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SHORT COMMUNICATION article

Effect of dietary flavonoids on amine incorporation activity of transglutaminase 2 enzyme

Mohamed E. Alshintari [1 *], **Philip L.R. Bonner** [2] and **Alan J. Hargreaves** [2]

[1] Libyan Biotechnology Research Center, Tripoli, Libya, [2] School of Science and Technology, Nottingham Trent University, Nottingham, United Kingdom
[*]Author to whom correspondence should be addressed

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Abstract: Transglutaminase 2 (TG2) is an inducible transamidating acyltransferase that catalyzes Ca^{2+}-dependent protein modifications. TG2 enzyme disruption has been implicated in several different disease processes and disorders such as Huntington and Parkinson's diseases, and cancers such as breast, ovarian and pancreatic cancers. Coeliac disease (CD) is the one disease state which TG2 activity plays a crucial role. To date, a gluten-free diet is the only accepted form of therapy for CD. Because of the important role of TG2 enzyme in the initiation of CD, therefore, this study was aimed at the identification of TG2 inhibitors from natural sources, as a potential intervention in CD therapy. Competitive amine inhibitors are the most widely used TG2 inhibitors because they are commercially available, chemically stable and relatively non-toxic in living systems. The natural products chosen for this study were dietary flavonoids. Flavonoids were extracted from different food samples. The flavonoid food extracts were subjected to the TG2 activity assays to examine their effect on the enzyme activity. The assays were carried out under optimal conditions of pH, Ca^{2+} and with N, N-dimethylcasein (acyl-donor) or casein (acyl-acceptor) as acyl-donor substrates and biotin cadaverine (acyl-acceptor) or TVQQEL as (acyl-donor) substrates. TG activity was measured by two different microplate assays, a Biotin-labeled cadaverine incorporation assay and Biotin-labeled peptide cross-linking assay. In the TG2 amine incorporation activity, all of food extracts display a significant inhibition effect towards the human recombinant transglutaminase2 (hrTG2) and guinea pig transglutaminase 2 (gplTG2) (20%-50% of inhibition). While in the TG2 cross-linking activity, the majority of food extracts displayed an inhibition effect on the gplTG2 cross-linking activity (50%-70% inhibition) but only the strawberry and kale extracts showed an effect on hrTG2 activity (40%-50% inhibition). The inhibition of TG2 activity can be considered as a potential therapeutic target in the treatment of CD.

Introduction

Transglutaminase 2 (TG2) is a multi-domain, multi-functional enzyme that posttranslational modifies proteins by catalysing the formation of intermolecular isopeptide bonds between glutamins and lysine side-chains. TG2 is widely distributed in the tissues and cell types; it is mainly a cytosolic protein but also has been shown to be present in the nucleus and on the plasma membrane of cells [1]. TG2 exhibits a number of functions with different molecules acting as substrates for this enzyme such as proteins, amines and water. TG2 disruption

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has been implicated in many diseases and disorders processes as Huntington's and Parkinson's diseases [2], and different cancers as breast, ovarian and pancreatic cancers [3]. In addition, during the diagnosis of type 1 diabetes mellitus, there is about 4.0% to 17.0% positive tests for TG2 antibodies (anti-tTG) [4, 5]. Coeliac disease (CD) is a disease state which TG2 activity plays a crucial role by mediating the deamidation of glutamine in 33mer gliadin peptide initiating chain of events leading to the destruction of small intestinal villi [6]. To date, the only accepted form of therapy for CD is the avoidance of gluten through dietary control [7]. In recent years, the high quality of gluten-free products has improved, however, the adherence to these products depends on different individual and environmental factors [8]. One study reported in the UK, that diet adherence in adult CD patients ranged from 36.0% to 96.0% [9]. It has been established that the CD patients who keep to a gluten-free diet shown improvements in vitamin deficiencies and abnormalities linked with the disease [10]. However, the majority of CD patients fail to adhere to a gluten-free diet. In some parts of the world, especially in developing or third world countries, gluten-free products are not readily available [11]. In addition, a gluten-free diet is usually more expensive. Therefore, it is challenging to adhere to a gluten-free diet for life and alternative therapeutic strategies are warranted for sufferers of CD [11]. The critical role of TG2 in CD makes inhibition of TG2 activity a potential therapeutic target. Natural products such as garlic and milk present less of toxicity problem, as they are ingested by many people, without adverse side effects. The existence of TG2 inhibitors has been found in several natural products such as garlic [12] and milk [13, 14], but their structures have not been revealed yet. It is, therefore, important to screen natural products with known medicinal attributes in order to assess them as potential TG2 inhibitors and moderators of CD. Natural products chosen for this study were dietary flavonoids due to their promising biological activity. Flavonoids have been reported in the literature to have different biological activities such as antioxidant, anti-inflammatory and protective against diseases such as cancers, diabetes, cardiovascular diseases and atherosclerosis [15, 16, 17, 18]. Certain flavonoids have been shown to moderate the esterase activity of trypsin and acetylcholinesters [19]. Screening these natural compounds to moderate TG2 activity is appropriate because TG2 possesses esterase activity [20]. Since the activity of TG2 is crucial to the initiation of CD, therefore decreasing the activity of TG2 in CD patients will supply a potential treatment choice. TG2 inhibitors targeted to reduce the TG2 activity have been considered potential therapeutic tools in the treatment of CD [21]. These natural products (flavonoids) were used in this study to evaluate their effect on TG2 activity as a potential intervention in treating CD. To the best of our knowledge, there are no published studies describing the effects of flavonoids on TG2 activity using transamidating or deamidating assay. Therefore, the aim of this work was to study the effect of dietary flavonoids on the amine incorporating the activity of TG2 as a potential method to moderate the initiation of CD and to evaluate any influence the dietary flavonoids can have on the enzyme.

Materials and methods

General laboratory reagents: Pure flavonoids (kaempferol, luteolin, epicatechin, apigenin, myricetin, cyanidin, morin, naringin, hesperetin, hesperidin, quercetin, catechin and taxifolin) were obtained from sigma-Aldrich (UK). Casein, N', N'-dimethylcasein and ExtrAvidin-horseradish peroxidase (HRP) were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, UK). The purified human recombinant TG2 with purified guinea-pig liver TG2 was obtained from Zedira GmbH (Darmstadt, Germany). Biotin cadaverine (N-(5 aminopentyl) biotinamide) was purchased from invitrogen (Loughborough, UK).

Transglutaminase transamidation activity assays: Biotin-labeled cadaverine incorporation assay: The amine incorporating activity of TG2 was measured by biotin-cadaverine incorporation into N, N' dimethylcasein. The assay was performed as described previously by Slaughter and others [22] with modifications [23]. Briefly, 96-well microtitre plates were coated overnight at 4°C with 250 µl of N′, N′ dimethylcasein (10 mg ml^{-1} in

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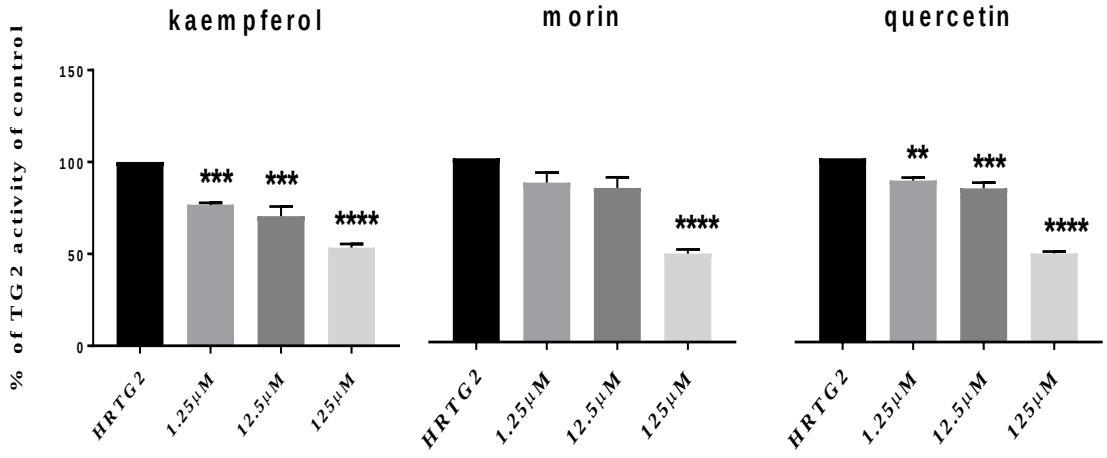
100 mM Tris-Hcl, pH 8.0). The plate was washed twice with distilled water and blocked with 250.0 µl of 3.0% (w/v) BSA in 100.0 mM Tris-HCl, pH 8.0 and incubated for 1.0 hr at a room temperature with gentle agitation. The plate was washed twice with distilled water before the application of 150.0 µl of either 6.67 mM calcium chloride (required for enzyme activity) or with 13.3 mM EDTA (used to detect background TG activity) assay buffer containing 225 µM biotin cadaverine (a widely used substrate to monitor TG amine incorporating activity) and 2.0 mM mercaptoethanol. The reaction was started by the addition of 50.0 µl of samples or positive control (50.0 ng/well of hrTG2) and negative control (100.0 mM Tris-HCl, pH 8.0). After incubation for 1.0 hr at 37°C, plates were washed as before. Then, 200.0 µl of 100.0 mM Tris-HCl pH 8.0 containing 1.0%w/v. BSA and ExtrAvidin®-HRP (1: 5000 dilution) were added to each well and the plate was incubated at 37°C for 45 min then washed as before. The plate was developed with 200.0 µl of freshly made developing buffer (7.5 µg per ml 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 0.0005%v/v, H_2O_2 in 100 mM sodium acetate buffer, pH 6.0) and incubated at room temperature for 15 min. The reaction was terminated by adding 50.0 µl of 5.0 M sulphuric acid and the absorbance read at 450 nm. One unit of TG2 activity was defined as a change in absorbance at 450 nm per minute.

Statistical analysis: For group comparison, one-way ANOVA followed by Dunnet's multiple comparison test and two-way ANOVA were performed for parametric and nonparametric data, respectively by using GraphPad Prism® software (GraphPad Software, Inc., USA). All sets of data were based on a minimum of three separate experiments and expressed as mean ± standard error of the mean (SEM) and p-value<0.05 was considered as statistically significant.

Results and discussion

The aim of this research was to evaluate the effect of flavonoids on the activity of TG2 as a potential treatment for CD. Sodium deoxycholate 0.5% (w/v) was used as a means to disperse thirteen flavonoids and they were subjected to an initial screen using the biotin cadaverine assay to measure the transamidating activity of TG2 [22]. Previous work at NTU has shown that TG2 is unaffected by sodium deoxycholate and this detergent is similar in structure to the detergents released by the bile duct in the digestion of food [24], which makes this more relevant to CD

Figure 1: Effect of flavonoids dissolved in sodium deoxycholate on the amine incorporation activity of hrTG2



Pure flavonoids (1.25, 12.5 & 125 µM) dissolved in sodium deoxycholate 0.5% (w/v) were then incubated with 5 µg per ml hr TG2, The TG2 activity was measured according to the method [22]. The data points represent mean±S.E.M from three independent experiments (**p<0.01, ***P<0.001 & ****P<0.0001). All the values are as compared to the control untreated TG2.

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The assessment of potential therapeutics to treat CD is typically achieved on simplified biological systems e.g. enzyme assays. Although, Stuven and others [25] have reported that some *in vitro* and *in vivo* models such as intestinal cell lines and human mucosal biopsy cultured have helped in understanding the pathophysiology of CD not all those models have been applied in testing new therapeutics for the disease. Currently, adherence to a gluten free diet represents the only therapy for CD that has been confirmed to alleviate the symptoms and prevent potential complications [26, 27]. The crucial role of the activity of TG2 in the initiation of CD has introduced a new approach to the treatment of this disease by exploring substances that have an inhibition effect on the activity of TG2. The side effects of using chemical TG2 inhibitors has directed this research towards the screening of TG2 inhibitors from natural sources. The natural products chosen for this study are dietary flavonoids due to their potent biological activity.

Thirteen pure flavonoids were selected to cover most of the flavonoid's subclasses, they divided into a different class such as flavonols (e.g., quercetin, kaempferol, morin, epicatechin, and myricetin,) flavones (e.g., apigenin, and luteolin), flavanones (e.g., hesperetin, and naringin), anthocyanin (cyanidin chloride), catechins (catechin and epicatechin), flavonolignans (taxifolin). All 13 flavonoids were dispersed in sodium deoxycholate 0.50 % (w/v) and applied to the TG2 assays in the final concentrations of 1.25, 12.5 and 125 µM (**Figure 1**). The reason for using sodium deoxycholate bile detergent was to try to mirror the environment of the human lower intestine where TG2 is located and initiates CD. As the bile salts occur in the duodenum of the small intestine which is the main part of the intestine where lipid digestion happens, bile salts help in emulsifying the fatty foods adsorbing on oil-water interfaces [28]. This would also help to solubilize the flavonoids in food released by maceration in the mouth and digestion in the stomach. The screening of the TG2 modulatory effects was done using a biotin cadaverine incorporation assay [22]. This assay is the most commonly used assay to study the transamidation activity of transglutaminases.

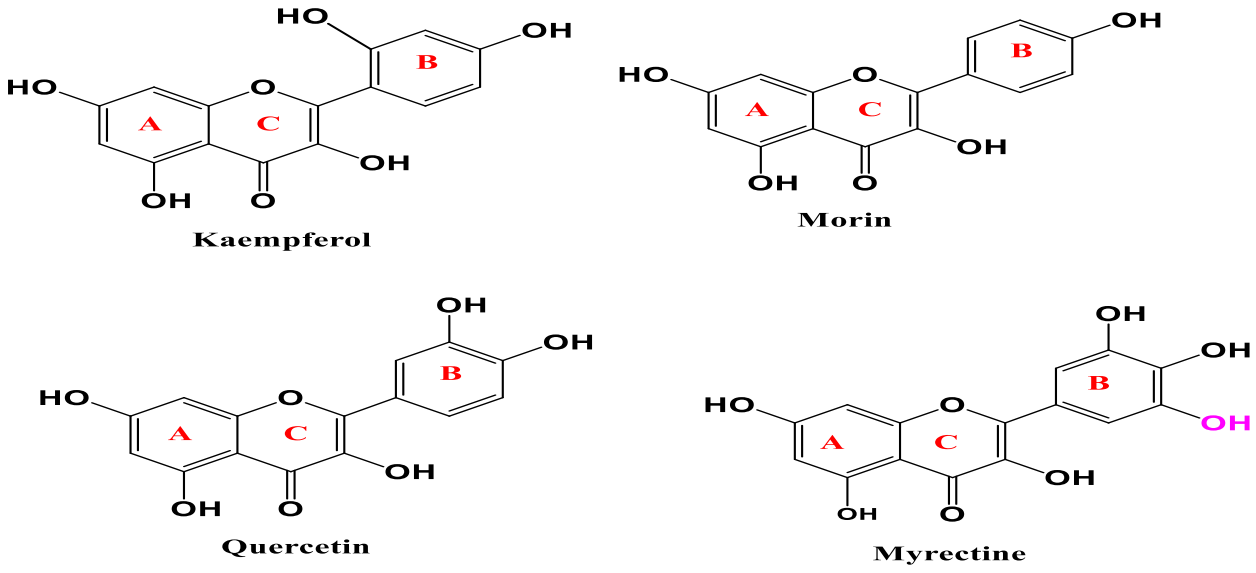

Figure 2: structures of flavonol subclasses with the key hydroxyl group

In **Figure 1**, three flavonoids (kaempferol, morin and quercetin) demonstrated inhibitory behavior against hrTG2 amine incorporation activity. At 125 µM, the reduction in activity was around 50.0% for the three flavonoids against hrTG2 activity. Those three flavonoids have a similar chemical structure and they are in the same subclass, which is the flavonol. The detailed structures of the flavonoids differ structurally in the position of their hydroxyl group. The unexpected finding is that myricetin which is flavonol has moderate inhibitory effect which is statistically insignificant, as the rings A and C are same for the 4 flavonols used, but

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the main difference in myricetin structure is that it contains an OH group at position 5 (**Figure 2**). As mentioned, in the literature, the B-ring hydroxyl configuration is the most significant determinant of the biological effects of the flavonoids in general [29, 30]. Therefore, it was hypothesized that the affinity binding between flavonoids and transglutaminase was related to the position of the hydroxyl group within the flavonoids. In the presence of calcium ions, TG2 exposes hydrophobic areas within its structure [31]. This suggests that the interaction between the flavonol may rely on the presence of calcium ions. If the flavonols disturb the binding of calcium to TG2, preventing the enzyme from completely opening up this, in turn, could alter the substrate from binding to the active site cys377. However, future studies are suggested to test those flavonoids on CD human biopsies to elucidate their effect on the activity of TG2.

Conclusion: This study shows kaempferol, morin and quercetin that belong to flavonol subclass of flavonoids at this concentration using the amine incorporation assay have inhibitory effect on the amine incorporation activity of hrTG2.

Author's contribution: MEA, PLB & AJH conceived, design the study and drafted the manuscript. MEA & PLB collected and analysed data. All authors approved the final version of the manuscript and agreed to be accountable for its contents.
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Ethical issues: Including plagiarism, informed consent, data fabrication or falsification and double publication or submission were completely observed by the authors.
Data availability statement: The raw data that support the findings of this article are available from the corresponding author upon reasonable request.
Author declarations: The authors confirm that all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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