Pressurized Hot Water Extraction and Enzyme-Catalyzed Conversion of Polyphenolic Glycosides in Onion Waste

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Pressurized hot water (PHW) has been used to quantitatively extract polyphenolic glycosides from onion waste. Of particular interest is quercetin, a potent antioxidant and anti-neurodegenerative compound, which is found in both red and yellow onions. Polyphenolic glycosides were extracted from onion waste within 15 min of extraction using water at 120°C and 5 MPa. Quercetin glycosides in the onion extracts were converted to quercetin and sugars by different types of recombinantly expressed thermostable β-glucosidases. These enzymes maintain activity at temperatures around 90°C, and are therefore ideal to use in combination with hot water extraction. Our results show that such thermostable glucosidases are able to convert quercetin glycosides to active quercetin within less than 10 min of reaction in 90°C water. Analysis of onion extracts and reaction products was performed by RP-C18 HPLC and UV detection at 350 nm with methanol/water (1:1) with 0.1% formic acid as mobile phase. LC-MS/MS analysis was also performed to identify several different types of glycosidic compounds including the aglycones quercetin and isorhamnetin. In summary, we have developed fast and efficient processes for modifying and quantifying high-value compounds in onion waste.

INTRODUCTION

Antioxidants are compounds with electron scavenging properties that may slow down or prevent the development of cancer [1,2]. Fruits and vegetables are rich in antioxidants [3], for example lycopene in tomatoes, β-carotene in carrots, anthocyanins in grapes and red onions, and quercetin in grapes, apples and onions. Furthermore, several recent studies have shown that different types of natural polyphenols may have neuroprotective effects both in vitro and in vivo [4-6], partly due to their electron scavenging properties. It has for example been shown in a few studies that quercetin may prevent or slow down the development of Alzheimer disease [5,7,8].

Quercetin is a polyphenolic compounds that occurs in vegetables and fruits mainly as different glycosides, although the skin of the fruit/vegetable commonly contains higher amounts of the quercetin aglycone. **Figure 1** shows the chemical structure of quercetin and the two most common glycosides in onion, quercetin-4'-glycoside and quercetin-3,4'-diglycoside. Several research projects have undertaken the isolation and identification of

quercetin and its glycosides in various fruits and vegetables, including onion [9], kale [10], broccoli [11], apple skin [12], green tea [13], and grapes and wine [14,15]. However, most of these studies use simple liquid/solid extraction techniques combined with chemically catalyzed hydrolysis reaction followed by liquid/liquid extraction of quercetin aglycone. These procedures are tedious and require the disposal of organic solvents, such as the commonly used ethyl acetate.

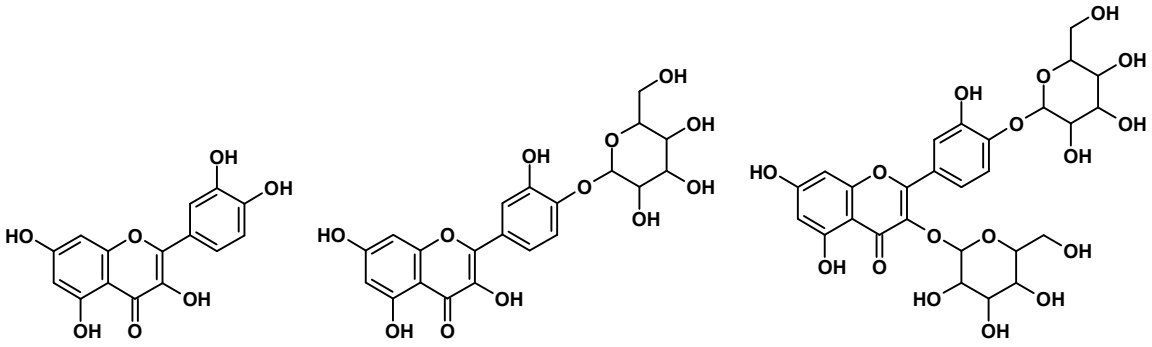

Figure 1. *Chemical structures of quercetin (A), quercetin-4′-glycoside (B) and quercetin-3,4′-diglycoside (C).*

The most environmentally friendly solvent that could be used for extraction of quercetin is water. However, since pure water at ambient condition is too polar to be a good solvent for quercetin, we instead use water at elevated temperatures as a solvent, i.e. subcritical water. It is well-known that the dielectric constant of water decreases significantly as the temperature of the water is increased above its atmospheric boiling point, while applying pressure to maintain water as a liquid [16]. Less polar compounds such as antioxidative compounds [17], polycyclic aromatic hydrocarbons [16], insecticides [18] and essential oils [19] have all been successfully extracted from various plant and soil samples using subcritical water at temperatures between 100 and 374°C.

As mentioned above, flavonoids occur in food as different glycosides, which is also the preferred form for uptake in the human intestine. After uptake, the glycosides are converted to aglycone and free carbohydrates in hydrolysis reactions mainly catalyzed by β-glucosidase and to some extent by lactase phloridzin hydrolase [20]. Hence, in quantitative analysis of flavonoid content in food, β-glucosidase is a useful catalyst for transforming the several different occurring flavonoid glycosides to a single measurable aglycone. Some of these glycosidic compounds are present at concentrations below 1% of the total flavonoids content, which naturally is difficult to quantify.

In this paper, we have evaluated the use of PHW for extraction of quercetin molecular species from onion waste. In addition, a method for hydrolysis of quercetin glycosides to active aglycon and carbohydrates was optimized using two different thermostable β-glucosidases. This paper demonstrates a fully “green” procedure, where the raw material is a biodegradable and renewable agricultural waste (onion waste), the extraction process uses only water as a solvent, and the hydrolysis reaction is catalyzed by enzymes rather than by chemicals or non-renewable catalysts. Furthermore, onion waste is largely produced in Sweden as a byproduct of no value for the producer. After quercetin has been extracted, the onion waste can still be used as animal feed or as an energy source.

MATERIALS AND METHODS

Onion waste was obtained from a local restaurant as well as from a Swedish onion industry. Methanol and formic acid was purchased from Merck (Darmstadt, Germany). Quercetin dehydrate (>98%), morin (puriss p.a.), citric acid monohydrate and disodium hydrogen phosphate were purchased from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water (MilliQ) was used at all times.

The genes encoding the β-glucosidases Bgl1A, and Bgl3B were amplified by PCR using genomic DNA from the hyperthermophile *Thermotoga neapolitana* (DSM strain 4359) as template. Primers were designed from sequence data available at NCBI server (http://ncbi.nlm.nih.gov) under the accession numbers AF039487 (Bgl1A) and Z77856 (Bgl3B), and included introduction of the restrictions-sites *Nde*I and *Xho*I, in the 5´- and 3´-end respectively, for cloning in vector pET22b+ (Novagen, Madison, Wi). *Escherichia coli* strain Tuner(DE3) (Novagen) was used for expression, and both recombinant enzymes were purified to more than 90 % purity by heat-treatment (70°C, 40 min), and immobilized metal ion affinity chromatography, using the vector encoded C-terminal His-tag cloned in frame with the respective gene, as described in more detail in Svensson et al, (manuscript in preparation for J. Mol. Catal. B: Enz.).

Onion waste was cut into small pieces (~1-10 mm in diameter) using a food processor. 4 g of onion samples were weighed into 33-mL stainless steel extraction cells containing a cellulose filter at the bottom. Extractions were performed on a Dionex ASE®-200 pressurized fluid extraction system using water as the only solvent. The pressure was set to 5 MPa and the initial heating was 5 min. Purging between extractions was performed with nitrogen. Collection was performed in 50-mL clear glass vials.

Enzymatic hydrolysis was achieved taking 1.2 mL of onion extract and mixed with 300 µL citrate buffer (0.1M, pH 3, 5 or 7). After heating to desired temperature for 5 min, an initial 100-µL fraction was taken and thereafter the reaction was started by adding the enzyme. The reaction took place during magnetic stirring, and samples of 100 µL were collected during the course of the reaction. To each collected 100 µL fraction, 400 µL formic acid (0.25%), 100 µL morin (50µL/mL, internal standard in methanol) and 400 uL methanol were added. These vials were analyzed by HPLC on a Waters Symmetri C18 column (150×2.1 mm, 3.5µm) using a methanol/water/formic acid (50:50:0.1) mobile phase. 10 µL portions were injected and detection was accomplished at 350 nm.

LC-MS/MS was accomplished using the chromatographic system described above coupled to a QTrap (Applied Biosystems). The flow rate from the column was split using T-coupled capillaries, resulting in a flow rate of 5 µL into the MS. An electrospray interface with positive ionization at 5500 V was used. Full ion trap scanning was applied, for all masses between 250-800 amu (250 amu/s, 0.03 amu step size) or for selected masses specific to quercetin/isorhamnetin detection (303.1, 317.1, 465.2, 479.2, 627.3, 641.3, 649.3 amu).

RESULTS AND DISCUSSIONS

A simple HPLC method was developed as described above. LC-MS/MS analysis was also performed to identify the different aglycone and glycosidic polyphenolic compounds. **Figure 2 A and B** show the chromatograms of an onion-waste sample before and after enzymatic treatment with Bgl1A. It is obvious that most of the glycosidic compounds are transformed to quercetin aglycone after only 10 minutes of reaction with the Bgl1A enzyme.

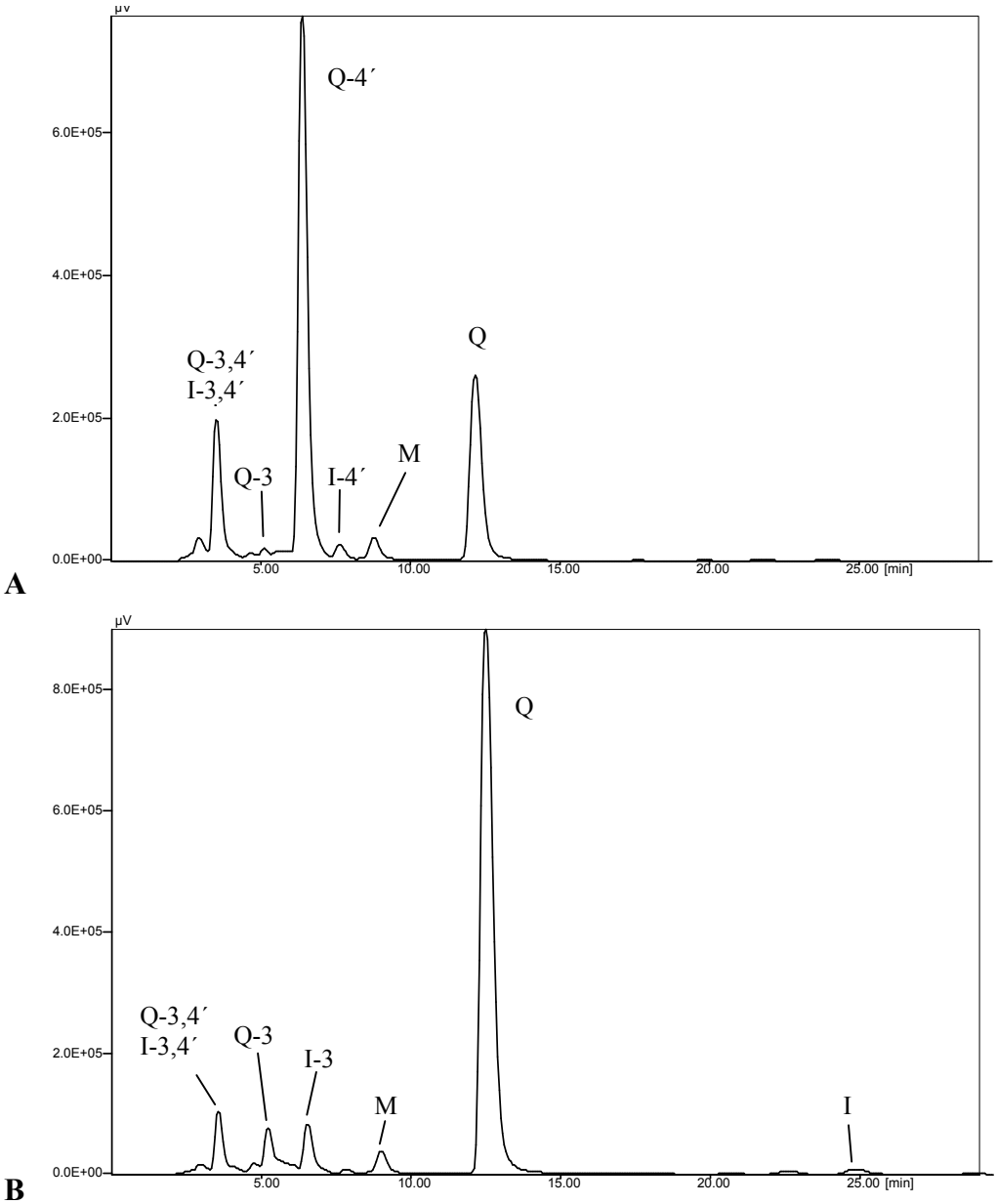

Figure 2. *HPLC chromatogram of an onion extract before enzymatic reaction (A) and after (B). The extraction was performed at 120°C, 2 cycles of 5 min each and the reaction with Bgl1A for 10 min at 90°C and pH 5.0. Compounds identified: quercetin-3,4´-diglycoside (Q-3,4´), isorhamnetin-3,4´-diglycoside (Q-3,4´), quercetin-3-glycoside (Q-3), isorhamnetin-3-glycoside (I-3), quercetin-4´-glycoside (Q-4´), isorhamnetin-4´-glycoside (I-4´), quercetin (Q), isorhamnetin (I), and morin (M, internal standard).*

The enzymatic reaction was optimized using a three-level full factorial design for the parameters pH (3, 5, 7) and temperature (40, 65, 90°C). The reaction time was 10 minutes and the amount of enzyme used was 136 pmol for Bgl1A and 35 pmol for Bgl3B, based on preliminary experiments with both enzymes. **Figure 3** below shows the results for Bgl3B as measured by quercetin concentration (mg/g onion waste). The effects were clearly significant at a 95% significance level for pH (p=0.000), temperature (p=0.004) and pH*temperature (p=0.000). Similar results were obtained for Bgl1A with significant effects of pH (p=0.002) and temperature*pH (p=0.001), but the effect of temperature was not significant on a 95%

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both enzymes.

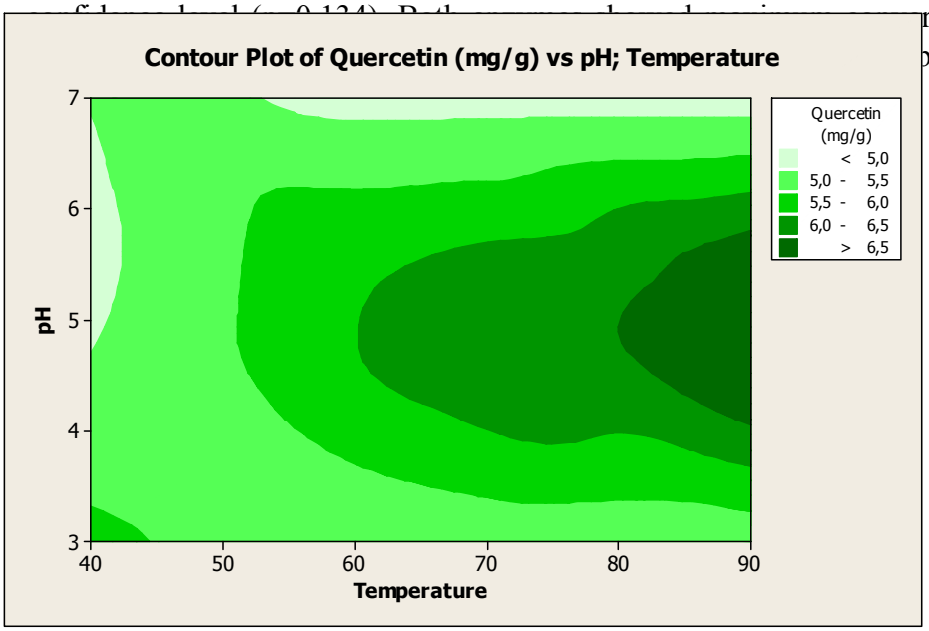

Figure 3. *Contour plot of quercetin yield (mg/g onion waste) vs. pH and temperature of the reaction catalyzed by Bgl3B.*

Effects of enzyme amount and reaction time were tested for both enzymes at 90°C and pH 5.0. Results showed that for Bgl1A 50 µL (=3400 pmol) was necessary to convert all the quercetin glycosides to quercetin aglycone in a 4-mL onion extract within 5 min of reaction, while only 5 µL of Bgl3B (=345 pmol) was needed to obtain the same result (data not shown). Hence, it was decided that the best method for conversion of quercetin glycosides to quercetin was to use 5 µL of Bgl3B per 4 mL of onion extract (~40 mg onion/mL) at 90°C and pH 5.0 for 10 min reaction.

The PHW extraction method was optimized using a three-level full factorial design for temperature (40, 80, 120°C) and number of 5-min extraction cycles (1, 2 and 3). The results are shown in **Figure 4**. The statistical evaluation showed that the main effects were significant at a 95% confidence level, giving p=0.000 for temperature and p=0.023 for number of cycles, but temperature*number of cycles gave p=0.175. The figure also shows that the highest temperature tested, 120°C, gave the highest quercetin yield, and two or three cycles give slightly better results than only one extraction cycle. In conclusion, the optimal extraction method was to use water at 120°C for 5 + 2×5 min extraction.

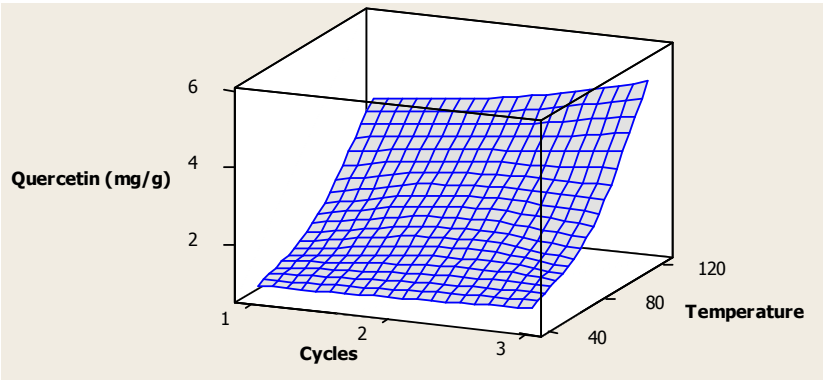

Figure 4. Response surface of quercetin yield (mg/g onion) vs. temperature and number of 5-min extraction cycles for the PHW extraction.

CONCLUSION

We have developed a fast and efficient method for extraction and hydrolysis of quercetin-glycosides from onion waste using subcritical water as a solvent and thermostable β-glucosidases as biocatalysts. The entire extraction method takes only 15 min per sample, and the enzymatic reaction only 10 min. Our results show that onion waste from a local restaurant as well as from a Swedish onion-peeling industry would give around 7 mg of quercetin per g of waste. The developed procedure is interesting from a sustainable chemistry point-of-view, and will lead to an increased value of Swedish industry biomass waste.

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