

## Contribution of Chlorogenic Acids to the Inhibition of Human Hepatic Glucose-6-phosphatase Activity in Vitro by Svetol, a Standardized Decaffeinated Green Coffee Extract

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Glucose-6-phosphatase (Glc-6-Pase) is a multicomponent system that exists primarily in the liver and catalyzes the terminal step in gluconeogenesis and glycogenolysis. Several studies have attempted to identify synthetic or natural compounds that inhibit this enzyme complex for therapeutic use in regulating blood glucose and type 2 diabetes. For this paper an in vitro structure–activity relationship study of several natural chlorogenic acids was conducted, and the active components of the natural decaffeinated green coffee extract Svetol were identified. Glucose-6-phosphate (Glc-6-P) hydrolysis was measured in the presence of Svetol or chlorogenic acids in intact human liver microsomes. Svetol significantly inhibited Glc-6-P hydrolysis in intact human liver microsomes in a competitive manner, and it was determined that chlorogenic acids (caffeoylquinic acids and dicaffeoylquinic acids) were the chief compounds mediating this activity. In addition, the structure–activity analysis showed that variation in the position of the caffeoyl residue is an important determinant of inhibition of Glc-6-P hydrolysis. This inhibition by Svetol contributes to its antidiabetic, glucose-lowering effects by reducing hepatic glucose production.

**KEYWORDS:** Decaffeinated green coffee extract; Svetol; chlorogenic acids; glucose-6-phosphatase

### INTRODUCTION

Coffee is one of the world's most popular beverages. The numerous beneficial health effects of coffee consumption have received significant scientific attention recently, because the results of epidemiological and experimental studies suggest that drinking coffee regularly helps prevent several chronic diseases, especially metabolic disorders, such as type 2 diabetes (1–3). Extensive investigations have revealed that most of these effects are attributed to the chlorogenic acids (CGAs) in coffee (4).

Green (or raw) coffee is a significant source of CGAs in nature (5–12 g/100 g) (5). In green coffee, the primary CGAs are 3-, 4-, and 5-caffeoylquinic acids (CQA) and 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids (diCQAs). Caffeoylferuloylquinic acids (FQAs) are minor CGA compounds also found in green coffee (6).

Weight loss is linked to the capacity of coffee to prevent type 2 diabetes; one prospective epidemiological study found that the consumption of coffee lowered the risk for diabetes, but only in participants who had lost weight (7). Two clinical studies distinguished the effects of caffeinated and decaffeinated coffee (8, 9) and suggested that there are noncaffeine compounds in coffee, such as CGAs, that enhance glucose tolerance and insulin sensitivity. In a recent study, Dellalibera et al. showed that

chronic consumption of Svetol, a decaffeinated green coffee extract that has high CGA content, decreased weight and increased lean/fat ratios in overweight volunteers (10).

One proposed mechanism of such effects is inhibition of glucose-6-phosphatase (Glc-6-Pase; EC 3.1.3.9), which forces lipids to be used as energy to compensate for the decrease in glucose release from glycogenolysis. Liver Glc-6-Pase is a multicomponent system that catalyzes the final step of hepatic glucose production, that is, the hydrolysis of glucose-6-phosphate (Glc-6-P) from glycogen breakdown or gluconeogenesis. The active site of Glc-6-Pase is in the lumen of the endoplasmic reticulum (ER); therefore, transporter proteins are required to shuttle Glc-6-P into this compartment and expel glucose and phosphate (11).

Glc-6-P hydrolysis appears to involve a Glc-6-P translocase (Glc-6-PT), which transports Glc-6-P across the ER, and a catalytic subunit, located on the luminal side of the ER (12). 5-CQA is a highly specific inhibitor of Glc-6-Pase (13), and several analogues of 5-CQA that effect greater inhibition (e.g., S3483) have been synthesized; they increase the latency of Glc-6-Pase by reducing its activity in intact microsomes or in the intact ER in situ (14).

The aim of this study was to determine the inhibitory activity of Svetol, a decaffeinated green coffee extract that has a specific ratio between 5-CQA and other CGAs, on Glc-6-P hydrolysis in intact human liver microsomes. In addition, we report the

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**Table 1.** Chlorogenic Acid Content in Svetol<sup>a</sup>

compound	typical content in Svetol (%)	sample (%)
3-CQA	6.53 ± 0.54	6.61
4-CQA	7.31 ± 0.43	7.66
5-CQA	14.72 ± 1.07	13.83
3,4-diCQA	3.57 ± 0.54	3.34
3,5-diCQA	2.38 ± 0.08	2.38
4,5-diCQA	4.22 ± 0.15	4.15
3-FQA	1.28 ± 0.11	1.30
4-FQA	1.50 ± 0.23	1.87
5-FQA	3.39 ± 0.36	3.39
3,4-caffeoylferuloylquinic acid	0.67 ± 0.06	0.77
3,5-caffeoylferuloylquinic acid	0.30 ± 0.02	0.31
4,5-caffeoylferuloylquinic acid	0.30 ± 0.29	0.81
caffeoyltryptophan	1.00 ± 0.80	1.23

<sup>a</sup> Typical content in five industrial batches (mean ± SD) and the sample used in this study (batch 252/10/A9; Naturex). Data are expressed as 5-CQA equivalents.

inhibitory effects of a series of structurally related compounds in Svetol, such as caffeoylquinic acids and dicaffeoylquinic acids.

## MATERIALS AND METHODS

**Chemicals.** Svetol (ref. GA501071, batch 252/10/A9) was supplied by Naturex (Avignon, France). Ascorbic acid, cacodylic acid, D-glucose 6-phosphate sodium salt, 5-CQA, ammonium molybdate tetrahydrate, potassium phosphate, and sodium dodecyl sulfate were purchased from Sigma (Saint Quentin Fallavier, France). Pooled human liver microsomes were obtained from BD Biosciences (Le Pont le Claix, France) and stored at -80 °C until use. Standards for caffeoylquinic and dicaffeoylquinic acids were supplied by Chengdu Biopurify Phytochemicals LTD (Chengdu, China).

**HPLC Analysis of CGAs in Svetol.** Analysis of CGAs in Svetol was performed using the HPLC—diode array detector gradient system (Agilent 1100 series). The chromatographic analysis was conducted with a Zorbax Eclipse XDBC<sub>18</sub> 4.6 × 50 mm column (1.8 μm). The solvents were H<sub>2</sub>O/acetic acid (96:4, v/v) as solvent A and methanol/acetonitrile/acetic acid (60:10:2, v/v/v) as solvent B, at a flow rate of 1.2 mL/min with the following gradient: 5% B (0–1 min), 5–15% B (1–4 min), and 15–70% B (4–25 min).

Quantification was performed at optimal wavelengths (330 nm) for the CGAs during chromatographic separation. Samples were filtered (0.45 μm), and 2 μL was injected directly. The standard deviation for three analyses of the same sample was <5% for all compounds.

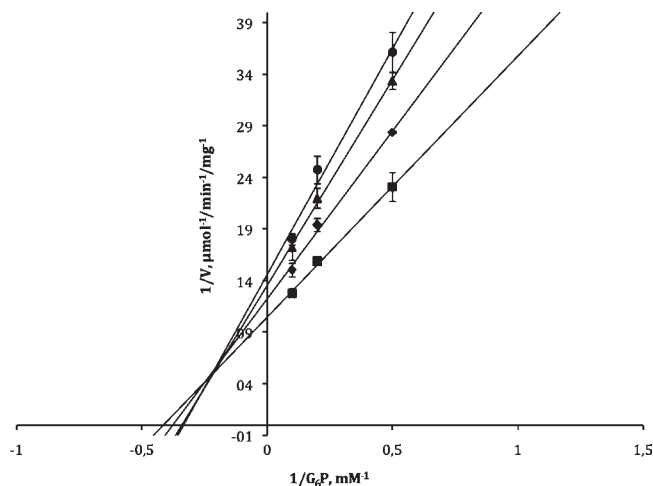
**Measurement of Glc-6-Pase Activity in Microsomes.** Microsomal Glc-6-Pase activity was measured on the basis of the rate of release of phosphate under the assay conditions that were described by Wallert et al. (15). The enzyme assays were performed at 37 °C in a final volume of 320 μL, containing 100 mM cacodylic acid, pH 6.5, and concentrations of the substrate Glc-6-P ranging from 2 to 10 mM.

The reaction was started by adding intact microsomes and was stopped with the addition of 3.2 mL of colorimetric reagent [9 volumes of molybdate (0.42% ammonium molybdate in 1 N H<sub>2</sub>SO<sub>4</sub>), 2 volumes of 5% SDS, and 1 volume of 10% ascorbic acid, freshly prepared and stored on ice for a maximum of 6 h]. All samples were incubated for 30 min at 45 °C, and the absorbance of the phosphate—molybdate complex was measured at 820 nm.

Microsomal intactness was quantified by measuring Man-6-Pase activity (16). In a preliminary study, Glc-6-Pase activity in intact human liver microsomes was determined on the basis of microsomal protein concentration and incubation time to obtain optimal experimental conditions, that is, 100 μg of microsomal proteins and 5 min of incubation (data not shown).

**Preparation of Test Compounds.** Stock solutions of test compounds were prepared in ultrapure water (pH 6.5) and diluted with assay reagent to the final concentrations.

**Data Analysis.** Enzymatic activity was expressed as micromoles of phosphate released per minute per milligram of protein. Results were expressed as means ± standard deviation (SD) of three independent experiments. Percentage of inhibition of Glc-6-Pase activity was calculated



**Figure 1.** Double-reciprocal or Lineweaver–Burk plot of inhibition of Glc-6-P hydrolysis by Svetol in human liver microsomes. Reaction mixture (pH 6.5) contained 2.0–10.0 mM Glc-6-P with 0 (■), 0.2 (◆), 0.4 (▲), or 0.6 mM (●) Svetol. Each bar represents the mean ± SD of three measurements.

by dividing the initial rate of reaction in microsomes that were treated with individual compounds by the initial rate of reaction in untreated microsomes. The contribution of individual CGAs to total inhibition by Svetol was calculated on the basis of their concentrations in Svetol and their own inhibition values by dividing the percentage of inhibition of each CGA by the percentage of inhibition of 0.6 mM total CGAs from Svetol.

Statistical analysis was performed using an ANOVA test followed by a post hoc Tukey test under a normality assumption (Shapiro Wilk) or Kruskal Wallis nonparametric test followed by Bonferroni adjusted Mann–Whitney test otherwise;  $p < 0.05$  was considered to be significant.

## RESULTS

**Determination of CGA Composition in Svetol.** Svetol is a commercial unroasted and decaffeinated green *Coffea canephora* extract, standardized to contain >45% CGAs and >10% 5-CQA. **Table 1** lists the average contents and standard deviations of CGAs in five industrial batches that have been quantified as 5-CQA equivalents (batches 252/10/A9, H43/17/A8, H37/40/A9, 327/23/A9, and 324/44/A9; Naturex). The sample that we used contained high levels of total CGAs (47.66% of dry weight), with a specific ratio (0.3) between 5-CQA and total CGAs.

**Inhibition of Glc-6-Pase Activity by Svetol.** We tested whether Svetol could inhibit the hepatic Glc-6-Pase system by measuring enzymatic activity in human liver microsomes. These experiments were conducted with or without Svetol at final CGA concentrations of 0.2, 0.4, and 0.6 mM. The effect of Svetol on Glc-6-Pase activity was tested as a function of Glc-6-P substrate concentration (2–10 mM).

The double-reciprocal plots in **Figure 1** show that Svetol decreased  $V_m$  values in a dose-dependent manner, but  $K_m$  was unchanged. By Michaelis–Menten kinetics, Svetol inhibited Glc-6-P hydrolysis in human liver microsomes in a significant and competitive manner (**Table 2**), which is consistent with previous studies of 5-CQA in rat liver microsomes (13).

**Inhibition of Glc-6-Pase Activity by CGAs.** Because studies have demonstrated that 5-CQA and its synthetic analogues inhibit Glc-6-Pase (13, 14, 17), we investigated whether other CGAs in coffee possess the same functions as 5-CQA. Therefore, the selected CQAs and di-CQAs were studied at their respective concentrations in Svetol (0.6 mM total CGAs) with 2 mM Glc-6-P (i.e., below the apparent  $K_m$ ) to facilitate detection of putative competitive inhibitors.

The percentages of inhibition of Glc-6-P hydrolysis of each compound and its contribution to the inhibitory activity of Svetol are shown in **Table 3**. Of the three CQAs in Svetol, 4-CQA inhibited Glc-6-P hydrolysis to the greatest extent (14% inhibition). In addition, 4-CQA contributed 40% of the inhibitory effect of Svetol.

4,5-diCQA effected similar inhibition as 4-CQA (13% inhibition) and contributed 35% of the inhibitory effect of Svetol.

We also examined inhibition by mixtures of CQAs and diCQAs (at their respective proportions in Svetol). When all CQAs and diCQAs were tested separately, we observed similar inhibition (approximately 20%). Moreover, when combined, the

inhibition of Glc-6-P hydrolysis by 0.6 mM total CGAs from Svetol (36%) was recovered (35%), suggesting that no other compounds participate in Svetol-mediated inhibition.

## DISCUSSION

Starvation and diabetes cause a 2–3-fold increase in Glc-6-Pase activity in the liver (18, 19), making this enzyme system a potential target for nutritional compounds that are intended, for example, to suppress hepatic glucose production to ameliorate diabetic hyperglycemia. Our study details the inhibition of Glc-6-P hydrolysis in intact human liver microsomes by Svetol; Svetol was found to be a competitive inhibitor of Glc-6-Pase in a dose-dependent manner.

Svetol is a decaffeinated green coffee extract that has a high CGA content and a specific ratio between CQAs and diCQAs. In this study, we showed that CQAs and diCQAs, at their respective concentrations in Svetol, have inhibitory effects similar to those of Svetol, suggesting that they are the compounds that are solely responsible for Svetol activity.

Our structure–activity analysis showed that variation in the position of the caffeoyl residue is important for the inhibition of Glc-6-P hydrolysis. Notably, two compounds (3-CQA and

**Table 2.** Kinetic Parameters of Glc-6-Pase in Human Liver Microsomes<sup>a</sup>

condition	$V_{\max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ of protein)	$K_M$ (mM)
control	0.095 $\pm$ 0.002	2.41 $\pm$ 0.33
Svetol (0.2 mM)	0.082 $\pm$ 0.003*	2.65 $\pm$ 0.18
Svetol (0.4 mM)	0.074 $\pm$ 0.007*	2.96 $\pm$ 0.47
Svetol (0.6 mM)	0.068 $\pm$ 0.001*	2.99 $\pm$ 0.25

<sup>a</sup>Data are expressed as mean of triplicate  $\pm$  SD. \* indicates values that are significantly different from control ( $P < 0.001$ ).

**Table 3.** Structure, Percentage of Inhibition, and Contribution of Chlorogenic Acids to Glc-6-Pase Inhibition by Svetol<sup>a</sup>

Compound	Structure	Concentration tested ( $\mu\text{M}$ )	Percentage inhibition of G6Pase	Contribution (%)
3-CQA		110	0	0
5-CQA		160	9.2 $\pm$ 1.4	25
4-CQA		120	14.4 $\pm$ 1.2	40
3,4-diCQA		33	6.9 $\pm$ 4.3	19
3,5-diCQA		20	0	0
4,5-diCQA		38	12.8 $\pm$ 2.6	35
All CQA			18.1 $\pm$ 5.5	50
All di-CQA			22.7 $\pm$ 1.5	62
All CQA + all di-CQA			34.8 $\pm$ 4.0	96

<sup>a</sup>Each compound was tested at its naturally occurring concentration in 0.6 mM Svetol.

3,5-diCQA) were apparently ineffective in suppressing Glc-6-P hydrolysis, and greater inhibition was achieved with 4-CQA and 4,5-diCQA. This result suggests that the caffeoyl residue at position 3 has an unfavorable effect, whereas at position 4, it appears to be beneficial.

The observed 36% inhibition by Svetol should contribute to its antidiabetic, glucose-lowering effects by reducing hepatic glucose production. On the basis of these and other published results (13, 14, 17), we propose a mechanism by which Svetol acts. In combination with diet, it inhibits glucose absorption from the small intestine (20). Furthermore, by inhibiting Glc-6-Pase activity, Svetol could limit the release of glucose from glycogen into general circulation and prevent insulinemia, as reported in vivo with the chlorogenic acid derivative S3483 (21, 22).

This mechanism, however, depends on the bioavailability of chlorogenic acid and its isomers. In rats, Lafay et al. showed that 5-CQA is not hydrolyzed in the stomach or small intestine but is absorbed in the stomach in its intact form and as caffeic and (iso)ferulic acids in the small intestine (23). Recently, Farah et al. (24) confirmed that CQA and diCQA are differentially absorbed and metabolized throughout the entire gastrointestinal tract. In addition, Farah et al. (24) also provide evidence that urination is not a major excretion pathway of intact CGA compounds and their metabolites.

In conclusion, our findings engender new research opportunities because they demonstrate the importance of the position of the caffeoyl residue in the inhibition of Glc-6-Pase by CGAs. Because few structure–activity relationship studies on the inhibition of Glc-6-Pase by synthetic analogues of 3-CQA exist (14, 17), additional complementary studies will provide new tools for investigating the molecular structure and function of the Glc-6-Pase system.

#### ABBREVIATIONS USED

CGAs, chlorogenic acids; CQAs, caffeoylquinic acids; diCQAs, dicaffeoylquinic acids; FQAs, feruloylquinic acids; Glc-6-Pase, glucose-6-phosphatase; Glc-6-P, glucose-6-phosphate; ER, endoplasmic reticulum; Glc-6-PT, glucose 6-phosphate translocase.

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