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High Performance Liquid Chromatography Methods for Purification of Recombinant Proteins

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Abstract: Recombinant proteins are frequently produced by overexpression in prokaryotic and eukaryotic host systems. Highly purified samples are an inevitable prerequisite for both research and therapeutic applications. High performance liquid chromatography (HPLC) is a frequently used technique in protein purification and its applications in protein science is very broad from analytical methods to the large scale preparative purifications of recombinant proteins used in biotechnology and pharmacology. The main types of HPLC protein separation are covered in the article including gel permeation chromatography (GPC), ion exchange chromatography (IEC), reverse phase chromatography (RPLC), hydrophobic interaction chromatography (HIC) and affinity chromatography (AC).

Keywords: HPLC, liquid chromotography, protein, purification, folding

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1. Introduction

Proteins belong to essential and the most important biological components, which participate in virtually every process within cells. Nowadays, recombinant proteins are frequently prepared by overexpression in prokaryotic and eukaryotic host systems with the aim to investigate protein structure and function as well as to produce biopharmaceuticals, diagnostics, fine chemicals and biocatalysts [1]. Proteins in a highly purified state are an inevitable prerequisite for all these purposes.

The type of purification employed depends on what the pure sample is required for. For the research purposes, two factors, the purity and the bioactivity are the most important. At an industrial production scale, in addition to the quality the price is necessary to be taken into consideration [2]. The main technique in protein purification technology is liquid chromatography, the highly efficient kind of this technique that utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

2. Principles and Application of HPLC

High performance liquid chromatography is a highly improved form of column chromatography, which uses pressures of up to 400 atmospheres. HPLC allows to use a smaller particle size for the column packing material and high flow rates. This results in a better separation of the components of the mixture. The main components of an HPLC in-

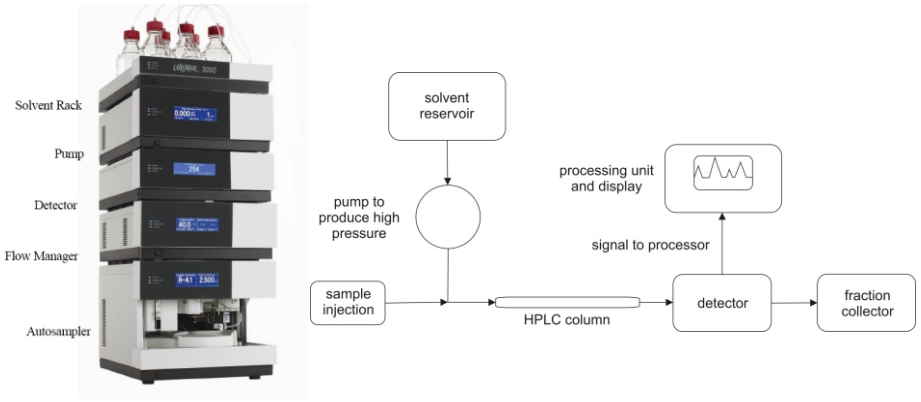

Fig. 1. Example (Ultimate 3000, Dionex) and general scheme of HPLC system.

strument are presented in Fig. 1. Apart from porous particle-based media in liquid chromatography monolithic columns become increasingly popular as efficient stationary phases for most of the important chromatographic separation modes, because of their resolution and capacity independent of the flow rate [3]. According to the principle of chromatographic separation and the type of chromatographic column several types are recognized including gel permeation chromatography (GPC), ion exchange chromatography (IEC), reverse phase chromatography (RPLC), hydrophobic interaction chromatography (HIC) and affinity chromatography (AC). For protein purification HPLC separation can be employed as either a single-step process or in combination [2].

Besides protein purification, HPLC can be used also for on-column protein refolding. By passing through the chromatographic column, the proteins produced in denatured forms might refold to gain their native structure and total activity. Suitable chromatographic medium and optimized operation system will affect conformational change and improve the yield of active proteins, which are valuable in both laboratory research and commercial productivity [4].

The gel permeation chromatography (or size exclusion chromatography) separates proteins according to their size directly related to their molecular mass. In principle, GPC columns are porous with pores of a size similar to the required proteins. The largest proteins cannot penetrate the beads because the pores are too small, so they flow quickly around the outside of the beads and elute out first. The smaller proteins are able to penetrate the pores and thus are slowered in the flow and are eluted later. An example is the MAbPac SEC-1column (Dionex) which is based on high-purity, spherical, porous (300 A), 5 μm silica particles that are covalently modified with a proprietary diol hydrophilic layer. The hydrophilic bonded layer results in minimal undesired interactions between the bio-molecules and the stationary phase. The chromatography can be used for analysis of monoclonal antibodies and for detection of aggregates in protein samples.

The universally used chromatographic technique in protein separation is the ion exchange chromatography. The retention of proteins on an IEC column depends on the strength of electrostatic interactions between the stationary phase and the charge of the

protein surface. A column of anion exchange chromatography (AEX) has a positive charge that attracts the negatively charged proteins, and under neutral pH conditions, most native proteins are negatively charged. For the minority of positively charged proteins cation exchange chromatography (CAX) is used. The IEC column can be chemically modified with various ligands to have four kinds of LC, strong cation exchange chromatography (SCX), with strong acidic e. g. sulfonate functional groups, weak cation chromatography (WCX) containing weak acidic, e.g. carboxylate, ligands, strong anion chromatography (SAX) based mainly on quaternary amine functional group and weak anion chromatography (WAX), based in most cases on tertiary amine ligands. The differences in selectivity between various functional groups provide flexibility in maximizing resolution of closely related protein species. Resolution of IEC separation is quite high, in some applications protein isoforms that differ by as little as one charged residue can be resolved [2]. At the same time anion exchange chromatography at a high pH is the most effective HPLC mode for the analysis of protein glycosylation [5].

In reverse phase liquid chromatography and hydrophobic interaction chromatography a similar separation principle is used. RPLC is one of the most important techniques for peptide separation. Because of its compatibility with mass spectrometry, RP-HPLC is an indispensable tool in proteomic research [6]. However, due to the strong hydrophobicity of the stationary phase, the native proteins often denature after purification by RPLC and this technique is thus used mainly for analytical separation of peptides and small proteins. The advantage of HIC, that separates based on differences in protein surface hydrophobicity, is that the chromatographic conditions are very close to the physiological conditions, such as a neutral pH, an aqueous salt solution, and room temperature, all of which are favourable to the maintenance of the proteins bioactivity.

The affinity chromatography is based on bio-specific interactions of proteins with ligand present in the stationary phase. Based on the various ligands, AFC can be divided into many types, such as inhibitor, lectin, nucleic acid, hormone, vitamin, sugar, and immunoaffinity chromatography. Although specific type of chromatography column is necessary for each protein, AFC is a frequently used method for protein purification, because of its high selectivity. The special subtype of AFC is the immobilized metal-ion affinity chromatography (IMAC) that is based on interaction of proteins containing surface-exposed histidine with metal ions (mainly Ni^{2+}, Cu^{2+}, Co^{2+}) immobilized on chelating chromatographic carrier [7, 8]. The typical HPLC column (e. g. ProPac® IMAC-10 from Dionex) is packed with 10 μm, nonporous, polymeric beads that are coated with a hydrophilic layer, then grafted with poly(imino-di-acetic acid) chains. The poly (IDA) grafts are converted into metal-containing nanoparticles when the column is loaded with metal cation. It is widely accepted that histidine, and also tryptophan and cysteine residues, as a result of strong interactions with metal-ions, are the key players in the binding of proteins in IMAC. Histidine residues are found in most of the natural protein amino acid sequences, but as a consequence of their mild hydrophobicity, only a few of them are located on the protein surface. In view of this and due to their importance in metal-ion adsorption processes, just a low number of naturally occurring proteins are potentially suitable for purification by this type of chromatography. On the other hand, histidine tags can be attached into C- or N-terminals of proteins by genetically engineering resulting in very efficient separation of the recombinant proteins with IMAC. Many different histidine tags have been employed, from very short ones to rather long extensions attached to various

model proteins. However, nowadays the most-used histidine tags consist of six consecutive histidine residues [7]. IMAC methodology can be operated between pH 2–12 and is compatible with commonly used protein purification reagents (non-ionic detergents, reducing agents). Its main advantage is that it can be used under native as well as denaturing HPLC conditions enabling purifications and subsequent refolding of recombinant proteins isolated from inclusion bodies.

3. Conclusion

Applications of HPLC in protein science are very broad covering methods for analysis of clinical (e. g. serum proteins) and research samples, checking for protein variants, aggregates and contaminants in commercially produced proteins and therapeutics [2, 9]. HPLC has been widely applied in proteomic investigations of all kinds of biological samples [10]. Other types of advanced methodologies are used for preparative purifications, especially for purifications of recombinant proteins used in biotechnology and pharmacology. The new techniques and separation chains are instantly developed and it is necessary to adjust thoroughly the used method for the particular application.

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