

### 3 Chapter III: Purification of $\beta$ -glucuronidases and preparation of their antibodies

#### 3.1 Introduction

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##### Purification of $\beta$ -glucuronidases

Purification of a variety of mammalian  $\beta$ -glucuronidases has been documented since the early 50's (see Harris et al. (1973), Himeno et al. (1974), and many references therein). In the early work, purification often involved multiple techniques, for example fractionation with ethanol or organic solvents, and different types of chromatography, such as gel filtration, isoelectric focusing, or ion exchange (Himeno et al. 1974). These purification schemes were therefore often laborious, and inferior in terms of yield and purity.

Affinity-based purification of  $\beta$ -glucuronidases has become a better alternative. Harris et al. (1973) reported the coupling reactions of saccharolactone (saccharo-1,4-lactone), a powerful competitive inhibitor of  $\beta$ -glucuronidase, to sepharose 4B. The coupled product has been used for affinity purification of bovine and *E. coli*  $\beta$ -glucuronidases (Harris et al. 1973; Rethinaswamy et al. 1994; Kim et al. 1995). Saccharolactone-linked agarose for  $\beta$ -glucuronidase purification is now available commercially from Sigma. Another ligand that has been used for affinity purification of  $\beta$ -glucuronidase is thiophenyl-glucuronide (PTG), an analog of a  $\beta$ -glucuronidase substrate that has strong affinity to, but is not cleaved by  $\beta$ -glucuronidase. The coupled product of this ligand to sepharose can be used for one-step affinity purification of  $\beta$ -glucuronidase to high homogeneity (Blanco & Nemoz 1987). Coupling reactions of a range of other ligands to Sepharose 4B, and the use of coupled products in affinity purification of bovine  $\beta$ -glucuronidase have also been reported (Iino & Yoshida 1992).

Immobilized-metal affinity chromatography (Porath et al. 1975) has become a method of choice for purification of many recombinant proteins. The method uses chelating ligands, like iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA), to immobilize metal ions such as  $Zn^{2+}$ ,  $Cu^{2+}$ , or  $Ni^{2+}$  on a gel matrix. These metal ions bind to histidine residues on the proteins, which are normally engineered to have a six consecutive histidine residues tag. Elution of proteins can be done with reduced pH, which causes protonation of histidines, and hence their dissociation from the metal ions; or with imidazole, which competes for binding sites on the ion-ligand complex; or with chelating agents like EDTA or EGTA, which elutes the whole proteins-metal complex. For detailed discussion of the method, see Porath et al. (1975); Sulkowski (1985).

The method is versatile and simple, offering high yield and purity. However, it has some limitations. Prior engineering of a fusion to the hexahistidine tag is required. Although the method is generally independent of the protein of interest, the necessary exposure of the hexahistidine tag to the ligand is by no means guaranteed. Fusions to the tag may affect physical and chemical properties of the proteins. For polyclonal antibody production using hexahistidine-tagged proteins, additional antibody species against the tag may be produced. Despite all these potential issues that need to be considered, the method has become very popular, due largely to its versatility and simplicity. A variety of products and protocols have been developed, and are available commercially.

### **Antibody for $\beta$ -glucuronidase**

The extreme popularity of the GUS reporter system has been due largely to the fact that it offers excellent histochemical analysis that is much simpler and more straightforward than antibody-based techniques, such as immunohistochemistry or *in situ* hybridization, to detect gene activity. However, one cannot underrate the power of antibody-based techniques in various analyses and optimization of the GUS reporter. Immunohistological techniques can be used to confirm histochemical results, and can

often pinpoint possible artifacts associated with the histochemical technique. More importantly, a GUS antibody is vital to many biochemical analyses, such as the detection of GUS post-translational modifications, or crosslinking to other proteins, when GUS is used as a marker to study protein targeting and transport. An important part of this PhD project is to study the secretion of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> in yeast and plants. Such studies will certainly depend on the availability of good antibodies against these proteins.

Some authors have reported the use of in-house or commercial polyclonal antibody against GUS<sup>Eco</sup> in their experiments (e.g. Jefferson 1985; Iturriaga et al. 1989; Yan et al. 1997). The GUS polyclonal antibody was commercially available from BD Biosciences Clontech (CA, USA) and is now available from Molecular Probes (OR, USA) although performance of the antibodies from these sources has been under question (various personal communications). I am not aware of any source of GUS<sup>Eco</sup> monoclonal antibody currently available.

## 3.2 Materials and Methods

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### 3.2.1 Chemicals, plasmids and bacterial strains

All chemicals were from Sigma, unless otherwise stated. pRAJ294 and pAKB51 contain native *gusA*<sup>Eco</sup> and native *gusA*<sup>Ssp</sup> genes, respectively, in the inducible pTTQ18 backbone (Stark 1987). pRAJA17.1 contains native *gusA*<sup>Ssp</sup> gene in pBlueScriptII. These plasmids were used for affinity purification using thiophenyl-glucuronide and saccharolactone. pLADF48 contains *gusA*<sup>Eco</sup> in a modular structure (section 2.3.4) with a hexahistidine tag, in pTTQ18. pTANE95.1 contains the corrected synthetic *gusA*<sup>Ssp</sup> gene with a hexahistidine tag in pLITMUS39 (AI variant – section 2.3.4). *E. coli* strain KW1, a GUS-operon-deleted strain (Wilson et al. 1995), was used as expression host.

### **3.2.2 Affinity purification of $\beta$ -glucuronidases using thiophenyl-glucuronide**

The coupling reaction of thiophenyl-glucuronide (PTG) to 6-aminohexanoic acid-sepharose 4B (AH sepharose 4B) was done as described in Blanco & Nemo (1987). Native GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were expressed in KW1 cell with the inducible pTTQ18 system (Stark 1987). Bacteria cells were washed with 1xM9 salt solution, and lysed in buffer A (20 mM Tris, pH=7.6, 1 mM DTT) with glassbead on a bead beater (Mixer-mill MM2, Retsch, Haan, FGR). Cell lysate was loaded on the PTG-linked sepharose column (pre-equilibrated in buffer A), wash extensively with buffer A (about 10 times of the lysate volume), and eluted sequentially with 0.1 M NaCl and 0.3 M NaCl.

### **3.2.3 Affinity purification of $\beta$ -glucuronidases using saccharolactone**

Saccharolactone-linked agarose was purchased from Sigma (CAT No. S5644). Native GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were expressed in KW1 cell with the inducible pTTQ18 system (Stark 1987). Bacteria cells were washed with 1xM9 salt solution, and lysed in buffer A (10 mM MES, pH=6.0, 0.1 % Triton X-100, 0.05 M NaCl) with glassbead on a bead beater (Mixer-mill MM2, Retsch, Haan, FGR). Cell lysate was loaded on the saccharolactone column (pre-equilibrated in buffer A), wash extensively with buffer A (about 10 times of the lysate volume), and eluted with buffer E (10 mM MES, pH=6.0, 0.1 % Triton X-100, 0.1 M NaCl).

### **3.2.4 Affinity purification of 6xhis-tagged $\beta$ -glucuronidases using Ni-NTA resin**

Ni-NTA agarose was purchased from QIAGEN (CAT No. 30230). Hexahistidine-tagged GUS<sup>Eco</sup> (pLADF48) and GUS<sup>Ssp</sup> (pTANE95.1) were expressed in KW1 cell. Bacteria cells were washed with 1xM9 salt solution, and lysed in lysis buffer with glassbead on a bead beater (Mixer-mill MM2, Retsch, Haan, FGR).

Purification under native conditions was done according to the manufacturer's recommendation with the following buffer: lysis buffer (50 mM NaPO<sub>4</sub>, pH=8.0, 300 mM NaCl, 10 mM imidazole), wash buffer (50 mM NaPO<sub>4</sub>, pH=8.0, 300 mM NaCl, 20

mM imidazole), elution buffer (50 mM NaPO<sub>4</sub>, pH=8.0, 300 mM NaCl, 250 mM imidazole). With these conditions, however, there were some contaminants in the purified fractions (see results). We have optimized the conditions, in which all buffers were at a reduced pH (pH=7.0 instead of 8.0), and the amount of imidazole in the wash was increased (75 mM instead of 20 mM). With these modifications, the proteins GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were highly purified.

### **3.2.5 SDS-PAGE**

Denaturing 7.5% SDS-PAGE was performed according to Laemmli (1970). Proteins were separated under reducing conditions. The gel was run with the Mini-PROTEAN II apparatus and dried with the Model 583 Gel Dryer. All equipment was from Bio-rad (CA, USA). All experimental steps were done according to recommended protocols from Bio-rad and QIAGEN.

### **3.2.6 Production of rabbit polyclonal antibodies against GUS<sup>Eco</sup> and GUS<sup>Ssp</sup>**

Polyclonal antibodies against GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were raised in rabbits using a commercial service provided by the Institute of Medical and Veterinary Science (SA, Australia). Two rabbits were injected each with 1.5 mg of purified hexahistidine-tagged GUS<sup>Eco</sup>, and one rabbit was injected with 1 mg of purified hexahistidine-tagged GUS<sup>Ssp</sup>. All protocols were standard, in which rabbits were inoculated on 5 occasions with equal amounts of antigen at 3-week intervals. Pre-bleed and test-bleed samples were collected and tested for antibody response to decide bleed-out time. Final yield was about 50-60 ml of serum/rabbit.

### **3.2.7 Affinity purification and titering of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> antibodies**

Affinity purification of rabbit polyclonal GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> antibodies from serum was based on the protocols by Board & Webb (1987). The titre of each batch of affinity-purified antibody was determined by a series of dot-blot with various antigen concentrations (typically 100, 10, 1, and 0.1 ng of antigen) and antibody dilutions

(1/1,000-1/32,000). The secondary antibody used was goat anti-rabbit IgG coupled with alkaline phosphatase. Colorimetric detection was done with NBT/BCIP.

### **3.2.8 Immunoblotting and immunodetection**

Protein gels were blotted onto either nitrocellulose or PVDF membrane using the Trans-Blot cell apparatus (Bio-rad, CA, USA) following recommended protocols. Blocking was done overnight with 5% powdered skim milk or 1% BSA in TBST. The primary antibodies were rabbit polyclonal antibodies against hexa-histidine-tagged GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> produced from GUS-negative *E. coli* strain KW1. The antibodies were affinity-purified (section 3.2.7). Another primary antibody tested was the commercial penta-his mouse monoclonal antibody (QIAGEN) to detect the hexa-histidine tag.

For colorimetric detection, the secondary antibodies used were biotin-anti-rabbit Ig F(ab')<sub>2</sub> (for GUS antibodies), and biotin-anti-mouse Ig F(ab')<sub>2</sub> (for penta-his antibodies). Both secondary antibodies were from Boehringer Ingelheim, Germany. For chemiluminescence detection, the secondary antibody used was the mouse monoclonal anti-rabbit antibody (clone AG-16) coupled with horseradish peroxidase (Sigma Cat. No. A2074).

Streptavidine-alkaline phosphatase and the substrate NBT/BCIP was used in colorimetric detection. For chemiluminescent detection, horseradish peroxidase and the chemiluminescent substrate Supersignal West Dura from Pierce (IL, USA) was used. Chemiluminescent signal was exposed on X-ray film.

### **3.2.9 Protein extractions**

Plant protein extraction was done using grinding/extracting buffer (100 mM Tris, pH=8.0, 1 mM EDTA, pH=8.0, 1 mM PMSF, 10 DTT, 1 ug/mL pepstatin A). The amount of buffer was 5 times (V) of the fresh plant materials (w). The extract was spun

at 10,000 g for 30 min at 4<sup>0</sup>C, and the supernatant (crude extract) kept at –80<sup>0</sup>C until used.

Yeast and bacteria were lysed in extraction buffer (20 mM Tris, pH=8.0, 1 mM DTT) with glassbead on a bead beater (Mixer-mill MM-2 Retsch, Haan, FGR). The extract was spun at 10,000 g for 30 min at 4<sup>0</sup>C, and the supernatant (crude extract) kept at –80<sup>0</sup>C until used.

## 3.3 Results and Discussions

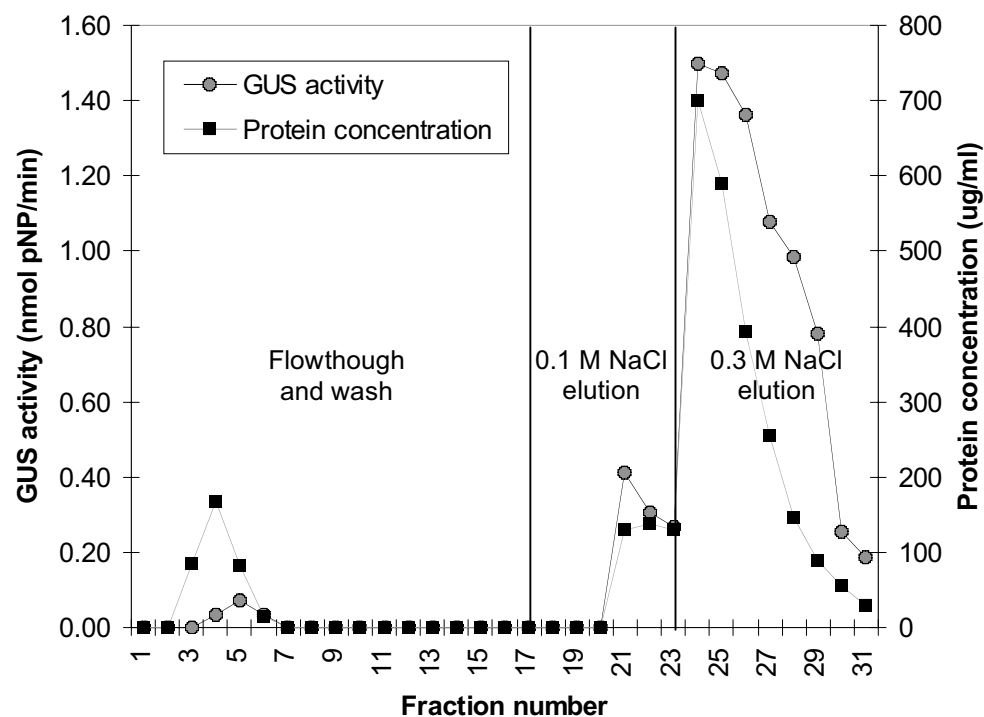
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### 3.3.1 Affinity purification of $\beta$ -glucuronidases with thiophenyl-glucuronide

Purification of GUS<sup>Eco</sup> using thiophenyl-glucuronide was not successful. Elution profiles for GUS and total protein were almost identical (Figure 3.1). GUS was retained on the column during washing step. However, the column also retained most other proteins. When GUS was eluted from the column with 0.1 M and 0.3 M NaCl, other proteins were also co-eluted. Therefore, there was no enrichment of GUS in the eluates, as confirmed on SDS-PAGE (data not shown).

Quantitative measurement (table 3.1) confirmed the above result, showing no significant purification of GUS in the eluates. In addition, it also revealed that a large amount of GUS (61.7%) was still retained on the column after elution with 0.3 M NaCl. However, the amount of total protein retained on the column was equally significant (54.4%). Since no selective elution of GUS was found with 0.1 and 0.3 M NaCl, it would be expected that GUS and other proteins would continue to co-elute, if further elution with higher salt concentrations were performed, leading to no significant further purification. This assumption was not confirmed experimentally.

It was almost certain that the failed purification was due to the unsuccessful coupling reaction of PTG to AH-sepharose 4B. I did try various coupling conditions, which resulted in products with different GUS-binding profiles, as judged by GUS batch binding test (data not shown). At that time, however, purification using saccharolactone was shown to be more successful (section 3.3.2). Since the goal of both PTG and saccharolactone purification methods was to purify native GUS proteins, and the saccharolactone method was more promising, the PTG experiments were not followed up further.



**Figure 3.1. Elution profiles of GUS<sup>Eco</sup> and total protein on PTG column.** The two profiles were almost identical. The column retained most proteins, including GUS, during the wash step. However, GUS and most proteins were co-eluted during the elution steps, resulting in no significant purification (table 3.1).

Fractions	Total GUS activity		Total protein		GUS specific	Purification fold
	(nmol pNP/min)	(%)	(mg)	(%)	activity (nmol pNP /min/mg protein)	
Crude extract	11991	100.0	1.49	100.0	8048	1.00
Flowthrough & wash	75	0.6	0.07	4.9	1033	0.13
0.1 M NaCl elution	368	3.1	0.10	6.8	3643	0.45
0.3 M NaCl elution	4143	34.5	0.50	33.6	8269	1.03
Total collected	4595	38.3	0.68	45.6		

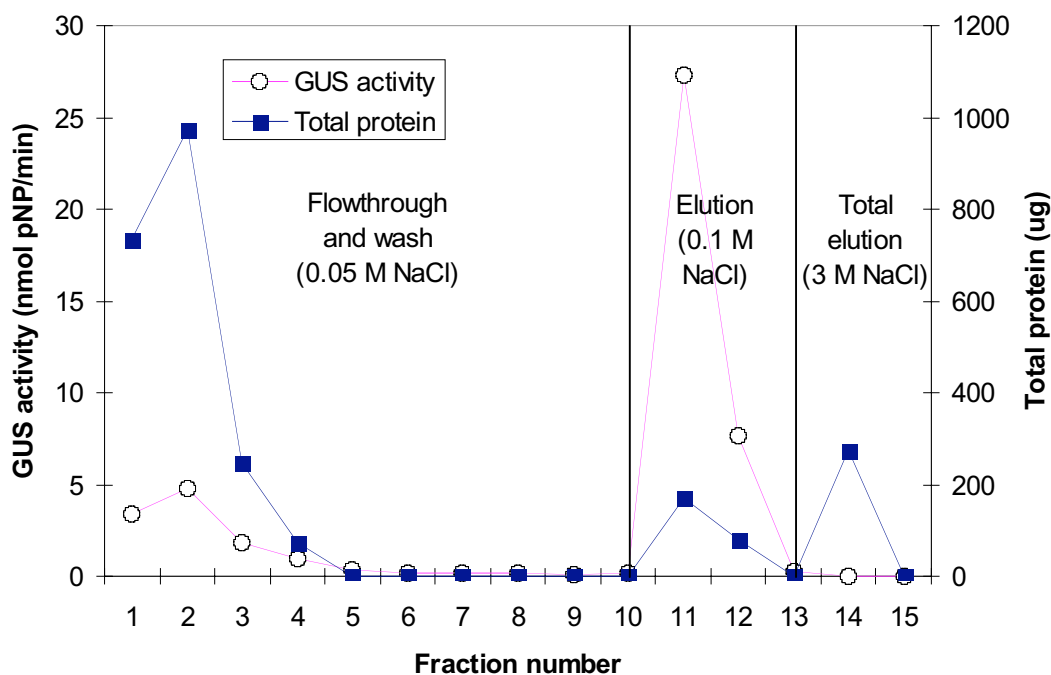
**Table 3.1. Summary of the GUS<sup>Eco</sup> purification using PTG column.** No significant enrichment of GUS was obtained, even in the best eluate (0.3 M NaCl). After elution with 0.3 M NaCl, a large amount of GUS (61.7%) was still retained on the column. However, the amount of total protein retained on the column was equally significant (54.4%). Since no selective elution of GUS was found with 0.1 and 0.3 M NaCl, it would be expected that GUS and other proteins would continue to co-elute, if further elution with higher salt concentrations were performed, leading to no significant further purification.

### 3.3.2 Affinity purification of $\beta$ -glucuronidases with saccharolactone

Purification of GUS<sup>Eco</sup> using saccharolactone was quite successful. As seen in figure 3.2, most of GUS was retained in the column, and eluted with 0.1 M NaCl, whereas a majority of other proteins came off in the wash. In the best eluate, about 50% of total GUS activity was recovered, with an eight-fold purification (table 3.2). Total elution of remaining proteins on the column was done with 3 M NaCl. The column could be reused, as described in Harris et al. (1973), although I have not tested this possibility.

The experiment was repeated several times, in which nearly ten-fold purification from crude extract was consistently obtained. Based on their specific GUS<sup>Eco</sup> activities, purified fractions were roughly estimated to be about 50% pure, as confirmed with SDS-PAGE (figure 3.3).

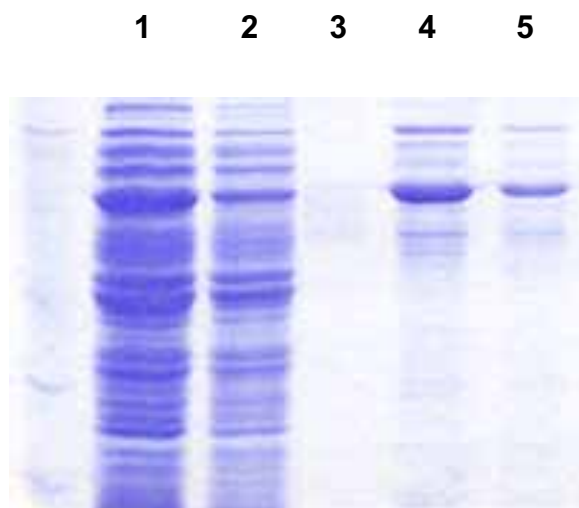
The purified protein was therefore not yet suitable to be used as antigen. Some fine-tuning of the purification scheme was carried out. Increasing the amount of Triton X-100 (to reduce the amount of contaminants via hydrophobic interactions) in the washing step from 0.1% to 0.2% and higher, caused the protein to elute from the column. Use of different buffers at different pHs (MES, pH=6.0-6.5; NaPO<sub>4</sub>, pH=7.0, and Tris, pH=8.0) did not have significant effects, except that NaPO<sub>4</sub> buffer seemed to disrupt binding of GUS on the column. The use of acetic acid (Harris et al. 1973), instead of NaCl for elution also did not have noticeable effects (data not shown). Elution with acetic acid would be appropriate with mammalian glucuronidases, which have acidic pH optima, but not with GUS<sup>Eco</sup>, which has neutral pH optimum. With GUS<sup>Eco</sup>, the use of NaCl would be milder. Increasing the concentration of NaCl in the wash would also be beneficial, although this was not tested experimentally.



**Figure 3.2. Elution profiles of GUS<sup>Eco</sup> and total protein on saccharolactone column.** Most of GUS was retained on the column, and eluted with 0.1 M NaCl (fractions 11-13). The majority of other proteins could be washed off. The column can be reused after a total elution with 3 M NaCl to get rid of all proteins retained on the column.

Fractions	Total GUS activity		Total protein		GUS specific activity (nmol pNP /min/mg protein)	Purification fold
	(nmol pNP/min)	(%)	(ug)	(%)		
Crude extract	20913	100.0	2801	100.0	7467	1.0
Flowthrough & wash	3489	16.7	2030	72.5	1719	0.2
Elution 1 (0.1 M NaCl)	10249	49.0	169	6.0	60487	8.1
Elution 2 (0.1 M NaCl)	2884	13.8	80	2.9	35915	4.8
Elution 3 (0.1 M NaCl)	91	0.4	0	-	-	-
Total elution (3 M NaCl)	16	0.1	276	9.9	58	0.0
Total collected	16728	80.0	2484	91.3		

**Table 3.2. Summary of the GUS<sup>Eco</sup> purification using saccharolactone column.** Purification was quite successful, recovering about 50% of total GUS activity with 8-fold enrichment (elution 1).



Lane	1:	Total cell lysate
	2:	Flowthrough
	3:	Final wash
	4:	Elution 1
	5:	Elution 2

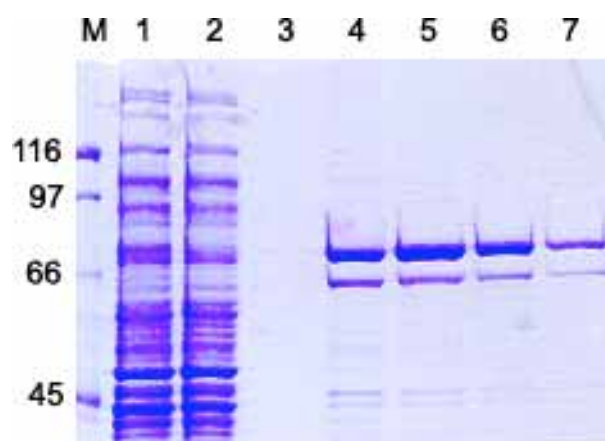
**Figure 3.3. SDS-PAGE of various fractions from purification of GUS<sup>Eco</sup> using saccharolactone column.**

Due to time constraints, optimization of the saccharolactone purification was not followed up fully. However, the initial observations mentioned-above would be useful if the optimization is repeated again in the future. It should also be noted that saccharolactone affinity purification can be used in combination with other methods, for example the PTG affinity column, or other non-affinity-based chromatography, to improve the purify of GUS.

Similar results were obtained with purification of GUS<sup>Ssp</sup> using saccharolactone (data not shown). However, the native *gusA*<sup>Ssp</sup> gene gave very low level of expression in *E. coli*. Since milligram amounts of the protein were needed for antibody production, it was decided not to scale up, but to postpone the purification until the codon-optimized version of the gene was constructed. The experiment was not repeated, however, as the hexahistidine-tagged protein purification method was used (section 3.3.3).

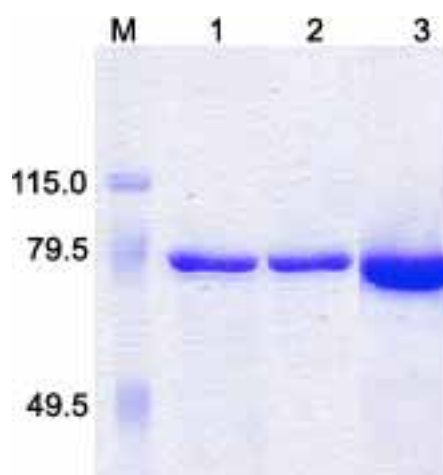
### **3.3.3 Affinity purification of 6xhis-tagged GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> with Ni-NTA resin**

IMAC purification of 6xhis-tagged  $\beta$ -glucuronidases using Ni-NTA resin was successful and straightforward. Both proteins GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were purified under native conditions to high homogeneity (figure 3.4). It should be noted that modification of the manufacturer's recommended protocols was necessary to obtain highly purified proteins. The modifications include lowering the pH of all buffers from 8.0 to 7.0, and increasing imidazole concentration in the wash from 20 mM to 75 mM. Purification was very quick and reproducible, with recovery normally higher than 80 % (data not shown). The method was used in numerous occasions to purify GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> for antibody production (section 3.3.4), and biochemical characterization (P. Wenzl and T. Nguyen, unpublished).



**A.** Purification of GUS<sup>Eco</sup> with Ni-NTA resin using QIAGEN's recommended protocols.

Lane 1: Lysate, 2: Flowthrough, 3: Last wash, 4-7: Elutions. M = Marker (in kDa). Note that the elution fractions were not very pure, with some prominent contaminated bands. Similar results were obtained with GUS<sup>Ssp</sup> (data not shown). Highly purified proteins were obtained with some modifications from the recommended protocols (see B).



**B.** Highly purified GUS<sup>Ssp</sup> and GUS<sup>Eco</sup> obtained using modified protocols: reduced pH and increased imidazole in the wash (see text for details).

Lane 1: purified GUS<sup>Ssp</sup> (2ug), 2: purified GUS<sup>Eco</sup> (2ug), 3: purified GUS<sup>Eco</sup> (10ug). M = Marker (in kDa). Lane 3 was intentionally overloaded to check for presence of contaminating bands.

**Figure 3.4. SDS-PAGE of hexa-histidine-tagged GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> using Ni-NTA column.**

### **3.3.4 Production, affinity purification, and titrating of rabbit polyclonal antibodies against GUS<sup>Eco</sup> and GUS<sup>Ssp</sup>**

Polyclonal antibodies against GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were produced in rabbits using a commercial service. Each rabbit was injected with a total of 1.0 - 1.5 mg of purified antigen. Two rabbits were used for GUS<sup>Eco</sup>, and one rabbit was used for GUS<sup>Ssp</sup>. Antigen response was monitored regularly on a series of test bleeds. Each rabbit produced about 50 mL of final bled-out serum.

Crude sera were affinity-purified according to the protocols by Board & Webb (1987). The titre of each batch of affinity-purified antibody was generally determined in a dot-blot. Normally, the affinity-purified antibodies, at a dilution of 1/1000- 1/4000, could detect about 1ng of GUS, using colorimetric detection method (data not shown – see 3.3.5 for performance of these antibodies in Western blot).

### **3.3.5 Optimization of immunodetection methods**

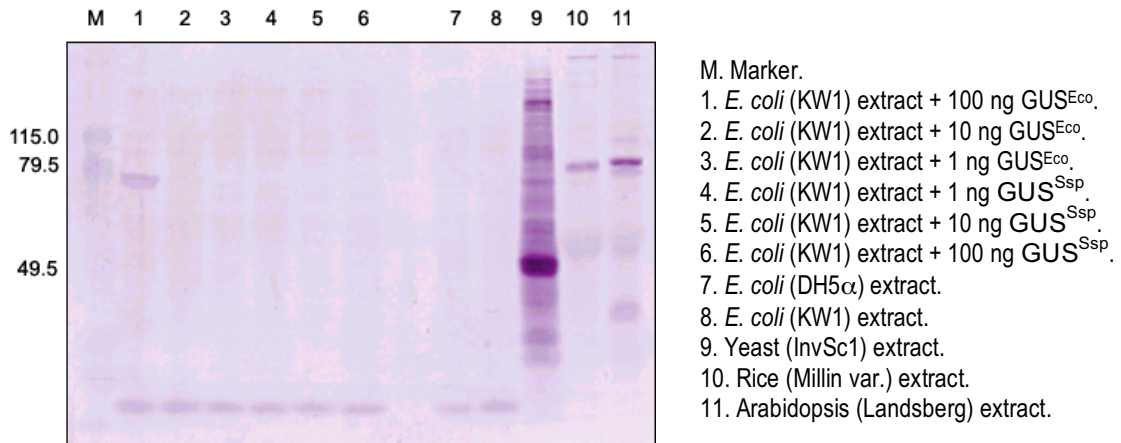
The sensitivity and cross-reactivity of the GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> antibodies in a given immunodetection scheme is crucial to the success of the protein crosslinking experiments to elucidate possible cysteine-mediated retention of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> when they are targeted into the ER. Therefore, optimization of immunodetection scheme to increase sensitivity and decrease possible cross-reactivity was an important part of my research.

I have tested a commonly used colorimetric immunodetection scheme using a biotin-linked secondary antibody, followed by streptavidine-alkaline phosphatase and subsequent colorimetric detection with the NBT/BCIP substrate (Figure 3.5.A). This scheme, however, was not satisfactory. First, it only detected an amount of GUS<sup>Eco</sup> around 100 ng or higher. Such sensitivity would not be adequate to detect any GUS if its expression level is less than 1% of total protein (equivalent to about 100 ng of GUS

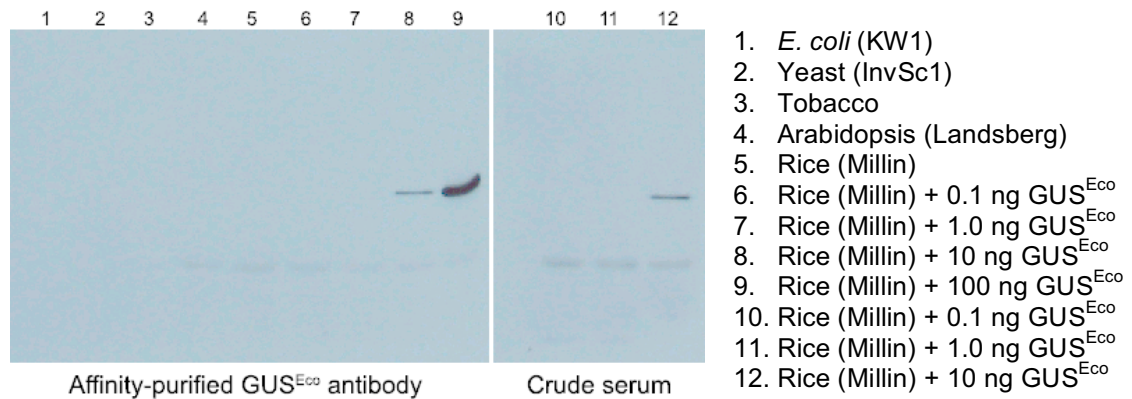
in 10 ug of total protein loaded on a gel). Very often, the amount of GUS protein found in GUS-expressing plant extracts would be lower than 1%, and therefore would not be detected.

Secondly, the detection scheme showed very high background with plants and yeast extracts. Cross-reactivity with yeast extract (InvSc1 strain) was very high. In GUS-negative rice (var. Millin) and *Arabidopsis* (Landsberg ecotype), there was strong non-specific signal detected very close to the size of GUS, and therefore, could be misinterpreted as (false) positives. Since the first antibodies were affinity-purified, it was suspected that the high cross-reactivity was caused by the secondary antibody. A secondary-antibody-alone control experiment was carried out to confirm that in fact, most of the background was due to the secondary antibody used (data not shown). Using the same detection scheme, I have also tested a commercial mouse monoclonal antibody raised against a penta-histidine peptide. The results, however, were very similar to those from using our in-house rabbit anti-GUS polyclonal antibodies (not shown).

A modified detection scheme was planned and tested. First, a monoclonal secondary antibody was used, to minimize possible cross-reactivity often caused by polyclonal secondary antibody. Secondly, chemiluminescent detection method was chosen. Chemiluminescence is intrinsically very sensitive, with detection limit about 0.1 ng of a 50 kDa protein (Harlow & Lane 1999). With this chemiluminescent detection scheme, the detection limit was lowered to about 1-10 ng of GUS. As expected, the levels of background were also very low against all various extracts (figure 3.5.B).



A. **Colorimetric detection scheme.** Various amount of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> were loaded to determine sensitivity. Bacterial, yeast, and plant extracts (10 ug each) were loaded to determine cross-reactivity. First antibody was affinity-purified GUS<sup>Eco</sup> antibody at 1/2000 dilution. Secondary antibody was biotin-anti-rabbit Ig F(ab')<sub>2</sub> at 1/1000. Colorimetric detection using streptavidine-alkaline phosphatase at 1/3000, and NBT/BCIP substrate. The method was not very sensitive, with detection limit about 100 ng of GUS<sup>Ssp</sup>. It also showed high cross-reactivity with yeast and plant extracts. This high cross-reactivity was found to be characteristics of the commercial secondary antibody (data not shown).



B. **Chemiluminescence detection scheme.** First antibody was affinity-purified GUS<sup>Eco</sup> antibody at 1/2000 or crude serum at 1/50,000. Secondary antibody was HRP-linked-mouse monoclonal anti-rabbit at 1/50,000 followed by chemiluminescence detection using supersignal west dura substrate. The method offered higher sensitivity, with detection limit about 1-10 ng of GUS<sup>Eco</sup>. It also had much lower cross-reactivity to various extracts, compared to the colorimetric detection scheme.

**Figure 3.5. Determination of sensitivity and cross-reactivity of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> antibodies in various bacterial, yeast and plant extracts.**

This chemiluminescent detection scheme was used in the protein cross-linking experiment in chapter V.

The nature of protein cross-linking experiments demands very sensitive detection to pick up various cross-linked products that are scattered in smaller quantities instead of condensed into discrete bands on a gel. To further increase the detection sensitivity, many dot-blot experiments were carried out to optimize various steps during the immunodetection process. For example it was found that blocking the blot with BSA, instead of the commonly used powdered skim milk, can significantly improve the signal intensity, leading to the detection of as low as 0.1 ng of GUS (data not shown).

One of the most important and interesting long-term goals of this project is to examine potential post-translational modifications, and/or interactions with other proteins, of various GUSes when they are targeted into different organelles/compartments. Such studies are no doubt strongly dependent on powerful immunodetection tools. The availability of GUS<sup>Eco</sup> and GUS<sup>Ssp</sup> antibodies, and of highly specific and sensitive immunodetection scheme described in this thesis will be very useful for many experiments in the future.