Soyabean glyceollins: biological effects and relevance to human health

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Glyceollins, one family of phytoalexins, are de novo synthesised from daidzein in the soyabean upon exposure to some types of fungus. The efficiency of glyceollin production appears to be influenced by soyabean variety, fungal species, and the degree of physical damage to the soyabean. The compounds have been shown to have strong antioxidant and anti-inflammatory activities, and to inhibit the proliferation and migration of human aortic smooth muscle cells, suggesting their potential to prevent atherosclerosis. It has also been reported that glyceollins have inhibited the growth of prostate and breast cancer cells in xenograft animal models, which is probably due to their anti-oestrogenic activity. In essence, glyceollins deserve further animal and clinical studies to confirm their health benefits.

Abbreviations: COX-2, cyclo-oxygenase 2; ER, oestrogen receptor; Keap1, Kelch-like enoyl-CoA hydratase-associated protein 1; LPS, lipopolysaccharide; Nrf2, nuclear factor-erythroid 2 p45 subunit-related factor 2; ROS, reactive oxygen species.

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Soybeans contain abundant isoflavones, a varied group of polycyclic compounds, which have been reported to have numerous biological activities including suppression of sex-hormone dependent cancers(16–19) and amelioration of post-menopausal complications, including osteoporosis and hot flashes(20). Isoflavonoids could be classified as phytoanticipins because they are stored in plant cells in anticipation of pathogenic attack(21). All soybeans and their products contain significant amounts of the isoflavones daidzein and genistein, either as the aglycone or as different types of glycoside conjugates. These include 6'-O-malonylglucosides, 6'-O-acetylglucosides and the β-glicosides of daidzein and genistein(22,23), all of which can be separated by reversed-phase HPLC. Smaller amounts of glyeollin conjugates are often found in soybean or soya protein, whereas conjugates of glycitein are abundantly present hypocotyledon or germ(23). The malonyl and acetyl glucosides are susceptible to heat and readily convert to the more stable β-glycoside(24). Therefore, the relative proportions of these conjugates can vary considerably among different soya foods depending on the extent of processing of the soyabean(25).

Glyeollins are de novo synthesised from daidzein in the soyabean in response to environmental stresses such as fungal infection. Although the glyeollins are detected at high concentrations in soybeans during stress, they have also been detected at trace levels in non-elicitor-treated soybean seeds(26).

The compounds possess pterocarpanoid skeletons with cyclic ether decoration originating from a C5 prenyl moiety. Enzymes involved in glyeollin biosynthesis have been thoroughly characterised during the early era of modern plant biochemistry, and many genes encoding enzymes of isoflavonoid biosynthesis have been cloned. However, some genes need to be identified for later biotechnological steps(27). According to a recent study by Akashi et al., dimethylallyl diphasphate: (6αS, 11S)-3,9,6α-trihydroxypterocarpan ((2)-glycinol) 4-dimethylallyltransferase appears to be involved in yielding the direct precursor of glyeollin I(27).

Glyeollins are derived from the precursor molecule, daidzein through several intermediate structures including glycinol(28). Glycinol, which was previously shown to be oestrogenic, is derived from daidzein via a pterocarpan by cyclisation and 6α-hydroxylation(29). For the biosynthesis of the glyeollins, glycinol is prenylated to produce glyeollidin I and II, followed by cyclisation into the corresponding glyeollins(30).

The most well-known elicitors that effectively stimulate biosynthesis of glyeollins include some fungal strains such as Pseudomonas glycinea, Meloidogyne incognita, Heterodera glycines, Aspergillus sojae, Aspergillus awamori, Aspergillus oryzae and Rhizopus oligosporous(21,23).

Whether an elicitor is a fragment of a fungal cell or a defined chemical molecule such as a β-glucan, host plants must recognise these phytoalexin-inducing factors. Such molecules from both pathogens and mutualists are presumably recognised by receptors located on the cell wall or membrane. Typical biotic elicitors causing glyeollin production in soybean include β-1,3,1,6-oligoglucoside, β-1,3-glucan and cyclic β-1,6,1,3-glucan, which are cell wall components of pathogenic or mutualistic symbiotic micro-organisms(32). For instance, cyclic β-1,6,1,3-glucans synthesised by both free-living cells and bacteroids of B. japonicum are active elicitors of glyeollins in soyabean(33,34).

Glyeollin synthesis is also induced by abiotic elicitors including iodoacetate, UV, Tx-100, and metal ions such as Fe, Cu, Hg and Ag(21) as well as during fungal infection. The mechanisms by which biotic and abiotic factors affect isoflavonoid phytoalexin formation in plants are unclear. Because elicitors have diverse structural features, they may act simply by injuring plant cells, which then stimulates the phytoalexin biosynthetic pathway. Or they may cause the host plant to release a constitutive elicitor that triggers phytoalexin formation(21).

**Antifungal activity**

According to our previous study glyeollins were shown to inhibit several lines of fungal species. More specifically, the glyeollins (200 and 600 μg/disk) revealed a remarkable antifungal effect against Phytophthora capsici and Sclerotinia sclerotiorum, and to a lesser degree Fusarium oxysporum and Botrytis cinerea, within the growth inhibition range of 10-9–61.0%, along with their respective minimum inhibitory concentration values ranging from 25 to 750 μg/ml. The glyeollins also had a strong suppressive effect on spore germination of all tested plant pathogens along with concentration- and time-dependent kinetic inhibition of P. capsici, which is responsible for pepper disease(35). Phytoalexins appear to exert their antifungal activity by altering membrane permeability.
although the presence of other mechanisms could not be excluded(36).

Antioxidant activity
In healthy aerobes, the production of reactive oxygen species (ROS) is approximately balanced with the antioxidant defence systems(37). Oxidative stress can be defined as a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage(38), or a serious imbalance between ROS production and antioxidant defences(37). Although ROS might be harmful at the high concentration, the generation of ROS, within certain boundaries, is essential to maintain homoeostasis. For instance, macrophages utilise ROS to combat infective agents. Likewise, cytosolic ROS may be involved in the regulation of some important cellular events such as proliferation, gene expression and signal transmission. However, it is generally agreed that an excessive production of ROS is associated with ageing, cancer development, atherosclerosis and neurodegenerative disorders(39,40).

Glyceollins isolated from soyabean that sprouted in the presence of \textit{A. sojae} showed strong antioxidant activity and ROS scavenging potential when assessed by an \textit{in vitro} model(13). The antioxidant activities of glyceollins were confirmed by measuring ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl radical scavenging, singlet oxygen quenching, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging, hydroxyl radical scavenging activity and lipid peroxidation inhibition. In addition, the antioxidant potential of glyceollins were measured by a fluorescent probe, 2,7-dichlorofluorescin diacetate, and dihydroethidium in mouse hepatoma hepa1c1c7 cells in which they were insulted with H$_2$O$_2$ to generate ROS. The compound showed a strong reducing power and inhibited lipid peroxidation and significant scavenging activities of radicals including singlet oxygen superoxide anion, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl(13). It was also found that glyceollins significantly suppressed H$_2$O$_2$-induced LDL oxidation production (Fig. 2), suggesting their potential as natural antioxidants and nutraceuticals.

Antioxidant enzyme induction
Many natural antioxidants have been found to induce phase 2/antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1, haem oxygenase 1, glutathione reductase, glutathione \textit{S}-transferase A1 (also known as glutathione \textit{S}-transferase Ya in mouse), and glutamate-cysteine ligase via the nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2)-mediated pathway(41–44). Our recent study indicated that glyceollins have the potential to induce antioxidant enzymes and phase 2 detoxifying enzymes through the Nrf2-Kelch-like enoyl-CoA hydratase-associated protein 1 (Keap1) pathway(11), although the mechanism of action how the compounds activate the Nrf2 signalling pathway needs to be defined (Fig. 3).

Under unstressed conditions, Nrf2 is present in cytosol in the form of a heterodimer with Keap1, and it is rapidly degraded by the proteosomal pathway. Once activated by oxidative stress or electrophiles, it migrates into the nucleus and binds to the antioxidant response element of specific genes, enhancing their transcription. Although the whole mechanism by which some natural compounds cause liberation of Nrf2 from Keap1 remains unclear, the conformational change of Keap1 by direct interaction with the compounds or an indirect signal generated from the
binding of the compounds to cellular membrane seems to be responsible for the nuclear translocation of Nrf2 following the transcriptional activation of antioxidant enzymes.

In particular, cysteine residues abundantly present in Keap1 could be modified by exogenous oxidants or antioxidants, facilitating Keap1 separation from Nrf2\(^{(45,46)}\). It is also speculated that the Nrf2–Keap1 complex is separated from each other by phosphorylation of either Nrf2 or Keap1 by certain signalling pathways including mitogen-activated protein kinase or phosphoinositide 3-kinase, which causes a conformational change\(^{(47–49)}\).

The importance of Nrf2 in cytoprotection has been proven from the result of when the knockout of Keap1 in mice led to juvenile lethality due to hyperkeratosis of the oesophagus\(^{(50)}\). Hepatocyte-specific knockout of the Keap1 gene consistently elevates the accumulation of Nrf2 in the nucleus and protects hepatocytes against acute drug toxicity and inflammatory liver injury\(^{(51,52)}\).

Halliwell pointed out that antioxidants would only significantly influence a disease process if free radicals or other reactive species caused or significantly contributed to the progression of the disease\(^{(37,25)}\). Evidence supports the view that increased free radical formation is usually a consequence of tissue damage by a disease or toxin\(^{(54)}\).

Nrf2-mediated phase 2 enzyme induction has been widely accepted as a promising approach for cancer chemoprevention as well as protection against oxidative stress\(^{(44,55–58)}\). Considering Nrf2-mediated antioxidant enzyme induction, glyceollins have the potential to prevent chemically induced carcinogenesis, neurodegenerative diseases such as Alzheimer’s disease and atherosclerosis.

**Anti-inflammatory activity**

Inflammation is a process that includes multiple steps, which are regulated by activating inflammatory or immune cells\(^{(59)}\). It is widely accepted that inflammation plays a key role in coronary artery disease and in the progression of atherosclerosis\(^{(60)}\). Similarly, inflammation has long been considered a major precursor for the development of cancer in both infectious and non-infectious conditions. Non-infectious chronic inflammatory disorders such as inflammatory bowel disease, which are typically associated with chemokine signalling, increased cell proliferation, reduced cell cycles, accumulation of mutations, and inadequate DNA repair are well-known harbingers of malignancy\(^{(61)}\). Thus, chronic inflammation is considered to be a common cause of atherosclerosis and may contribute to the incidence and/or promotion of certain types of cancer\(^{(62)}\).

Macrophages play a central role in the regulation of the inflammatory response by releasing pro-inflammatory cytokines, chemokines and other mediators that induce migration of additional cells to inflamed tissue\(^{(63)}\). The
activated macrophages secrete inflammatory cytokines such as TNFα, IL-6, and induce ROS and PGE₂ (64).

Phyto-oestrogen is known to have antioxidative, and anti-inflammatory properties by the inhibition of inducible NO synthase gene expression and NO production, as well as inhibition of the expression of inflammatory cytokines (65). The molecular mechanisms are implicated in the oestrogen receptor (ER)-dependent pathway or by the activation of the inflammatory NF-κB transcription factor through mitogen-activated protein kinases (66,67).

Our recent study showed that glyceollins markedly suppressed the inflammatory response in lipopolysaccharide (LPS)-activated murine macrophages and the skin in vivo (68). Interestingly, glyceollins suppressed LPS-dependent secretion of IL-6, NO and expression of cyclo-oxygenase 2 (COX-2) from RAW264.7 cells. NO is a molecule produced by inducible NO synthase in a reaction that converts arginine and O₂ into citrulline and NO (69). It plays an important role in inflammation (69). Upregulation of COX-2 expression by the transcriptional activation of the COX-2 gene, results in the increased production of PGE₂ which is a critical factor in the inflammatory condition (70).

It seems that the anti-inflammatory activity of glyceollins is associated with the suppression of LPS-dependent phosphorylation and the induction of NF-κB (68). It is known that inactive NF-κB normally stays bound to inhibitory κB in the cytosol. NF-κB can be activated by LPS and pro-inflammatory cytokines, which induce increased protein phosphorylation and proteolysis of inhibitory κB protein (71). The activated NF-κB is, in turn, translocated into the nucleus, binding to NF-κB-binding sites in the promoter regions of target genes and inducing the transcription of pro-inflammatory mediators such as inducible NO synthase, COX-2, TNFα, IL-1β, IL-6 and IL-8 (72). Activation of macrophages plays an important role in the initiation and propagation of inflammatory responses by the production of cytokines and mediators, such as IL-1β, TNFα, NO and COX-2 (73,74).

Glyceollins also suppress LPS-dependent phosphorylation of extracellular-signal-regulated kinase 1 and 2 and p38, suggesting their ability to inhibit the essential targets responsible for the inflammation process (68). Thus, glyceollins appear to exert anti-inflammatory activity via regulating the NF-κB signalling pathway (Fig. 4).

**Anti-cancer and oestrogen-modulating activities**

Oestrogen antagonists have been exploited to prevent or treat oestrogen-positive breast cancer. Soya isoflavones have been extensively studied for their potential to prevent and/or treat sex-hormone-dependent tumours (14,17–20). This key finding led to the proposal that a diet containing soyabean may be beneficial in the prevention or treatment of hormone-dependent diseases because of the presence of these bioactive non-nutrients (17–20,75). Soyabeans and their products have long been copiously consumed by the people of East Asian countries including Korea, China and Japan (76–78), countries where the incidence of breast cancer is relatively low (79,80). Therefore, it is quite plausible that isoflavones could prevent breast cancer by antagonising oestrogen action. Salvo et al. reported that treatment with

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**Fig. 4.** (Colour online) Hypothetical anti-inflammatory mechanism of glyceollins. Pro-inflammatory agents such as lipopolysaccharides (LPS) interact with Toll-like receptors (TLR) and relay signals to activate NF-κB, which acts as transcriptional activator to induce several pro-inflammatory mediators. Glyceollins seem to inhibit both expression and nuclear translocation of NF-κB, and thereby suppress LPS-induced inflammation. ARE, antioxidant response elements; ERK, extracellular-regulated protein kinase; IKK, inhibitor of NF-κB; MCP, monocyte chemoattractant protein-1.
Soyabean glyceollins and human health

Glyceollin suppressed E2-stimulated tumour growth of MCF-7 cells (−53.4%) and BG-1 cells (−73.1%) in ovariectomized athymic mice (15). These tumour inhibitory effects corresponded with significantly lower E2-induced progesterone receptor expression in the tumours, suggesting that the glyceollin mixture may be functioning as selective ER modulators, selectively antagonising ER function in a tissue type-specific manner (14, 15). In contrast to these reports, our data consistently suggested the oestrogenic activity of glyceollins, as shown in the E-screen assay and in the enhanced proliferation of MCF-7 cells carrying ER, and the increased expression of pS2, a typical biomarker for oestrogenic activity (11). Therefore, we speculate that like isoflavones, glyceollins appear to exert both oestrogen-like and anti-oestrogenic activities depending on the physiological condition. That is, glyceollins could act as oestrogen agonists under conditions that are lacking oestrogen, as could be seen in post-menopausal women. Conversely, these compounds could act as anti-oestrogens in the presence of endogenous oestrogen, and may also compete with oestradiol for binding to ER.

Meanwhile, glyceollines were found to inhibit the proliferation and cause the apoptosis of murine hepatoma cells (W-K Kim and J-S Lim, unpublished results). This has nothing to do with the oestrogenic activity of the compounds, but it may be associated with their inhibitory activity on the cell signalling pathway related to apoptosis. In particular, apoptotic activity of the compounds is believed to be associated with their stimulation of the c-Jun N-terminal kinase signalling pathway because cytochrome c release, increased by glyceollins, was suppressed by c-Jun N-terminal kinase inhibitor (W-K Kim and J-S Lim, unpublished results). Cytochrome c release from the mitochondria is known to be essential for the activation of caspase 3, one of the terminator enzymes executing the apoptotic process.

**Anti-atherosclerosis**

Due to their antioxidant properties (12), glyceollins may have an effect on preventing and/or treating atherosclerosis. In addition, glyceollins inhibited LPS-induced inflammation in macrophage cells and suppressed the proliferation and migration of human aortic smooth muscle cells, suggesting that the compounds had potential to retard the atherosclerotic process (60, 81). However, currently there is no direct evidence supporting anti-atherosclerotic effect in human subjects.

**Other beneficial effects**

**Skin whitening effect**

Melanin is a dark pigment produced by melanocytes and plays an important role as a natural protection agent of skin from UV light. However, the accumulation of over-produced melanin causes serious dermatological disorders such as melasma, freckles, post-inflammatory melanoderma, and solar lentigo (82, 83). Melanogenesis has been reported to be controlled by not only tyrosinase but also several factors including cytokines, growth factors, microphthalmia-associated transcription factor, melanocortins (α-melanocyte-stimulating hormone/MCR-1 and β-melanocyte-stimulating hormone), adrenocorticotropic hormone β-endorphin, catecholamines, acetylcholine, corticosteroids and oestrogens with their specific receptors (84).

Glyceollin inhibited tyrosinase and tyrosine related protein-1 expression. Additionally, glyceollin effectively inhibited intracellular cAMP levels in B16 melanoma cells stimulated by α-melanocyte stimulating hormone (85).

These results suggest that the whitening activity of glyceollin may be due to the inhibition of cAMP involved in the signal pathway of α-melanocyte stimulating hormone in B16 melanoma cells (85). **Insulinotropic action**

Park et al. hypothesised that glyceollins play an important role in glucose homeostasis by regulating glucose utilisation in adipocytes and improving β-cell function and survival (86). Glyceollins increased insulin-stimulated glucose uptake in 3T3-L1 adipocytes without affecting the PPARγ agonist (86). The compounds also reduced TAG accumulation in adipocytes. In addition, glyceollins slightly promoted glucose-stimulated insulin secretion without palmitate treatment in MIN6 cells, and they potentiated insulinotropic actions in the presence of 500 μM of palmitate used to induce β-cell dysfunction. This insulinotropic action might be associated with decreased β-cell apoptosis through the attenuation of endoplasmic reticulum stress, as assessed by mRNA levels of X-box binding protein-1, activating transcription factor-4 and -6 and C/EBP homologous protein. Glyceollins also increased glucagon-like peptide-1 secretion, resulting in insulinotropic actions in enterocortocine cells (86). These data suggest that glyceollins help normalize glucose homeostasis by potentiating β-cell function and survival and improving glucose utilisation in adipocytes.

**Conclusion**

Although some studies implicated the potential health benefits of glyceollins, there is not yet solid evidence that the compounds have significant pharmacological and/or physiological effects in human subjects. In particular, it is not understood how well they are absorbed in the digestive tract. Unlike isoflavones that are mainly present in glycose form in the soyabean and absorbed in large intestine after being metabolised by intestinal microflora, glyceollins, which are present in aglycone form in elicited soyabean and more hydrophobic than aglycone forms of isoflavones, are more likely to be better absorbed than soya isoflavones. However, it remains to be determined whether the compounds can be absorbed in the small intestine by passive diffusion and if they require any transporter for absorption. In relation to their bioactive function, glyceollins are just starting to draw attention because they may pose potential preventive actions from some chronic diseases, such as cancer, and merit further animal and human studies to evaluate their dietary and/or medical usefulness.
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Soyabeans glyceollins and human health

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