OPTIMISATION OF PEROXIDASE ADSORPTION ON CONCANAVALIN A-AGAROSE

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Abstract— **Many publications describe methods for peroxidase purification from plant material. When the goal was to obtain a high purity enzyme every purification method included an affinity chromatography step using Concanavalin A as the ligand. However, the adsorption step was carried out under quite different conditions with regard to pH, ionic strength and metal cation content in the binding buffer, thus leading to a rather confuse situation. To establish the best conditions for purification of peroxidases from horseradish root (HRP) and soybean hull (SBP), equilibrium adsorption isotherms were fitted to the Langmuir-type model, where ionic strength, pH and cation concentration were chosen as the variables. For SBP, our results showed that Kd rounded 10^{-6} M in all cases (pH 5.0 - 7.0, 1 and 5 mM Mn^{2+} / Ca^{2+}, 0 - 0.75 M NaCl). For HRP, Kd ranged between 10^{-5} M and 10^{-6} M depending on the parameters. Under optimised binding conditions, 84.3% SBP was recovered after elution carried out with 0.74 M α-methyl-D-mannopyranoside and 0.37 M NaCl. For HRP, the recovery was lower (75%) and 0.36M α-methyl-D-mannopyranoside was necessary for the elution step.**

Keywords-- **Peroxidase, Adsorption, Concanavalin A-Agarose, Chromatography.**

I. INTRODUCTION

Peroxidases (EC 1.11.1.7) are ubiquitous oxidoreductases that use hydrogen peroxide or organic hydroperoxides as oxidants. Most peroxidases are glycoproteins containing N-linked oligosaccharide chains (Dunford and Stillman, 1976; Gray and Montgomery, 1997). Horseradish peroxidase, extracted from *Armoracia rusticana* roots, is a commercially important enzyme that occurs as a large family of isoenzymes (Kay *et al.*, 1967). Later, a peroxidase extracted from soybean hulls, an inexpensive food industry by-product usually used as poultry feed, was described. This peroxidase was extracted from soybean seeds as a single isoenzyme (Gillikin and Graham, 1991). The unique thermal properties and activities of soybean peroxidase make it particularly suited for different industrial applications (McEldoon and Dordick, 1996).

Several methods for peroxidase purification have been reported. Both plant peroxidases are obtained by homogenising crude material with buffer or water; the homogenate is filtered and further purified by precipitation, centrifugation and different chromatographic steps depending on their intended application. When the goal was to obtain a high purity enzyme every purification method included an affinity chromatographic step. In this way, the revised publications use a lectin chromatography to achieve a pure enzyme preparation taking advantage of the fact that HRP and SBP are glycoproteins (Paradkar and Dordick, 1993; Gillikin and Graham, 1991; Casl and Kostrencic, 1987; Tijssen, 1985). In order to reduce the global process cost - especially for SBP - we reported other possibilities based on partitioning in aqueous two-phase systems, but in every proposed scheme an affinity chromatography step was necessary to obtain high-purity products (Miranda *et al.*, 1998).

Plant lectins bind monosaccharides with a relatively low affinity (Ka ~ 1 x 10^3 M), and oligosaccharides much more tightly (Ka ~ 1 x 10^6 M) [Sanders, *et al.*, 2001]. Concanavalin A (Con A), from the jack bean *Cannavalia ensiformis*, is the most extensively studied member of the lectin family. It consists of 26.5 kDa subunits that readily form tetramers. The association of subunits is pH-dependent. Each subunit has binding sites for one Mn^{2+} or Mg^{2+}, one Ca^{2+} and one saccharide. Mn^{2+} or Mg^{2+} must be bound before Ca^{2+} binding, and both metal ions must be present for saccharide binding (Reeke *et al.*, 1974). Con A binds molecules containing α-D-mannopyranosyl and α-D-glucopyranosyl residues, and has been extensively utilised for isolation, fractionation, structural characterisation and immobilisation of glycoproteins carrying these kind of residues. Con A-agarose is commercially supplied. The complex Con A-peroxidase is desorbed with α-methyl-D-mannopyranoside, a sugar that competes with the enzyme at the binding sites.

The aim of this work was to establish the best conditions for purification of peroxidases from horseradish root and soybean seed coat on Con A-agarose affinity chromatography. Ionic strength, pH and cation concentration were chosen as the variables for this study.

II. METHODS

A. Materials

Horseradish peroxidase (P-8000), soybean peroxidase (P-1432), Concanavalin A-agarose and α-methyl-D-mannopyranoside were from Sigma-Aldrich, St. Louis, USA. Both enzyme standards were used without further purification. Guaiacol was from Mallinkrodt Chemical Works, USA. All other reagents were AR grade.

B. Peroxidase assay

Peroxidase activity was measured by the method of Tjissen (1985). The assay mixture contained 3 ml of 100 mM potassium phosphate buffer (pH 7.0) for HRP or 100 mM sodium acetate buffer (pH 5.0) for SBP, 50 μl of guaiacol (2.45 mg/ml) and 10 μl of 8 mM hydrogen peroxide. The absorbance at 470 nm was recorded within 1 min. Activity calculations were also made as described by the above author.

C. Adsorption isotherms

Different isotherms were measured to assess the influence of ionic strength (NaCl concentration), presence of cations (Mn^{2+}, Ca^{2+}) and pH on peroxidases adsorption behaviour in lectin affinity chromatography. The adsorption isotherms for both enzymes binding to Con A-agarose were measured in batch systems, basically as described by Chase (1984). Aliquot samples containing 0.1, 0.25, 0.5, 1.0, 2.5 and 3.5 mg were taken from a stock of concentrated enzyme solution. pH (5.0, 6.0 and 7.0) was adjusted with an adequate buffer containing cations at the required concentration (1 and 5 mM). NaCl was added when corresponded. To equilibrate the matrix, Con A-agarose was packed into a column, washed extensively with water and then equilibrated with the appropriate buffer. This procedure was repeated for each experiment. An aliquot of conditioned Con A- Agarose (200 μl, 50% suspension) was then added to each flask. The suspensions in a final volume of 1 ml were gently agitated at 20°C for 15-20 hours to allow the system to reach its equilibrium. After this time, an aliquot of supernatant was removed from the flasks. The peroxidase concentration in the solution (c*) was determined spectrophotometrically at 403 nm (absorbance of the prosthetic heme group). The equilibrium concentration of peroxidase bound to the adsorbent per unit of total adsorbent volume (q*), was calculated as the total amount of enzyme present at the beginning of the experiment less the amount still in the soluble phase at equilibrium.

D. Fast Protein Liquid Chromatography

A column (0.5 x 4 cm) of Con A-agarose was prepared and equilibrated with the selected buffer. New columns were prepared for each application instead of regeneration and reusing of the same column. Chromatography was performed at room temperature. In the case of SBP, the matrix was equilibrated with 20 mM acetate buffer, pH 5.0, 1 mM $CaCl_2$ and $MnCl_2$ and 50 μl of the peroxidase solution (12.4 mg/ml) was

loaded. Fractions of 1 ml were collected at a linear flow rate of 3.21 cm/min. Elution was carried out by using a dual gradient of α-methyl-D-mannopyranoside /NaCl (0 - 1 M and 0 - 0.5 M, respectively). For HRP, the matrix was equilibrated with 20 mM Tris-HCl buffer, pH 7.0, 5 mM $CaCl_2$ and $MnCl_2$, 0.75 M NaCl, and 50 μl of peroxidase solution (12.4 mg/ml) was loaded. Fractions of 0.5 ml were collected at a linear flow rate of 1.53 cm/min. In this case, elution was carried out by using a α-methyl-D-mannopyranoside gradient (0 - 1 M).

III. RESULTS AND DISCUSSION

In assessing the effect of different variables (pH, cation and NaCl concentration) on the peroxidase binding to Con A-agarose, adsorption isotherms were developed. The binding data were fitted to a Langmuir-type isotherm in which q* varies with c* as shown in Eqn. (1):

$$q^* = q_m . c^* / (c^* + K_d) \qquad (1)$$

where q_m is the maximum bound peroxidase concentration at equilibrium and K_d is the equilibrium dissociation constant (Chase, 1984).

A. Effect of pH

For SBP, in all conditions assayed Kd was around 10^{-6} M. pH had an important influence on Kd when isotherms were developed in the absence of salt (NaCl) and in the presence of 1 mM ion concentration. The corresponding adsorption isotherms at different pH values in the absence of NaCl are shown in Figure 1A, where the influence of this variable on Kd is evident ($2.4 . 10^{-6}$ M at pH 5.0, and $5.0 . 10^{-6}$ M at pH 6.0 and 7.0). At higher NaCl concentration a maximum was observed at pH 6 (Figure 3A).
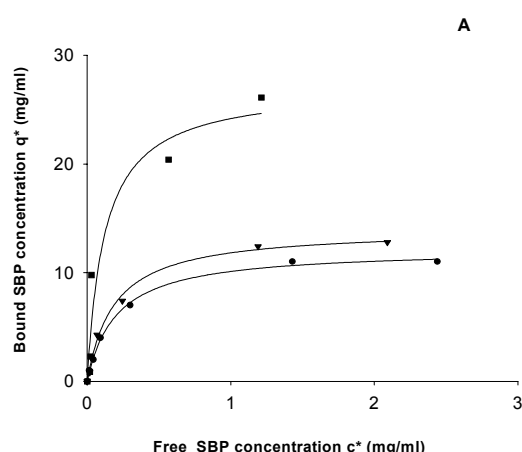

Figure 1A. Adsorption isotherms for SBP binding to Con A-agarose, at pH 5.0 (■), 6.0 (▼) and 7.0 (●). Buffer: 20 mM sodium acetate, pH 5.0, or 20 mM sodium phosphate, pH 6.0 or 7.0. Ions concentration: 1 mM. Details in Methods.
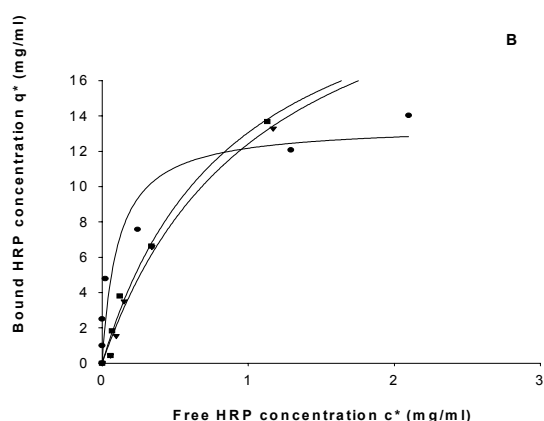
Figure 1B. Adsorption isotherms for HRP binding to Con A-agarose at pH 5.0 (■), 6.0 (▼) and 7.0 (●). Buffer: 20 mM sodium acetate, pH 5.0, or 20 mM sodium phosphate, pH 6.0 or 7.0. Ions concentration: 5 mM. NaCl concentration: 0.75 M. Details in Methods.

For HRP -in contrast-, pH has a strong influence on Kd, especially at high NaCl concentration. In the presence of 0.75 M NaCl and 5 mM ion, Kd value decreased from $2.6 \cdot 10^{-5}$ M to $9.5 \cdot 10^{-6}$ M when the pH was raised from 5.0 to 7.0 (Figure 1B). This effect was not evident at 1 mM ion concentration.

B. Effect of NaCl Concentration
In the case of SBP, results indicate that, specially at pH 6.0 and 7.0, NaCl concentration has not influence on Kd (Figure 3A) . In contrast, at pH 5.0 and 1 mM ion concentration, in the absence of NaCl, a slight positive effect on Kd is apparent (Figure 2A).

For HRP, results indicate a strong influence of salt on Kd at pH 7.0 and 5 mM ion concentration. No significant influence of salt, at pH 5.0 or 6.0 was shown. Figures 2B and 3B illustrate these results.

C. Effect of Ion Concentration
For SBP ion concentration was not an important variable that modifies Kd values. For HRP only at pH 7.0 ion concentration showed a positive influence on Kd. The higher ion concentration allowed a better adsorption of enzyme to the matrix (data not shown).

D. Selection of the Best Adsorption Conditions
Tables 1 and 2 show Kd and q_m values obtained in each experiment for a given pH and NaCl at a constant concentration. Figure 3 (A and B) is a 3D-graphic of Ka (1/Kd) vs. pH and NaCl concentration that facilitates the finding of the best adsorption conditions for each enzyme.

E. Optimisation of the Elution Step
Under the optimised binding conditions, a sample was loaded on a Con A-Agarose column. In the case of SBP, chromatography was performed in 20 mM sodium

acetate buffer, pH 5.0, containing 1 mM ion concentration. Results indicate that 84.3 % SBP was recovered without protein loss during the wash step. For elution it was necessary 0.74 M α-methyl-D-mannopyranoside and 0.37 M NaCl. Figure 4A illustrates these results. Addition of salt into the elution buffer accelerated the enzyme desorption with a lower α-methyl-D-mannopyranoside concentration.
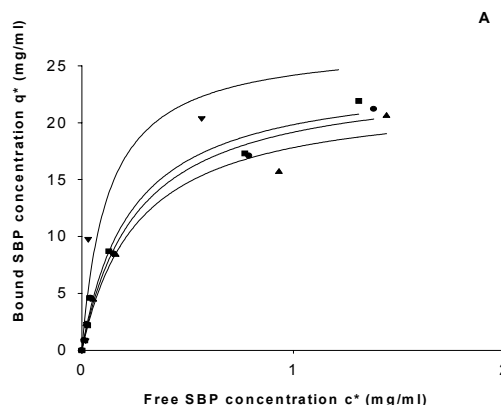

Figure 2A. Adsorption isotherms for SBP binding Con A-agarose at pH 5.0, and 0.25 (■), 0.50 (●), 0.75 M NaCl (▲), and absence of salt (▼).Buffer: 20 mM sodium acetate, pH 5.0, or 20 mM sodium phosphate, pH 6.0 or 7.0. Ions concentration: 1 mM. Details in Methods.
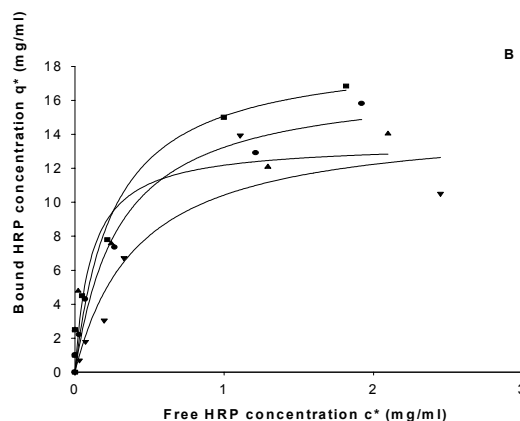

Figure 2B. Adsoption isotherms for HRP binding Con A-agarose at pH 7.0, and 0.25 (■), 0.50 (●), 0.75 M NaCl (▲), and absence of salt (▼). Buffer: 20 mM sodium acetate, pH 5.0, or 20 mM sodium phosphate, pH 6.0 or 7.0. Ions concentration: 5 mM. Details in Methods.

In the case of HRP, as high NaCl concentration helps enzyme binding to Con A-Agarose, the elution was performed only by a gradient of 0 - 1 M α-methyl-D-mannopyranoside. HRP eluted at 0.10 - 0.36 M α-methyl-D-mannopyranoside. The chromatographic

pattern is shown in Figure 4B. Therefore a step elution with 0.36 M α-methyl-D-mannopyranoside was developed and the enzyme recovery was 75.0 % - in contrast with SBP- 20-25 % of peroxidase activity passed through during the wash step.

Table 1. K_d and q_m values obtained in SBP adsorption isotherms.

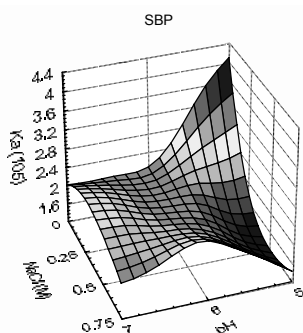|		Kd (10^{-6} M)	
pH	NaCl (M)	Ions 1 mM	Ions 5 mM
5	0	2.4	ND
	0.25	6.3	5.1
	0.50	7.0	6.4
	0.75	7.1	5.9
6	0	5.0	ND
	0.25	4.8	5.0
	0.50	4.8	4.5
	0.75	4.0	5.4
7	0	5.0	ND
	0.25	3.7	3.7
	0.50	4.4	5.1
	0.75	5.1	2.2
		qm (mg/ml Con A-agarose)	
pH	NaCl (M)	Ions 1 mM	Ions 5 mM
5	0	27.13	ND
	0.25	24.45	22.13
	0.50	24.14	23.60
	0.75	22.53	23.90
6	0	14.00	ND
	0.25	14.32	14.03
	0.50	14.75	13.14
	0.75	13.18	13.86
7	0	16.01	ND
	0.25	16.10	14.47
	0.50	14.08	16.90
	0.75	11.90	11.37

Figure 3A. 3-D plot showing the influence of pH and NaCl concentration on the Ka value obtained from adsoption isotherms for SBP at 1 mM cation concentration binding to Con A- Agarose.
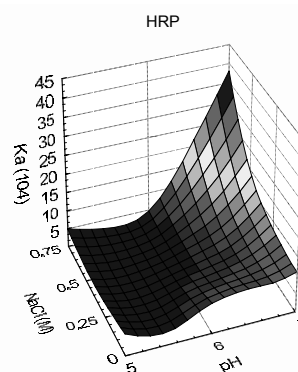

Figure 3B. 3-D plot showing the influence of pH and NaCl concentration on the Ka value obtained from adsoption isotherms for HRP at 5 mM cation concentration binding to Con A- Agarose.

Table 2. K_d and q_m values obtained in HRP adsorption isotherms.

		Kd (10^{-5} M)	
pH	NaCl (M)	Ions 1 mM	Ions 5 mM
5	0.25	2.2	2.6
	0.50	4.4	3.0
	0.75	2.0	2.0
6	0.25	2.0	1.8
	0.50	2.3	2.5
	0.75	2.1	2.4
7	0	ND	0.95
	0.25	1.6	0.67
	0.50	1.7	0.57
	0.75	3.0	0.26
		qm (mg/ml Con A-agarose)	
pH	NaCl (M)	Ions 1 mM	Ions 5 mM
5	0.25	24.84	23.75
	0.50	36.18	24.69
	0.75	23.02	29.29
6	0.25	27.00	23.54
	0.50	27.92	29.50
	0.75	27.62	25.89
7	0	ND	14.78
	0.25	18.19	17.17
	0.50	23.20	18.92
	0.75	28.63	13.50

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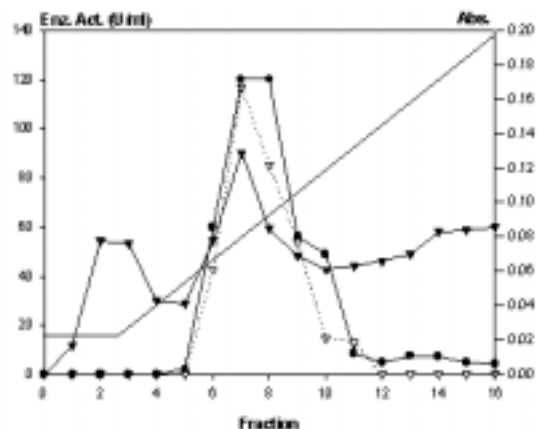

Figure 4A: Elution profile of SBP on a Con A- Agarose column. Absorbance at 280 nm (▼), at 403 nm (▽) and enzyme activity (●). Details in Methods. Elution dual gradient (–): 0 - 1M α-methyl-D-mannopyranoside and 0 - 0.5 M NaCl.
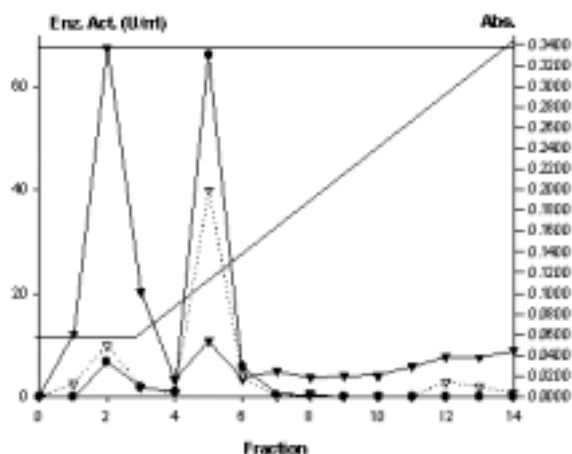

Figure 4B: Elution profile of HRP on a Con A-agarose column. Absorbance at 280 nm (▼), at 403 nm (▽) and enzyme activity (●). Details in Methods. Elution gradient (–): 0 - 1M α-methyl-D-mannopyranoside.

IV. CONCLUSIONS

Optimised binding conditions for peroxidases (HRP and SBP), a group of relevant industrial enzymes, has been established through a detailed quantitative study based on adsorption experiments. It was possible to establish the influence of factors that govern the performance of the adsorption stage of the separation procedure on Con A- Agarose.

It has been shown different optimised chemical environment for HRP and SBP. While SBP was relatively insensitive to buffer changes, HRP showed clear operational windows. This information was employed in a rational scheme for selecting operating conditions for adsorption and elution stages.

Finally, the parameter calculated from batch isotherm (Kd) was useful to predict the enzymatic behaviour in batch and packed bed chromatography.

These results are expected to be applied in the design of adsorption processes using affinity (Con A) fluidised adsorbents.

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