Partial characterization of goat brain proteins involved in bilirubin binding

Mohd. Mushahid Khan* and Saad Tayyab
Interdisciplinary Biotechnology Unit, Aligarh Muslim University,
Aligarh 202 002, India

In order to characterize in terms of bilirubin binding, major proteins in the goat brain were separated into five different peaks, namely P₀, P₁, P₂, P₃ and P₄ on a Seralose-6B column (90.5 x 2.5 cm). The peak P₀ was eluted with the void volume of the column. The molecular weights and Stokes radii of the remaining peak proteins were: P₁ (1,06,727 and 4.11 nm), P₂ (59,256 and 3.25 nm), P₃ (18,713 and 1.71 nm) and P₄ (11,928 and 1.14 nm). Bilirubin binding studies indicated that three out of the five peak proteins, namely P₁, P₂ and P₃ showed bilirubin binding as characterized by the blue shift and hyperchromism in the visible absorption spectra and quenching of the protein fluorescence upon addition of bilirubin to these peak proteins.

HYPERBILIRUBINEMIA is an important pathological condition in the newborn and the possibility of low grade brain damage due to bilirubin toxicity is of interest to clinicians¹. Decreased albumin binding capacity, increased bilirubin concentration or low albumin levels, can account for increased bilirubin deposition in the brain thus leading to the development of the clinical syndrome, kernicterus which may result in infant death². Bilirubin neurotoxicity may be mediated by a number of mechanisms due to its increased permeability in neuronal membranes. Recently, it was reported that the high neonatal serum bilirubin levels adversely affect hearing³, while in newborn piglets its prolonged infusion modified the N-methyl-D-aspartate (NMDA) receptor/ion channel complex in the cerebral cortex⁴. In spite of extensive research, the mechanisms of bilirubin toxicity in the brain and characterization of the bilirubin binding proteins remain elusive. In this paper, we report our data on the characterization of some bilirubin binding proteins in goat brain.

Fresh goat brain, obtained from a local slaughterhouse, was dissected and made free of membranous tissues and circulatory debris. Then, it was cut into small pieces and homogenized in a Remi mixer for about 5 min with three volumes of 0.06 M sodium phosphate buffer, pH 7.0. The crude homogenate was centrifuged at 6000 g for 30 min. The supernatant was filtered through millipore filter (0.25 mm) and the residue was discarded. It was then applied directly on a Seralose-6B column (90.5 x 2.5 cm). The different peak fractions, obtained


ACKNOWLEDGEMENTS. This work was supported by the DST (SP/SD/D/39/93). T.S. is thankful to CSIR for the financial support and to Prof. Faizan Ahmad, Department of Biosciences, JMI, New Delhi, for his guidance and encouragement.

Received 20 March 1999; revised accepted 2 June 1999

*For correspondence. (e-mail: btiisamua@nds.vsnl.net.in)

CURRENT SCIENCE, VOL. 77, NO. 3, 10 AUGUST 1999
from the same column, were used for preliminary characterization.

Protein concentration was determined by the method of Lowry et al.\textsuperscript{5} using BSA as the standard. The molecular weights and Stokes radii of different peak fractions of goat brain proteins obtained on a Seprose-6B column were determined by analytical gel filtration according to the standard procedure\textsuperscript{6,7}.

Fresh bilirubin solution was prepared by dissolving few of its crystals in 5 mM NaOH solution containing 1 mM EDTA and diluting it to the desired volume with 0.06 M sodium phosphate buffer, pH 8.0. The concentration of bilirubin was determined spectrophotometrically using a molar extinction coefficient of 47,500 M\textsuperscript{-1} cm\textsuperscript{-1} at 440 nm (ref. 10). Binding of bilirubin to different fractions of goat brain proteins was studied using both visible absorption spectroscopy and fluorescence quenching. Visible absorption spectra of free bilirubin and a complex of bilirubin with different goat brain proteins were recorded in the wavelength range 380–510 nm. All the binding studies were performed in 0.06 M sodium phosphate buffer, pH 8.0, ionic strength 0.15. The molar ratio of bilirubin to protein was kept as 1:1 and the spectra were recorded 20 min after adding the desired amount of stock bilirubin solution to the protein solution. Bilirubin binding was also studied by the fluorescence quenching method after adding increasing amount of stock bilirubin solution to a fixed amount of protein solution. The molar ratios between bilirubin and proteins were fixed as 0.0, 0.5, 1.0, 1.5 and 2.0. The final volume was adjusted to 5 ml by adding desired amount of 0.06 M sodium phosphate buffer, pH 8.0, ionic strength 0.15. The fluorescence emission spectra were recorded in the wavelength range of 300–450 nm by exciting the protein at 280 nm. The slit width was 2 nm and 5 nm for the excitation and emission spectra, respectively.

Five distinct peaks, namely P\textsubscript{0}, P\textsubscript{1}, P\textsubscript{2}, P\textsubscript{3} and P\textsubscript{4} were obtained when goat brain homogenate was passed on a Serrose-6B column (90.5 x 2.5 cm). The peak P\textsubscript{0} was eluted with void volume of the column. Of the remaining four peaks, P\textsubscript{1} seemed to have higher molecular size followed by P\textsubscript{2} and P\textsubscript{3}, whereas P\textsubscript{4} had the lowest (Figures 1 and 2). Treatment of gel filtration data of marker proteins as well as different proteins of goat brain according to Andrews\textsuperscript{8} and Porath\textsuperscript{7} (see Figure 1 a, b) yielded the following straight line equations.

\[
V_c / V_o = 4.92 - 0.58 \log M,
\]

\[
M^{1/3} = 223.11 - 205.65 Kd^{1/3}.
\]

To determine the Stokes radius, gel filtration data were also treated according to Laurent and Killander\textsuperscript{4} and Ackers\textsuperscript{5} which fit the following straight line equations (see Figure 2 a and b).

\[
(- \log K_{av})^{1/2} = 0.08 \text{ Stokes radius} + 0.14,
\]

\[
\text{Stokes radius} = 9.22 \text{ erfc}^{-1} Kd + 0.84.
\]

The values of molecular weights (from equations (1) and (2)) and Stokes radii (from (3) and (4)) for different goat brain proteins calculated from these equations are listed in Table 1.

Visible absorption spectrum of bilirubin showed an absorption maxima at 440 nm. Addition of different goat brain proteins to the biureum solution shifted its absorption maxima to the blue region and enhanced the absorbance (Figure 3 c). Occurrence of a blue shift and enhancement in the absorbance of bilirubin solution upon addition of different goat brain proteins were indicative of the binding of bilirubin to these proteins. Similar type of blue shift and enhancement was also observed with different trypic and peptic fragments of serum albumin.

![Figure 1](image1.png)

**Figure 1.** Treatment of gel filtration data for marker proteins according to: a, Andrews\textsuperscript{8} and b, Porath\textsuperscript{7} obtained on a Seprose-6B column (90.5 x 2.5 cm). Different marker proteins used along with their molecular weights and Stokes radii are: (1) α-chymotrypsinogen [25,000 and 2.24 nm]; (2) ovalbumin [45,000 and 2.73 nm]; (3) BSA monomer [67,000 and 3.55 nm]; (4) BSA dimer [135,000 and 4.30 nm]; and (5) γ-globulin [205,000 and 5.30 nm]. The positions of different peak proteins of goat brain are shown by arrows.

![Figure 2](image2.png)

**Figure 2.** Treatment of gel filtration data for marker proteins according to: a, Laurent and Killander\textsuperscript{4} and b, Ackers\textsuperscript{5}. The representation of various marker proteins is the same as given in the legend to Figure 1. The positions of various peak proteins of goat brain are shown by arrows.
Table 1. Molecular weights and Stokes radii of different peak proteins of goat brain obtained from gel filtration results at pH 7.0 and ionic strength 0.15

<table>
<thead>
<tr>
<th>Protein fractions</th>
<th>Molecular weight</th>
<th>Stokes radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_e$ (ml)</td>
<td>From eq. (1)</td>
</tr>
<tr>
<td>$P_1$</td>
<td>330</td>
<td>1.03,276</td>
</tr>
<tr>
<td>$P_2$</td>
<td>355</td>
<td>57.069</td>
</tr>
<tr>
<td>$P_3$</td>
<td>400</td>
<td>19.647</td>
</tr>
<tr>
<td>$P_4$</td>
<td>415</td>
<td>13.237</td>
</tr>
</tbody>
</table>

Figure 3. a. Visible absorption spectra of bilirubin in the absence and presence of different peak proteins of goat brain. The spectra are: (O) free bilirubin, (●) $P_1$ + bilirubin, (▲) $P_2$ + bilirubin, and (△) $P_3$ + bilirubin; b. Fluorescence quenching results of different peak proteins of goat brain at different bilirubin to protein molar ratios. The molar ratios are: 0.0, 0.5, 1.0, 1.5 and 2.0. Different peak proteins are; (O) $P_1$, (△) $P_3$, and (△) $P_3$.

upon their interaction with bilirubin\(^1\). Although these protein fractions were qualitatively similar in terms of bilirubin binding, they varied in terms of the extent of blue shift as well as the magnitude of absorbance. A blue shift of 20 nm was observed with peak proteins $P_1$ and $P_2$, whereas $P_3$ showed a blue shift of only 15 nm. On the other hand, increase in absorbance was maximum with $P_2$ followed by $P_1$ and minimum with $P_3$. Peak $P_4$ did not show any indication of bilirubin binding with this method.

All the three peak proteins, $P_1$, $P_2$ and $P_3$ gave the fluorescence spectra in the wavelength range 300–450 nm with the emission maxima at 330–334 nm. Addition of bilirubin to these proteins caused quenching in their emission spectra. The bilirubin-induced fluorescence quenching was suggestive of the binding of bilirubin to these proteins as observed earlier with serum albumin\(^1\). Bilirubin binding to these proteins was studied at different molar ratios between bilirubin and protein and the results were plotted as relative fluorescence against bilirubin to protein molar ratio (Figure 3 b). As is evident from the figure, maximum quenching was observed with peak $P_2$ at all molar ratios studied, followed by $P_1$, whereas peak $P_3$ showed relatively little quenching. Taken together, the data on bilirubin-induced fluorescence quenching and blue shift and enhancement in the visible absorption spectroscopic results suggested that all the three proteins bind bilirubin to a significant extent. Studies are in progress to characterize these proteins in detail in terms of bilirubin binding.


ACKNOWLEDGEMENTS. Facilities provided by the Aligarh Muslim University, Aligarh are gratefully acknowledged. M.M.K. is a Senior Research Fellow of the Council of Scientific and Industrial Research, New Delhi, India.

Received 5 February 1999; revised accepted 17 April 1999