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Screening of Garlic Water Extract for Binding Activity with Cholera Toxin B Pentamer by NMR Spectroscopy – An Old Remedy Giving a New Surprise

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Binding between a component of the crude hot water extract obtained from *Allium sativum* crushed bulbs (ASw) and cholera toxin B pentamer (CTB) was detected by STD NMR experiments. Bioassay-oriented fractionation allowed the partial identification of a high molecular weight polysaccharide mainly composed of galactose as the bioactive complex against CTB. This work represents the first example of screening of a medicinal plant by NMR against a specific disease, and corroborates traditional medical uses of the species.

Introduction

The potential of NMR approaches for the screening of natural product mixtures obtained from medicinal species has been reported previously.[1] In addition, direct screening of infusions or decoctions obtained from medicinal plants as sources of possible bioactive ligands can be performed by this technique, since water is the ideal solvent for both components (ligands and protein) in this type of analysis. Water-based extracts are standard ways to administer natural medicines and are the most quoted in modern ethnopharmaceutical literature, where traditional medical uses of species are described.[2–4] The use of garlic (*Allium sativum*) as a basic remedy against cholera infection has been described, as least in Bulgarian traditional medicine.[5] Unfortunately, this report, like many others from the past, lacks a detailed ethnopharmaceutical discussion. The existence of direct antimicrobial activity of garlic extracts against *Vibrio cholerae* has also been reported.[6]

It can be presumed that the anti-cholera effect of garlic could be due to the presence of potential inhibitors of the cholera toxin B pentamer (CTB) anchorage to the epithelial cells in the intestine, and this hypothesis has been investigated in this work as a direct application of NMR methods for ligand binding screening. Galactose derivatives and their glycomimetics are known to bind CTB,[7,8] and since garlic water extract is known to contain a galactan[9] we felt that this garlic/CTB system might be a suitable one to provide further evidence of the usefulness of NMR methods[1] for evaluation of the binding activity of a given ligand mixture (ASw) against a given receptor (CTB).

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Results and Discussion

STD (Saturation Transfer Difference) NMR experiments have become a robust method with which to detect the binding of ligands to a given receptor[10] under a variety of experimental conditions,[11] even when ambiguous responses in the control experiments are found.[12]

The first step in our analysis was the application of STD to the analysis of the interaction between the hot water mixture obtained from garlic bulbs and the B pentamer of cholera toxin. Figure 1 shows proton spectra of the garlic water extract (Figure 1, a) in comparison with STD analysis of the ASw/CTB ligand/receptor system and the corresponding controls. The STD experiments resulted in the following observations. Only some background signals from CTB pentamer are observed from the sample containing CTB alone (Figure 1, b), whilst STD on ASw alone (Figure 1, c) gives some positive responses. Since no NMR signals of this sample are evident at the irradiation point (ca. 0.2 ppm), these resonances probably arise from large molecules contained in the crude hot water garlic extract.[12] The STD experiment was performed on the actual ASw/CTB complex (Figure 1, d) under identical experimental conditions, including the CTB and ASw concentrations, and clear STD signals were observed. In fact, evident differences in signal intensities and chemical shift values between the STD responses for the complex and for the ligands alone were observed (see zoomed area in Figure 1). The STDD experiment[12] allowed us to deduce the actual signals stemming

from the CTB-interacting molecules present in the ligand mixture (Figure 1, e). Thus, one or more interacting carbohydrate-containing molecules that interact with CTB must be present in the ASw mixture.

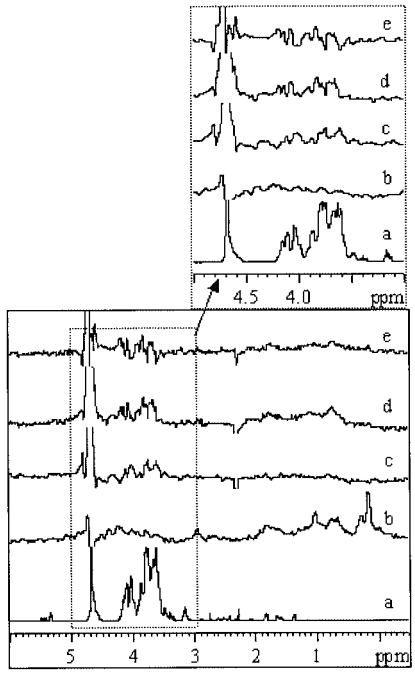

Figure 1. Comparison between the following NMR spectra: a) ^{1}H NMR of ASw. b) STD of CTB. c) STD of ASw. d) STD of CTB with ASw. e) STDD of the ASw/CTB complex (difference between the STD spectra of ASw/CTB and the STD of ASw alone). An expansion of the spectra is given in the dotted box.

The next move in the analysis was the application of a tr-NOESY analysis, which is indeed helpful to identify small molecules interacting with the receptor. In fact, given the appropriate kinetic conditions for the ligand–receptor association process, NOE-cross peaks for small ligands, in their free states, show a different sign (positive cross peaks) than those recorded, for their bound states, in the presence of the receptor (negative cross peaks). This change is due to the large change in rotational correlation time that a rapidly rotating ligand, in the free state, experiences upon binding to a large macromolecular receptor. However, the observed NOE patterns for the ligand mixture in the absence and in the presence of CTB were basically identical and showed negative cross peaks (data not shown), so the CTB-interacting molecule, even when free in solution, is already in the slow tumbling regime and gives rise to negative NOE cross peaks. On the basis of the NMR conditions used,[1] this constituent has a molecular weight (MW) higher than 1–2 kDa.

A different approach therefore had to be used to continue the evaluation, and so target identification and bioassay-oriented fractionation, also based on NMR analysis, was chosen.[1] The STD spectrum showed the chemical shifts of the interacting protons between 3.6 ppm and 4.2 ppm, within the resonance region of ASw alone (Figure 1, e). In the following step, and in order to evaluate the sizes of the different components of the garlic water extract, a variety of 1D-DOSY experiments[13] were performed, with different values of gradient strength (10%, 45% and 80%; see parts a–c of Figure 2). It is well known that the intensities of the proton signals from low-MW compounds are strongly reduced or even eliminated with increasing gradient strength. In this way, it was deduced that ASw extract contains three major classes of molecules, in terms of their different MWs. In particular, the proton signals with δ from 1.28 to 3.30 ppm and δ from 5.40 to 6.00 ppm belong to low-MW constituents, the proton signal at δ = 5.33 ppm is from a medium-sized saccharide, while those resonating at δ = 5.02 and 4.57 ppm come from a higher polysaccharide contained in the ASw mixture. However, the signals present in the STD spectrum (chemical shifts between 3.6 ppm and 4.2 ppm) are also visible in all three 1D-DOSY spectra. Figure 3 shows the results obtained after a preliminary simple fractionation of the ASw crude extract (see Exp. Sect., Fractionation 1) with the corresponding 2D-DOSY analyses performed on the obtained fractions. In this manner additional data on the chemical constituents were obtained, which allowed further optimisation of the purification approach. A precipitate (ASw_p) and a soluble part (ASw_s) were obtained from ASw after centrifugation in methanol/water 4:1 (see Exp. Sect.). The MW values from the 2D-DOSY analyses were deduced by reference to a calibration curve obtained with different dextrans.[13] Fraction ASw_p contains the high-MW polysaccharides (>20 kDa), while the low-MW derivatives (<1 kDa) are retained in fraction ASw_s. The medium-sized polysaccharide is shared between both fractions. We estimated a MW of 2 kDa for this medium-sized component by the 2D-DOSY analysis, performed on fraction ASw_s under conditions selected to filter out signals from low-MW constituents.[13] In this way, the overlapping phenomenon observed in 2D-DOSY spectra measured under standard conditions was avoided. This

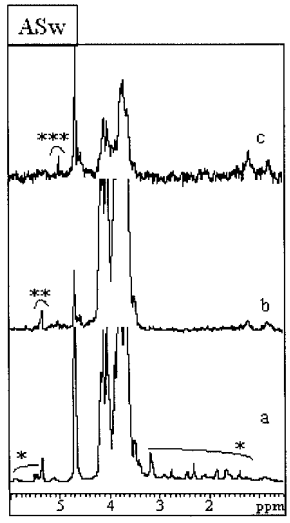

Figure 2. Comparison between the 1D-DOSY spectra of ASw performed with big delta = 0.4 s, little delta = 2 ms, and with the following values of gradient strength: a) 10%, b) 45%, c) 80%. * Low-MW compounds. ** Medium MW. *** High MW.

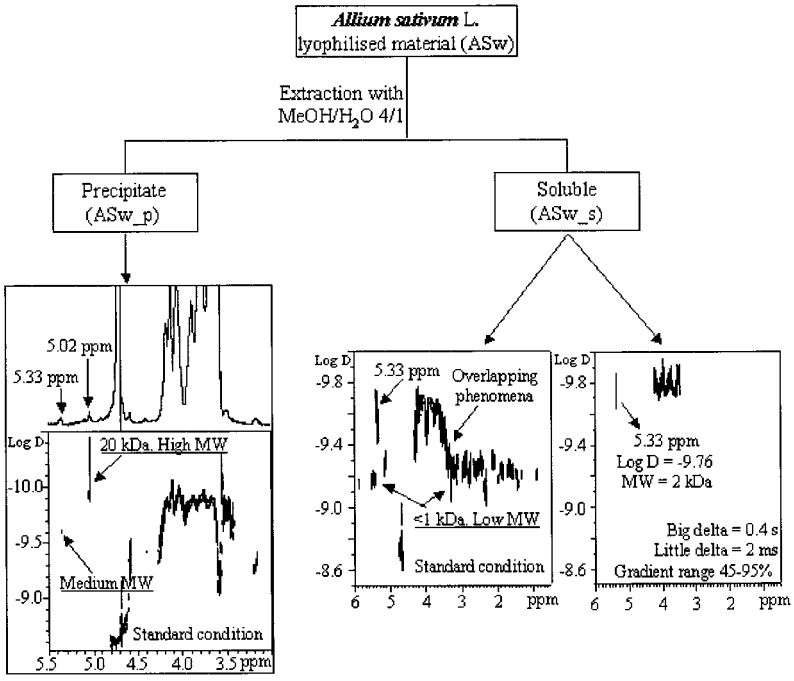

Figure 3. Fractionation 1 scheme of ASw extract (see Exp. Sect.) and corresponding 2D-DOSY of the soluble (ASw_s) and precipitate fractions (ASw_p). The standard 2D-DOSY parameters (big delta = 0.1 s, little delta = 1 ms, and gradient range from 2% to 95%) were used to analyse both fractions (ASw_s and ASw_p). The conditions to filter out the signals from low-MW compounds (big delta = 0.4 s, little delta = 2 ms, and gradient range from 45% to 95%) were also used in the second 2D-DOSY analysis of fraction ASw_s (spectra on the right). Approximate MW values of the components, calculated from the 2D-DOSY data, are shown in the panels.

analysis thus supplied relatively accurate values for the MWs of the different molecules and allowed us to build up a fractionation scheme based on dialysis by use of different membrane MW cut-offs (MWCOs). Since the membrane MWCO values are usually optimised for protein material, the ASw crude extract was fractionated with dialysis membranes of 13 kDa and 3.5 kDa cut-offs. Figure 4 shows the optimised ASw fractionation scheme (see Exp. Sect., Fractionation 2) and the corresponding NMR analyses. In two simple steps, an almost pure medium-sized polysaccharide was found in fraction ASw_d_r (Figure 4, c; proton signal at δ = 5.33 ppm alone). On the other hand, a mixture of the medium- and high-MW polysaccharides was obtained in fraction ASw_r_s (Figure 4, b; proton signal at δ = 5.33 ppm together with those at δ = 5.02 and 4.57 ppm).

Fraction ASw_d_r was hydrolysed and the released monosaccharides were identified and quantified by GC of their alditol acetates (Table 1). Only mannose and glucose (arising from fructose after borohydride reduction[14]) were detected. Full NMR analysis (not shown), monosaccharide composition and comparison with literature data[15] allowed the identification of a medium-sized fructan in fraction ASw_d_r. Such a constituent had been identified in garlic water extract previously.[14,16] Maldi MS of this fructan (not shown) confirmed the utility of the 2D-DOSY data to predict the MWs of certain constituents in a natural mixture. As expected for a polysaccharide, the fructan is polydisperse, with an average molecular weight, as detected by MALDI-MS, of 1662 Da, corresponding to a decasaccharide. Hydrolysis of fraction ASw_d_r with TFA (0.15 M) released a large proportion of fructose, whilst when the TFA concentration was raised to 3 M the fructan was degraded, and no other sugars were detected (Table 1), thus suggesting that only fructan is present in this fraction. Hydrolysis of fraction ASw_r_s (medium and high molecular weight polysaccharides) with TFA (0.15 M) also released fructose, but when the TFA concentration was raised to 5 M the fructan was again degraded and about 5% of galactose was detected (Table 1), so a galactan is also present in the mixture. 1D-DOSY experiments (spectra to the left in Figure 4) indicated that fraction ASw_r_s contained a high-MW galactan with α and β anomeric ^{1}H NMR signals at δ = 5.02 and 4.57 ppm, respectively. The rest of the protons signals overlapped in the chemical shift region from 3.6 ppm to 4.2 ppm. ^{1}H NMR spectra of the other two fractions, ASw_d_d and ASw_r_p, are shown in Figures S1 and S2, respectively (see Supporting Information). Fraction ASw_d_d contains a mixture of low-MW derivatives including the single unit from the fructan.[16] ^{1}H NMR spectra of fraction ASw_r_p (performed in DMSO) showed the signals from a (poly)peptide constituent. The presence of this peptide could explain the positive response in the STD analysis on the ligand mixture ASw, mentioned above.

STD and STDD analysis[12] (Figure 5) was performed on the two fractions ASw_d_r (the fructan alone; Figure 5, A) and ASw_r_s (containing the mixture of fructan and galactan; Figure 5, B). Clear differences between the complexes and the corresponding controls were observed (see Figure S3 in the Supporting Information). In the STDD spectra of ASw_d_r/CTB, only the ^{1}H NMR signals from the

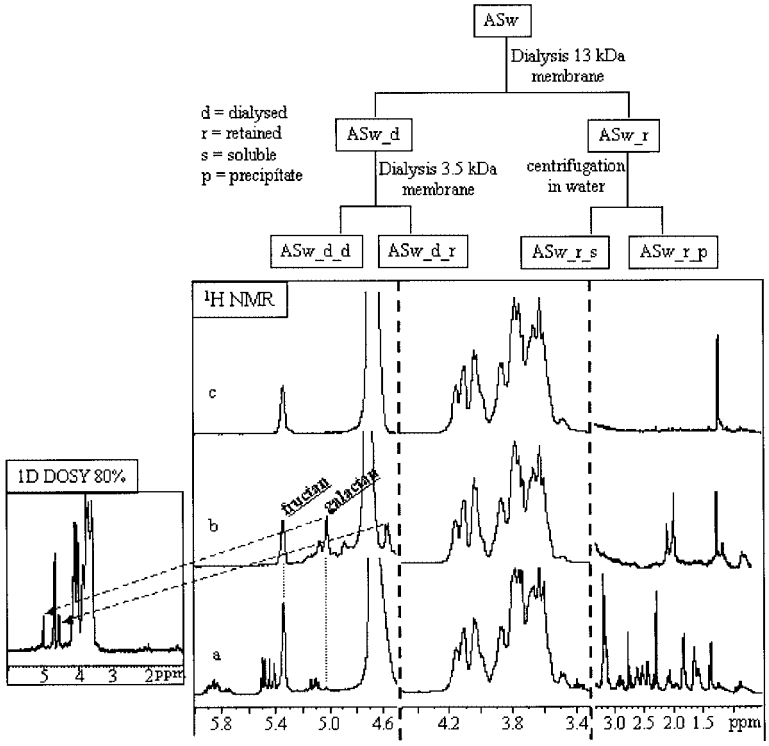

Figure 4. Fractionation 2 scheme of ASw extract (see Exp. Sect.) and related NMR analysis. Spectra a), b), and c) show the ^{1}H NMR analyses of ASw, ASw_r_s and ASw_d_r, respectively. Signal intensities are different between the three chosen chemical shift areas. In the 1D-DOSY analysis on fraction ASw_r_s (spectra to the left. Big delta = 0.4 s, little delta = 2 ms, gradient strength 80%), only proton signals from the galactan (the higher MW compound in the mixture ASw_r_s) are detected.

Table 1. Neutral sugars (detected as alditol acetates) from the fractions ASw_d_r and ASw_r_s under different hydrolytic conditions.

	Fructose mannose[a]	glucose[a]	Galactose
ASw_d_r			
TFA 0.15 M 1 h, 100 °C	26.3	35.5	0.0
TFA 3 M 1 h, 120 °C	10.6	17.6	0.0
ASw_r_s			
TFA 0.15 M 1 h, 100 °C	34.8	44.1	0.0
TFA 3 M 1 h, 120 °C	4.4	6.3	2.0
TFA 5 M 1 h, 120 °C	3.9	5.3	4.9

[a] These two sugars are produced from fructose after borohydride reduction. The amount of fructose in the polysaccharides is the sum of these two components.

CTB appear, indicating that the fructan alone is not able to bind CTB. On the other hand, STDD performed on ASw_r_s/CTB gave rise to the galactan signals, indicating that the bound ligand is contained in fraction ASw_r_s and it is thus possible to confirm that the high-MW galactan is able to bind to CTB. Moreover, although it may be ambitious to describe a well defined epitope, given the polymeric nature of the galactan, the sugar signals that showed the highest STD were those at δ = 3.61 ppm and 3.73 ppm, and these should correspond to those saccharide protons closest to the CTB lectin.

In order to support the NMR results further, the CTB binding features of the two fractions ASw_d_r (fructan) and ASw_r_s (fructan and galactan) were measured by fluorescence analysis (Figure 6). Initial analysis of the isolated fractions, without CTB, showed small levels of fluorescence in both cases. Addition of increasing amounts of ASw_d_r to CTB resulted in a monotonic and regular decrease in the fluorescence, due to the interfering impurities in the ASw_d_r sample. In contrast, addition of ASw_r_s to CTB did not result in an analogous reduction in the fluorescence of CTB. Instead, a sharp decrease in the CTB fluorescence was observed upon titration with minute concentrations of ASw_r_s (up to 100 $\mu g \cdot mL^{-1}$). This behaviour suggests that one of the components of ASw_r_s indeed binds to CTB and quenches its fluorescence. The observed decrease in CTB fluorescence in the presence of ASw_r_s is similar to those observed for weak-binding monosaccharides.[17] The strong-binding GM1, in contrast, was reported to promote an increase in CTB fluorescence with a blue shift of the fluorescence maximum.[17]

The midpoint of the ASw_r_s titration was estimated as 50 $\mu g \cdot mL^{-1}$. In any case, this qualitative fluorescence data support the NMR results and indicate that the ASw_r_s fraction is the only one enriched in the compound(s) that bind(s) CTB. If we assume that the ASw_r_s fraction contains just 5% of a 60 kDa ligand (as estimated from the 2D-DOSY spectra of ASw_r_s; not shown), then the midpoint

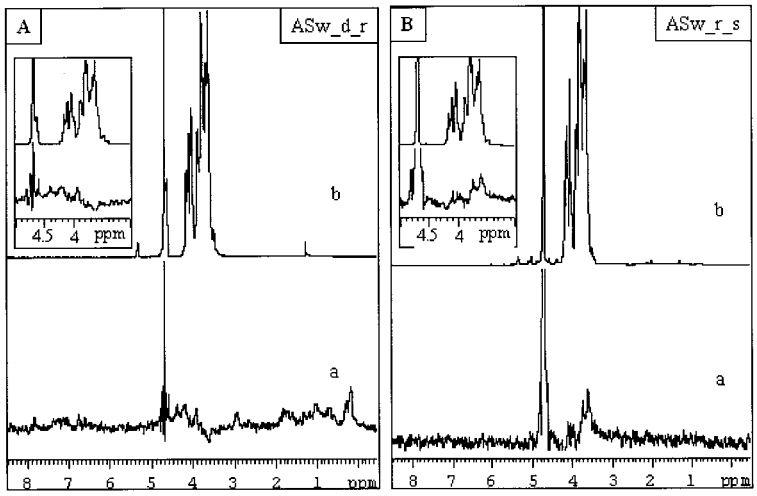

Figure 5. **A**) Comparison between the following NMR spectra: a) STDD of the complex ASw_d_r/CTB (difference between the STD spectra of ASw_d_r/CTB and the STD of ASw_d_r alone). b) ^{1}H NMR of ASw_d_r. **B**) Comparison between the following NMR spectra: a) STDD of the complex ASw_r_s/CTB (difference between the STD spectra of ASw_r_s/CTB and the STD of ASw_r_s alone). b) ^{1}H NMR of ASw_r_s.

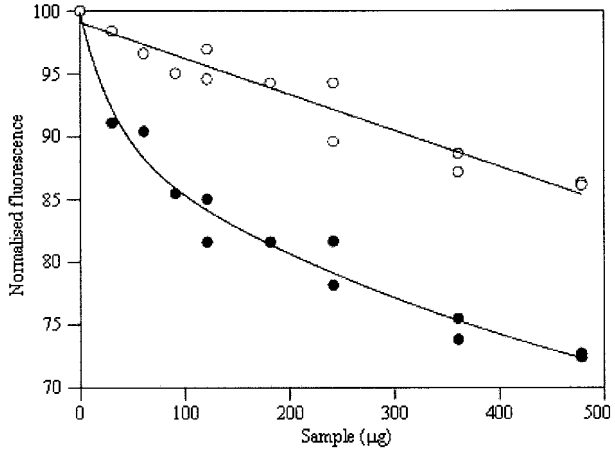

Figure 6. Fluorescence analysis for measurement of the binding activity between the two fractions ASw_d_r (open circles) and ASw_r_s (close circles) and CTB. The ASw_d_r data are least-squares fitted to a straight line, whereas the ASw_r_s line fit is for illustration.

of the titration can be converted from 50 μg·mL^{-1} to 42 nM, which effectively binds 1 *mM* CTB at the midpoint. This value provides an approximate estimate of one active molecule binding to 24 CTB protein monomers. Although merely qualitative, this high estimate for the stoichiometry suggests the presence of multiple binding sites in the ligand (as might be expected for a polysaccharide), which are in turn interacting with a large number of CTB pentamers. Nevertheless, it is also possible that other compounds in the ASw_r_s mixture could bind CTB. In fact, a variety of high molecular weight polyphenols from other natural sources such as apples, hop bract and the Chinese rhubarb rhizome have also been reported to be cholera toxin inhibitors,[18] forming aggregates with the A and B subunits. Further purification of the garlic galactan, followed by complete structural elucidation and detailed binding activity studies, was beyond the scope of this work and was not further pursued.

Conclusions

The galactan detected here as a bioactive ligand against CTB is a minor component of the crude ASw extract (less than 2%). Nevertheless, the NMR-detected interaction was evident from the first screening step (Figure 1), so the NMR-based approach can be used to detect the binding of a ligand in a complex mixture even when the ligand is a minor component in the mixture, which strengthens the efficacy of NMR approaches for the screening of crude natural extracts.

To the best of our knowledge this work represents the first example of screening of a medicinal plant by NMR against a specific disease that corroborates the traditional medical uses of the species, and indicates a second possible pharmacological mechanism for the treatment of cholera infection with garlic products. New inputs for research into medicinal plants from the direct investigation of their water-based extracts may be expected in the near future.

Experimental Section

Plant Material: Semi-fresh bulbs of *Allium sativum* L. (garlic) were bought directly from a market in Madrid, Spain, in March 2005.

Extraction: The semi-fresh crushed bulbs (50 g) were extracted with hot water (200 mL) for 3 h; the extract was then lyophilised to provide crude material (ca. 7 g), labelled as ASw.

Fractionation 1: ASw (200 mg) was dissolved in a methanol/water mixture (4:1, 5 mL), producing a soluble material and a precipitate, which were separated by centrifugation. The precipitate was washed in methanol/water (4:1, 5 mL) and centrifuged, and the

process was repeated three times, finally to provide soluble material (ASw_s, 130 mg) and precipitate (ASw_p, 48.6 mg).

Fractionation 2: ASw (1.5 g) was dissolved in water (15 mL) and dialysed in a membrane (protein MWCO = 13 kDa) for 24 h with agitation in a tube containing water (about 250 mL) at 25 °C, and dialysates (× 2) were collected, combined, concentrated and freeze-dried (fraction ASw_d, 0.46 g). The retained material was further dialysed against tap water for 24 h and freeze-dried (ASw_r; 0.58 g). ASw_d (200 mg) was dissolved in water (5 mL) and dialysed for 48 h in a membrane (protein MWCO = 3.5 kDa) by the previous procedure. After removal of solvent, a retained material labelled ASw_d_r (72.0 mg) and a dialysed one labelled ASw_d_d (91.2 mg) were obtained. ASw_r (200 mg) was dissolved in water (5 mL) and centrifuged to provide a soluble part labelled ASw_r_s (120 mg) and a precipitate labelled ASw_r_p (60.0 mg).

Materials: Highly purified, salt-free, lyophilized CTB (MW ca. 15 kD) was obtained from the *Vibrio cholerae* O395-tacCTB strain, kindly provided by the firm IRIS Chiron Srl. This strain was obtained by transformation of the O395-NT strain[19] with a plasmid derived from pMMB67[20] that expresses the cholera toxin B gene under the control of the *tac* promoter. *V. cholerae* O395-tacCTB strain was inoculated in LB medium (100 mL) supplemented with ampicillin (LB+, 200 μg·mL^{-1}) and cultured overnight at 37 °C in an orbital shaker. One millilitre of the overnight culture was added to LB+ medium (400 mL) and incubated at 37 °C. When the culture reached an optic density (O.D. 600 nm) of 0.7 (approximately 3 h). CTB expression was induced by addition of isopropyl IPTG (β-D-1-thiogalactopyranoside) to a final concentration of 1 mM. After 20 h of incubation at 37 °C in an orbital shaker, the bacterial culture was centrifuged for 5 min at 12,000 g, and the supernatant was transferred to a flask. We added sodium hexametaphosphate (2.5 g·L^{-1}), adjusted the pH to 4.5 with concentrated HC1 and stirred the mixture for 2.5 h at room temperature to precipitate the CTB. The precipitate was collected by centrifugation at 12,000 g for 30 min and resuspended in sodium phosphate (0.1 M, pH 8.0). After dialysis of the protein sample three times against sodium phosphate (10 mM, pH 7.0), the insoluble material was removed by centrifugation at 25,000 g for 30 min. The cleared sample was loaded into a 30 mL carboxymethyl Sepharose CL-6B column and washed with 6 column volumes of sodium phosphate (20 mM, pH 7.5). Elution of the CTB was performed with 1 column volume of sodium phosphate buffer (100 mM, pH 8.3). The purity of the protein was then checked by polyacrylamide SDS gel stained with Coomassie.

The Na_2HPO_4 and NaH_2PO_4, were of reagent quality. Before the NMR experiments were performed, the affinity of CTB was tested with lactose and it was deduced that most of the protein was intact.

The dextrans used for the calibration curve were purchased from Pharmacia Fine Chemicals (Sweden): dextran T10 (MW 10,000), Dextran T70 (MW 70,000), Dextran T500 (MW 500,000). Dextran with MW of 38,900 was purchased from Sigma.

Preparation of the Samples: Samples ASw_s and ASw_p (10 mg) were dissolved in D_2O (0.5 mL). All other NMR tubes (*I*–*VII*) contained the following D_2O phosphate buffer solutions (75 mM, pH = 7.5, 0.5 mL):

I) CTB (75 μM), *II*) ASw (10 mg), *III*) CTB (75 μM) with ASw (10 mg), *IV*) ASw_d_r (10 mg), *V*) CTB (75 μM) with ASw_d_r (10 mg), *VI*) ASw_r_s (10 mg), *VII*) CTB (75 μM) with ASw_r_s (10 mg).

Sugar Analyses: Fractions ASw_r_s and ASw_d_r were hydrolysed with TFA under different conditions (Table 1). Released sugars were converted into their corresponding alditol acetates[21] and were then identified and quantified by gas-liquid chromatography (GLC) with a SP-2380 fused silica column (30 m × 0.25 mM I.D. × 0.2 μm film thickness) with a temperature program (210 °C to 240 °C, initial time 3 min, ramp rate 15 °C·min^{-1}, final time 7 min) and a flame ionization detector.

MALDI-MS: The laser desorption/ionisation experiments were performed on a BIFLEX III time-of-flight instrument (Bruker–Franzen Analytik, Bremen, Germany) operated in the positive mode. Samples were analysed in the reflectron mode, and 100 laser shots were summed into a single mass spectrum. External calibration was performed, with use of the monoisotopic peaks of ACTH (*m*/*z* 2465.2), angiotensin (*m*/*z* 1046.5) and the matrix CCA (*m*/*z* 379) recorded in a single spectrum. Aqueous 2,5-dihydroxybenzoic acid (10 mg·mL–1) was used as the matrix. Equal volumes (0.5 mL) of the sample solution and the matrix were spotted on the target and air-dried. The masses of the oligosaccharides were observed as the [M + Na]+ ions, and their relative amounts were calculated according to peak area.

Emission Fluorescence: Fluorescence experiments were performed with buffer (1.64 mL, 0.1 M NaCl, 0.025 M NaH_2PO_4, pH 7) containing CTB (2 μM) in a 1-cm light-path cuvette. The samples were continuously stirred with a 0.5 mL magnetic stirrer. The experimental parameters were λ_{ex} = 280 nm, four averaged scans between 300 and 400 nm over at 1 nm·s^{-1}, excitation and emission slits of 2 nm and datapoints collected each 1 nm. Aliquots of ligand were added to the protein solutions. Protein dilution, which was less than 2%, was not corrected. Fluorescence changes were followed at a fixed wavelength of 357 nm, which provided the maximum difference between free and bound protein spectra.

NMR Analyses: All the NMR analyses were performed at 298 K with a Bruker Avance 500 MHz spectrometer fitted with a triple resonance ^1H, ^{13}C, ^{15}N probe. Chemical shifts are in ppm with respect to the 0 ppm point of the manufacturer's indirect referencing method. The saturation transfer difference (STD) experiments were recorded (number of scans 4096) by use of the sequence proposed by Meyer and co-workers.[10] A cascade of soft Gaussian-shaped pulses of 50 ms (with a power level of 50 Hz) was used for the 2.5 s saturation time. On-resonance irradiation was done at 0.2 ppm while the off-resonance was set at δ = +50 ppm. A short spin-lock period (15 ms) was used prior to the acquisition in order to reduce background protein signals. For the 2D-DOSY experiments, the standard Bruker protocol was used for processing, with the ledbpg2s pulse sequence, and a linear gradient of 32 steps. For samples ASw_p and ASw_s we used a gradient range between 2% to 95%, a diffusion time (big delta) of 0.1 s, and little delta of 1 ms. For sample ASw_s we also modified the parameters with use of a gradient range between 45% to 95%, big delta 0.4 s, and little delta 2 ms. Diffusion edited ^1H NMR spectra (1D-DOSY) were acquired with the ledbpg2s1d pulse sequence, big delta 0.4 s, little delta 2 ms, with use of different values of the gradient strength (see captions of the figures).

Supporting Information (for details see the footnote on the first page of this article): Figure S1. ^1H NMR of fraction ASw_d_d. Figure S2. ^1H NMR of fraction ASw_r_p. * Protein proton signals. Figure S3. A) Comparison between the following NMR spectra: a) STD of ASw_d_r. b) STD of ASw_d_r + CTB. c) ^1H NMR of ASw_d_r. B) Comparison between the following NMR spectra: a) STD of ASw_r_s. b) STD of ASw_r_s + CTB. c) ^1H NMR of ASw_r_s.

Acknowledgments

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