

WEB348

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Introduction

Question

What do pH profiles of the reaction velocities look like for three different substrates (eriodictyol, iso-ferulic acid and caffeic acid) under conditions where Mg is present and under no-Mg conditions? Is there an influence of magnesium on the catalysis?

Data

HPLC profiles of the reactions were analyzed. Substrate and product-peaks were integrated and the initial velocities were calculated from the slopes. For the sake of uniformity the actual data that is being worked with here is only concerned with the appearance and disappearance of SAM and SAH. These products are the same in every reaction. The other products (ferulic acid, homo-eriodictyol and dimethyl caffeic acid) have different molar extinction coefficients from each other. This adds bias to the analysis.

	label	substrate	Mg
1	A	eriodictyol	FALSE
2	B	eriodictyol	TRUE
3	C	iso-ferulic acid	FALSE
4	D	iso-ferulic acid	TRUE
5	E	caffeic acid	FALSE
6	F	caffeic acid	TRUE

Table 1: Experiment key.

	sample	pH	key	V_AUpermin	sd_AUpermin
1	A	5.50	Eriodyctiol	-30890.10	17512.51
2	A	5.50	Homoeriodictiol	3143.07	94.60
3	A	5.50	SAH	0.00	0.00
4	A	5.50	SAM	1129.98	2392.07
5	A	6.50	Eriodyctiol	-212683.60	10112.91
6	A	6.50	Homoeriodictiol	218413.10	5255.67

Table 2: The first rows of the velocities that were calculated from HPLC runs.

	sample	pH	key	V_AUpermin	sd_AUpermin	Mg
1	A	5.50	SAH	0.00	0.00	FALSE
2	A	5.50	SAM	1129.98	2392.07	FALSE
3	A	6.50	SAH	43492.40	2286.97	FALSE
4	A	6.50	SAM	-34469.43	5547.25	FALSE
5	A	7.50	SAH	57777.43	2995.79	FALSE
6	A	7.50	SAM	-44773.30	1839.22	FALSE

Table 3: Only the velocities of SAM-disappearance and SAH-appearance that were calculated from HPLC runs. Another column Mg was added to describe, whether Mg was added or not.

pH profiles

The pH profiles show a clear pH-dependency of the PFOMT reaction. This is true when magnesium is present or absent. The reaction is quick for the catecholic substrates caffeic acid and eriodictyol. However the reaction is slow for iso-ferulic acid (margin).

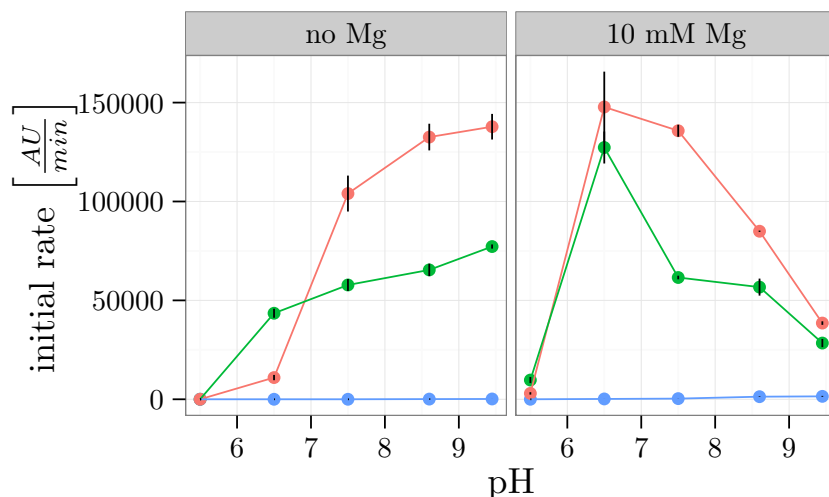


Figure 1: pH-profiles for SAH production. Substrates: red – caffeic acid, green – eriodictyol, blue – iso-ferulic acid

Iso-ferulic acid is not catechol, rather it bears a (4'-O-methyl-3'-hydroxyl)-moiety. But clearly iso-ferulic acid is methylated at higher pH values, especially when Mg is present.

THE pH-OPTIMUM of the enzyme seems to shift to lower pH-values with addition of Mg. When no Mg is added the initial velocity increases with pH. However, upon Mg addition the maximum is reached at a pH of around 6.8 for the catecholic derivatives. After that the rate drops drastically.

FOR ISO-FERULIC ACID this effect is not present. The rates are much higher, when Mg is added. Even at high pH-values. When no Mg is added there is virtually no conversion at low pH values. This could correlate with the pKa.

Regression tree

Linear models

The motif plays an important role for catalysis. Thus, the data set was split up into two separate ones. One including only iso-ferulic acid and one including the other two catecholic substrates.

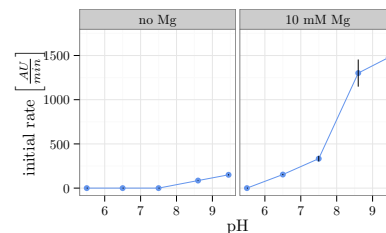
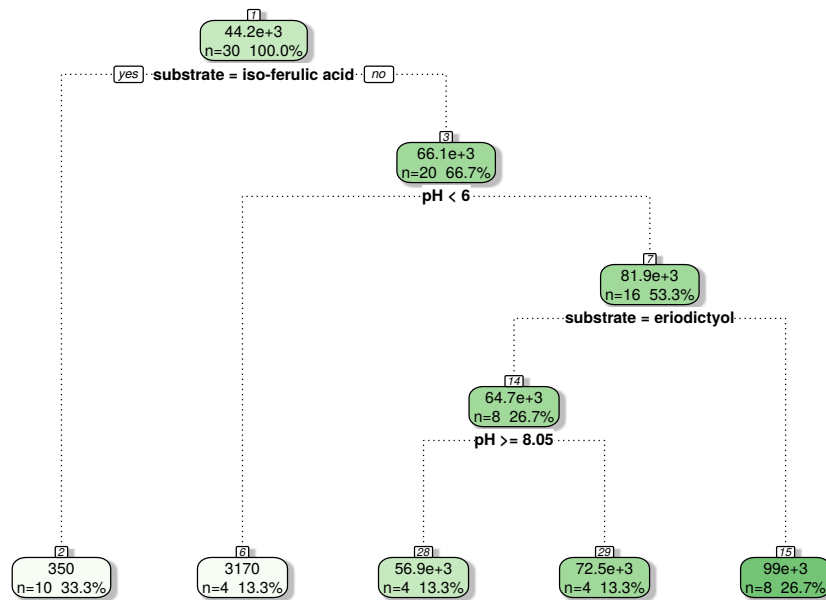


Figure 2: pH-profiles for the substrate iso-ferulic acid. The reaction occurs much slower than for the catecholic substrates.



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ENZYMATIC REACTIONS ARE HARD TO MODEL using simple linear models. This is due to the fact that enzymatic mechanisms are hardly linear. In fact enzymatic mechanisms are highly complex and non-linear. However for a close approximation it should do.

Iso-ferulic acid dataset

93.55% of the variance in the data can be described by the model $\text{rate} \sim \text{pH} * \text{Mg}$, including both main effects and interaction terms. Both main effects (pH and Mg), as well as the interaction term seem to be significant, as suggested by their p-values below 0.01 when compared by ANOVA. However, the model would deem the main effect of pH not significant (p-value 0.51).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH	1	1016694.74	1016694.74	33.60	0.0012
Mg	1	918173.79	918173.79	30.34	0.0015
pH:Mg	1	699519.42	699519.42	23.12	0.0030
Residuals	6	181570.88	30261.81		

Table 4: ANOVA-table for the simple model for iso-ferulic acid. The interaction term is included.

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	-241.4238	420.1485	-0.57	0.5864
pH	38.4239	54.9778	0.70	0.5108
MgTRUE	-2201.3084	594.1797	-3.70	0.0100
pH:MgTRUE	373.8131	77.7503	4.81	0.0030

The model predicts the data sufficiently correct. Plus it is not the goal to make predictions, rather than draw inferences.

Catechols dataset

The catecholic substrates were modelled accordingly. However, because the non-linear nature of the data a quadratic term was included in the model. The model formula was $\text{rate} \sim \text{Mg} * \text{pH} + \text{I}(\text{pH}^2)$. The substrate was not incloed in the model for the sake of simplicity, although it undoubtetly has an influence.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Mg	1	207399999.37	207399999.37	0.19	0.6695
pH	1	7699725667.36	7699725667.36	7.06	0.0188
I(pH^2)	1	11409722262.76	11409722262.76	10.46	0.0060
Mg:pH	1	8909537802.01	8909537802.01	8.17	0.0127
Mg:I(pH^2)	1	5057251759.85	5057251759.85	4.64	0.0492
Residuals	14	15273527656.71	1090966261.19		

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	-421929.9946	356063.7085	-1.18	0.2557
MgTRUE	-839999.8874	503550.1257	-1.67	0.1175
pH	103271.3345	97739.1728	1.06	0.3086
I(pH^2)	-4977.7406	6512.6996	-0.76	0.4574
MgTRUE:pH	266920.7964	138224.0638	1.93	0.0740
MgTRUE:I(pH^2)	-19830.2264	9210.3481	-2.15	0.0492

THE TREND OF THE CURVE is described by the model sufficiently correct. Together with the data from the ANOVA table, it can be concluded that there is an interaction effect between pH and Mg. However, the linear model is hardly sufficient to predict the correct curve. In fact it merely accounts for 68% of the variance ($R^2=0.6855$).

Table 5: Model summary for the linear model (lm()).

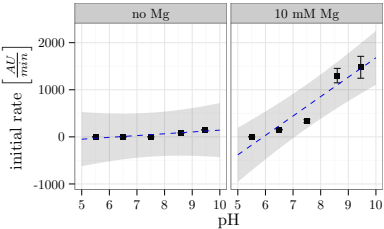


Figure 3: pH profiles for iso-ferulic acid with predicted data from the model. The grey ribbon displays the 95% prediction interval.

Table 6: ANOVA-table for the simple model foir iso-ferulic acid. The interaction term is included.

Table 7: Model summary for the linear model (lm()).

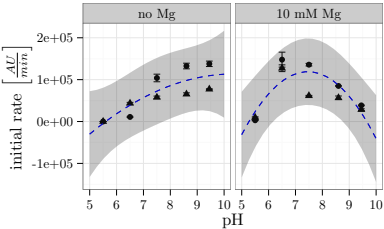


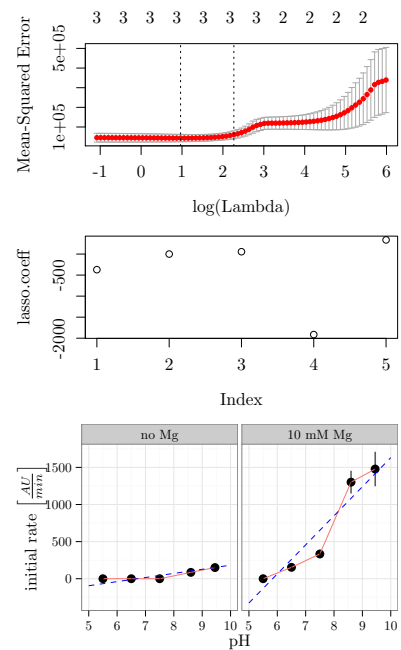
Figure 4: pH profiles for catecholic substrates with predictions and 95% prediction interval.

Cross validation lasso regression

The split datasets were also modelled using lasso regression, together with a cross validation approach.

CV lasso on iso-ferulic acid

Since only non-zero coefficients are shown, it can also be concluded here that pH and Mg display both main and an interaction effect for iso-ferulic acid. Diagnostic plots that show the optimization for the tuning parameter lambda are displayed on the margin. The final shrunken model includes only three variables (Mg, pH and Mg:pH interaction).



	variable	coefficient
1	(Intercept)	-371.3745
2	pH	55.3578
3	MgTRUE	-1913.8456
4	pH:MgTRUE	336.2752

Table 8: Variables and coefficients that were retained. Non-zero coefficients not shown.

CV lasso on catechols

The same CV method was also applied for the catecholic substrate data. The model formular was $\text{rate} \sim \text{pH} * \text{Mg} * \text{I}(\text{pH}^2)$. After shrinkage the model still contained a lot of factors. All the main effects, as well as 2 two-way interaction and one 3-way interaction effect. This makes the model very complex. However from the predicted curves it is clear that the model roughly describes the data. This can be seen as evidence that the relationship of pH and rate includes a quadratic term.

Conclusion

- big difference in rate of catecholic and non-catecholic substrates
- rate is influenced by pH, Mg
- pH and Mg have interaction effect
- pH/rate relationship includes a quadratic term → common form for pH-profiles of enzymes
- Mg addition shifts pH optimum of enzyme to pH around 7 for catechols
- Mg addition is beneficial for conversion of iso-ferulic acid

Specific activity

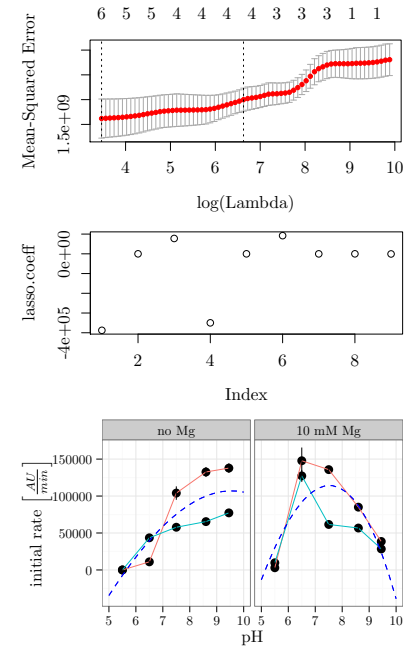
The specific activity can be calculated. Therefore we approximate that the molar extinction coefficient for SAM and SAH are the same and the combined area-under-the-curve (AUC) of the SAM and SAH peak are 100% of substrate or 500 uM. The concentration for either one of SAM or SAH can then be estimated from that relationship.

From the concentration and the injection volume (10 uL) the amount (moles) of substrate or product can be estimated. From that the specific activity can be calculated from the slope of the P-t-diagram and the amount of enzyme used (0.2 ug/uL). First the above equations were employed to calculate the concentration of SAH and SAM in each sample. This was plotted against the area. The curve displays a linear relationship, although the errors seem to get larger with increasing area. The errors seem to be non-normally distributed. However as a rough estimation it will suffice.

The data for each SAH and SAM were fitted with a linear model concentration~area.

$$c = a \times A + b \quad (1)$$

For SAH the values for a and b were 1.272e-04 and 6.219e-02 respectively.



	variable	coefficient
1	(Intercept)	-387606.9260
2	pH	77725.3554
3	MgTRUE	-349735.8685
4	I(pH^2)	-0.0045
5	pH:MgTRUE	92049.1328
6	pH:I(pH^2)	-284.3251
7	pH:MgTRUE:I(pH^2)	-715.0114

Table 9: Variables and coefficients that were retained. Non-zero coefficients not shown.

$$A_{\text{SAM}} + A_{\text{SAH}} = 1 \approx 500\text{uM}$$

$$x_{\text{SAH}} = \frac{A_{\text{SAH}}}{A_{\text{SAM}} + A_{\text{SAH}}}$$

$$c_{\text{SAH}} = x_{\text{SAH}} \times 500\text{uM}$$

$$n_{\text{SAH}} = c_{\text{SAH}} \times V^{\text{inject}}$$

Figure 5: Calculation of specific activity.

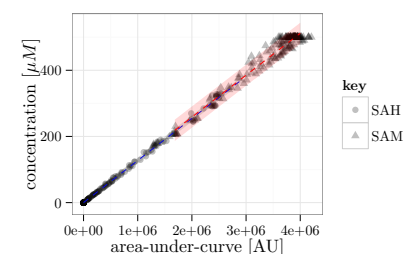


Figure 6: The calculated concentrations from the areas. The error looks as though it is not normally distributed.

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	0.0622	0.4018	0.15	0.8772
area	0.0001	0.0000	292.71	0.0000

	max.U.mg	max.pkat.mg	at.pH	substrate	Mg
1	49.42	823.73	9.45	eriodictyol	FALSE
2	81.26	1354.41	6.50	eriodictyol	TRUE
3	0.41	6.78	9.45	iso-ferulic acid	FALSE
4	1.25	20.85	9.45	iso-ferulic acid	TRUE
5	87.96	1465.93	9.45	caffeic acid	FALSE
6	94.30	1571.61	6.50	caffeic acid	TRUE

The maximum enzyme activities that were calculated here are comparable to the ones that were obtained previously (DIM paper PFOMT, wt(caffeic acid) $\approx 72 \text{ nmol min}^{-1} \text{ mg}^{-1}$, Vogt (2003) nativ(6-OH-kaempferol) $\approx 830 \text{ pkat per mg}$).

Table 10: Linear fit for SAH area and concentration.

Table 11: The maximal activities obtained for each sample (substrate/Mg combination). Activities are given in $\text{nmol min}^{-1} \text{ mg}^{-1}$

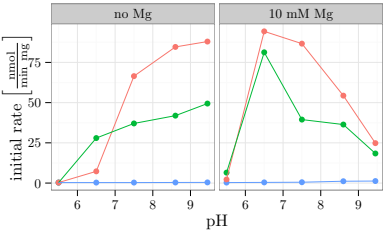


Figure 7: pH-profiles with specific activities.

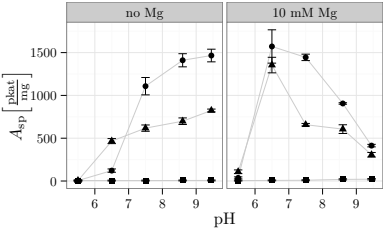


Figure 8: pH-profiles with specific activities.