Investigation of the Stereoselectivity of an Anti-Amino Acid Antibody Utilizing Tryptophan Fluorescence

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- Change in intrinsic tryptophan fluorescence is used to measure the binding of an antibody to its antigen
- Stereoselective antibodies specifically bind to the D-enantiomer of amino acids
- Effect of pH, temperature, and concentration on intrinsic tryptophan fluorescence

Introduction

The binding sites of proteins such as antibodies are known to often contain tryptophan (Trp) residues, whose fluorescent properties may be altered upon ligand binding. Conformational changes within the binding site or simply the presence of the ligand can result in either fluorescence quenching or enhancement, which may be utilized to quantitatively investigate protein-ligand interactions.[1] We have previously described the production of highly stereoselective antibodies to amino acids.[2,3] These antibodies have been used in a variety of analytical techniques for the sensitive detection of enantiomeric impurities and for enantiomer separation.[4] The objective of this study was to test if tryptophan fluorescence can be used to determine the affinity of an anti-D-amino acid antibody toward a variety of standard and non-standard amino acids.
In order to examine the utility of BMG LABTECH's FLUOstar for measuring Trp fluorescence (Figure 1), experimental conditions were first optimized using the free amino acid as analyte.

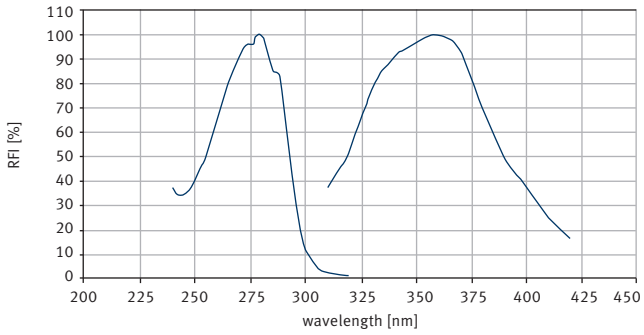

Fig. 1: Excitation and emission spectra of Tryptophan (dissolved in 50 mM PBS buffer, pH 7.4) performed on the POLARstar Omega.

Materials and Methods

- FLUOstar plate reader
- Reacti-Bind White Opaque 96-well plates (PIERCE 15042)
- D,L-Tryptophan (Sigma T3300)
- D-Phenylalanine (Sigma P1751), L -Phenylalanine (Sigma P2126)
- Phosphate buffers (pH values between 2 and 12)
- Monoclonal anti-D-amino acid antibody

96-well microtiter plates were blocked with 1% gelatin in PBS/0.05% Tween 20 (250 µl/well; 2 h at 37°C), followed by washing with PBS/0.05% Tween 20. Samples containing either Trp (200 µl/well) or antibody at varying concentrations in phosphate buffer (100 µl/well) were excited at 280 nm; emission was detected at 350 nm. For ligand binding studies, 50 µl/well of antibody at a fixed concentration (30 µg/mL) was pre-incubated for 2 hours at RT with ligand in varying concentrations (50 µl/well) before fluorescence measurement.

Results and Discussion

It is well known that experimental conditions strongly affect Trp fluorescence. Here, the effect of analyte concentration, temperature, and pH was investigated in order to establish optimal conditions to be used with BMG LABTECH's FLUOstar. As seen in Fig. 2, a clear concentration-dependent increase in Trp fluorescence was observed at concentrations ranging between about 1 µM and 1 mM. Excellent signal-to-noise ratios with minimal background fluorescence were obtained upon excitation at 280 nm and detection of fluorescence emission at 350 nm. In contrast, no fluorescence was observed using phenylalanine in the same range of concentrations (not shown).

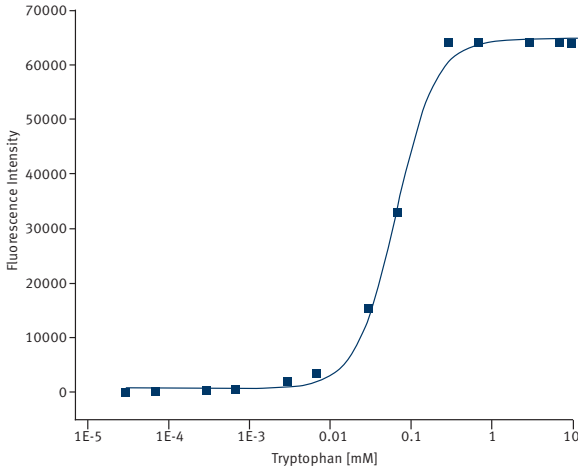

Fig. 2: Fluorescence intensities obtained with Trp at varying concentrations in phosphate buffer, pH 7.4.

The effect of temperature and pH was investigated using Trp at a concentration of 70 µM as analyte. As seen in Fig. 3, fluorescence intensity significantly decreases at higher temperatures.

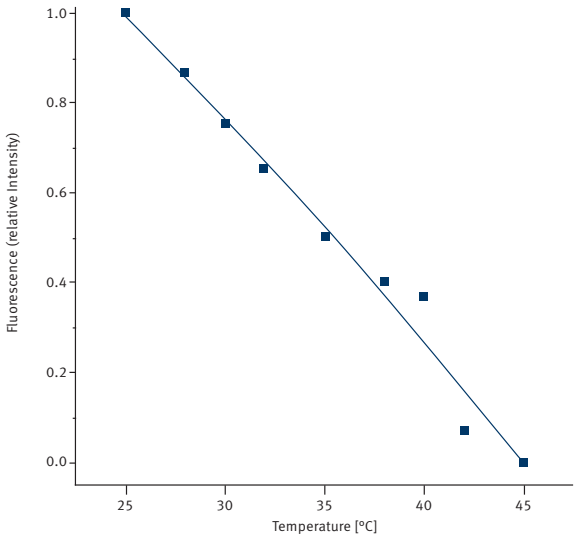

Fig. 3: Effect of temperature on Trp fluorescence.

Also changes in the pH have a considerable effect on Trp fluorescence, which is strongest at a pH around 11 (Fig. 4). Both results are in good agreement with previous reports.[5,6]

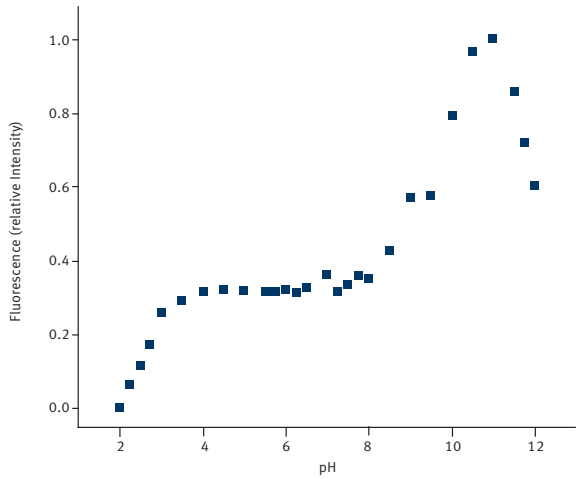

Fig. 4: Effect of pH on Trp fluorescence.

Investigating antibody stereoselectivity by measuring Trp fluorescence

As seen in Fig. 5, the FLUOstar plate reader can be used to determine the intrinsic Trp fluorescence of the anti-D-amino acid antibody used in this study. Increasing concentrations of the antibody in phosphate buffer, pH 7.4, resulted in increasing fluorescence emission at 350 nm upon excitation at 280 nm.

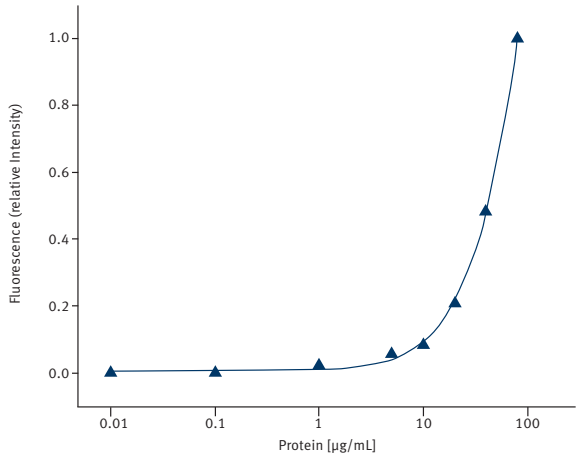

Fig. 5: Intrinsic Trp fluorescence of an anti-D-amino acid antibody at varying concentrations.

For protein-ligand studies, a fixed concentration of the antibody was incubated with the D- or L-enantiomers of a variety of amino acids. Fig. 6 shows the results obtained with D- and L-phenylalanine, respectively.

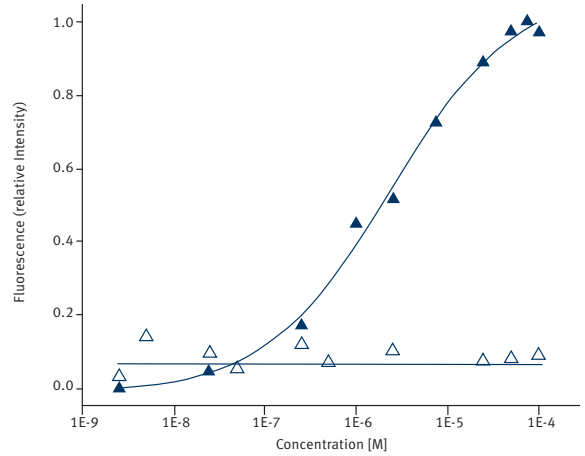

Fig. 6: Binding of D-phenylalanine (filled triangles) to an anti-D-amino acid antibody causes a concentration-dependent enhancement of the protein's intrinsic Trp fluorescence, while no such effect is observed using L-phenylalanine (open triangles) in varying concentrations.

While the interaction of the antibody with D-Phe causes a concentration-dependent increase of the antibody's intrinsic Trp fluorescence, no such effect is observed using the L-enantiomer. Similar results were obtained with the enantiomers of cyclohexylalanine, histidine, norleucine, leucine, and norvaline (not shown)[7]. In all cases, the stereoselective interaction of the antibody with the D-enantiomers of these amino acids caused a concentration-dependant enhancement of the protein's intrinsic Trp fluorescence, while no change in fluorescence was caused by the L-enantiomers. As observed using other analytical techniques,[3,4] the affinity of the antibody is strongest to D-amino acids having aromatic or bulky side chains, while aliphatic amino acids are bound more weakly. E.g., the affinity of the antibody for D-phenylalanine was determined to be 1.4 µM, while the antibody's affinity toward D-norvaline is 3.2 mM.

Conclusion

The FLUOstar plate reader allows measurement of Trp fluorescence with high sensitivity and good signal-to-noise ratios. Excitation of appropriate proteins at 280 nm and measurement of fluorescence emission at 350 nm can be employed to investigate protein-ligand interactions and to deduce, e.g., binding affinities. As experiments are performed in standard microtiter plates, only small sample volumes are needed, which is especially useful for the study of valuable proteins.

References

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