Electronic Supplementary Information

Affinity resins as new tools for identifying target proteins of ascorbic acid

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I. Supplementary methods

Specificity of AA-affinity resins for ascorbate oxidase. All subsequent manipulations of affinity chromatography were done at a temperature of about 4°C. AA-affinity resins 4 and 8 and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. Solutions of ascorbate oxidase (AO) from Cucurbita sp. (Wako Pure Chemical Industries, Osaka, Japan) in binding buffers (total protein of 30 µg) without or with 0.1 mM and 1.0 mM AA derivative 5 were each applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative 5 solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative 5 solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100°C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit.
Recyclability of AA-affinity resins by using ascorbate oxidase (AO). All subsequent manipulations of affinity chromatography were done at a temperature of about 4°C. AA-affinity resins 4 and 8 and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. A solution of AO in binding buffer (total protein of 30 µg) was applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative 5 solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative 5 solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100°C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit. The affinity resins already used were re-equilibrated with more than 3 bed volumes of binding buffer and reused in the same manner.
Competitive binding assay of oxd-cyt c with AA-affinity resins. All subsequent manipulations of affinity chromatography were done at a temperature of about 4°C. AA-affinity resins 4 and 8 and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. Solutions of oxd-cyt c in binding buffers (total protein of 20 µg) without or with 0.1 mM AA and 0.1 mM derivative 5 were each applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins, and the wash fractions were collected by 1.0-ml fractions. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative 5 solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative 5 solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the No.1 and No.3 of the three wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100°C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit.
II. Supplementary figures

Figure S1. Structures of control resin and reducing agents. (a) Control resin. (b) Reducing agents used in the oxd-cyt c reduction assay and FRAP assay.
**Figure S2.** Comparison of UV spectra of AA-affinity resin 4 with AA derivative 9 and control resin or AA-affinity resin 8 with AA derivative 10 and control resin in binding buffer/glycerol (1:1, v/v). (a) AA-affinity resin 4 (red solid line), 50 µM AA derivative 9 (red dotted line) and control resin (black solid line). (b) AA-affinity resin 8 (blue solid line), 50 µM AA derivative 10 (blue dotted line) and control resin (black solid line).
**Figure S3.** Specificity of AA-affinity resins for ascorbate oxidase (AO). Affinity chromatography of AO for AA-affinity resins 4 and 8 and control resin were carried out without or with 0.1 mM and 1.0 mM AA derivative 5 as a competitor. Lane 1, molecular weight marker; lane 2, AO standard; lanes 3, 6, and 9, wash fractions; lanes 4, 7 and 10, 0.1 mM AA derivative 5 elution fractions; lanes 5, 8 and 11, 1.0 mM AA derivative 5 elution fractions.
Figure S4. Recyclability of AA-affinity resins by using ascorbate oxidase (AO).

Affinity chromatography of AO for AA-affinity resins 4 and 8 and control resin were carried out by three times repetitive experiments using the same respective resins. Lane 1, molecular weight marker; lane 2, AO standard; lanes 3, 6, and 9, wash fractions; lanes 4, 7 and 10, 0.1 mM AA derivative 5 elution fractions; lanes 5, 8 and 11, 1.0 mM AA derivative 5 elution fractions.
Figure S5. Competitive inhibition assay of oxd-cyt c without or with AA and AA derivative 5 for AA-affinity resin 4 (a), AA-affinity resin 8 (b) and control resin (c). Lane 1, molecular weight marker; lane 2, oxd-cyt c standard; lanes 3, 7, and 11, wash fractions (No. 1); lanes 4, 8 and 12, wash fractions (No. 3); lanes 5, 9 and 13, 0.1 mM AA derivative 5 elution fractions; lanes 6, 10 and 14, 1.0 mM AA derivative 5 elution fractions.
$^1$H- and $^{13}$C-NMR spectra of main AA derivatives

Figure S6. $^1$H- and $^{13}$C-NMR of 2-deoxy-2-amino-L-ascorbic acid (3).
Figure S7. $^1$H- and $^{13}$C-NMR of 2-0-methyl-L-ascorbic acid (5).
Figure S8. $^1$H- and $^{13}$C-NMR of 6-amino-6-deoxy-2-$O$-methyl-L-ascorbic acid (7).
Figure S9. $^1$H- and $^{13}$C-NMR of 2-acetylamino-2-deoxy-L-ascorbic acid (9).
Figure S10. $^1$H- and $^{13}$C-NMR of 6-acetlamino-6-deoxy-2-\textit{O}-methyl-\textit{L}-ascorbic acid (10).