The production and characterization of a coumarin-bovine serum albumin (BSA) conjugate and its use for antibody production.

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Coumarin is a constituent of many plants. It has found use in industry mainly due to its strong fragrant odour. Coumarin has been used in the treatment of a range of ailments such as cancer, burns, brucellosis and rheumatic disease [1]. The most commonly used quantitative method for coumarin analysis in biological samples is high performance liquid chromatography (HPLC). However, this method requires substantial sample preparation prior to assay [2]. It was, therefore, decided to develop an enzyme-linked immunosorbent assay (ELISA) for the measurement of coumarin and its metabolites. To facilitate conjugation, coumarin was first derivatised as follows. It was converted to 6-nitrocoumarin using methods reported by Clayton [3] and Kondo and Tetsukichi [4]. The melting point (mp) of this compound was found to be 170°C. Infra red (IR) analysis revealed bands at 1500 and 1300 cm\(^{-1}\). 6-Nitrocoumarin was used to prepare 6-aminocoumarin using methods reported by Morgan and Micklethwait [4]. The mp of this compound was found to be 176°C. Also, IR analysis revealed the presence of bands at 3400 and 3300 cm\(^{-1}\). Diazotization and conjugation of 6-aminocoumarin to BSA was performed using a modification of methods by Morgan and Micklethwait [5] and Baker [6]. This involved addition of 6-aminocoumarin to hydrochloric acid and a cold sodium nitrite solution. The pH was maintained at 7.9 by the simultaneous addition of cold 0.5 N sodium hydroxide. A red colour developed and the solution was maintained at 4°C for 24 hours while mixing slowly. The conjugate was purified by dialysis against distilled water for 24 hours and freeze-dried and characterised by electrophoresis, HPLC, UV and IR spectroscopy. The molecular weight was estimated using polyacrylamide gel electrophoresis (PAGE) and HPLC [7]. UV spectroscopy of the conjugate, coumarin and BSA was carried out to determine their respective absorption maxima. The absorption maxima were 350, 280 and 280 nm, respectively. The molar extinction coefficient of coumarin was determined and used to calculate the number of moles of coumarin per mole of BSA [8]. Potassium bromide discs for the three compounds were prepared and scanned from 4000-800 cm\(^{-1}\).

The mp results and IR analysis of 6-nitrocoumarin and 6-aminocoumarin produced results that were very similar to reported values for these compounds. The molecular weight of the conjugate was estimated using PAGE and found to be 69,800, approximately. Results show that between 10 and 15 molecules of coumarin are conjugated to each BSA molecule. A UV scan of the conjugate revealed an absorption maximum of 350 nm, while an IR scan contained an extra band at 1405 cm\(^{-1}\), both of which indicate the presence of an azo link. The conjugate was used to raise monoclonal and polyclonal antibodies in mice and rabbits, respectively. A 1 mg/ml stock solution of conjugate was prepared in phosphate buffered saline (Oxoid, Dulbecco A), pH 7.3. A 0.1 ml aliquot of this was emulsified with 0.3 ml of Freund's adjuvant (100 mg). An intra peritoneal injection of this emulsion was administered to seven week old Balb/C mice. Also, a 2.0 ml aliquot of the stock solution of conjugate in phosphate buffer saline was used to reconstitute a vial of Ribi adjuvant. A 1 ml dose of this emulsion was administered as follows: 0.4 ml intra muscularly, 0.3 ml sub-cutaneously and 0.2 ml intra peritoneally to New Zealand white rabbits. All animals were boosted on two occasions and bled. Serum antibody titers were estimated by ELISA.

The procedures developed for the production of the coumarin conjugate and subsequent immunization have resulted in successful antibody production.

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