

## INHIBITION OF GLYCOHYDROLASE ENZYMES BY AQUEOUS EXTRACTS OF CHINESE MEDICINAL HERBS IN A MICROPLATE FORMAT

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### Summary

A microplate assay, for use with a variety of glycohydrolase enzymes, was developed to aid the screening of Chinese medicinal herb extracts for the presence of potential anti-viral and anti-lymphoma compounds. The microplate assay method described offers greater convenience, speed and reproducibility over existing methods. The enzymes tested were  $\alpha$ -glucosidase,  $\beta$ -glucosidase and  $\beta$ -glucuronidase. The assay can be easily adapted for use with other glycohydrolase enzymes. Of the 12 herb extracts examined four did not inhibit any of the enzymes (<50% inhibition), one inhibited  $\alpha$ -glucosidase only (>50% inhibition), six inhibited  $\beta$ -glucuronidase only, and one inhibited both  $\alpha$ -glucosidase and  $\beta$ -glucuronidase. None of the extracts were capable of inhibiting  $\beta$ -glucosidase to any significant extent.

Key words: enzyme assay, microplate, glycohydrolase, inhibition, Chinese herbs

### Introduction

Eukaryotic cells contain a number of enzymes known as glycohydrolases. These enzymes catalyse the stereospecific cleavage of sugar-aglycon conjugates. They are located in the Golgi apparatus of the endoplasmic reticulum and in lysosomes where they may function in the processing of host protein glycosylation as the products of translation traverse the Golgi stack (1,2). They also contribute to viral protein processing as the virus particle matures within the host cell. It has been found that  $\alpha$ -glucosidase contributes to the glycosylation of human immunodeficiency virus type 1 (HIV-1) gp120 (3). Inhibitors of glycoprotein processing may be useful anti-viral compounds by interfering with the maturation of virions (4). The compound N-butyl-deoxynojirimycin is a potent  $\alpha$ -glucosidase inhibitor that has been tested in phase I clinical trials as an anti-HIV-1 compound (5). This compound appears to act by impairing viral entry at the level of post-CD4 binding by affecting viral envelope components (3). The glycosylation inhibitor 2-deoxy-D-glucose has also been reported to interfere with the expression of viral glycoproteins from HIV-1 infected cells (6). Inhibition of intestinal  $\alpha$ -glucosidase has been used successfully to treat patients with both type I and type II diabetes mellitus (7).

The traditional colorimetric method of assaying for glycohydrolase activity, using a variety of synthetic substrates, typically involves long incubations (up to 60 mins) and the handling of large volumes (8-11). Although the assay is not a burden in terms of the expense of reagents it is cumbersome and time-consuming to perform. Consequently, the assay was adapted for use in a 96-well microplate allowing greater convenience, reproducibility and speed and reducing the amount of waste produced and reagents required. This assay is more convenient than other microplate assay systems that have been described previously (11) and is the first report of such a colorimetric microassay specifically for  $\alpha$ - and  $\beta$ -glucosidases using *p*-nitrophenyl- glucopyranosides as substrates.

Our interests in this laboratory concern the screening of aqueous extracts of plants traditionally used in Chinese herbal medicine for the presence of potential anti-viral and anti-lymphoma compounds and so a rapid reproducible assay was required. We report here the rapid screening of 12 aqueous extracts of plant material against three commonly assayed glycohydrolase enzymes,  $\alpha$ -glucosidase from yeast,  $\beta$ -glucosidase from almonds and  $\beta$ -glucuronidase from bovine liver.

### Materials and Methods

The herbs were obtained from local vendors. For *Fritillaria* sp., *Coptis chinensis*, *Sophora flavescens*, *Panax ginseng* and *Paeonia suffruticosa* the dried root, rhizome or corm was used. For the others the dried leaves or stems were used. For *Prunus persica* and *Lithospermum erythrorhizon* the dried seed was used. Each herb (2.5g) was cut into small pieces and soaked overnight in 50ml sterile distilled water at 4°C. The preparations were filtered through cheesecloth and Whatman No:1 paper before dialysis against excess distilled water using a MWCO 1000 membrane (Spectra/Por 6, Spectrum Medical Industries, Inc., CA, USA). After dialysis the solutions were lyophilised. The residue was resuspended in sterile distilled water to 10mg/ml and stored at -20 °C. The enzymes  $\alpha$ -D-glucosidase (E.C. 3.2.1.20) from brewer's yeast,  $\beta$ -D-glucosidase (E.C. 3.2.1.21) from almonds and  $\beta$ -glucuronidase (E.C. 3.2.1.31) from bovine liver were obtained from Sigma Chemical Co. (St.Louis, MO, USA). Immulon™ flat-bottomed 96-well microplates were from Dynatech Laboratories, Inc. (Chantilly, VA, USA). The substrates *p*-nitrophenyl- $\alpha$ -D-glucopyranoside, *p*-nitrophenyl- $\beta$ -D-glucopyranoside and *p*-nitrophenyl- $\beta$ -D-glucuronide were obtained from Sigma Chemical Co. Other reagents were the best commercially available.

Substrates and enzymes were dissolved in 50mM buffer appropriate for each enzyme (Mes-NaOH, pH 6.5 for  $\alpha$ -glucosidase, sodium acetate pH 5.5 for  $\beta$ -glucosidase and sodium acetate, pH 5.0 for  $\beta$ -glucuronidase). To test enzyme inhibition by the aqueous extracts each well of the microplate contained 8 $\mu$ mol buffer, 0.2mg/ml test compound and sufficient enzyme to cause a measurable change in absorbance at 405nm (0.02units/well for  $\alpha$ -glucosidase, 0.01 units/well for  $\beta$ -glucosidase and 20 units/well for  $\beta$ -glucuronidase). The extract was allowed to interact with the enzyme for five minutes at room temperature before the reaction was started by the addition of substrate to concentration of 0.1-0.2K<sub>M</sub> of the respective enzyme. The total reaction volume was 0.2ml. The plate was incubated at room temperature for 15 minutes before the reaction was stopped by the addition of 50 $\mu$ l 2M glycine-NaOH, pH 10. The plate was read on a Bio Rad model 3550 microplate reader at 405nm with a reference at 490nm. The samples were assayed in duplicate. The results did not vary by more than  $\pm$ 5% of the mean.

Carbohydrates were detected using the colorimetric method described by Dische (12). Briefly, hexoses liberated by partial acid hydrolysis of polysaccharides are converted to 5-hydroxymethylfurfural which gives a red complex with resorcinol that can be detected spectrophotometrically.

## Results

The performance of the microplate assay was monitored by examining standard enzyme assay parameters.

### Reaction rate is linear with time

The reaction was stopped at various times between 0 and 15 minutes and the absorbance at 405nm was measured. The observed rate was linear for the duration of the reaction for each enzyme (data not shown).

### Reaction rate is proportional to amount of enzyme

The reaction mix was incubated with increasing amounts of enzyme and the reaction followed as described above. The reaction was proportional to the amount of enzyme in each case (data not shown).

### Determination of optimum pH

The enzymes were incubated with different buffers in the range 5.0 - 8.5. Figure 1 shows the pH curve obtained with each enzyme. The observed pH maxima for each enzyme were as follows:  $\alpha$ -glucosidase pH 6.5,  $\beta$ -glucosidase pH 5.5 and  $\beta$ -glucuronidase pH 5.0. This corresponds to the pH optima previously determined for each enzyme (9, 13). Consequently all experiments reported here were done at the observed pH optima for each enzyme.

### $K_M$ values agree with previous estimates

The  $K_M$  values for each enzyme were calculated by varying the substrate concentration in the range 0.1-10 $K_M$ . The  $K_M$  values calculated from Lineweaver-Burk plots were as follows:  $\alpha$ -glucosidase 2.5mM,  $\beta$ -glucosidase 5.6mM and  $\beta$ -glucuronidase 0.38mM (data not shown). All the kinetic parameters calculated agree with previously determined values (8-10).

### Inhibition of glycohydrolases by aqueous plant extracts

Table 1 shows how each enzyme was affected by the partially purified aqueous herb extracts. Each extract was present at 0.2mg/ml. A reduction in enzyme activity of >50% was considered significant. Some extracts did not inhibit any of the enzymes (e.g. *Fritillaria* sp., *Prunus persica* and *Coptis chinensis*). One extract inhibited only  $\alpha$ -glucosidase, i.e. *Paeonia suffruticosa*. None of the extracts were capable of inhibiting  $\beta$ -glucosidase to any significant degree. Most extracts (7/12) were capable of inhibiting the  $\beta$ -glucuronidase. Specific inhibitors of  $\beta$ -glucuronidase included extracts of

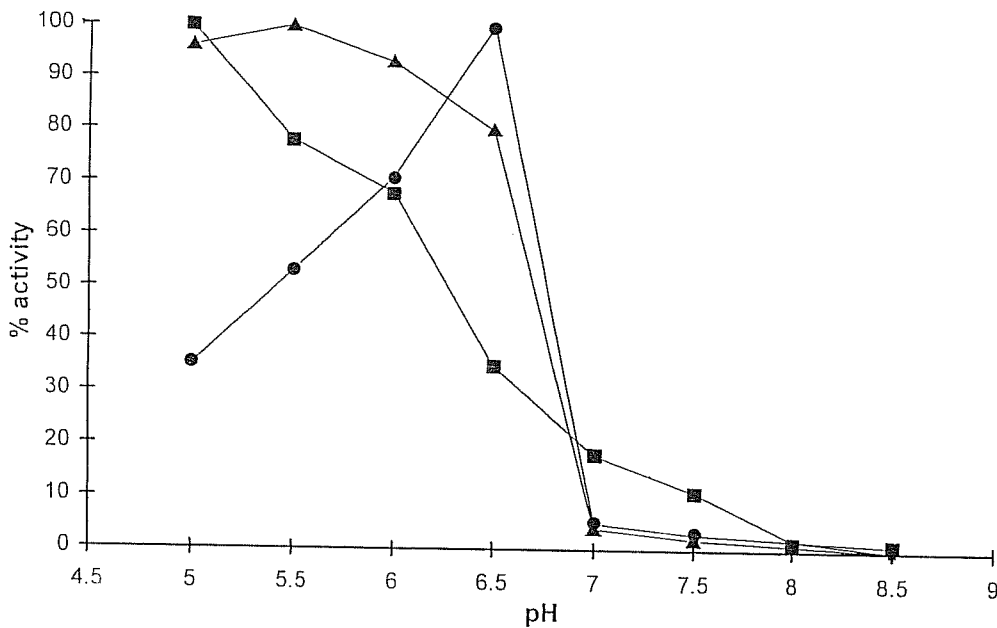


Figure 1

**Reaction rate as a function of pH.**

The enzymes  $\alpha$ -glucosidase (●),  $\beta$ -glucosidase (▲) and  $\beta$ -glucuronidase (■) were incubated at the indicated pH and assayed as described in Materials and methods. The rates are normalized with respect to the maximum observed rate in each case. For pH values below 5.5 sodium acetate buffer was used, for pH 6.0-6.5 Mes-NaOH buffer was used, and for pH 7.0 and above Tris-HCl buffers were used.

*Andrographis paniculata*, *Panax ginseng* and *Lonicera japonica*. One extract inhibited both  $\alpha$ -glucosidase and  $\beta$ -glucuronidase (i.e. *Lithospermum erythrorhizon*).

**Carbohydrate assay**

Dialysed extracts of the Chinese herbs, containing 50-200 $\mu$ g material, were tested for the presence of carbohydrate. All the samples, except *Coptis chinensis*, tested positive indicating the presence of a high molecular weight hexose polymer.

**Discussion**

This assay is a simple, reliable, robust method for the reproducible screening of compounds for the presence of inhibitors of glycohydrolase enzymes. The reaction rate for each enzyme is linear with respect to time and proportional to the amount of enzyme. The assay can easily be modified to enable any similar enzyme to be analysed. The consumption of reagents is kept to a minimum and the amount of waste produced compared to the previously used methods is greatly reduced. The kinetic

Table 1

## Inhibition of glycohydrolase enzymes by aqueous extracts of Chinese medicinal herbs

Herb	% inhibition*		
	$\alpha$ -glucosidase	$\beta$ -glucosidase	$\beta$ -glucuronidase
<i>Fritillaria</i> sp.	0	0	11.3
<i>Prunus persica</i>	6.2	4.2	0
<i>Coptis chinensis</i>	0	0	17.0
<i>Lithospermum erythrorhizon</i>	94.5	16.2	62.0
<i>Paeonia suffruticosa</i>	83.7	6.0	40.4
<i>Paris chinensis</i>	41.3	7.2	33.2
<i>Viola yedoensis</i>	32.8	0	71.0
<i>Sophora flavescens</i>	4.5	19.1	57.0
<i>Panax ginseng</i>	0	0	68.7
<i>Andrographis paniculata</i>	0	0	66.5
<i>Senecio scandens</i>	9.6	0	58.2
<i>Lonicera japonica</i>	0	0	56.9

\* extract present at 0.2mg/ml in each case.

Values are means of duplicate measurements that did not vary by more than 5%.

parameters determined for the enzymes using the microplate assay method compare very well with those determined by more traditional methods. Many modern microplate readers contain built-in software for this purpose.

The microplate assay was used to determine if any of the 12 partially purified aqueous herb extracts contained inhibitors of the enzymes. This is the first description of an  $\alpha$ - and  $\beta$ -glucosidase microplate assay. The microplate assay previously described did not examine the  $\alpha$ - or  $\beta$ -glucosidase reactions and the conditions of the  $\beta$ -glucuronidase assay were significantly different from those described here (11). Several extracts contained potent inhibitors of one or more of the enzymes tested (Table 1). Most significantly, for further anti-viral studies, potent inhibitors of  $\alpha$ -glucosidase were found (*Paeonia suffruticosa* and *Lithospermum erythrorhizon*), which may, like N-butyl-deoxyojirimycin, have potential for use in anti-HIV-1 therapy. Whether each herb contains the same inhibitory compound awaits further study. No extract was capable of inhibiting  $\beta$ -glucosidase to any significant extent and gives an indication as to the stereochemistry of the inhibitory compounds present in the extracts. The most susceptible enzyme to inhibition was the bovine liver  $\beta$ -glucuronidase where 7/12 extracts were capable of causing significant inhibition (Table 1). This susceptibility may

correspond to its broad substrate specificity and reflect a relatively non-discriminatory active site allowing ease of access by molecules of differing shapes and sizes. The greatest degree of inhibition of  $\beta$ -glucuronidase was caused by the extract of *Viola yedoensis* (Table 1). A heat-stable sulphonated polysaccharide of 10-15kDa isolated from *V.yedoensis* and capable of inhibiting HIV-1 at a minimal concentration of 3 $\mu$ g/ml has been described (14). Previous studies on the *V.yedoensis* extract failed to identify the nature of the HIV-1 inhibition (14,15). The *V.yedoensis* extract does not inhibit HIV-1 reverse transcriptase or interfere with the HIV-1 gp120/CD4 receptor interaction (16). It may be the reduction in correctly processed viral glycoproteins caused by the inhibition of host cell glycohydrolases that is responsible for the efficacy of the *V.yedoensis* extract. The compound responsible for the observed inhibition of HIV-1 and the active constituent of the *V.yedoensis* extract produced in this report are likely to be the same or highly related.

The plants containing potent  $\alpha$ -glucosidase inhibitors may prove useful as sources of anti-viral compounds in a manner analogous to the anti-viral  $\alpha$ -glucosidase inhibitor N-butyl-deoxyojirimycin (3). MAR-10, a substituted polysaccharide isolated from an aqueous extract of *Hyssop officinalis*, was able to inhibit HIV-1 replication as judged by a reduction in p24 antigen and syncytia formation. It was also found to be a broad spectrum glycohydrolase inhibitor (17). Interestingly, inhibition of  $\alpha$ -glucosidase in the intestinal lumen has been reported to be beneficial in the treatment of both type I and type II diabetes mellitus (7). The highly soluble nature of the extracts described may prove useful in the therapy of this disease. Inhibitors of  $\alpha$ -glucosidase and  $\beta$ -glucuronidase may be of further pharmacological interest. The true chemical and physical properties of the compounds present in the various extracts awaits more detailed analysis. The observed inhibition is not due to contaminating mono- or disaccharides, or other low molecular weight compounds, as these were removed by extensive dialysis prior to testing. The inhibiting compounds are likely to be highly polar polysaccharides of quite substantive molecular mass analogous to the the compounds MAR-10 (17), prunellin (18) and *V.yedoensis* extract (14,15). The identity of the inhibitory compounds is the subject of further study.

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