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Review Separation and Purification of Proteins with Chromatography Applications

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The separation and purification of recombinant proteins are an important issue on bioprocessing. It not only contacts our lives, but associates with genetic engineering and expression. Because it is a junction of chemistry and biology, researchers should find a method to have a common effect on them for the separation and purification of protein. Though chromatography technology develops very quickly, it is difficult to meet requires of the separation and purification of protein. Despite these difficulties, chromatography, having been updated to solve the separation and purification of proteins constantly, which have been found widespread applications in the large scale purification of proteins from mammalian cells and microbial feedstocks in industrial bioprocessing. And others methodologies also are studied on the separation and purification of proteins.

Key words: Review, protein, Chromatography, Bioprocessing.

Nowadays, chromatography purification is widely used in the downstream processing of the fermentation, biological process and so on. Chromatography has many advantages since it is an easy, fast and high-efficiency procedure for capturing of the target protein and removing impurities. And chromatography may have many selections including chromatofocusing, SEC, IEX, LAC, IAC, HPC, CCC(TY Zhang and Berthod, 2002) and HA. Due to its selectiveness for the AC, an affinity-purification step early in the purification chain is commonly introduced[1]. Thereby, the number of successive unit operations can be reduced(Harakas, 1994; MacLennan, 1995). Because proteins, playing an important role in our daily lives, which are used in many fields such as nutrition, medical and immune diagnosis, etc. With the development of chromatography technology, the separation and purification of the protein also become easier relatively than before. But each protein has their physical and chemical properties, and that makes their separation and purification very difficult. Indeed, proteins can have different structures, the tertiary and quaternary ones are the most important because they show the specific three-dimensional structure of the protein. However, proteins are very easy to denature and loses their activity. So the separation and purification of the protein ought to be taken considered in the problem of activity.

At present, the demand of amount of proteins has been increased rapidly for the downstream purification product processes. Larger volumes to be processed and harder requirements for the cleaning-in-place (CIP) protocols are some of the features that need to be solved. The most applied affinity system for the purification of antibodies is the Staphylococcal (SPA) and smaller ligands derived thereof(Hober *et al*., 2007). For the

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protein of the special functions, some reasonable chromatography was applied that affinity ligand used for IgG-purification and originating from a bacterial species, is the streptococcal protein G (SPG)(L. Björk and Kronvall, 1984). If the protein was expressed by histidine fusion tags, purification of histidine-tagged proteins can be achieved in a single capture step using immobilized metal-ion affinity chromatography (IMAC)(Ford *et al*., 1991; Lanio *et al*., 2000; Porath, 1992; R. Janknecht *et al*., 1991). And other chelating groups for IMAC purification were described such as Tris-(carboxylmethyl) ethylenediamine(Porath and Olin, 1983)or iminodimethylphosphonic acid(V .P. Varlamov *et al*., 1986). But the yields have usually been lower than with chromatographic systems and a still time-consuming coupling step of the Ni^{2+} ions to the beads, so Andre´Frenzel found the novel purification system for 6XHis-tagged proteins by magnetic affinity separation(Frenzel *et al*., 2003)ÿwhich could posses substantial advantages compared with the conventional Ni-NTA-based methods.

This review will not only present different protein separation and purification, but also discuss the advantages and disadvantages of chromatography for the separation and purification of protein.

Protein

In nature, there are an infinite number of proteins you could take. And these proteins, originated from different areas such as animal protein from mammalian expression systems, protein expressed from different bacterial systems and filamentous fungi, have many various functions and properties. Heterologous proteins produced in microbial systems are either secreted into periplasmic space and/ or into the extal conditioned medium, in soluble form or in aggregates requiring one additional step-refolding(J. Evangelism Dyr and Suttnar, 1997). But their physical and chemical properties have some similarity.

So far, current popular choices of expression systems have been yeast (S. cerevisiae), bacteria (E.coli), and Chinese hamster ovary (CHO) mammalian cells. This is unlikely to change significantly in industry in the near future, at least in the US, because of the extensive background data already submitted on these host organisms to the US Food and Drug Administration (Hodgson, 1993) and a number of approved protein products secreted from yeast and CHO cells or refolded from E. coli(J. Evangelism Dyr and Suttnar, 1997). So the separation and purification of the protein is a formidable task in order to research on downstream product.

In the purification of IgG, protein A used had high affinity to IgG from various species, for instance human, rabbit and guinea pig but only weak interaction with bovine and mouse (Table 1) (Biotech, 2000; Richman *et al*., 1982).

Table 1. IgG binding of SPA(Biotech, 2000; L. Bjork and Kronvall, 1984; Richman et al., 1982)

Species Subclasses Protein	Subclasses	Protein A
Human	IgG1	++
	IgG2	++
	IgG3	“
	IgG4	++
	IgA	Variable
	IgD	“
	IgM	Variable
Rabbit	No distinction	++
Guinea pig	IgG1	++
	IgG2	++
Bovine		+
Mouse	IgG1	+
	IgG2a	++
	IgG2b	+
	IgG3	+
	IgGM	Variable
Chicken	IgY	“

Strong binding ++, medium interaction +, weak or no interaction

According to the purpose of the protein used, the separation and purification of protein have different methods. If protein is served as crystallization, crystallized protein is used to determine the protein’s three-dimensional structure. X-ray crystallography(Rosenberger, 1996), NMR spectroscopy, neutron crystallography, surface plasmon resonance (Natsume *et al*., 2000), microchip self-interaction chromatography(Garcia *et al*., 2003)and electron microscopy are the principal methods used in order to find the protein structure. Table 1 shows the characteristics, advantages and disadvantages of

each technique, and reveals that X-ray crystallography is the most common technique based on the number of papers published in the last 10 years (1996–2006)(A. Navarro *et al*., 2009). These studies are contributed to basic research for the protein. However, many high quality proteins should be produced, when the protein is used in the industry and life. But high-throughput and high yield for high-quality protein produced is still a worldwide difficult problem.

Table 2. Summary of analytic techniques of crystal proteins

Techniques	Characteristics	Advantages	Disadvantages	Papers published last 10years[a]
X-ray diffraction				2174
Single-crystal X ray	Solve complete structure of crystalline materials	1.Detect all structure 2.No destructive sample	Difficulty to have only one crystal and to orient it	
Powder diffraction	Crystallite size	1.Easier to prepare a powder sample 2.No destructive sample	Measure intensity more difficult	
NMR spectroscopy				590
Protein NMR	High resolution 3D structures of protein<25kDa	1.Use a solution 2.Possibility to study time-dependent phenomena	Prefer for small molecules	
Electron microscopy(EM)				940
Transmission EM	2D structure	Very small specimen	1.Long exposition time 2.Radiation damage Under vacuum	
Scanning EM	3D structure	1.Good resolution 2.Easy to use		
Cryo-EM	3D image Analysis	1.High-resolution 10A 2.Short exposition time 3.Less damage	Low image contrast	

[a] Research done on Scopus with the key words: protein crystals, X-ray diffraction; protein crystals, NMR spectroscopy and protein crystals, electron microscopy.

Major separation methodologies

Sample preparation

In the aspect of the sample preparation, preparation methods improved are much faster than the development of sophisticated methodologies and instruments dedicated to the separation and detection of recombinant proteins. If the properties of protein are known such as hydrophobicity and isoelectric point, sample preparation depends on these properties to separate and purify. But the suitable step is the most tedious and time-consuming and the source of much of the imprecision and inaccuracy of the overall analysis. And sample preparation cannot destroy its structure unless need and try to keep the activation.

When cells are broken in order to make the protein secreted, combinations of heat and enzyme pretreatment were followed by high-pressure homogenization for the release of a heat-

stable enzyme from Bacillus cereus(Vogels G. and M.-R., 1992). Enzymatic pretreatment of Candida utilis and Saccharomyces cerevisiae improved cell disruption relative to that of untreated(Baldwin and Robinson, 1994). The process should be a low operating pressure in short times, low energy consumption to prevent protein denatured. Meanwhile, cell solution has some inclusion body contaminants, and various guanidine HCl and Triton X-100 pre-treatments were investigated. Pretreatment of recombinant E. coli with 1.5 M guanidine HCI and 1.5% triton X-100 prior to high-pressure homogenization does not have an adverse effect on the physical characteristics of the cell homogenate. Pre-treatment with this combination of guanidine HCI and Triton X-100 not only allows the use of one pass through the homogenizer at a low operating pressure but also removes the need for an additional inclusion body washing step during downstream processing since the inclusion bodies are released and washed during the disruption step(J. Evangelism Dyr and Suttnar, 1997). Soluble recombinant proteins that may be sensitive to guanidine HC1 can be released from cells without pretreatment by using one pass through the high-pressure homogenizer at 83 Mpa(aldwin C. and CW., 1990; Bailey *et al*., 1995).

Separation and purification technology

In the last several years, partitioning of phase-system were widely used in the separation method for the protein purified. There were solid-phase extraction including liquid-solid extraction and sorbent extraction, which has occurred as a preparation step. And it is similar to that of liquid-liquid extraction, involving a partitioning of compounds. A review with discussion on new solid phases, chromatographic modes, experimental configurations and off-line and on-line automated devices and on the basic principles and recent developments in the solid phase extraction has been published(L. A. Berrueta *et al*., 1995). However, an affinity extraction method combining an aqueous two-phase system and affinity ligand-bound fine magnetic particles is proposed as a novel protein separation technique(M. Suzuki *et al*., 1995). And a novel nickel–silica matrix for the generation of magnetic beads for metal-ion affinity chromatography had been research on improving purification and yield of histidine-tagged proteins from crude bacterial extracts achieved(Frenzel *et al*., 2003).

With the development of chromatography and chromatographic filler, various chromatography technologies are applied to the separation and purification of proteins. Slow and steady improvement marks biopurification methods development. IEC and RPC or AC are recommended as a general set-up for a two-dimensional separation. This should allow a mild and efficient separation, starting very close to the crude material. But these methods used independently cannot play an efficient part in the purification of proteins. Sometimes, proteins purified are based on multi-step procedure of chromatographic methods. But there are advantages and disadvantages in multi-step procedure for the separation and purification of proteins. Its advantage is that purity of protein is improved. On the contrary, it has a low yield and high time consumed procedure. Rational design of purification for recombinant proteins have been suggested including integrated expert systems (Leser and Asenjo, 1992). So the separation of these molecules is regarded as one of the most challenging areas of chromatography (Guiochon, 1993).

Bogdan Gologan considered protein identification, separation, and purification with retention of biological activity that soft-landing efficiency for multiply-charged hexokinase ions was found to be some four times higher for a glycerol/fructose liquid surface than for a fluorinated self-assembled monolayer surface(B Gologan *et al*., 2004). This method showed protein purified can be easy to be identified. But it had a low yield. K.L. Carson used the expanded bed chromatography to capture either the product or a major contaminant in early steps involved affinity ligands. It could dispose a mass of proteins. Consequently, chromatography and other technologies make a rapid progress with the separation and purification of protein, but it cannot meet the requirement of all protein purified and separated.

CONCLUSION

Though the chromatography used in the separation and purification of proteins has matured in recent years, it is a difficult task to separate and purify protein for large capacity and high yield at short notice. So the way is that protein synthesis and folding, and secretion will be opened for

further development and applications of protein expression strategies. Thus, in the future, optimized protein production and purification will be possible to design the common methods in order to reduce cost and time. This paper regroups many separation and purification technologies of protein. These strategies are very important and necessary for the separation and purification of protein. Indeed, the engineering in separation of proteins is a domain which a lot of researchers and industries have been made and still many others keep to be made. Protein has many benefits for our body in our life. So we still have a lot of work left undone to do and explore an effective method as early as possible.

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