Supporting Information

Ascorbylation of a Reactive Cysteine in the Major Apple Allergen Mal d 1

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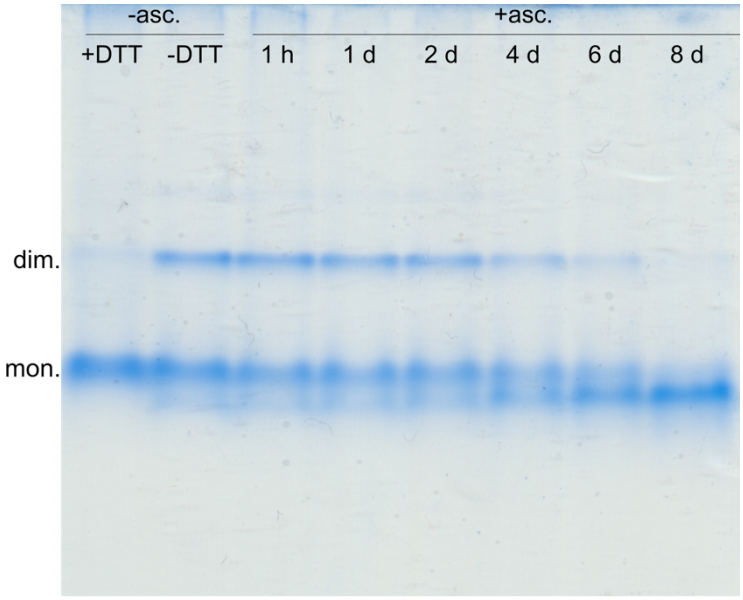

Figure S1: Native polyacrylamide gel electrophoresis (PAGE) for monitoring the effect of incubating Mal d 1 with L-ascorbate. Experimental conditions: 0.025mM Mal d 1.0101 was incubated with a 10-fold molar excess of L-ascorbate in 10 mM sodium phosphate buffer pH 6.9 at 25°C. A 15% polyacrylamide gel was used for PAGE. Samples taken after 1 hour, 2, 4, 6 and 8 days (lanes 3-8) show that during this time the Mal d 1 band gradually shifts. Lanes 1 and 2 show that dimerization of Mal d 1, which was previously reported (Kaeswurm, J.A.H.; Nestl, B.; Richter, S.M.; Emperle, M.; Buchweitz, M.: Purification and characterization of recombinant expressed apple allergen Mal d 1. *Methods Protoc.* **2021**, *4*, 3), can be reverted by the addition of 1mM 1,4-dihiothreitol (DTT).

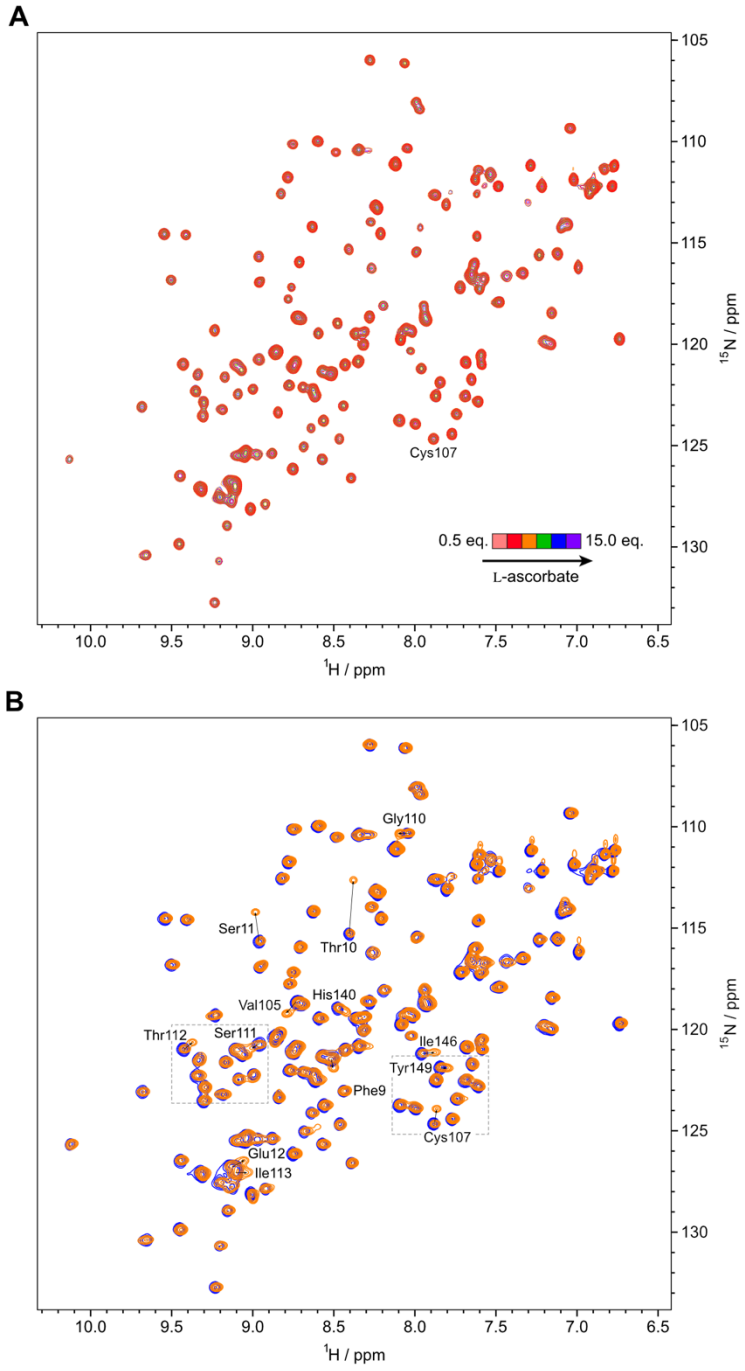

Figure S2: (**A**) Backbone amide $^{1}H^{15}N$-HSQC spectra of ^{15}N-labeled Mal d 1.0101 in the presence of variable amounts of L-ascorbate (up to 15-fold molar excess). All spectra were recorded immediately after addition of L-ascorbate, 500 MHz. Sample conditions: 0.4 mM Mal d 1, 10 mM sodium phosphate buffer pH 6.9, 9% D_2O, 25 °C. Concentrations of L-ascorbate: 0.2 mM, 0.4 mM, 0.8 mM, 2.0 mM, 4.0 mM, 6.0 mM. To ensure identical experimental conditions in all titration steps, L-ascorbate was dissolved in the same buffer as the protein and the pH was adjusted to pH 6.9 before addition. (**B**) $^{1}H^{15}N$-HSQC spectra before (blue) and after (orange) incubation with L-ascorbate (15-fold molar excess) for one week at 25 °C. Residues with $^{1}H/^{15}N$ chemical shift changes exceeding 0.2 ppm are labeled. Gray dashed boxes are drawn for sections of the spectrum that are shown in Figure 1.

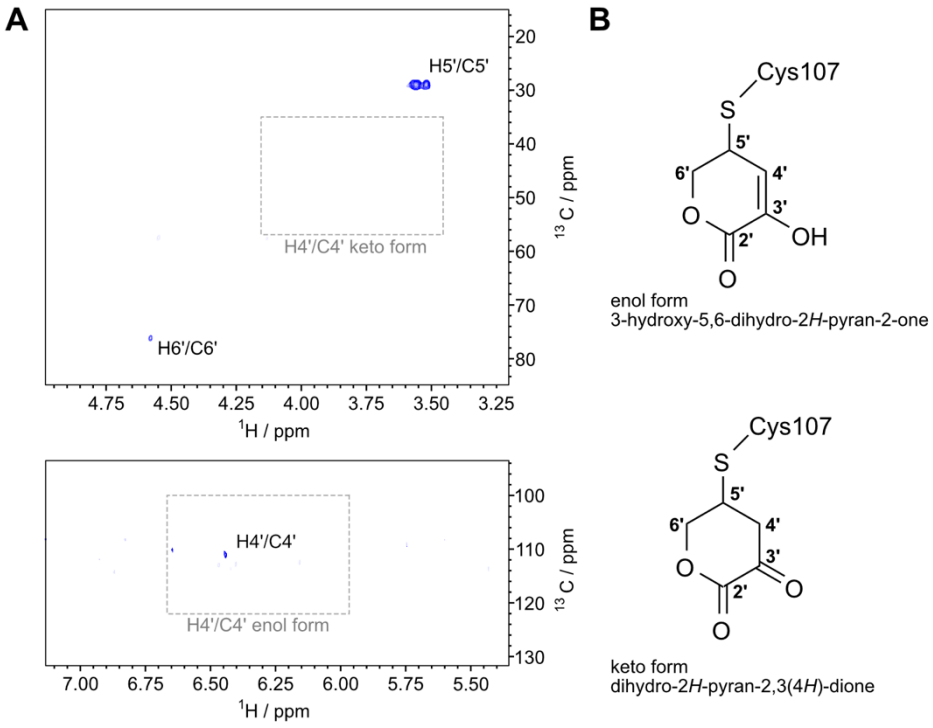

Figure S3: (**A**) Sections from a two-dimensional ^{13}C-filtered ^{1}H^{13}C-HSQC spectrum of Mal d 1 after reaction with $^{13}C_6$-labeled L-ascorbate, recorded at 700 MHz, in 99.9% D_2O, at pH 6.9, 10 mM sodium phosphate. The observed resonances in the aliphatic (top) and olefinic (bottom) region of the ^{1}H-^{13}C-HSQC spectrum are consistent with the enolic form (**B**, top) but not with the keto form (**B**, bottom) of the six-membered ring structure, which was proposed for glutaredoxin (Flandrin, A.; Allouche, S.; Rolland, Y.; McDuff, F. O.; Richard Wagner, J. R., Klarskov, K.: Characterization of dehydroascorbate-mediated modification of glutaredoxin by mass spectrometry. *J. Mass Spectrom.* **2015,** *50* (12), 1358-1366). The observation of two distinct ^{1}H resonances for H5' is indicative for the presence of two diastereomers. Resonance assignments for H4'/C4', H5'/C5' and H6'/C6' are based on the observed ^{1}H and ^{13}C chemical shifts. Resonance intensities for H4'/C4' and H6'/C6' are low, possibly due to conformational heterogeneity.

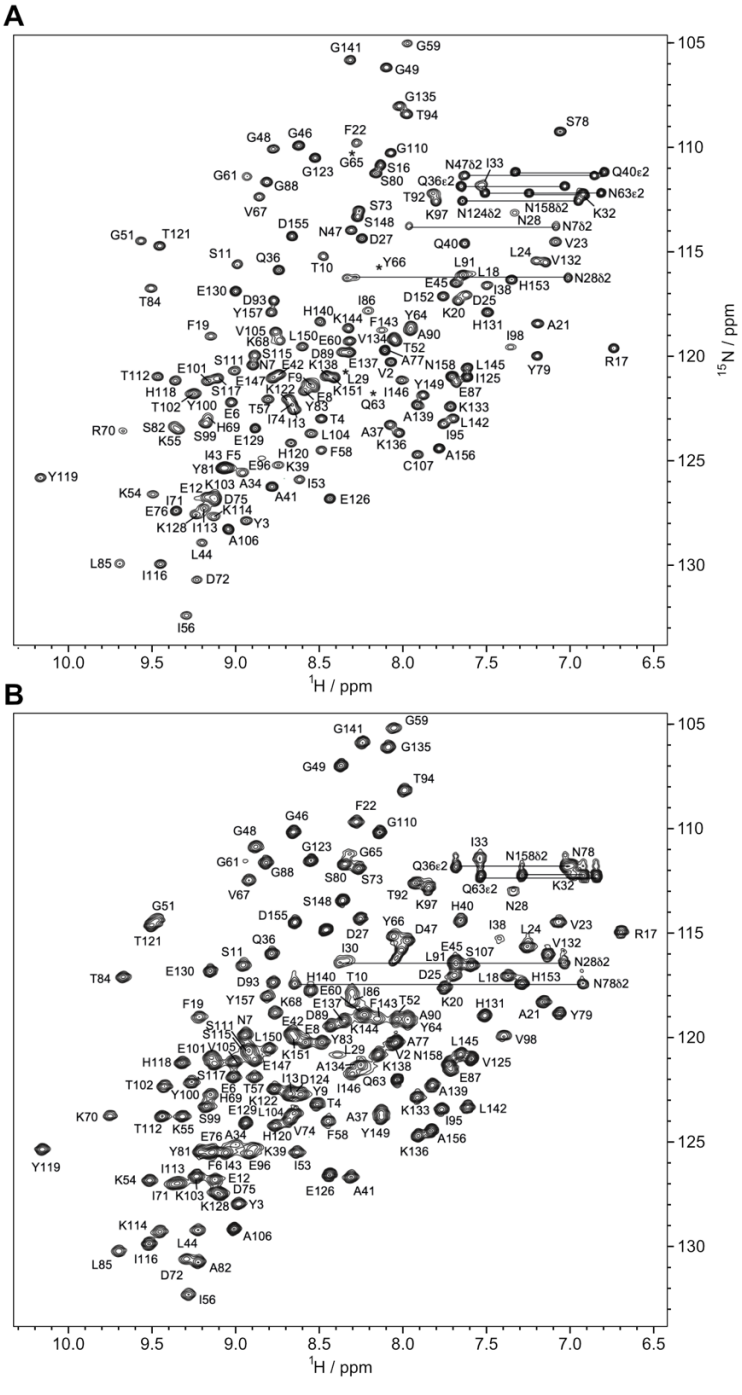

Figure S4: Backbone amide $^{1}H^{15}N$-HSQC spectra and resonance assignments of isoforms (**A**) Mal d 1.0101 and (**B**) Mal d 1.0201 of the major apple allergen. Sample conditions: 0.5 mM ^{15}N-labeled protein, 10 mM sodium phosphate buffer pH 6.9, 7.0 mM L-ascorbate, 9 % D_2O. (**C**) Comparison of amino acid sequences of Mal d 1.0101 and Mal d 1.0201. Stars indicate identical residues, while colons and dots indicate conservation of residues with strongly and weakly similar properties, respectively.

	Mal d 1.0101							
	1	2	3	4	5	6	**mean**	**std. dev.**
patient 1	0.131	0.120	0.127	0.145	0.132	0.150	**0.134**	**0.011**
patient 2	0.063	0.062	0.081	0.051	0.054	0.062	**0.062**	**0.010**
patient 3	0.082	0.105	0.097				**0.095**	**0.012**
patient 4	0.054	0.058	0.063				**0.058**	**0.005**
patient 5	0.071	0.070	0.082				**0.074**	**0.007**
patient 6	0.052	0.053	0.071				**0.059**	**0.011**
patient 7	0.107	0.110	0.109				**0.109**	**0.002**
patient 8	0.076	0.067	0.080				**0.074**	**0.007**
patient 9	0.101	0.110	0.096				**0.102**	**0.007**
patient 10	0.143	0.141	0.197				**0.142**	**0.001**

	Mal d 1.0101 + asc.							
	1	2	3	4	5	6	**mean**	**std. dev.**
patient 1	0.115	0.103	0.113	0.102	0.099	0.107	**0.107**	**0.006**
patient 2	0.073	0.057	0.067	0.049	0.057	0.073	**0.063**	**0.010**
patient 3	0.055	0.050	0.047				**0.051**	**0.004**
patient 4	0.059	0.052	0.065				**0.059**	**0.007**
patient 5	0.077	0.073	0.096				**0.082**	**0.012**
patient 6	0.048	0.053	0.065				**0.055**	**0.009**
patient 7	0.059	0.066	0.058				**0.061**	**0.004**
patient 8	0.061	0.060	0.079				**0.067**	**0.011**
patient 9	0.065	0.064	0.069				**0.066**	**0.003**
patient 10	0.103	0.095	0.087				**0.095**	**0.008**

Table S1: IgE reactivity to the isoform Mal d 1.0101, measured by ELISA using blood sera of 10 birch pollen and apple allergic patients, with and without prior incubation of the allergen with oxidized ascorbate. OD values recorded at a wavelength of 650 nm are reported. For patients 1 and 2, two experiments in triplicates (columns 1-3 and 4-6) were performed, while for patients 3-10 one experiment in triplicates (columns 1-3) was performed. For patient 10, one OD value (shown in gray) was identified as outlier (>95% confidence) and excluded from further analysis. Mean values and standard deviations were used for generating Figure 3. Mean OD values for negative serum samples were 0.045 (standard deviation: 0.003) before and 0.047 (0.004) after S-ascorbylation.

	Mal d 1.0201							
	1	2	3	4	5	6	**mean**	**std. dev.**
patient 1	0.105	0.096	0.095	0.110	0.112	0.136	**0.109**	**0.015**
patient 2	0.058	0.056	0.069	0.042	0.044	0.049	**0.053**	**0.010**
patient 3	0.047	0.055	0.059				**0.054**	**0.006**
patient 4	0.049	0.056	0.061				**0.055**	**0.006**
patient 5	0.081	0.074	0.074				**0.076**	**0.004**
patient 6	0.053	0.077	0.069				**0.066**	**0.012**
patient 7	0.070	0.063	0.063				**0.065**	**0.004**
patient 8	0.081	0.067	0.093				**0.080**	**0.013**
patient 9	0.073	0.079	0.066				**0.073**	**0.007**
patient 10	0.093	0.076	0.074				**0.081**	**0.010**

	Mal d 1.0201 + asc.							
	1	2	3	4	5	6	**mean**	**std. dev.**
patient 1	0.112	0.103	0.127	0.099	0.094	0.117	**0.109**	**0.012**
patient 2	0.074	0.054	0.080	0.056	0.048	0.053	**0.061**	**0.013**
patient 3	0.046	0.056	0.068				**0.057**	**0.011**
patient 4	0.055	0.051	0.071				**0.059**	**0.011**
patient 5	0.089	0.095	0.079				**0.088**	**0.008**
patient 6	0.053	0.066	0.074				**0.064**	**0.011**
patient 7	0.068	0.079	0.065				**0.071**	**0.007**
patient 8	0.070	0.061	0.074				**0.068**	**0.007**
patient 9	0.063	0.083	0.063				**0.070**	**0.012**
patient 10	0.095	0.083	0.078				**0.085**	**0.009**

Table S2: IgE reactivity to the isoform Mal d 1.0201, as measured by ELISA using blood sera of 10 birch pollen and apple allergic patients. OD values recorded at a wavelength of 650 nm are reported. For patients 1 and 2 two experiments in triplicates (columns 1-3 and 4-6) were performed, while for patients 3-10 one experiment in triplicates (columns 1-3) was performed. Mean values and standard deviations were used for generating Figure 3.