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Short communication

Purification of the crude solution from *Helix pomatia* for use as β-glucuronidase and aryl sulfatase in phytoestrogen assays

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Abstract

Phytoestrogens occur in a variety of foods and are thought to offer a protective effect against a number of complex diseases. Due to the diversity of phytoestrogen conjugates formed in the human body, most assays include an enzymatic hydrolysis step prior to analysis. β-Glucuronidase from *Helix pomatia*, which also contains sulfatase activity, is popular for this task but contains appreciable levels of some phytoestrogens and related compounds, which could affect accurate quantification at low concentrations. Use of solid phase extraction on a polymeric resin has been found to remove the majority of these compounds from the enzyme, without affecting the enzyme activity for almost all of the analytes tested.

Keywords: Phytoestrogens; Isoflavones; Lignans; *Helix pomatia*; Glucuronidase; Sulfatase; Solid phase extraction; LC/MS/MS

1. Introduction

Phytoestrogens are diphenolic compounds that naturally occur in a variety of plants that can form part of the human diet. Interest in phytoestrogens stems from the belief that they may offer a protective effect against a number of human conditions including cardiovascular disease, certain forms of cancer, and menopausal symptoms [1–4]. Investigation of these potential health benefits relies to a large part on the use of analytical methods that are capable of accurately quantifying the levels of phytoestrogens in biological fluids.

In plants, phytoestrogens occur mainly as glycosides with the carbohydrate often being esterified with acetyl or malonyl groups at the 6″ position. Upon entering the human body, these glycosides are hydrolysed in the gut to aglycones, which are further transformed in the liver to β-glucuronic acid or sulfate conjugates (Fig. 1). Whilst phytoestrogen aglycones can be detected in human urine, studies of the conjugated forms of seven different phytoestrogens by Adlercreutz et al. revealed that aglycones were present in very low amounts (<3% of the total phytoestrogen content) [5]. Due to the diversity of phytoestrogen conjugates formed in the human body, most assays include an enzymatic hydrolysis step, prior to analysis, to convert the glucuronic acid and sulfate conjugates back to the aglycone form. An enzyme preparation that is popular for this task is β-glucuronidase from *Helix pomatia* digestive juice, which also contains aryl sulfatase activity. However, it has been noted that *H. pomatia* contains appreciable levels of some phytoestrogens and related compounds, which could affect accurate quantification [6–9]. Other sources of β-glucuronidase and sulfatase are available; however, it has been shown that these alternatives also suffer from phytoestrogen contamination in one form or another [9]. If the levels of contamination are low, allowances can be made, either by running a blank and subtracting the amount found from each sample analysed, or by spiking calibration samples with the same amount of enzyme as the samples. However, when the levels of analytes in the enzyme mixture are high, it becomes more difficult to accurately quantify low levels of these analytes in real samples. In this case, it is preferential to remove the interferences, but without causing a loss of enzyme activity. Use of solid phase extraction (SPE) on a polymeric resin has been found to remove the majority of these compounds from the enzyme, without affecting the enzyme activity for almost all of the analytes tested.

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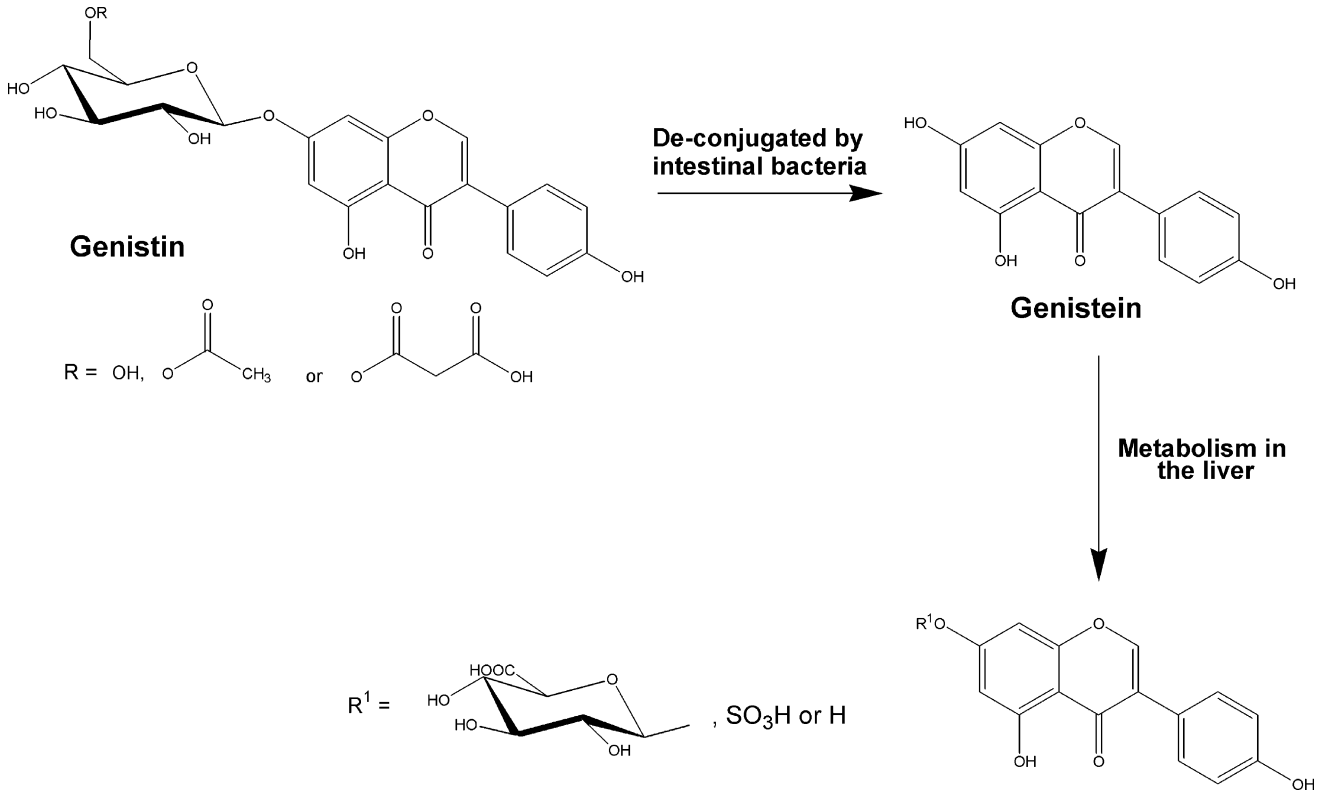

Fig. 1. Metabolism of the isoflavone genistein in the human body.

2. Experimental

2.1. Chemicals

Phytoestrogen standards were purchased from Plantech UK (Reading, Berkshire, UK) and [$^{13}C_3$]-labelled internal standards were purchased from Dr. Nigel Botting (University of St. Andrews, Fife, UK). β-Glucuronidase from *H. pomatia*, type HP-2, was purchased from Sigma (Poole, Dorset, UK).

2.2. Comparison of C18 and polymeric sorbents for purification of H. pomatia

Strata C18-E (50 mg, 1 mL) and Strata-X (30 mg, 1 mL) SPE cartridges were purchased from Phenomenex (Macclesfield, Cheshire, UK). SPE cartridges were conditioned with methanol (1 mL) followed by sodium acetate buffer (140 mM, pH 5, 1 mL). Two millilitres of a 10% (v/v) solution of *H. pomatia* digestive juice in sodium acetate buffer (140 mM, pH 5) was then passed through the cartridge and the eluate collected in a clean tube. To HPLC grade water (200 μL) was added 250 μL of either 10% enzyme purified by C18, 10% enzyme purified by the polymeric sorbent, or a 10% enzyme solution which had not been passed through either SPE cartridge ($n = 5$ for each). Samples were then diluted with methanol (200 μL), extracted using Strata-X (30 mg), washed with 40% methanol, and eluted in 1:1 methanol/acetonitrile (600 μL). Samples were dried under nitrogen and redissolved in 40% methanol (100 μL) prior to LC/MS/MS analysis. The extent of purification offered by C18 and polymeric SPE cleanup was determined by comparing the concentrations of phytoestrogens in the purified samples to those that had not been purified.

2.3. Comparison of the hydrolysis efficiency of an enzyme purified via polymeric SPE with a non-purified enzyme

A Strata-X SPE cartridge (1 g, 20 mL) was conditioned with methanol (10 mL), followed by sodium acetate buffer (140 mM, pH 5, 10 mL). Nine millilitres of a 10% (v/v) solution of *H. pomatia* digestive juice in sodium acetate buffer (140 mM, pH 5) was then passed directly through the cartridge and collected in a clean tube. To the purified 10% enzyme solution (7.2 mL) was added a mix of [$^{13}C_3$]-labelled internal standards in methanol (1 μg/mL, 300 μL). This solution was vortexed and 250 μL added to each of 10 aliquots of a well characterised urine sample (200 μL). To a 10% enzyme solution that had not been purified by SPE (7.2 mL) was added a mix of [$^{13}C_3$]-labelled internal standards in methanol (1 μg/mL, 300 μL). This solution was also vortexed and 250 μL added to each of 10 more aliquots of the same characterised urine sample (200 μL).

2.4. Liquid chromatography/mass spectrometry

Analysis was carried out as published previously [10], with modifications that selected reaction monitoring (SRM) transitions for apigenin (*m/z* 269 → 151), coumestrol (*m/z* 267 → 211), naringenin (*m/z* 271 → 119), matairesinol (*m/z* 357 → 83), and secoisolariciresinol (*m/z* 361 → 165) were

Table 1
Concentrations of 12 phytoestrogens and related compounds in a sample of β-glucuronidase from *H. pomatia*

Analyte	Concentration in *H. pomatia* (ng/mL)
Apigenin	81
Coumestrol	ND
Daidzein	ND
O-Desmethylangolensin	ND
Enterodiol	ND
Enterolactone	2
Equol	ND
Genistein	11
Glycitein	ND
Naringenin	16
Matairesinol	12
Secoisolariciresinol	19

ND indicates that the analyte was not detected.

included, and Strata-X was used for the final extraction of all samples prior to LC/MS analysis as described in Section 2.2. LC/MS/MS analysis was carried out using a Quattro Premier triple quadrupole mass spectrometer interfaced via an electrospray probe to a 1525 μ HPLC pump and a 2777 autosampler (Waters, Milford, MA, USA). The column was a Targa C18 (2.1 mm × 150 mm, 3 μ; Higgins Analytical, CA, USA). HPLC separation was performed as described previously [10].

3. Results and discussion

3.1. Determination of phytoestrogen contamination in H. pomatia digestive juice

The concentrations of a number of phytoestrogens and related compounds in *H. pomatia* digestive juice were calculated by diluting an aliquot of crude enzyme mixture with water and analysing the resulting solution via LC/MS/MS, using calibration standards which had not been spiked with enzyme or extracted. The concentrations of three isoflavones (genistein, daidzein, and glycitein), two metabolites of daidzein (equol and *O*-desmethylangolensin), two lignans (secoisolariciresinol and matairesinol), two lignan metabolites (enterolactone and enterodiol), one coumestan (coumestrol), one flavone (apigenin), and one flavanone (naringenin) were quantified in a sample of *H. pomatia* (Sigma, Poole, UK). The results are shown in Table 1. The concentration of genistein, the major isoflavone contaminant, was similar to that found previously [9]. Although the concentrations of phytoestrogens have been found to vary from batch to batch of enzyme, every batch so far tested has contained significant amounts of at least four of the analytes described (data not shown).

3.2. Purification of H. pomatia with C18 and polymeric sorbents

A method that has been suggested for selectively removing phytoestrogens from *H. pomatia* solution is to pass the enzyme through a C18 cartridge [7,8]. However, practical details of this cleanup procedure and the effect, if any, on enzyme activity have not been published. In order to investigate whether phytoestrogen interferences could be selectively removed from the enzyme, 10% (v/v) solutions of *H. pomatia* in sodium acetate buffer (140 mM, pH 5) were passed through SPE cartridges containing either C18 (Strata C18-E, Phenomenex, Macclesfield, UK) or polymeric sorbent (Strata-X, Phenomenex) and collected. Sorbents had been pre-conditioned with methanol followed by sodium acetate buffer (140 mM, pH 5). To test each enzyme mixture, to 200 μL of HPLC grade water was added 250 μL of 10% enzyme solution (either purified by C18, purified by Strata-X, or not purified, $n = 5$ for each). Solutions were extracted by SPE prior to LC/MS/MS analysis. The percentage of contaminant phytoestrogen removed from the enzyme was calculated by comparing the concentration of analyte in samples with unpurified enzyme, with those that had been purified by either C18 or Strata-X SPE. The results are shown in Table 2. The most effective method to remove phytoestrogens from the enzyme solution was to use the Strata-X SPE procedure, as this sorbent was found to remove between 94 and 100% of the contaminant phytoestrogens, whereas the C18 sorbent removed 52–99%.

Table 2
Percentage of contaminant phytoestrogens removed from crude *H. pomatia* solution using C18 or Strata-X SPE

Analyte	% Removed using C18 SPE	% Removed using Strata-X SPE
Apigenin	52	94
Enterolactone	55	100
Genistein	56	95
Naringenin	55	99
Matairesinol	99	100
Secoisolariciresinol	95	100

3.3. Enzyme activity of purified and crude H. pomatia digestive juice

In order to assess the suitability of a purified enzyme for phytoestrogen assays, it was necessary to determine whether the purification step had affected the enzyme activity with respect to the hydrolysis of any of the target analytes. To test the enzyme activity, a previously well characterised urine sample, which was known to contain naturally occurring high levels of all of the analytes of interest, was hydrolysed using either crude enzyme solution, or enzyme solution that had been purified using SPE on a Strata-X column ($n = 10$ for each). By using a sample that contained high concentrations of all of the analytes, the effect of phytoestrogen contamination from the enzyme was negligible. Spiked calibration lines were treated in the same way as samples which meant that the additional levels of phytoestrogens present as contaminants in the non-purified enzyme samples were accounted for. A *t*-test was used to compare the two sets of data and to discover whether they were statistically different (indicating a possible change in enzyme activity) or identical (indicating no change

Table 3
Mean concentrations ± standard deviation of a urine sample after hydrolysis with either crude β-glucuronidase from *H. pomatia* or *H. pomatia* that had been purified using Strata-X ($n = 10$ for each)

Analyte	Concentration of analytes in urine ± standard deviation (ng/mL)		p
	Crude enzyme	Enzyme cleaned with Strata-X	
Apigenin	99 ± 8	103 ± 6	0.153
Coumestrol	22 ± 1	18 ± 1	6.8×10^{-7}
Daidzein	834 ± 30	823 ± 40	0.509
O-Desmethylangolensin	71 ± 2	72 ± 3	0.156
Enterodiol	493 ± 6	497 ± 10	0.238
Enterolactone	1466 ± 29	1453 ± 33	0.381
Equol	76 ± 1	78 ± 3	0.052
Genistein	328 ± 16	324 ± 26	0.697
Glycitein	105 ± 4	104 ± 2	0.511
Naringenin	597 ± 26	588 ± 53	0.648
Matairesinol	64 ± 4	60 ± 4	0.082
Secoisolariciresinol	294 ± 10	284 ± 10	0.051

p-Values represent the results of a two tailed t-test. Results were considered to be statistically equivalent if $p > 0.05$.

in enzyme activity). Analyte concentrations were assumed to be equivalent between the two sets of data if $p > 0.05$. Table 3 shows the mean concentrations found to be present in the sample (±standard deviation), plus the p-values from the t-test.

The concentration of coumestrol when the purified enzyme was used was found to be 17% lower than when the crude enzyme solution had been used, thus indicating a decrease in enzyme activity for that compound. The intra-assay CV for the 10 replicates of crude enzyme and purified enzyme in coumestrol measurements was 6.1 and 4.9%, respectively, which indicated that this loss was consistent. However, no other analytes showed any significant increase or decrease in concentration, with all p-values >0.05, thereby indicating that enzyme activity had been maintained. The reason for the reduced activity of the purified enzyme for coumestrol, but not for the other phytoestrogens tested, has not yet been established. The β-glucuronidase preparation is a crude solution from *H. pomatia* digestive juice and a possibility is that one component of this complex mixture is removed, or reduced, during purification which subsequently affects the deconjugation of coumestrol. An example would be if the purification procedure reduced the activity of aryl sulfatase, without affecting β-glucuronidase activity. A previous study showed that the vast majority of phytoestrogen conjugates exist as glucuronic acid conjugates, with lesser amounts present as sulfate conjugates and free aglycones [5]. Although, there is no evidence to suggest that a reduction in sulfatase activity had adversely affected the enzyme kinetics of any other analyte, the conjugates of coumestrol in human fluids have not been studied and so it is not known whether that compound exists in the body with a higher relative amount of sulfate conjugates. In order to ascertain the reasons for the decrease in enzyme activity for coumestrol, further work is required which is outside of the scope of this study. However, this finding emphasises the need to test applicability of this purification procedure if additional analytes were to be included in any assay for which it was used.

4. Conclusion

This study indicates that β-glucuronidase and aryl sulfatase from *H. pomatia* can be purified using SPE with a polymeric sorbent to remove phytoestrogen contamination, without affecting the enzyme activity for the majority of analytes. However, an enzyme purified in this way should not be used if coumestrol is to be included as an analyte. This highlights the importance of testing all potential analytes when any kind of enzyme purification is to be attempted. The methodology outlined in this communication should prove useful in studies where β-glucuronidase/aryl sulfatase from *H. pomatia* is to be used for the analysis of phytoestrogens and related compounds in human biofluids. The procedure is applicable not only for assays where mass spectrometric detection is used, but also for assays where other techniques, such as HPLC with coulometric array detection or immunoassay, are used.

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