Thermodynamic analysis of binding of 4-methylumbelliferyl-α- and β-D-galactopyranosides to *Momordica charantia* lectin

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Binding of 4-methylumbelliferyl-α-D-galactopyranoside (MeUmbαGal) and the corresponding β-anomer (MeUmbβGal) to the *Momordica charantia* (bitter gourd) lectin (MCL) has been investigated by fluorescence spectroscopy. Binding of MeUmbαGal to MCL resulted in a decrease in the fluorescence intensity of the ligand. Saturation binding at 25°C resulted in a 17.8% decrease in the fluorescence intensity of the ligand. Quenching of the ligand fluorescence intensity was temperature-dependent and decreased with increase in temperature. Addition of lactose reversed the quenching due to binding, indicating that decrease in the fluorescence intensity of MeUmbαGal is due to the interaction of its carbohydrate moiety with the lectin. The changes in the fluorescence intensity of MeUmbαGal resulting from the binding were analysed to obtain the association constants for the binding process at different temperatures. At 25°C, the association constant, K_a, was determined to be 1.14×10^4 M^{-1}, and from the temperature dependence of the K_a values the enthalpy and entropy of binding were estimated as $\Delta H^\circ = -25.9$ kJ mol^{-1} and $\Delta S^\circ = -9.1$ J mol^{-1} K^{-1}. A comparison of these values with the ΔH° and ΔS° values obtained for the binding of MeUmbβGal revealed that the higher affinity of the β-anomer is due to a larger enthalpy of binding, which overrides a larger negative entropy of binding for the latter.

LECTINS, the carbohydrate-binding proteins of non-immune origin, have been the subject of intense investigations in view of their interesting biological properties such as preferential agglutination of transformed cells, blood-group specificity, mitogenicity and hormone-mimicking activity[1–3]. All these properties are manifested through the carbohydrate-binding activity of lectins, and hence it is of great interest to investigate their saccharide specificity and to delineate the forces that govern it.

The *Momordica charantia* lectin (MCL) is a galactose-specific lectin present in the seeds of bitter gourd, which is used as a part of the diet in the tropics. MCL has been purified by affinity chromatography and characterized in considerable detail with regard to its macromolecular properties[4–6]. Chemical modification studies have indicated that tryptophan and tyrosine residues are important for the sugar-binding activity of this lectin. Chemical modification, intrinsic fluorescence quenching and time-resolved fluorescence studies indicate that the tryptophan residues are in a heterogeneous environment, with at least two populations of tryptophan residues with different degrees of exposure being present in the protein[6,7]. In addition to haemagglutination-inhibition studies, the binding of a few saccharides has been investigated by monitoring saccharide-induced changes in the intrinsic fluorescence emission spectrum of the protein[4]. The binding of 4-methylumbelliferyl-β-D-galactopyranoside (MeUmbβGal) was investigated at different temperatures by monitoring the changes in the fluorescence intensity of the ligand upon titration with MCL in order to determine the thermodynamic parameters associated with the binding[5], whereas the binding of 4-methylumbelliferyl-α-D-galactopyranoside (MeUmbαGal), the corresponding α-anomer was not studied. In this study the interaction of MeUmbαGal and MeUmbβGal with MCL was investigated at different temperatures, in order to delineate the thermodynamic basis for the higher affinity of the protein for the β-linked galactose moiety.

M. charantia lectin was purified by affinity chromatography on cross-linked guar gum[6,8]. The protein gave a single band in PAGE[9] and two bands corresponding to Mr ~28 kDa and ~30 kDa in SDS–PAGE[10], consistent with literature reports[6]. Fluorescence measurements were performed on a Hitachi F-3010 fluorescence spectrometer, equipped with a water-jacketed cuvette holder that was maintained at constant temperature by means of a circulatory water bath. Titrations were performed by the addition of small aliquots of the protein from a concentrated stock solution (ca. 30 mg/ml) to 1.0 ml of a 5 μM solution of the fluorescent sugar. Samples were excited at 318 nm and emission spectra were recorded in the wavelength range of 330–450 nm. Slits of 5 nm were used for both excitation and emission monochromators. All the binding data reported here correspond to the average values obtained from two different titrations.

Fluorescence spectra of MeUmbαGal alone and in the presence of different concentrations of MCL, recorded at 25°C, are shown in Figure 1. From Figure 1 it is evident that addition of the lectin decreases the fluorescence intensity of MeUmbαGal. Further, changes in the fluorescence intensity resulting from protein addition could be reversed by the addition of lactose or galactose, demonstrating that the binding is mediated through the saccharide moiety of the labelled sugar. Similar results were reported for the titration of MeUmbβGal with MCL[5].

A binding curve, depicting the change in fluorescence intensity of the ligand (ΔF) as a function of the protein concentration, obtained from the above fluorescence spectra is shown in Figure 2. Here, it is seen that the binding curve displays saturation behaviour, clearly indicating that binding occurs at specific binding sites on the

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protein. A plot of $1/\Delta F$ as a function of $1[P]_t$, where $[P]_t$ is the total protein concentration, is given in the inset to Figure 2. The data yield a linear fit and from the *Y*-intercept of this plot, the change in fluorescence intensity at infinite protein concentration, ΔF_∞ was obtained. It was observed that the ΔF_∞ value was temperature-dependent and decreased with increase in temperature. At 15°C, the fluorescence intensity of MeUmbαGal decreased by 25%, whereas at 30°C, the decrease was only 15.2% (Table 1).

The titration data were then analysed according to eq. (1) in order to obtain the association constants, K_a (ref. 11).

$$\log\{\Delta F/(F_c - F_\infty)\} = \log K_a + \log[P]_f, \quad (1)$$

where ΔF is the fluorescence change at any point of the titration, F_c is the fluorescence intensity at any point of the titration corrected for dilution, ΔF_∞ is the change in fluorescence intensity at infinite protein concentration and $[P]_f$ is the free protein concentration. A more detailed description of the method can be found elsewhere[11,12].

Association constants for the binding of the umbelliferyl sugar (K_a) were obtained from the abscissa of the plot of log $\{\Delta F/(F_c - F_\infty)\}$ vs $\log[P]_f$. A representative plot for the titration data obtained at 25°C is given in Figure 3. From the intercept of this plot the K_a value has been estimated as $1.14 \times 10^4\ M^{-1}$. Association constants for the MeUmbαGal–MCL interaction have been determined at several temperatures and the K_a values obtained as described above are listed in Table 1.

As mentioned above, the binding of MeUmbβGal to MCL was investigated earlier by Khan *et al.*[5]. However, in order to compare the data obtained for both α- and β-anomeric derivatives of the sugar under similar conditions and with the same lot of the purified protein, the binding of MeUmbβGal has also been investigated in this study. Experiments were performed essentially as described above for MeUmbαGal and it has been observed that the fluorescence intensity of the ligand is totally quenched at saturation binding at all temperatures investigated, an observation consistent with the results of Khan *et al.*[5]. The titration data were analysed as described above for the MeUmbαGal–MCL interaction, in order to determine the K_a values for the binding of MeUmbβGal to MCL. At 25°C, the association constant was obtained as $2.12 \times 10^4\ M^{-1}$, which is in excellent agreement with the value of $2.1 \times 10^4\ M^{-1}$ obtained from equilibrium dialysis measurements, and also compares well with the value of $1.98 \times 10^4\ M^{-1}$, obtained from fluorescence titrations by Khan *et al.*[5]. The K_a values obtained at different temperatures are listed in Table 1.

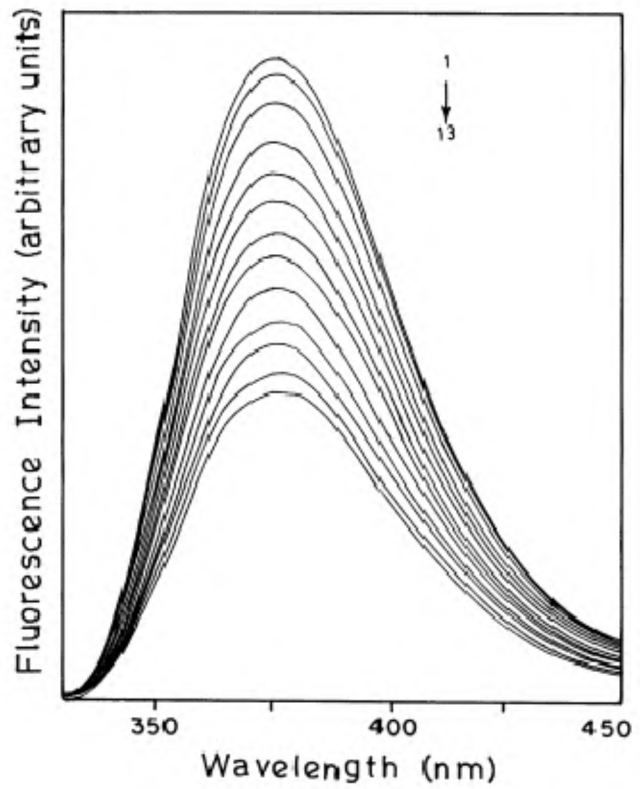

Figure 1. Fluorescence spectra of MeUmbαGal in the absence and presence of *M. charantia* lectin at 25°C. Spectrum 1 is that of MeUmbαGal alone and spectra 2 to 13 were recorded after the addition of increasing concentrations of MCL.

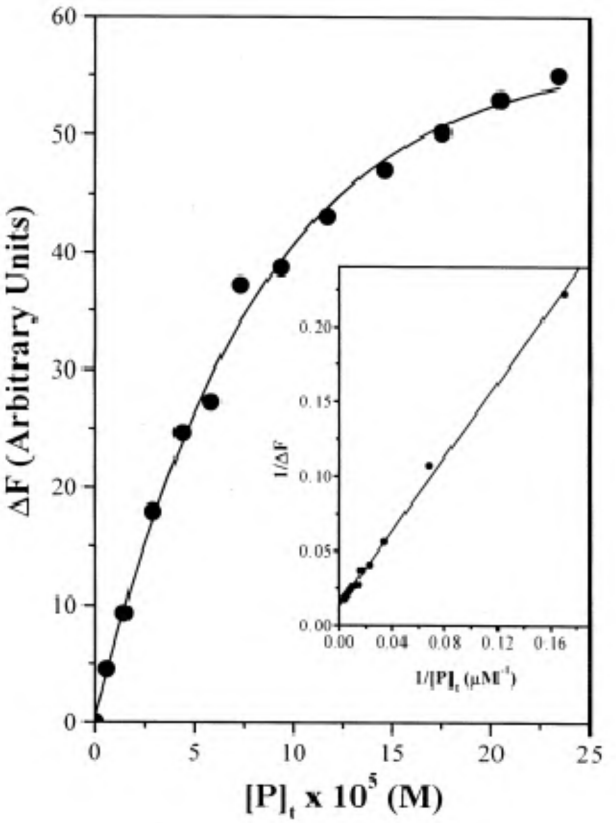

Figure 2. Binding curve for titration of MeUmbαGal by MCL at 25°C. Change in fluorescence intensity at 376 nm (ΔF) was plotted as a function of the added protein concentration. (Inset) Plot of $(1/\Delta F)$ as a function of reciprocal protein concentration. From the *Y*-intercept of this plot, fluorescence intensity of the ligand at saturation binding was determined. See text for further detail.

From the K_a values obtained at different temperatures it is clear that the interaction of MeUmbβGal with MCL is characterized by a stronger affinity than that of MeUmbαGal; the K_a values for the former sugar being 1.6 to 2.0 times higher than the latter (Table 1). These results are consistent with the earlier observations that MeβGal is 1.5 times more potent than MeαGal in its ability to inhibit the haemagglutination by MCL[6].

Table 1. Maximum changes in fluorescence intensity (ΔF_∞), association constants (K_a) and thermodynamic parameters ($\Delta H°$, $\Delta S°$) associated with the binding of MeUmbαGal and MeUmbβGal to *Momordica charantia* lectin, determined from the fluorescence spectral titrations

T (°C)	ΔF_∞ (%)	$K_a \times 10^{-3}$ (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
MeUmbαGal				
15	25.0	16.9	– 25.9	– 9.1
20	19.2	14.1		
25	17.8	11.7		
30	15.2	9.9		
MeUmbβGal				
15	100	34.8	– 36.3	– 39.3
20	100	26.6		
25	100	21.2		
30	100	16.3		

The temperature dependence of the association constants for MeUmbαGal and MeUmbβGal has been analysed by Van't Hoff plots of $\ln K_a$ vs $1/T$ (see Figure 4) and linear fits have been obtained in each case. From the slope and intercept of these plots, enthalpy of binding ($\Delta H°$) and entropy of binding ($\Delta S°$) have been determined according to eq. (2):

$$\ln K_a = -\Delta H°/RT + \Delta S°/R. \quad (2)$$

The enthalpy and entropy of binding for MeUmbαGal–MCL interaction were obtained as $\Delta H° = -25.9$ kJ mol^{-1} and $\Delta S° = -9.1$ J mol^{-1} K^{-1}, whereas the corresponding values for the binding of MeUmbβGal to MCL have been determined to be $\Delta H° = -36.3$ kJ mol^{-1} and $\Delta S° = -39.3$ J mol^{-1} K^{-1}. These values are also given in Table 1.

An analysis of the above thermodynamic parameters indicates that the enthalpy of binding for MeUmbβGal is considerably larger than the value obtained for MeUmbαGal, clearly indicating that binding of the β-anomer is favoured by a larger enthalpy value. In addition, binding of the α-anomer is associated with a smaller negative entropy compared to the β-anomer. Therefore, the larger $\Delta H°$ value associated with the binding of MeUmbβGal more than compensates for the negative

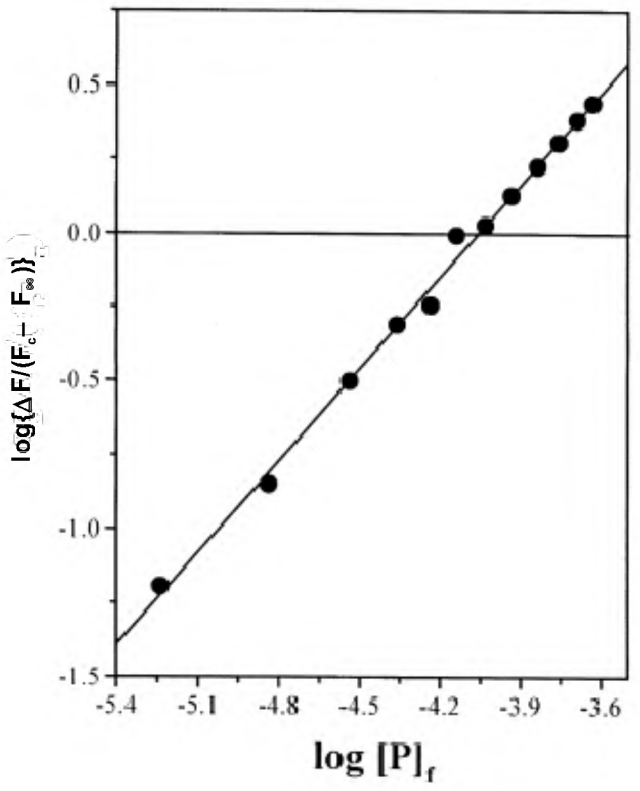

Figure 3. Chipman plot for binding of MeUmbαGal to MCL at 25°C. The Y-intercept of the plot yielded pK_a, from which the association constant, K_a is determined.

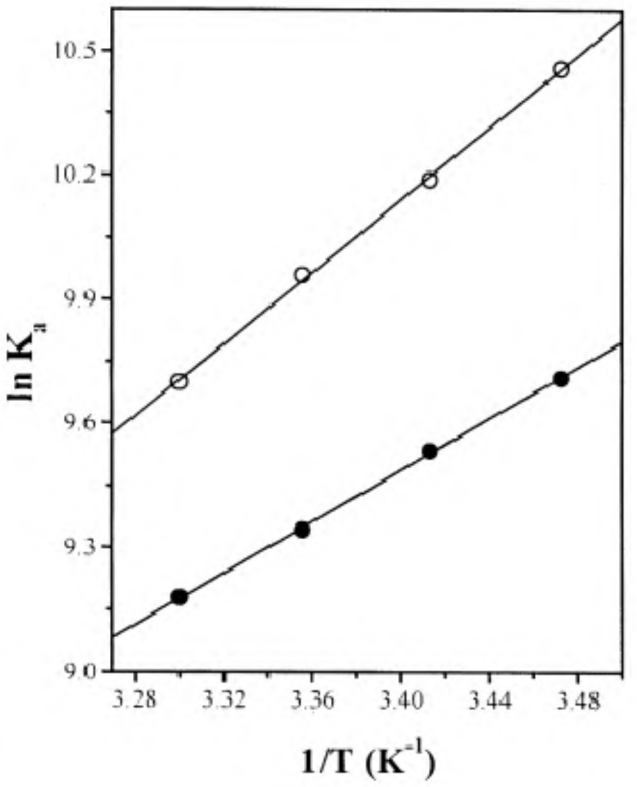

Figure 4. Van't Hoff plot for binding of MeUmbαGal (•) and MeUmbβGal (○) to MCL. Thermodynamic parameters, enthalpy of binding ($\Delta H°$) and entropy of binding ($\Delta S°$) were determined from the slope and intercept respectively, of this plot according to eq. (2). See text for further detail.

contribution from the entropy of binding, leading to a larger K_a value for the binding of MeUmbβGal than for MeUmbαGal. It is possible that binding of MeUmbβGal is associated with additional hydrogen-bonding interactions of the anomeric oxygen atom with the combining site of the lectin, which are absent in the binding of MeUmbαGal. While this leads to an increase in the binding enthalpy, the additional ordering could lead to an increase in the entropy of binding. The thermodynamic data presented in Table 1 are consistent with this model.

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Bud break and plantlet regeneration *in vitro* from mature trees of *Pinus roxburghii* Sarg.

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Terminal and axillary buds of 30-year-old mature *Pinus roxburghii* Sarg. trees were collected to test their *in vitro* response. Five different types of shoots containing these buds were available in a year. Shoots collected between February and May gave the best response. Bud sprouting could be obtained on solid half strength DCR basal medium (1/2 DCR), fortified with BA from 0.44 to 11.1 µm, 11.1 µm being the optimum concentration used. On taking these buds for elongation, however, only those transferred from 0.44 µm elongated on plain 1/2 DCR after three passages of 45 days each. The initial concentration of BA therefore had an influence on the elongation of shoots. Rooting was obtained in 5% of the shoots on the elongation medium. Optimum rooting (54%) was obtained after a 24 h treatment on 12.25 µm IBA and the plantlets survived upon transfer to polyhouse.

PINUS roxburghii Sarg., commonly known as 'chir pine', is one of the five species of pines occurring wild in the Himalaya and hills of Assam in India[1] and in the monsoon belt of northeastern Pakistan to Bhutan. It grows to a height of 45 to 54 m and is known to be fairly drought-resistant[2]. The wood is widely used for making furniture, door and window frames, railway sleepers, paper, pulp, etc. Oleoresin from chir pine is the main source of turpentine in India, which is chiefly used as a solvent for thinning, paints and varnishes besides having medicinal uses[1]. Natural regeneration is through seeds. Trees less than 30 years old rarely bear cones. A good seed year occurs only once in four to five years. Considering its uses, seeding problems and depletion of the natural stocks, there is an urgent need to have alternative methods for the establishment of plantation. This can be achieved through two methods of vegetative propagation. One involves rooting of juvenile cuttings from seedlings raised from hybrid seeds, as is done in the case of radiata pine[3]. The other involves rooting of stem cuttings from superior phenotypes. Application of tissue culture techniques would be another method for large-scale cloning of mature trees[4]. In the case of conifers this is more important, as the rooting frequency declines when the parent plant is more than ten years old[5].

In the last 15 years, there have been several reports on *in vitro* propagation of conifers. In recent years, there has been success in conifer propagation through both somatic embryogenesis[6–9] and induction of adventitious buds[10–13]. Most of these reports deal with studies using juvenile explants (seeds, seedlings, young cotyledons, etc.). However, there are few reports on the *in vitro* propagation from mature tree-derived explants[14–16]. The large genome sizes of pines and the recalcitrance of tissues from mature trees to *in vitro* manipulation continue to present challenges to researchers[17]. In the case of *P. roxburghii*, only adventitious-bud proliferation has been reported so far[18]. To our knowledge, there are no reports on *in vitro* vegetative propagation of this species from mature tissues.

The present study is part of an ongoing programme to develop micropropagation methods for *Pinus* species.

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