and applied aspects*, vol 16. Springer, Dordrecht, The Netherlands, pp 353–358
- Ninomiya M, Itoh T, Ishikawa S, Saiki M, Narumiya K, Yasuda M, Koshikawa K, Nozawa Y, Koketsu M (2010) Phenolic constituents isolated from *Fragaria ananassa* Duch inhibit antigen-stimulated degranulation through direct inhibition of spleen tyrosine kinase activation. *Bioorg Med Chem* 18:5932–5937
- Sirover MA (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1432:159–184
- Sugahara T, Shirahata S, Sasaki T, Murakami H (1995) The mode of actions of glyceraldehyde-3-phosphate dehydrogenase identified as an immunoglobulin production stimulating factor. *FEBS Lett* 368:92–96
- Yamaji R, Chatani E, Harada N, Sugimoto K, Inui H, Nakano Y (2005) Glyceraldehyde-3-phosphate dehydrogenase in the extracellular space inhibits cell spreading. *Biochim Biophys Acta* 1726:261–271
- Yano S, Umeda D, Yamashita T, Ninomiya Y, Sumida M, Fujimura Y, Yamada K, Tachibana H (2007) Dietary flavones suppresses IgE and Th2 cytokines in OVA-immunized BALB/c mice. *Eur J Nutr* 46:257–263
- Zhang Y, Seeram NP, Lee R, Feng L, Herber D (2008) Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *J Agric Food Chem* 56:670–675