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Affinity extractants for the selective recovery of IgG from proteins

ABSTRACT

Due to the required high purity and low concentrations in product streams from current cell lines, downstream processing represents the major part of the overall production costs for monoclonal antibodies. Extractive separations offer potential for significant cost reductions through process intensification compared to currently-applied chromatographic separations. This work presents a new methodology for the direct extraction of IgG into an apolar organic solvent (iso-octane). This methodology is based on the integration of affinity chromatography ligands into selective extractants with apolar tails. Experimental evaluation demonstrated that with the synthesised affinity extractants recovery of IgG, up to 70% can be achieved in one extraction step, while no extraction of proteins such as myoglobin (MYO) or human serum albumin (HSA) was observed. In mixtures of IgG with the myoglobin and real fermentation product the overall selectivity was reduced due to competitive interactions resulting in a decreased extraction of IgG.

INTRODUCTION

Immunoglobulin G (IgG) is an important antibody with numerous biotechnological and pharmaceutical applications. However, due to the required high purity and low concentrations in the product streams from current cell lines, downstream processing represents the major part of the overall production costs [1]. Commonly, this purification process consists of an initial concentration step (precipitation or membrane filtration) followed by several chromatographic isolation and purification steps. For these reasons, there is a driving force to develop alternative technologies that can increase the efficiency and capacity of downstream processing to reduce production costs. Extractive separations offer potential for significant cost reductions through process intensification compared to the currently applied chromatographic separations. It is, however, well known that organic solvents are not the most suitable environments for proteins [2]. Moreover, proteins are insoluble in organic solvents. Therefore the presence of an agent (host) able to interact with proteins (guest) is required [3].

This work presents a new methodology based on the integration of affinity chromatography ligands into selective extractants with apolar tails. The extractants were synthesised by combining A2P and MEP ligands with a JMT Primene[®] and AOT support (**Figure 1**) and applied for the selective extraction of the antibody Immunoglobulin G (IgG) from Human Serum Albumin (HSA) and Myoglobin (MYO), as important examples of large proteins. For the AOT support only one of the phenolic A2P ligand rings was attached in order to simplify the structure and minimize solubility problems. For each extractant the capacity to extract IgG has been evaluated in relation to pH influence and salt concentration. Furthermore, synthetic mixtures of MYO with IgG and a Chinese Hamster Ovary fermentation derived supernatant were used to determine the selectivity of extraction.

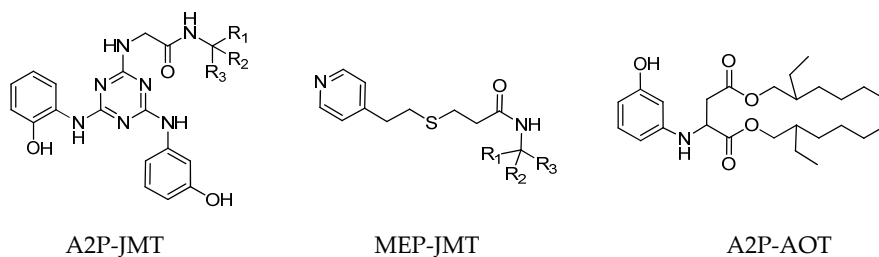


Figure 1 Structure of the synthesised extractants where R1, R2 and R3 are C6-C8 alkyl chains

METHODOLOGY

Materials

Human Serum Albumin (HAS) and Myoglobin (MYO) were purchased from Sigma-Aldrich (Saint Louis, USA). Immunoglobulin G (IgG) was purchased from Sigma-Aldrich (Saint Louis, USA). Isooctane (analytical grade) was purchased from Merck (Darmstadt, Germany). NaCl (99.5%) was purchased from Acros (s'-Hertogenbosch, The Netherlands). A Chinese Hamster Ovary (CHO) cell supernatant containing 0.1 g/l IgG was purchased from Excellgene (Monthey, Switzerland).

EXTRACTANT SYNTHESIS

MEP-JMT was synthesised in three steps by addition of 3-mercapto propionic acid to vinylpyridine. Reaction of the acid with DCC/NHS afforded the activated succinimide ester which was subsequently treated with primene to yield MEP-JMT. The target material was purified by column chromatography and isolated in 52% over-all yield.

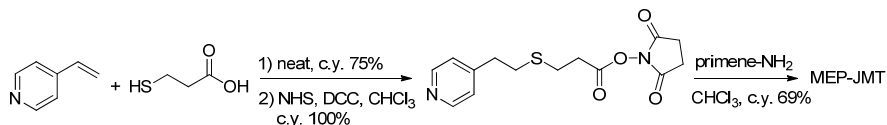


Figure 2 Reaction scheme 1: The synthesis of MEP-JMT

A2P-JMT was prepared by starting from the commercially available NHS-ester of benzyloxy carbonyl-protected Glycine (Z-GlySuc). The NHS-ester was substituted by primene and the amino group deprotected by reduction with hydrogen and Palladium on Carbon. Reaction of the amine with bis-aminophenol substituted cyanuric chloride affording the target material in 27% yield.

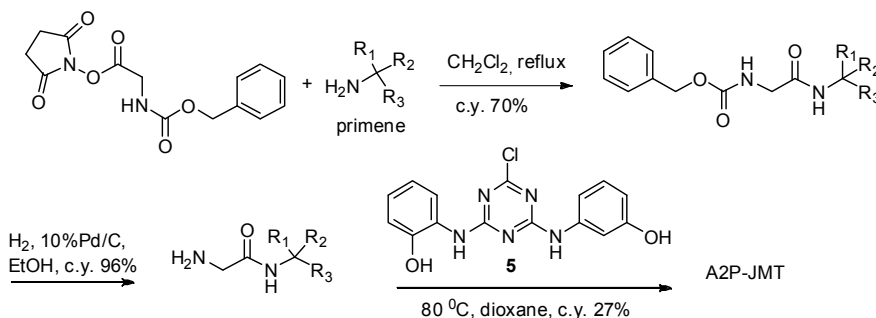


Figure 3 Reaction scheme 2: The synthesis of A2P-JMT

A2P-AOT was synthesised in two steps by esterification of 2-bromo-succinic acid with 2-ethyl-1-hexanol followed by substitution of the bromide with 3-aminophenol.

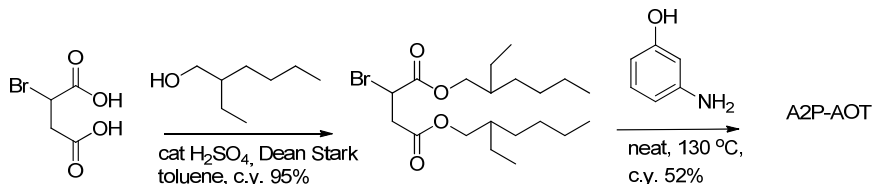


Figure 4 Reaction scheme 3: The synthesis of A2P-AOT

Extraction experiments

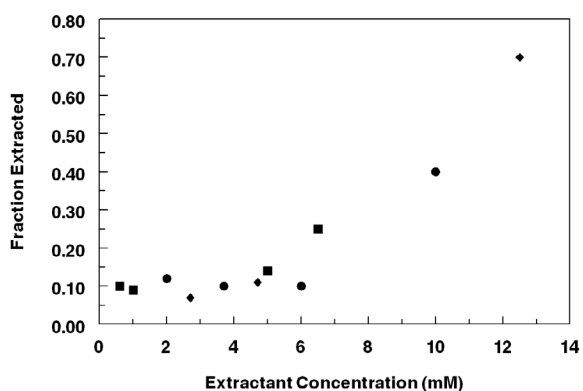
Experiments were carried out in 10 ml vials. The experiments were carried out at a constant temperature of 25 °C in a Julabo SW23 thermostated shaking bath. 1.0 g/l aqueous solutions of the protein (HSA and myoglobin) were prepared. 0.2 g/l aqueous

solutions of IgG with a 150 mM NaCl solution were prepared. The pH was adjusted to the desired value with diluted solutions of H₂SO₄ and NaOH. The solvent used was isoctane. The volume ratio was 1:1 using 4 ml of solution per phase. Shaking speed was 200 rpm. After 4 hours equilibrium was reached. The aqueous phase was analysed using HPLC, Varian Pro Star Size Exclusion Chromatography at 280 nm for Human Serum Albumin and Myoglobin and 211 for IgG. The concentration in the organic phase was calculated by a mass balance. Accuracy of the HPLC analysis was 3%.

RESULTS AND DISCUSSION

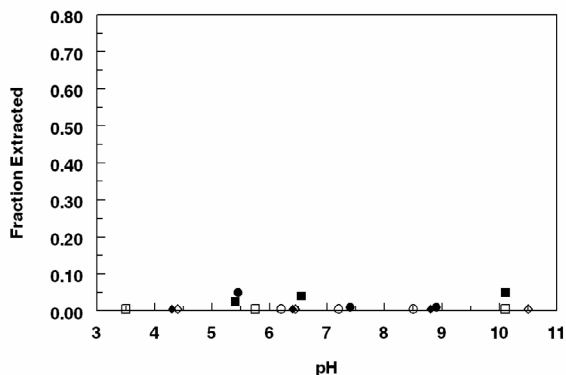
Influence of extractant concentration on IgG extraction

Experiments varying the concentration of extractant were carried out in order to determine the influence on the extraction capacity. Initial experiments demonstrated that for IgG the best conditions to avoid denaturation were between pH 5 and 11, which was verified by SDS-page analysis [3,4]. Therefore all experiments have been conducted at a pH 5.9–6.5. **Figure 2** shows that for all extractants a low extraction capacity is obtained at concentrations below 5 mM. Above this ‘threshold’ extractant concentration the fraction extracted increases rapidly and significant amounts of IgG are extracted into the solvent phase. This ‘threshold’ concentration indicates that a certain minimum amount of extractant is required to form reverse micellar like aggregates that are able to solubilise the IgG into the solvent phase. Dynamic light scattering (DLS) confirmed that the A2P-AOT forms reverse micellar like structures with a diameter of 20–30 nm. Although DLS showed no spontaneous micelle formation for the other two extractants, the obtained results indicate that in the presence of guest molecules (e.g., IgG) these extractants can form reverse micelles like aggregates. Furthermore the results demonstrate that all three extractants studied are able to extract the IgG to a similar extend.



A2P-AOT (■), A2P-JMT (●), MEP-JMT (♦) pH=5.9-6.5. [IgG] initial 0.2 g/l

Figure 5 Influence of the extractant concentration on the fraction of IgG extracted



A2P-AOT (■: MYO, □: HSA) A2P-JMT (●: MYO, ○: HSA), MEP-JMT (◆: MYO, ◇: HSA),
[A2P-AOT]: 5 mM, [A2P-JMT] and [MEP-JMT]: 10 mM

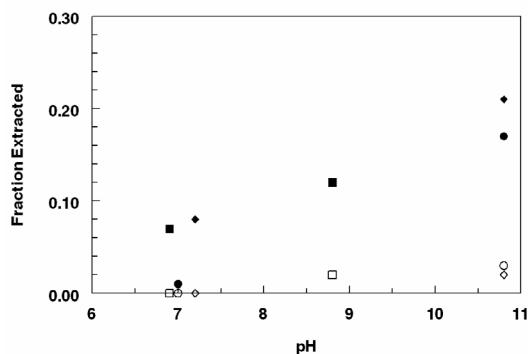
Figure 6 Fraction extracted for MYO and HSA as a function of pH

Human serum albumin and myoglobin extraction

In addition to the extraction of IgG, it is important to test the capacity of the extractants for other proteins to obtain indications about the selectivity. Knowing that in a fermentation broth typically a large number of secondary products and other components are present, human serum albumin (HSA) and myoglobin (MYO) were the proteins chosen in this work. **Figure 3** illustrates that in the range of 5–10 mM extractant no significant extraction is obtained with any of the extractant materials used for both proteins. Furthermore, changes in pH do not appear to have any effect on the extraction of the proteins. In all the cases the fraction extracted was below 0.05 indicating low affinity of the extractants for both proteins. This may result in considerable selectivities towards IgG when extracted from a mixture.

Selectivity in synthetic mixtures

The selectivity of the extractants was determined by extraction from a synthetic model mixture of MYO (1.0 g/l) and IgG (0.2 g/l). The concentration of IgG was chosen 5 times smaller to reproduce the common ratios of IgG/contaminants obtained in the production of IgG. Mixtures with HSA were not evaluated due to incompatibilities with the analytical method. **Figure 4** shows that, surprisingly, the amount of MYO extracted is considerably higher than without the presence of IgG. Furthermore the extraction of IgG is significantly decreased in the presence of MYO. Nevertheless all three extractants still exhibit reasonable (5–20) selectivity for IgG over MYO. Apparently, there are competitive effects between MYO and IgG occurring which must be taken into account.



A2P-AOT (■: IgG, □: MYO), A2P-JMT (●: IgG, ○: MYO), MEP-JMT (◆: IgG, ◇: MYO),
[A2P-AOT]: 5 mM, [A2P-JMT] and [MEP-JMT]: 10 mM, [IgG] = 0.2 g/l, [MYO] = 1.0 g/l

Figure 7 Fraction extracted from a synthetic binary mixture of IgG and MYO as function of pH

Application to a real fermentation product

Finally the extractants were evaluated on their capability to extract IgG from a real fermentation product being the Chinese Hamster Ovary (CHO) supernatant. **Figure 5** shows that the A2P-AOT extractant failed to extract the IgG present in the supernatant. A2P-JMT and MEP-JMT have some capacity to extract IgG present in the cell supernatant. However, the amount extracted is considerably lower than with IgG in pure solution. This is most likely due to the presence of other proteins which interfere (compete) with the extractants.

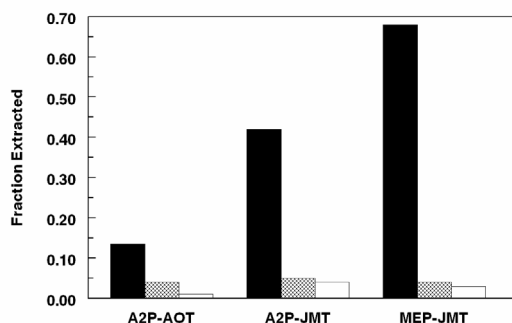


Figure 8 Fraction IgG extraction from pure solution (black), mixture with MYO (dashed) and CHO supernatant (white). [A2P-AOT]: 5 mM, [A2P-JMT] and [MEP-JMT]: 10 mM. [IgG]: 0.1 g/l. pH: 5-7

CONCLUSION

A new methodology based on the integration of affinity ligands obtained from affinity chromatography with apolar tails leading to a surfactant-like structure. With the synthesized A2P and MEP ligand containing extractants up to 70% extraction of IgG could be achieved in one extraction step, while, no affinity for proteins such as myoglobin or

human serum albumin was observed. In mixtures, competitive interactions with other proteins result in a decreased selectivity and decreased IgG extraction.

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