Supplementary Information to "**Substrate specificity of an oxygen dependent sulphur transferase in ovothiol biosynthesis**"

Gabriel T. M. Mashabela[1, 2] and Florian P. Seebeck[1*]

[1]Department for Chemistry, University of Basel, St. Johanns-Ring 19, 4056, Basel, Switzerland

[2] Department of Chemistry, University of Cape Town, 7700, Rondebosch, South Africa

*To whom correspondence should be addressed. phone +41 612671143. E mail: florian.seebeck@unibas.ch

Material. OvoA from *Erwinia tasmaniensis* was produced and purified as previously described.[1] Amino acids and amino acid derivatives were purchased from Sigma-Aldrich. 2-thio-L-histidine was purchased from Bachem (Bubendorf) Samples of 2- and 4-fluoro-L-histidine was a gift from Dr. Kenneth Kirk (NIH).[2] Chloro- bromo- and iodohistidines were synthesized following published procedures.[3]

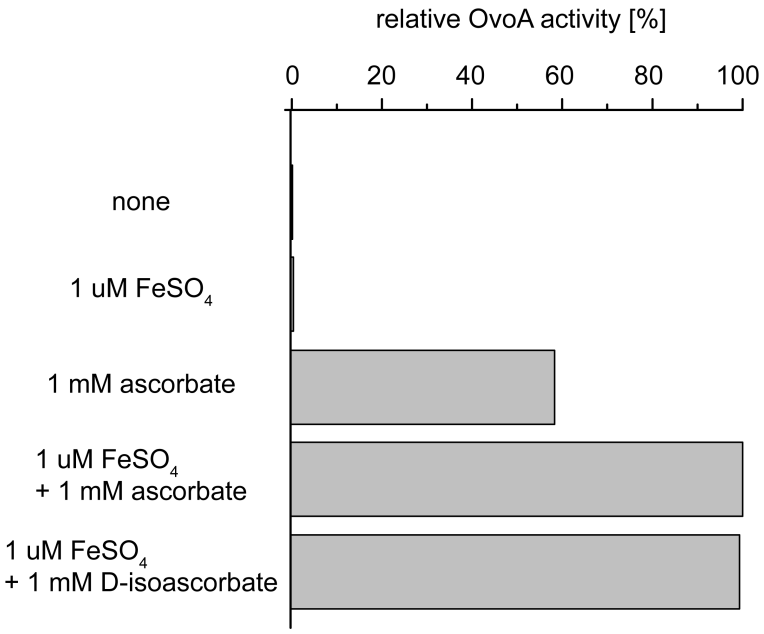


Figure S1. Addition of 1 uM iron (II) and 1 mM ascorbate or iso-D-ascorbate enhances *in vitro* OvoA activity by 100-fold. OvoA was assayed in the presence of 1 mM L-cysteine and L-histidine, 50 mM Tris HCl at pH 8.0 and 50 mM NaCl, at 26°C. At 1, 2, 4 and 8 minutes, 30 ul aliquots of the reactions were quenched by addition of 15 ul 1 M phosphoric acid and analyzed by cation exchange HPLC using 50 mM phosphoric acid at pH 2 as a mobile phase. Compounds were eluted in a NaCl gradient. The chromatograms were recorded at 220 nm. The displayed data represent averages of three independent measurements. Standard deviations are less than 10 percent points. The data was fitted by linear regression to obtain d[product]/d[time] of the OvoA catalyzed conversion of L-histidine and L-cysteine to L-5-histidyl-L-cysteine sulfoxide.

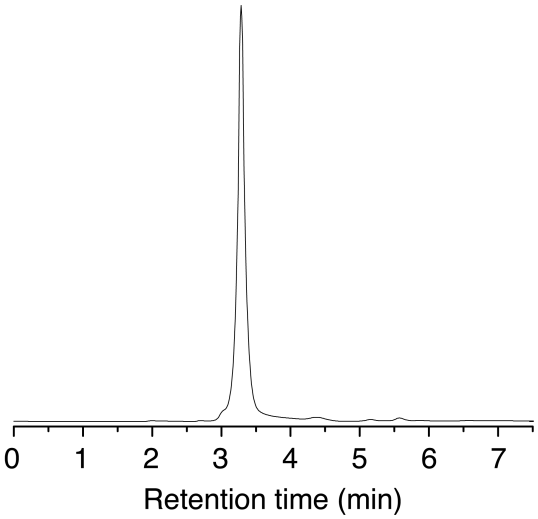


Figure S2. HPLC chromatogram recorded at 220 nm of 5-L-histidyl-L-cysteine sulfoxide prepared on a 100 mg scale. A 800 ml reaction containing 1 mM L-histidine and L-cysteine, 20 mM NaCl, 2 mM TECEP, 1 mM ascorbate, 1 uM $FeSO_4$, 20 mM Tris HCl pH 7.5, and 10 mM OvoA was incubated at room temperature (22 – 25 °C) over night. The solution was acidified to pH 3.0 with formic acid and then incubated with 20 ml Dowex resin. The resin was loaded on a column and washed with 25 mM ammonium formate solution pH 3.0 until no ascorbate was detected by HPLC. 5-L-histidyl-L-cysteine sulfoxide was eluted with 50 mM ammonium formate solution at pH 5.0. Product containing fractions were pooled an lyophilized. Residual ammonium formate was removed by repeated freeze-drying. From this procedure we obtained 100 mg 5-L-histidyl-L-cysteine sulfoxide.

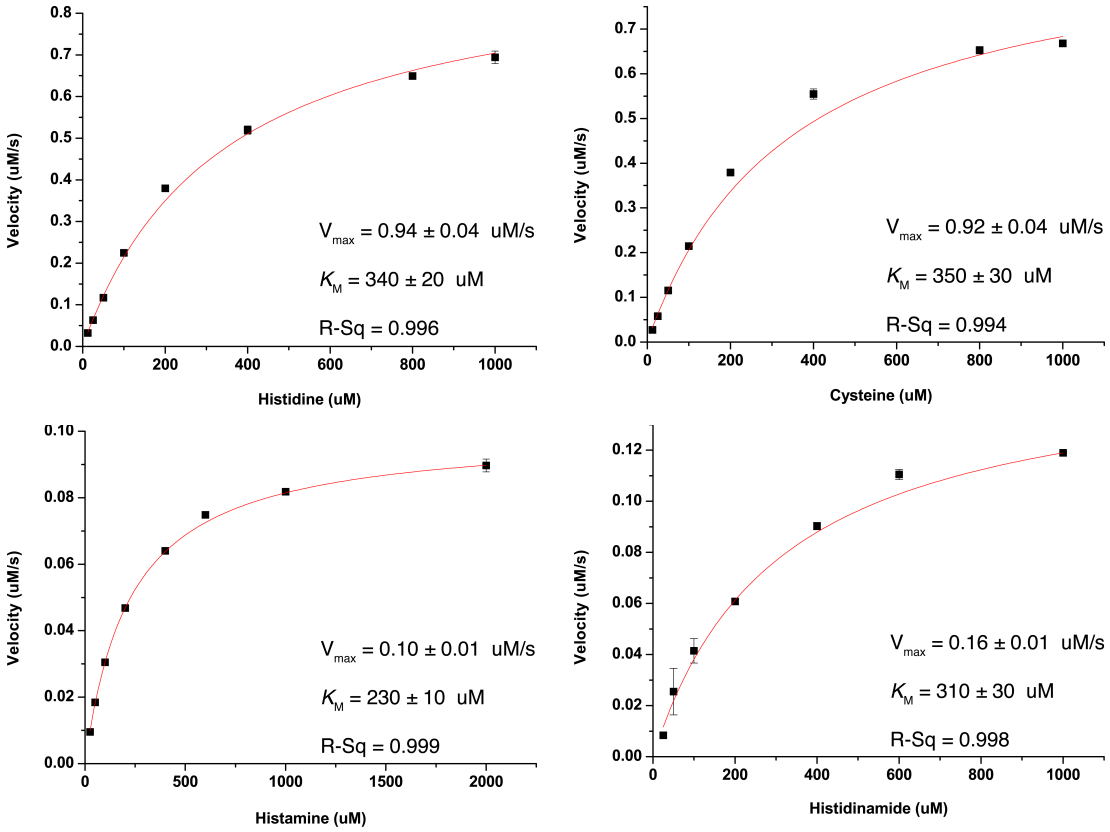


Figure S3. Michaelis-Menten kinetic analysis. Standard conditions for this assay were as follows: Reaction mixtures containing 20 mM Tris HCl pH 8.0, 20 mM NaCl, 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 0.8 mM L-cysteine, L-histidine or L-histidinamide (**6**, Figure 2) and histamine (**7**) (12.5 – 1000 uM), 0.28 uM OvoA in final volume of 250 ul was incubated at 26°C. At 1, 2, 4 and 8 minutes, 30 ul aliquots of the reactions were quenched by addition of 15 ul 1 M phosphoric acid and analyzed by cation exchange HPLC using 50 mM phosphoric acid at pH 2 as a mobile phase. Compounds were eluted in a NaCl gradient. All HPLC chromatograms were recorded at 220 nm. The data was fitted to the function $v = V_{max}[s]/(K_M + [s])$. For measurements Michaelis-Menten kinetic analysis of histidine analogs the concentration of L-cysteine was kept constant at 0.8 mM. The displayed data represent averages of two independent measurements.

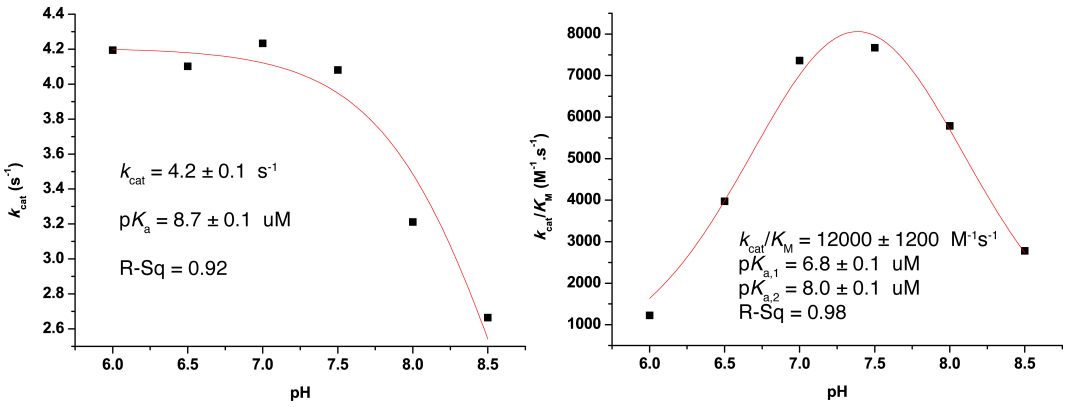


Figure S4. pH dependence of $k_{cat,his}$ (**left**) an $k_{cat,his}/K_{M,his}$ (**right**). OvoA catalyzed product formation in reactions containing 0.8 mM L-cysteine, L-histidine (12.5 – 1000 uM), 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 20 mM NaCl and 0.28 uM OvoA, in 50 mM Britton-Robinson buffer was characterized at pH 6 – 8.5. At each pH we performed a full Michaelis-Menten analysis as described above (Figure S3). The pH vs. $k_{cat,his}$ plot was fitted to $k_{cat,his} = k_{A^-} \times 10^{(pH - pKa)}/(1 + 10^{(pH - pKa)})$. The pH vs. $k_{cat,his}/K_{M,his}$ plot was fitted to $k_{cat,his}/K_{M,his} = k_{HA}/(1 + 10^{(pKa1 - pH)} + 10^{(pH - pKa2)})$.

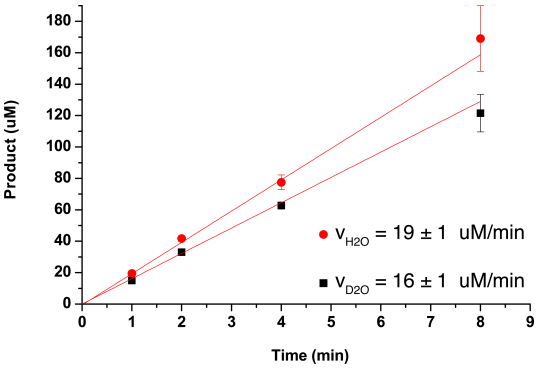
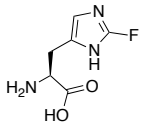
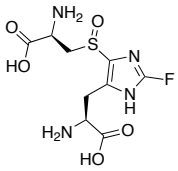
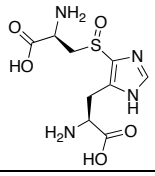
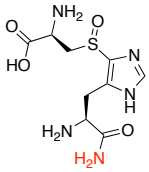
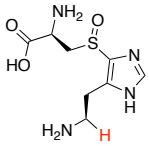
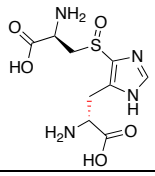
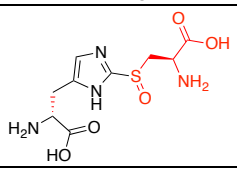
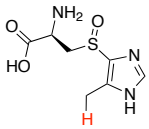
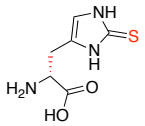
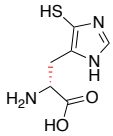
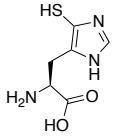


Figure S5. The solvent isotope effect of OvoA catalyzed conversion of L-histidine and L-cysteine to 5-L-histidyl-L-cysteine sulfoxide was quantified in D_2O or H_2O solutions containing 0.8 mM L-cysteine and L-histidine, 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 20 mM NaCl and 0.28 uM OvoA, 20 mM Tris HCl pH 8.0. The reactions were monitored as described above. The displayed data represent averages of three independent measurements and was fitted by linear regression to obtain d[product]/d[time].

Table S1. ESI MS identification of OvoA product analogs. Structural deviations from the native OvoA substrates/products are indicated in red.

structure	compound	Cald. [Da]	Meas. [Da]	HRMS [Da]
	2-fluoro-L-histidine	174.0679		174.0674
	5-(2-fluoro)-L-histidyl-L-cysteine sulfoxide	309.0669	309.1	309.0663
	5-L-histidyl-L-cysteine sulfoxide	291.07	291.0	
	5-L-histidinamidyl-L-cysteine sulfoxide	290.08	290.0	
	5-histaminyl-L-cysteine sulfoxide	247.08	269.1 (M^+ + Na)	
	5-D-histidyl-L-cysteine sulfoxide	291.0763	291.0	291.0757
	2-D-histidyl-L-cysteine sulfoxide	291.0763	291.0	291.0757
	4-methyl-5-imidazyl-L-cysteine sulfoxide	218.05	218.1	
	2-thio-D-histidine	188.04	188.1	
	5-thio-D-histidine	188.04	188.0	
	5-thio-L-histidine	188.04	188.0	

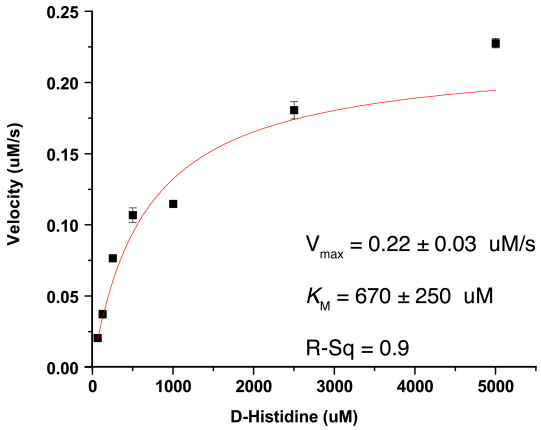


Figure S6. Michaelis-Menten kinetic analysis of OvoA catalyzed turnover of D-histidine. The HPLC analysis of OvoA catalyzed turnover of D-histidine shows that OvoA generates a mixture of 2-L-histidyl-L-cysteine sulfoxide and 5-L-histidyl-L-cysteine sulfoxide. The two products are difficult to separate by analytical HPLC and are characterized by different extinction coefficients at 220 nm, which makes conversion of HPLC integrals to concentrations difficult. We therefor monitored D-histidine consumption and determined k_{cat} and k_{cat}/K_M for this reaction analogously to method described above (Figure S3).

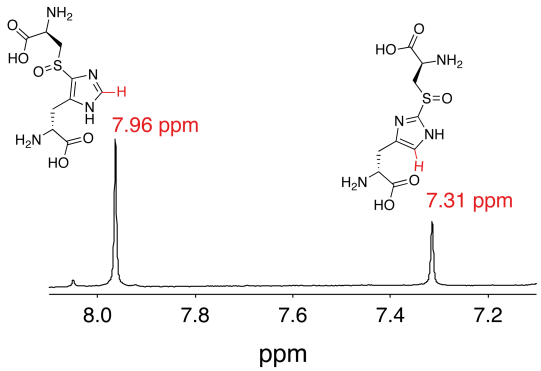


Figure S7. ^{1}H NMR analysis of the HPLC purified product mixture (Figure S7, right, reaction **a**) showed that OvoA produces 5-D-histidyl-L-cysteine sulfoxide and 2-D-histidyl-L-cysteine sulfoxide in a ratio of 1:0.6 (600 MHz, D_2O, 20°C): ^{1}H NMR δ 7.96 (s, 1 H, C_2-H, integral = 1) and δ 7.31 (s, 1 H, C_5-H, integral = 0.6).

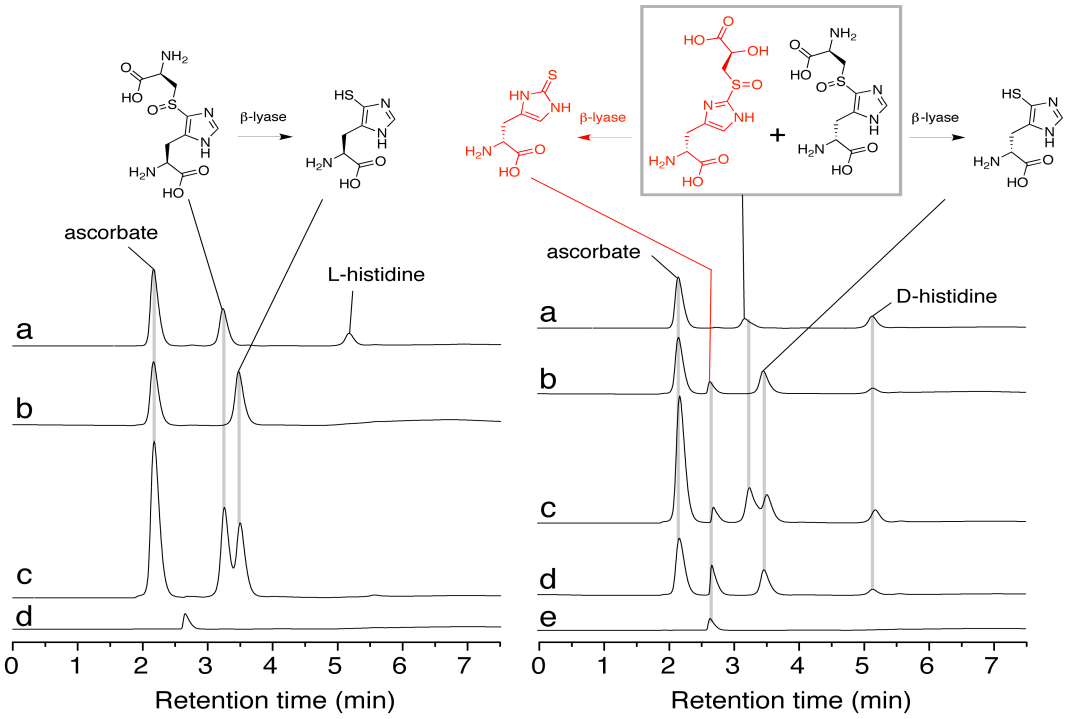


Figure S8. OvoA sulphurizes D-histidine at the 2- and 4-position. All reaction products were purified and characterized by ESI MS (Table S1). **Left:** A reaction containing 20 mM Tris HCl pH 8.0, 20 mM NaCl, 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 0.8 mM L-cysteine and L-histidine was incubated at 26°C and analyzed by HPLC (→ **a**). Addition of a non-specific pyridoxal phosphate dependent β-lyase converts 2-L-histidyl-L-cysteine sulfoxide to 5 thio-L-histidine (→ **b**). Coinjection of reactions **a** and **b** shows that the products 2-L-histidyl-L-cysteine sulfoxide and 5-thio-L-histidine are separated by HPLC (→ **c**). HPLC comparison of reaction **c** with an authentic sample of 2-thio-L-histidine confirms that the β-lyase product is 5-thio-L-histidine (→ **d**). **Right:** A reaction containing 20 mM Tris HCl pH 8.0, 20 mM NaCl, 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 0.8 mM L-cysteine and L-histidine was incubated at 26°C and analyzed by HPLC (→ **a**). Addition of a non-specific pyridoxal phosphate dependent β-lyase converts 2-D-histidyl-L-cysteine sulfoxide to 2-thio-D-histidine; and 5-D-histidyl-L-cysteine sulfoxide to 5-thio-D-histidine (→ **b**). Coinjection of reactions **a** and **b** shows that the products 2-D-histidyl-L-cysteine sulfoxide, 5-D-histidyl-L-cysteine sulfoxide, 2-thio-D-histidine, and 5-thio-D-histidine are separated by HPLC (→ **c**). Coinjection of reaction **b** with authentic 2-thio-L-histidine identifies one of the β-lyase products as 2-thio-D-histidine (→ **d**). Authentic 2-thio-L-histidine (→ **e**) . All HPLC chromatograms were recorded at 220 nm.

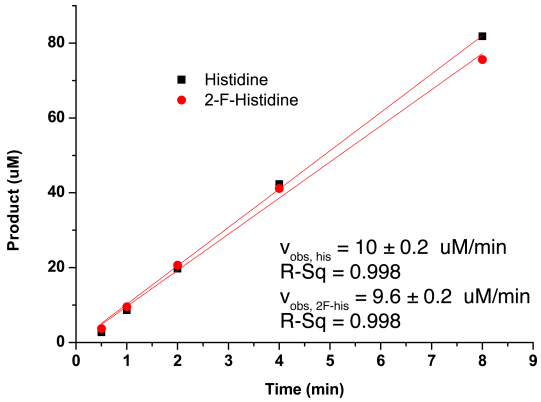


Figure S9. Comparison of OvoA catalyzed L-histidine and 2-fluoro-L-histidine turnover. A reaction mixture containing 20 mM Tris HCl pH 8.0, 20 mM NaCl, 2 mM TCEP, 1 mM ascorbate, 1 uM $FeSO_4$, 0.8 mM L-cysteine, 1 mM L-histidine and 1 mM 2-fluoro-L-histidine (**24**, Figure 2) and 0.28 uM OvoA was incubated at 26°C. At 1, 2, 4 and 8 minutes, 30 ul aliquots of the reactions were quenched by addition of 15 ul 1 M phosphoric acid and analyzed by cation exchange HPLC using 50 mM phosphoric acid at pH 2 as a mobile phase. Compounds were eluted in a NaCl gradient. The chromatograms were recorded at 220 nm. The data was fitted by linear regression.

References

(1) Braunshausen, A.; Seebeck, F. P. *J. Am. Chem. Soc.* **2011**, *133*, 1757.
(2) Kirk, K. L.; Cohen, L. A. *J. Am. Chem. Soc.* **1973**, *95*, 4619
(3) Jain, R. K.; Avramovitch, B.; Cohen, L. A. *Tetrahedron* **1998**, *54*, 3235