Supplementary information

Modification of *Rapana thomasiana* hemocyanin with choline amino acid salts significantly enhances its antiproliferative activity against *MCF*-7 human breast cancer cells

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1. Methods

Synthesis and characterization of cholinium - based amino acid ionic liquids [Chol][AA]

In the first step, chloride anion in cholinium chloride was exchanged to hydroxide anion on the ion exchange resin (Dowex Monosphere 550 A UPW (OH form) resin). For this purpose, an aqueous solution of cholinium chloride was placed on the top of glass column filled with ion

exchange resin and then cholinium hydroxide was slowly eluted with deionized water. In collected eluates the content of chloride anions was checked with $AgNO_3$ and eluates without chloride anions were combined and concentrated under vacuum to half volume. So obtained aqueous solution of cholinium hydroxide was used in the second synthesis step. The concentration of cholinium cation in this solution was determined by titration method in biphasic system in the presence of bromophenol blue indicator and sodium tetraphenylborate as titrant.

In the second step, amino acid (1.2 equivalents) was dissolved in the aqueous solution of cholinium hydroxide (1 equivalent). The mixture was stirred at room temperature for 24 h. Then, the water was evaporated at 60°C under vacuum by using rotary evaporator. The excess of amino acid was precipitated from residue with absolute ethanol and filtered. Then, ethanol was distilled off from filtrate under vacuum. The target cholinium salt of amino acids [Chol][AA] were dried in vacuum oven at 60°C to content of water below 800 ppm.

Cholinium salts of amino acids were identified by ^{1}H-NMR. The ^{1}H-NMR spectra were recorded on a Bruker DPX-400 spectrometer operating at 400.13 MHz in DMSO-d_6 as solvent. The chemical shifts were referred to tetramethylsilane as internal standard.

The water content in [Chol][AA] was determined by coulometric Karl Fischer titration, using Metrohm 831 KF Coulometer.

Optical rotation measurements were taken with Autopol IV Polarimeter (Rudolph Research Analytical) for aqueous solutions of [Chol][AA] (c=1%(m/V)).

Thermogravimetric analysis was performed on a TG 209 F1 Libra - Thermo Microbalance (Netzsch) at a heating rate of 10ºC/min to a maximum temperature 500ºC and in air atmosphere (gas flow 25 mL/min), in Al_2O_3 crucible. Temperatures corresponding to 5% and 50% weight loss ($T_D^{5\%}$ and $T_D^{50\%}$) were determined.

(2-hydroxyethyl)trimethylammonium L-methionate [Chol][Met]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 1.44-1.49 (m, 1H, CH_3-S-CH_2-C$\underline{H}_2$-; Met), 1.77-1.83 (m, 1H, CH_3-S-CH_2-C$\underline{H}_2$-; Met), 2.00 (s, 3H, C$\underline{H}_3$-S-; Met), 2.48 (t, ov, 2H, J=8.0 Hz, CH_3-S-

$C\underline{H_2}$-CH_2-; Met), 2.88 (dt, 1H, J=4.5 Hz, J=3.3 Hz, -$C\underline{H}(COO^-)NH_2$; Met), 3.14 (s, 9H, $(C\underline{H_3})_3N$-; cation), 3.45 (t, 2H, J=5.0 Hz, -$C\underline{H_2}$-N-; cation), 3.83-3.87 (m, 2H, -$C\underline{H_2}$-OH; cation), [α] = +2.30, $T_D^{5\%}$= 179.9°C, $T_D^{50\%}$= 213.1°C.

(2-hydroxyethyl)trimethylammonium glycinate[Chol][Gly]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 2.75 (s, 2H, -$C\underline{H_2}$-; Gly), 3.13 (s, 9H, $(C\underline{H_3})_3N$-; cation), 3.44 (t, 2H, J=5.0 Hz, -$C\underline{H_2}$-N-; cation), 3.82-3.86 (m, 2H, $C\underline{H_2}$-OH; cation), $T_D^{5\%}$= 165.9°C, $T_D^{50\%}$= 209.4°C.

(2-hydroxyethyl)trimethylammonium L-valinate [Chol][Val]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 0.68 (d, 3H, J=6.7 Hz, $C\underline{H_3}$-; Val), 0.80 (d, 3H, J= 6.7 Hz, $C\underline{H_3}$-; Val), 1.85-1.87 (m, 1H, -$C\underline{H}(CH_3)_2$; Val), 2.66 (s, ov, 1H, -$C\underline{H}COO^-$; Val), 3.13 (s, 9H, $(C\underline{H_3})_3N$-; cation), 3.44 (t, 2H, J=4.9 Hz, -$C\underline{H_2}$-N-; cation), 3.83-3.86 (m, 2H, $C\underline{H_2}$-OH; cation), [α] = +6.81, $T_D^{5\%}$= 168.9°C, $T_D^{50\%}$= 213.3°C.

(2-hydroxyethyl)trimethylammonium L-leucinate [Chol][Leu]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 0.95 (d, ov, 6H, J=6.6 Hz, J=6.6 Hz, $(C\underline{H_3})_2$-; Leu), 1.02 (t, ov, J=7.1 Hz, 1H, -$C\underline{H_2}$-; Leu), 1.33-1.37 (m, 1H, $(CH_3)_2C\underline{H}$-; Leu), 1.67 (t, 1H, J=6.8 Hz, -$C\underline{H_2}$-; Leu), 2.77 (t, ov, J=4.4 Hz, 1H, H_2N-$C\underline{H}$-; Leu), 3.11 (s, 9H, $(C\underline{H_3})_3N$-; cation), 3.42 (t, 2H, J=5.0 Hz, -$C\underline{H_2}$-N-; cation), 3.80-3.84 (m, 2H, $C\underline{H_2}$-OH; cation), [α] = +2.80, $T_D^{5\%}$= 171.2°C, $T_D^{50\%}$= 208.2°C.

(2-hydroxyethyl)trimethylammonium L-threoninate [Chol][Thr]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 0.93 (d, 3H, J=6.2 Hz, CH_3-; Thr), 2.85 (d, 1H, J=4.0 Hz, -$C\underline{H}$-COO-; Thr), 3.13 (s, 9H, $(C\underline{H_3})_3N$-; cation), 3.44 (t, 2H, J=4.9 Hz, -$C\underline{H_2}$-N-; cation), 3.48-3.51 (m, 1H, $C\underline{H}(OH)CH_3$-Thr); 3.85 (m, ov, 2H, $C\underline{H_2}$-OH; cation), [α] = -3.05, $T_D^{5\%}$= 177.2°C, $T_D^{50\%}$= 213.7°C.

(2-hydroxyethyl)trimethylammonium L-isoleucinate [Chol][Ile]

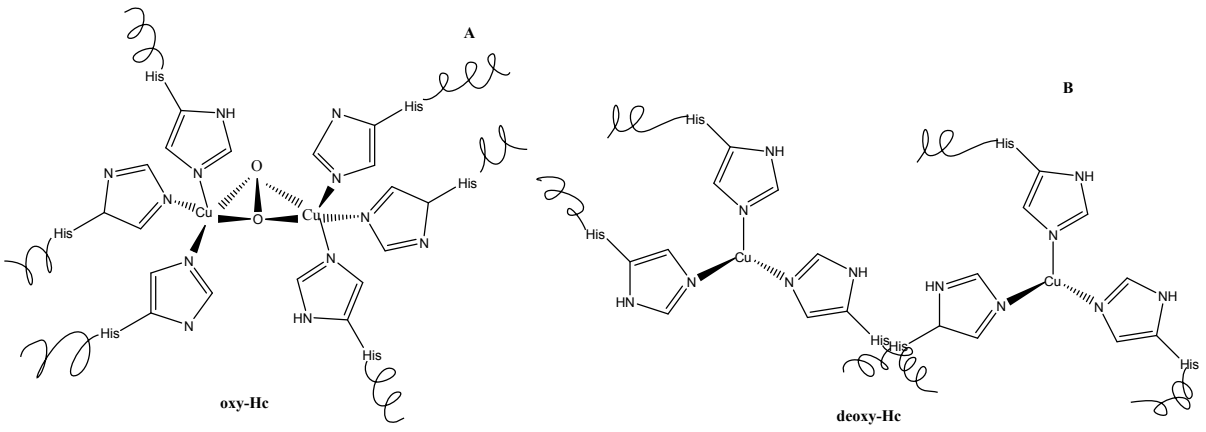
^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 0.75-0.79 (t, ov, J=7.5 Hz, d, ov, J=6.8 Hz, 6H, C$\underline{H}_3$-Ile), 0.99-1.05 (m, 1H, -C$\underline{H}_2$-; Ile), 1.50 (m, ov, 1H, -C$\underline{H}_2$-; Ile), 1.53-1.55 (m, 1H, CH_3-C$\underline{H}$-; Ile), 2.72 (d, 1H, J=3.9 Hz, H_2N-C$\underline{H}$-; Ile), 3.13 (s, 9H, (C$\underline{H}_3$)$_3$N-; cation), 3.44 (t, 2H, J=4.9 Hz, -C$\underline{H}_2$-N-; cation), 3.82-3.85 (m, 2H, C$\underline{H}_2$-OH; cation), [α] = +6.07, $T_D^{5\%}$= 176.3°C, $T_D^{50\%}$= 209.8°C.

(2-hydroxyethyl)trimethylammonium L-tryptophanate [Chol][Trp]

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 3.10 (s, 9H, (C$\underline{H}_3$)$_3$N-; cation), 3.12 (d, 2H, J=3.6 Hz, -C$\underline{H}_2$-Trp), 3.39-3.45 (t, ov, 2H, -C$\underline{H}_2$-N-; cation, t, ov, 1H, H_2N-C$\underline{H}$-; Trp), 3.82-386 (m, 2H, -C$\underline{H}_2$-OH; cation), 6.92 (t, 1H, J=7.4 Hz, -C$\underline{H}$-; Trp), 7.01 (t, 1H, J=7.4 Hz, -C$\underline{H}$-; Trp), 7.12 (s, 1H, -HN-C$\underline{H}$-; Trp), 7.31 (d, 1H, J=8.1 Hz, -C$\underline{H}$-; Trp), 7.66 (d, 1H, J=7.8 Hz-C$\underline{H}$-; Trp), 11.06 (s, 1H, N$\underline{H}$(Ar)-; Trp), [α] = -2.55, $T_D^{5\%}$= 115.1°C, $T_D^{50\%}$= 239.0°C.

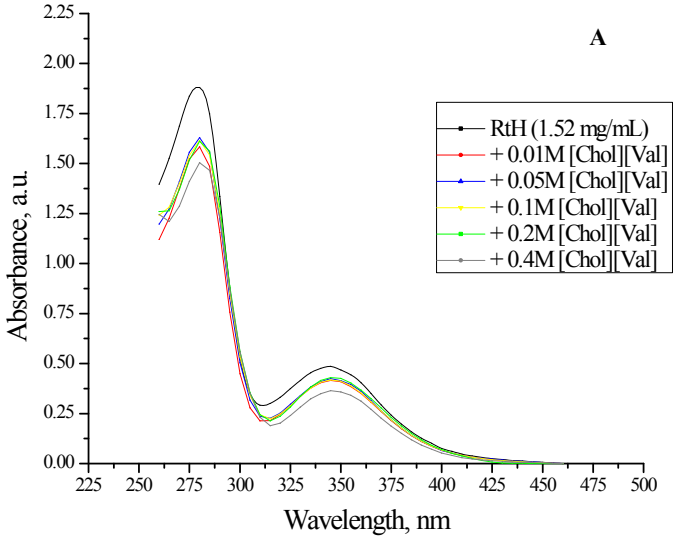
2. Results

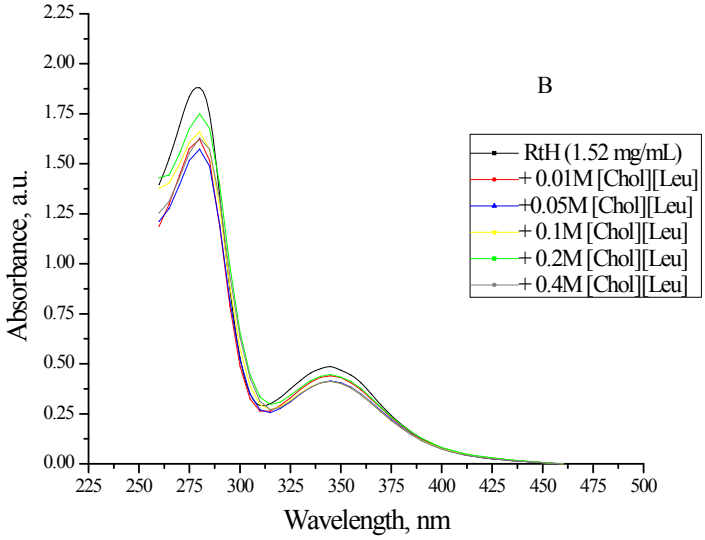
Active site of hemocyanins (type-3 copper proteins)

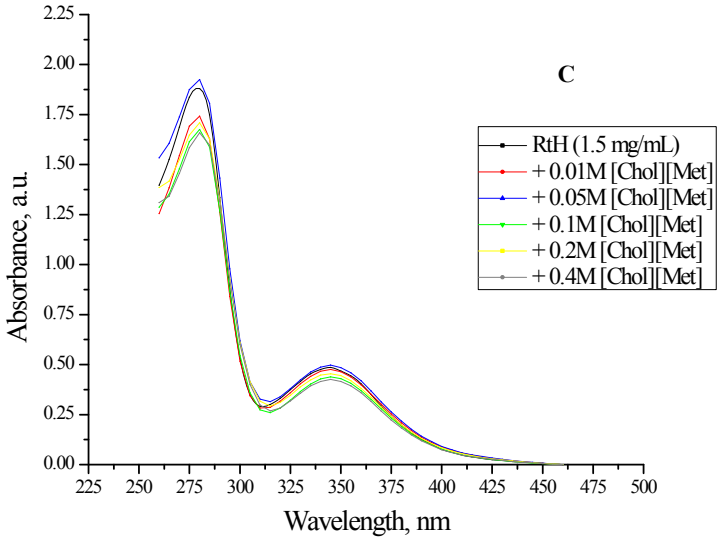


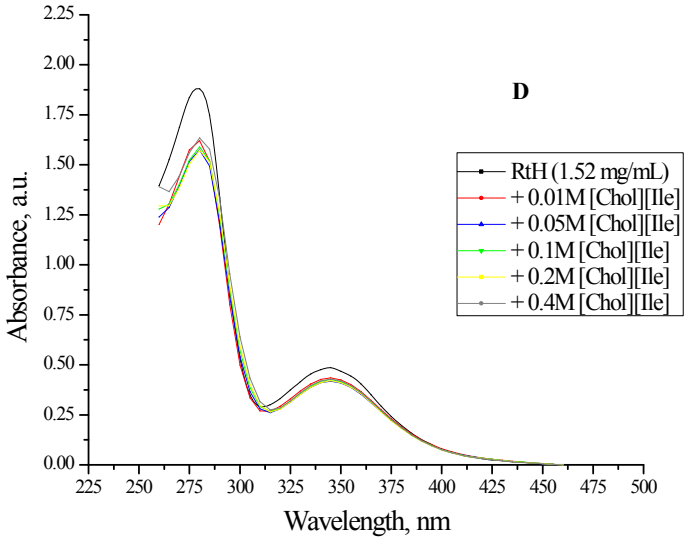
Scheme S1. Active site of Hc with two copper ions coordinated six histidine residues shown in O_2-bound form (oxy-Hc) and in absence of O_2 (deoxy-Hc).

2.1. UV-vis spectroscopy






C
Absorbance, a.u.
Wavelength, nm
RtH (1.5 mg/mL)
+ 0.01M [Chol][Met]
+ 0.05M [Chol][Met]
+ 0.1M [Chol][Met]
+ 0.2M [Chol][Met]
+ 0.4M [Chol][Met]


D
Absorbance, a.u.
Wavelength, nm
RtH (1.52 mg/mL)
+ 0.01M [Chol][Ile]
+ 0.05M [Chol][Ile]
+ 0.1M [Chol][Ile]
+ 0.2M [Chol][Ile]
+ 0.4M [Chol][Ile]

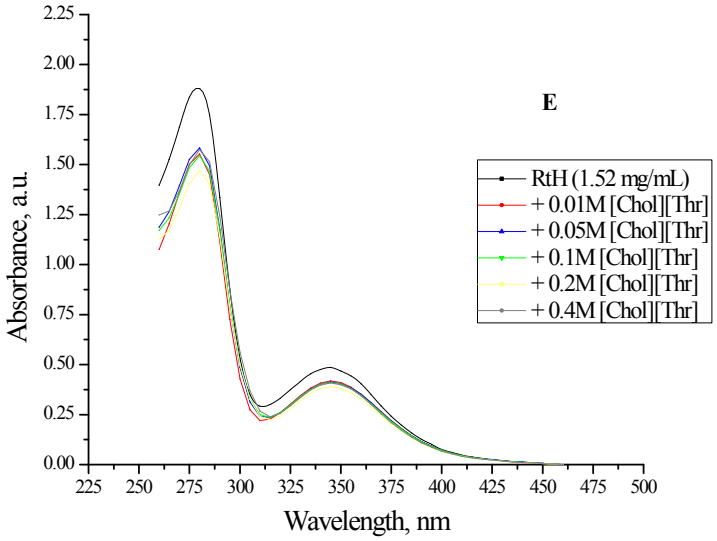
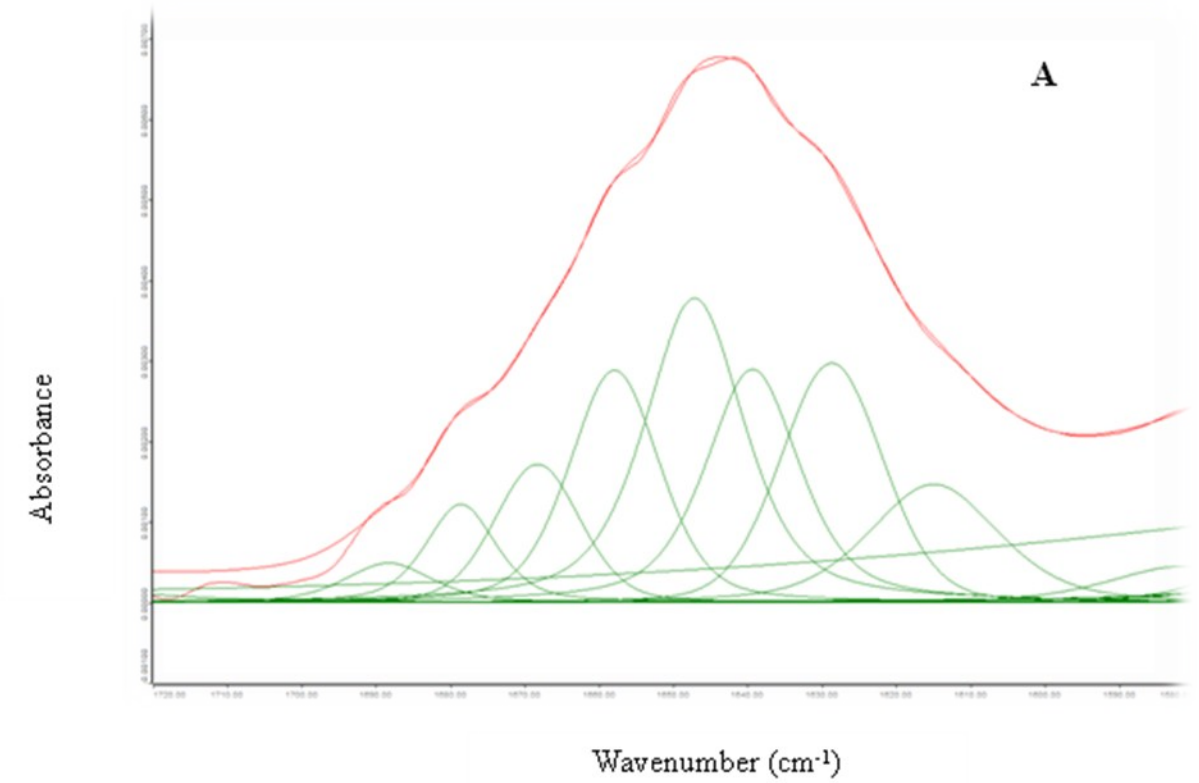
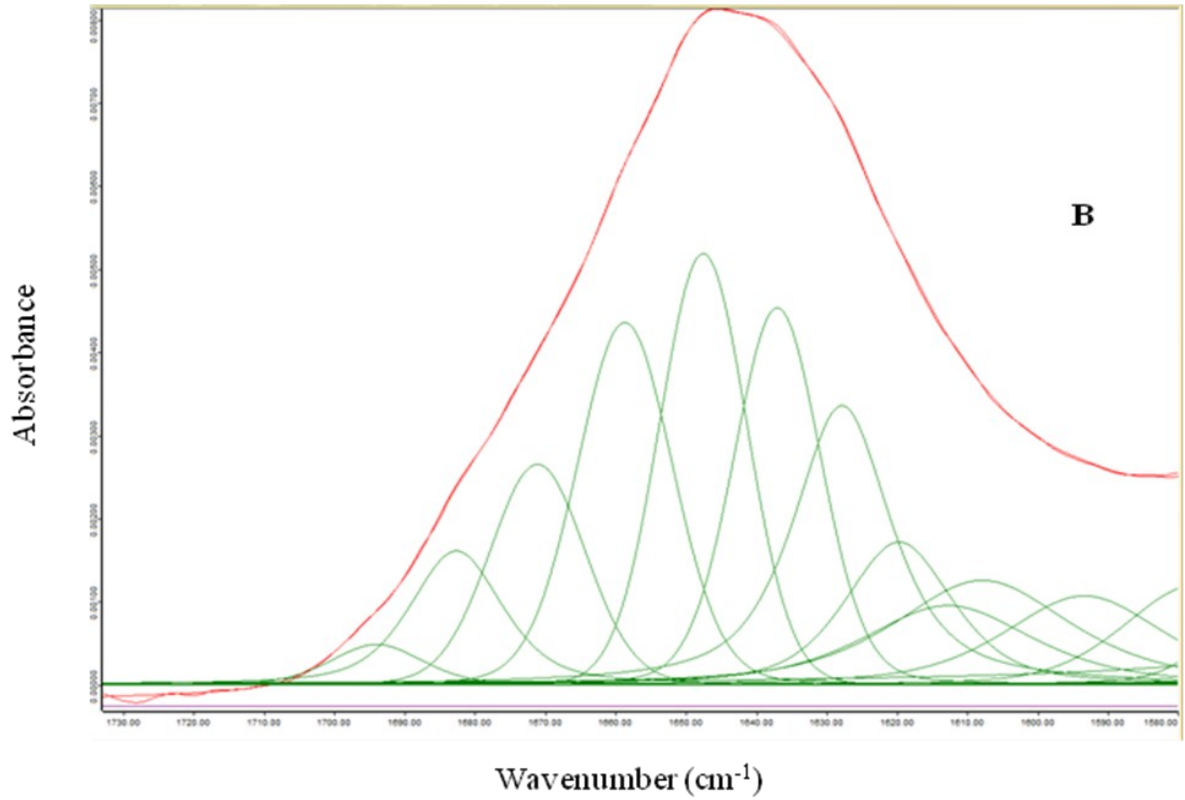


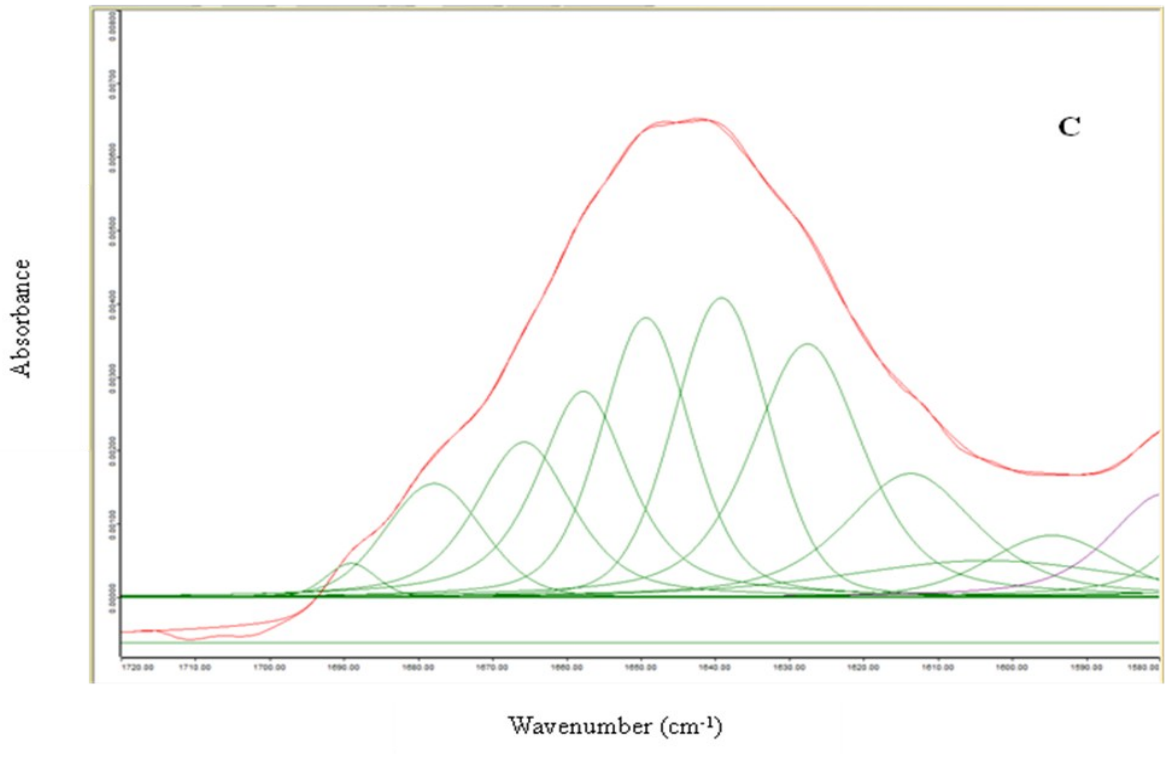
Fig. S1. UV-vis absorbance of RtH (1.52 mg/mL), dissolved in sodium phosphate buffer (pH 7.2, 5 mM) containing various amounts of [Chol][Val] (A), [Chol][Leu] (B), [Chol][Met] (C), [Chol][Ile] (D) and [Chol][Thr] recorded immediately after protein-IL mixing.

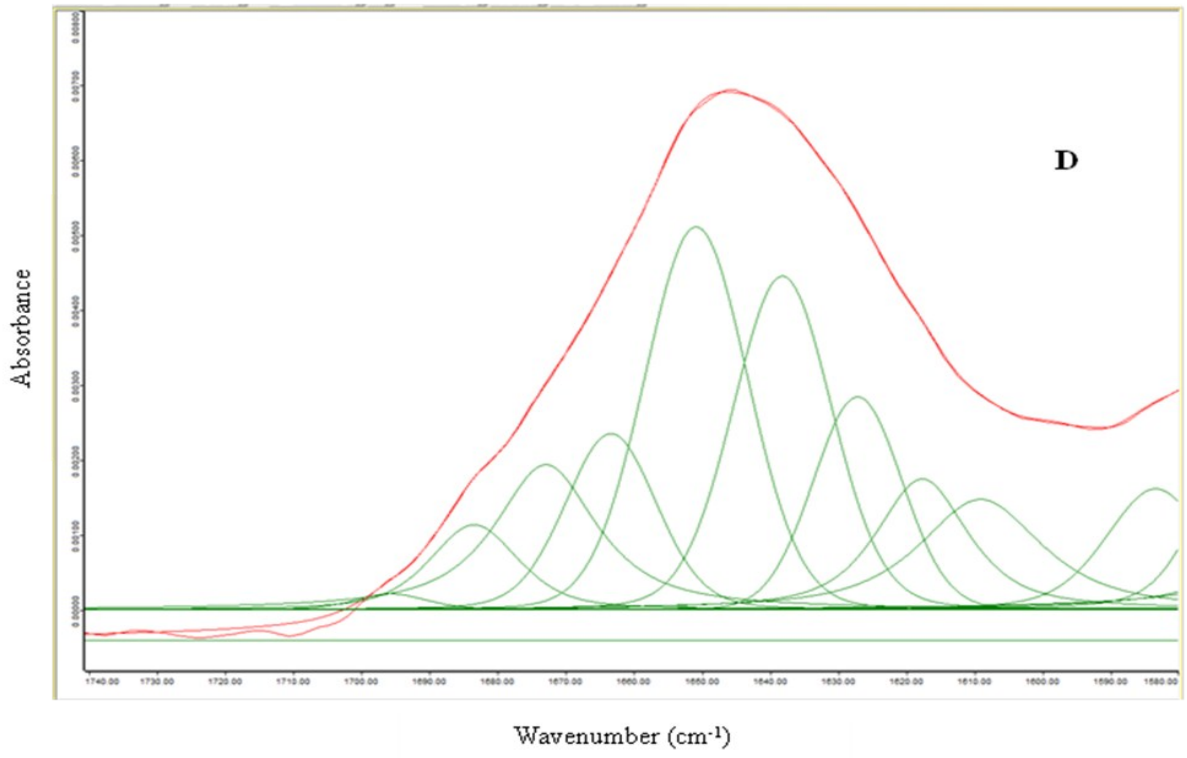
[#] The spectra of RtH-[Chol][Trp] are not shown. The absorbance of [Chol][Trp] in the region 260–460 nm was too high, i.e. unreliable, within the concentration range of the assay (10–400 mM).

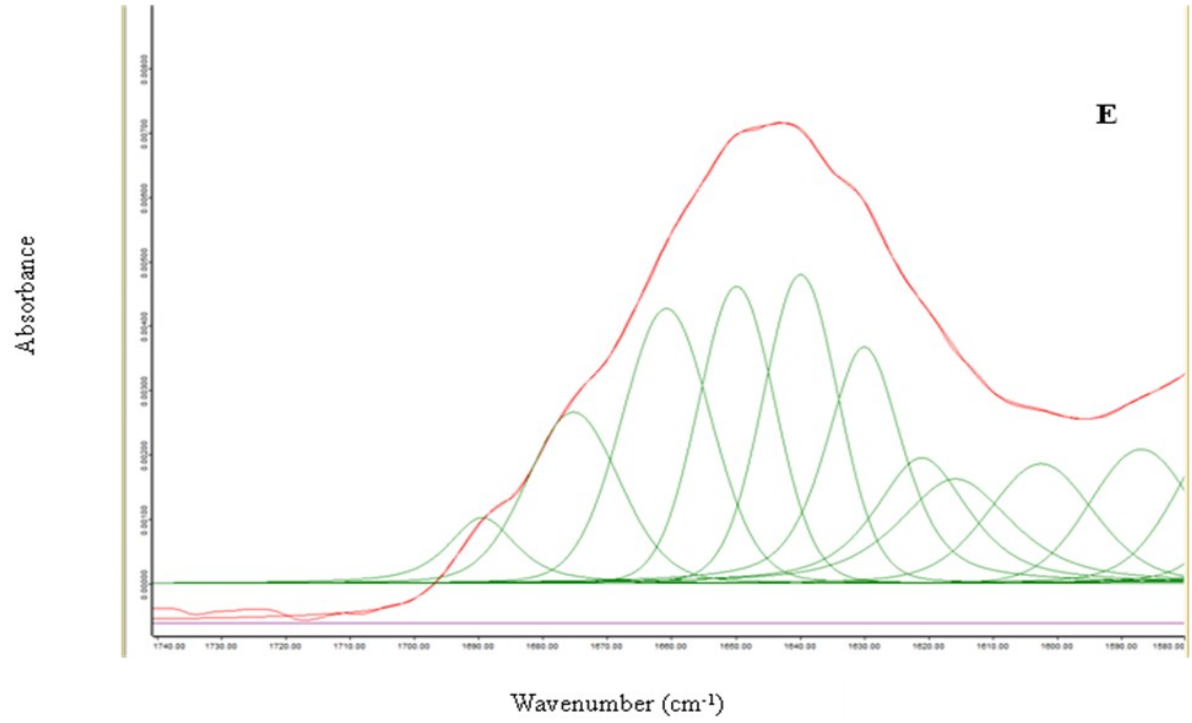
2.2. FTIR spectra of RtH and complexes of RtH with choline amino acid salts

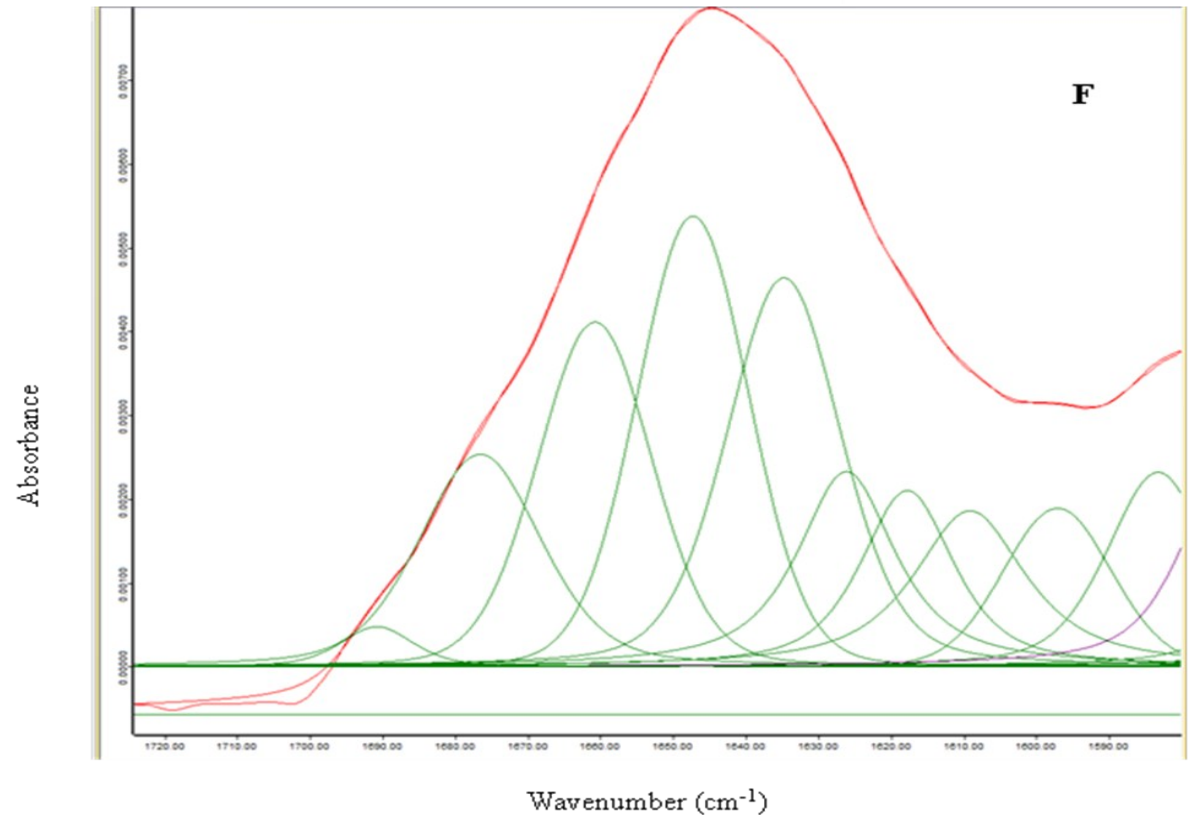

A
Absorbance
Wavenumber (cm-1)

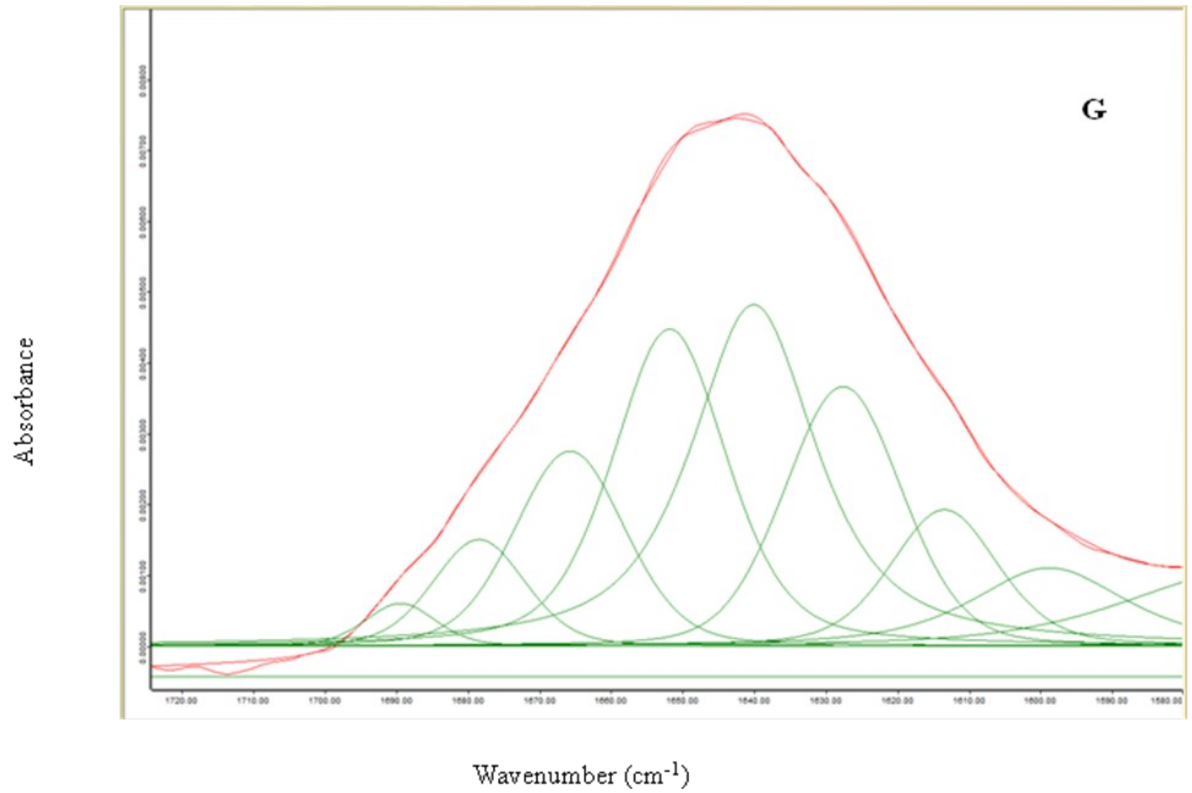

B
Absorbance
Wavenumber (cm-1)


C
Absorbance
Wavenumber (cm-1)


D
Absorbance
Wavenumber (cm-1)


E
Absorbance
Wavenumber (cm-1)


F
Absorbance
Wavenumber (cm-1)


G
Absorbance
Wavenumber (cm-1)

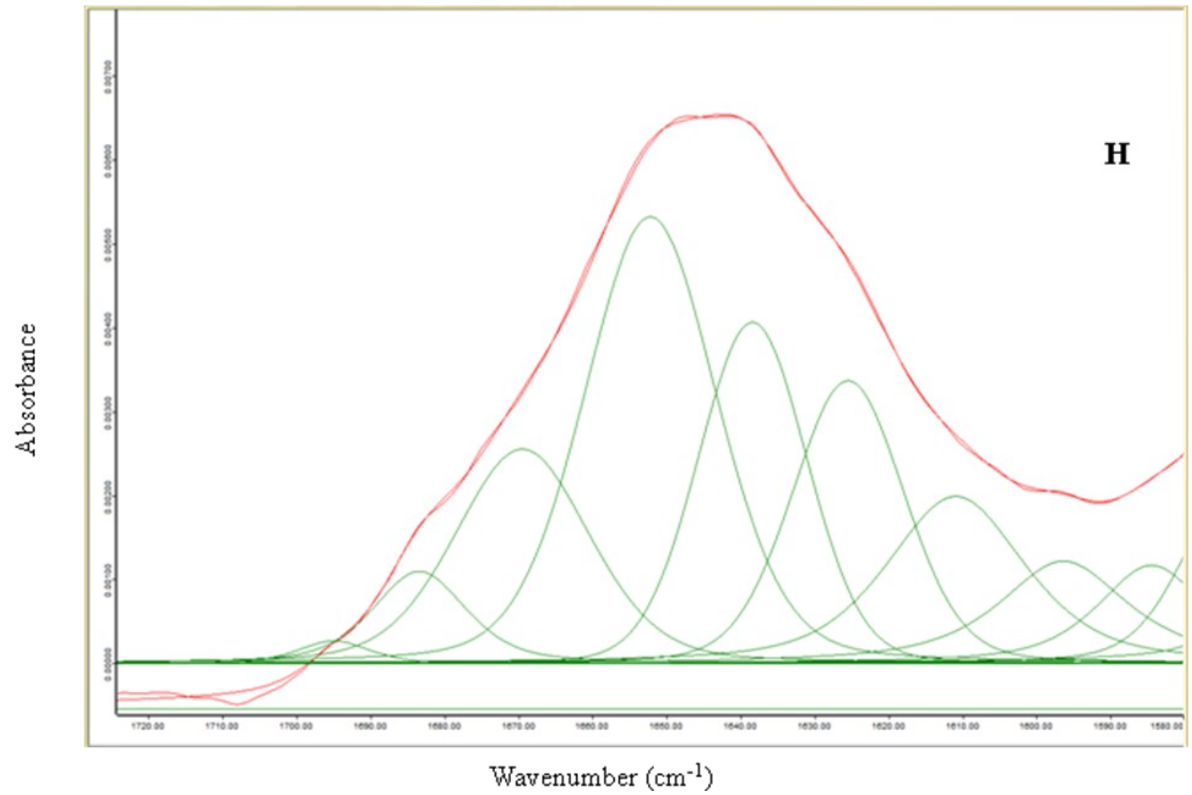

H
Absorbance
Wavenumber (cm-1)

Fig. S2. Fourier-deconvoluted infrared spectra in Amide I region and reconstituted spectra after curve fitting of hemocyanin from *Rapana thomasiana* in sodium phosphate buffer (pH 7.2, 5 mM) (A) and in presence of [Chol][Gly] (B), [Chol][Val] (C), [Chol][Leu] (D), [Chol][Ile] (E), [Chol][Met] (F), [Chol][Thr] (G) and [Chol][Trp] (H).

Table S1. Secondary structure of RtH in sodium phosphate buffer (pH 7.2, 5mM) and in 0.2M choline amino acid salts.

Band position wavenumber (cm^{-1})	Relative area	Assignment of the components [Barth 2007]
A) RtH (in buffer)		
1615	11.35	aggregated strands
1629	16.44	β-sheets
1639	17.18	β-sheets
1647	23.86	unordered/random coils
1658	15.16	α-helices
1668	8.09	β-turns
1679	5.30	β-turns
1688	2.62	β-turns/antiparallel β-sheets
B) RtH-[Chol][Gly]		
1608	9.72	amino acid side chain residues
1613	6.98	aggregated strands
1620	7.71	aggregated strands
1628	14.73	β-sheets
1637	14.07	β-sheets
1648	15.38	unordered/random coils
1659	14.67	α-helices
1671	8.85	β-turns
1683	6.19	β-turns/antiparallel β-sheets
1694	1.71	β-turns/antiparallel β-sheets
C) RtH-[Chol][Val]		
1603	7.10	amino acid side chain residues
1614	11.26	aggregated strands
16.28	18.64	β-sheets
16.39	16.51	β-sheets
1649	15.53	unordered/random coils
1658	13.47	α-helices
1666	10.20	β-turns
1678	6.32	β-turns
1689	0.98	β-turns/antiparallel β-sheets

Band position wavenumber (cm^{-1})	Relative area	Assignment of the components [Barth 2007]
D) RtH-[Chol][Leu]		
1609	10.42	amino acid side chain residues
1618	8.85	aggregated strands
16.27	10.79	β-sheets
16.38	19.55	β-sheets
1651	22.88	α-helices
1663	9.72	β-turns
1673	11.70	β-turns
1683	5.19	β-turns/antiparallel β-sheets
1696	0.79	β-turns/antiparallel β-sheets
E) RtH-[Chol][Ile]		
1602	8.72	amino acid side chain residues
1616	9.46	aggregated strands
1621	9.36	aggregated strands
1630	13.62	β-sheets
1640	14.85	β-sheets
1650	14.36	α-helices
1661	15.60	β-turns
1675	10.41	β-turns
1689	3.62	β-turns/antiparallel β-sheets
F) RtH-[Chol][Met]		
1609	9.78	amino acid side chain residues
1618	8.61	aggregated strands
1626	10.61	β-sheets
1635	20.09	β-sheets
1647	20.88	unordered/random coils
1661	16.73	β-turns
1677	11.90	β-turns
1691	1.40	β-turns/antiparallel β-sheets
G) RtH-[Chol][Thr]		
1613	8.97	aggregated strands
1628	17.57	β-sheets
1640	30.56	β-sheets
1652	22.78	α-helices
1666	12.55	β-turns
1678	5.77	β-turns
1689	1.80	β-turns/antiparallel β-sheets
H) RtH-[Chol][Trp]		
1611	13.75	aggregated strands

Band position wavenumber (cm^{-1})	Relative area	Assignment of the components [Barth 2007]
1625	16.16	β-sheets
1638	18.24	β-sheets
1652	30.66	α-helices
1669	15.28	β-turns
1683	4.99	β-turns/antiparallel β-sheets
1695	0.92	β-turns/antiparallel β-sheets

[a]Prior to measurements, RtH (30.4 mg/mL) were incubated with sodium phosphate buffer (pH 7.2, 5 mM) containing 0.2M [Chol][AA] for 60 min at 25°C.

A. Barth, Infrared spectroscopy of proteins, Biochimica et Biophysica Acta 1767 (2007) 1073 - 1101.

2.3. Gel-electrophoresis analyses

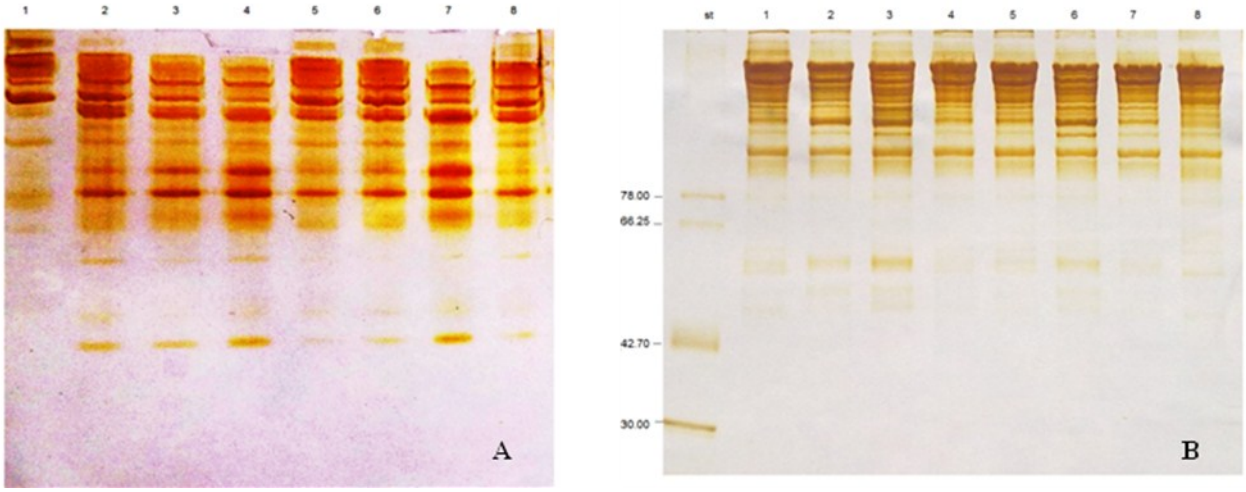
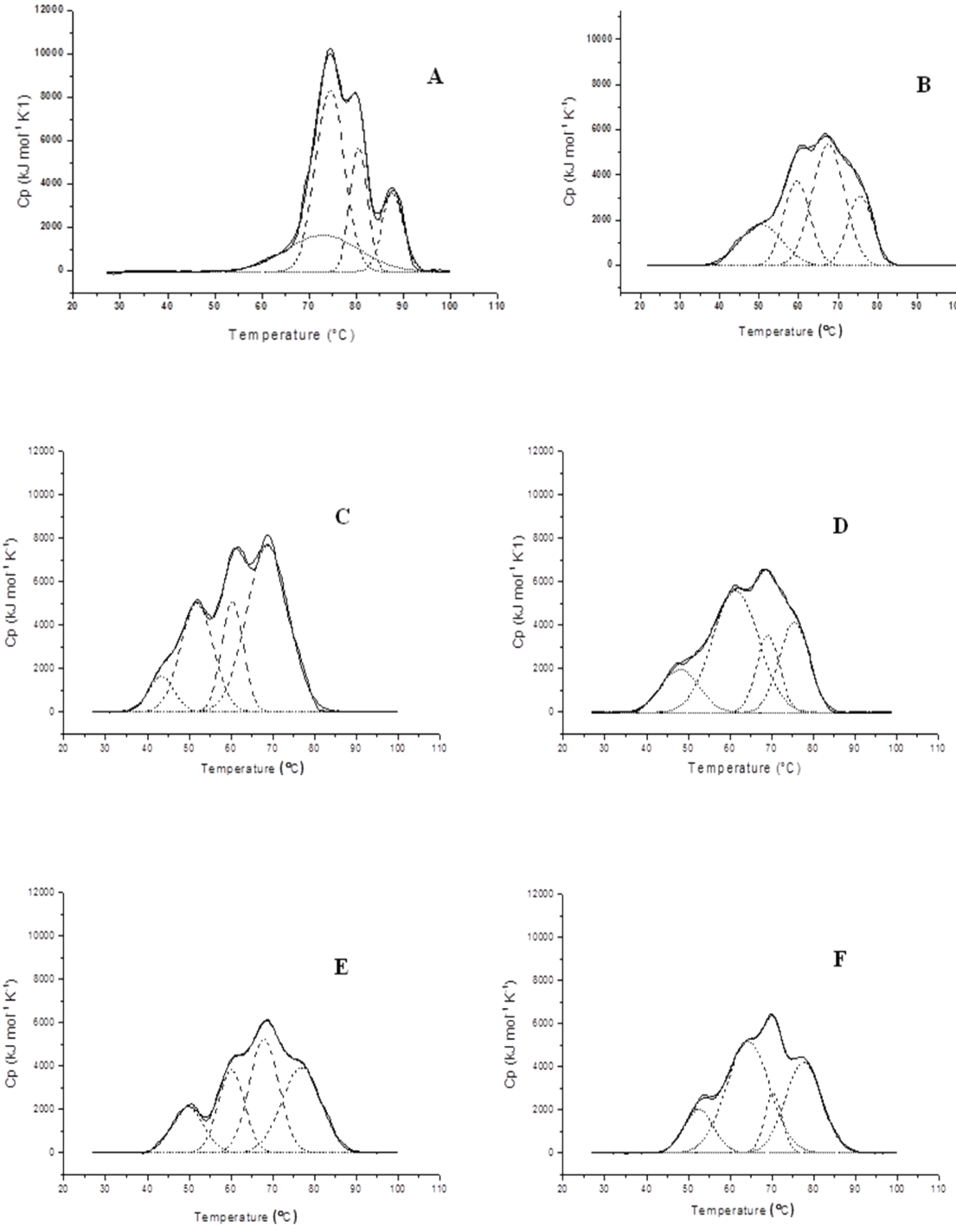


Fig.S3. A. SDS-PAGE on 7.5% running gel. Protein standards (st); lane 1, native RtH; lane 2, RtH-[Chol][Leu]; lane 3, RtH-[Chol][Trp]; lane 4, RtH-[Chol][Gly]; lane 5, RtH-[Chol][Ile]; lane 6, RtH-[Chol][Thr]; lane 7, RtH-[Chol][Met]; lane 8, RtH-[Chol][Val]. B. Native PAGE on 7.5% running gel. Lane 1-8 as described above.

2.4. Differential scanning calorimetry of RtH and RtH-[Chol][AA] complexes



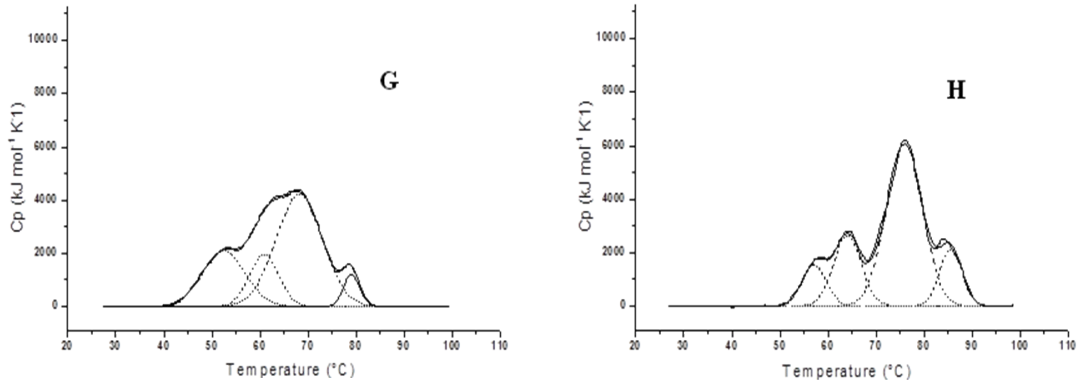


Fig. S4. The curve fitting of DSC profile for hemocyanin from *Rapana thomasiana* in sodium phosphate buffer (pH 7.2, 5 mM) (A) and in presence of [Chol][Gly] (B), [Chol][Val] (C), [Chol][Leu] (D), [Chol][Ile] (E), [Chol][Met] (F), [Chol][Thr] (G) and [Chol][Trp] (H).

Table S2. Transition parameters for RtH in sodium phosphate buffer (pH 7.2, 5 mM) with and without added cholinium-based amino acid salts.

Sample	T_{m1} (°C)	Cp1 (kJ/mol. K)	$\Delta H1_{cal}$ (kJ/mol)	T_{m2} (°C)	Cp2 (kJ/mol. K)	$\Delta H2_{cal}$ (kJ/mol)	T_{m3} (°C)	Cp2 (kJ/mol. K)	$\Delta H3_{cal}$ (kJ/mol)	T_{m4} (°C)	Cp4 (kJ/mol. K)	$\Delta H4_{cal}$ (kJ/mol)	ΔH_{tot} (kJ/mol)
RtH IL-free	72.9	1675.6	32688	74.5	8364.9	64386	80.6	5728.1	26183	87.8	3599.6	20222	141571
RtH + [Chol][Gly]	50.5	1814.3	27511	59.6	3754.4	31055	67.5	5383.6	55586.4	75.6	3080.5	24852	136354
RtH + [Chol][Val]	43.4	1639.9	12473	51.9	4968.0	48882	60.4	5088.1	33136.2	68.8	7670.6	95958	190456
RtH + [Chol][Leu]	48.0	1983.2	22791	61.4	5612.4	78061	69.2	536.6	24610.9	75.5	4149.4	37716	161668
RtH + [Chol][Ile]	49.7	2127.6	19994	60.1	3822.8	29986	67.9	5230.7	46464.2	76.9	3929.5	46322	142182
RtH + [Chol][Met]	52.5	2023.9	18001	64.3	5159.7	67103	70.3	2761.7	15087.5	77.6	4217.6	45737	144917
RtH+ [Chol][Thr]	52.5	2080.2	23126	60.9	1954.5	14852	68.2	4210.7	51060.9	79.1	1214.2	5087	93801
RtH + [Chol][Trp]	57.1	1548.1	9660	64.0	2626.9	18978	75.9	6048.2	57351	85.6	2096.1	12312	98641