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Chromatographic fractionation of yeast extract: A strategy to identify physicochemical properties of compounds promoting CHO cell culture

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ABSTRACT

The study proposes to get a better knowledge of the physicochemical properties of yeast extract (YE) molecules involved in the improvement of CHO cell growth and to reduce YE complexity without losing positive effects. Various chromatographic processes were implemented for fractionation of a nanofiltrated YE (nYE). The nYE was first fractionated by one-step preparative chromatography, either with anion exchange (AEC), hydrophobic interaction (HIC) or size exclusion (SEC) methods. After analysis of its main components, each fraction was added in a control chemically defined medium to assess its impact on CHO cell growth. Results mainly underlined that AEC was the most selective separation process to purify nYE in one step without decreasing cell growth promoting effect. A three-step chromatographic process including successive AEC, HIC, and SEC was then developed to refine the physicochemical properties of nYE compounds. Among fractions that triggered similar cell growth promoting effect than nYE, one also improved IgG specific production. It mainly included cationic and hydrophilic peptides with a great proportion of lysine and arginine, low quantities of polysaccharides and no nucleic acids. Thus, this study allowed us to deepen the YE contribution to animal cell culture as well as to evaluate fractionation strategies to simplify such a complex mixture.

1. Introduction

Nowadays, the production of therapeutic recombinant proteins represents the fastest growing segment of the pharmaceutical industry [1]. Some recombinant proteins are produced in microbial expression systems, but most of them require extensive post-translational modifications and need to be produced in mammalian expression systems [2]. However, mammalian cells are very sensitive to culture operating conditions and often need promoting growth agents to reach appropriate cell density and recombinant protein level. Due to high cost and lot-to-lot variability of bovine serum, historically added in culture medium, the development of serum substitutes has been encouraged. Interesting results were obtained with purified or recombinant proteins, such as insulin and transferrin [3], with hydrolysates from animal proteins [4], or with milk-derived products [5]. Nevertheless, the detection of prions and the potential viral or microbial contamination induced a new step for media formulations free from any compounds from animal source [6].

Hydrolysates from yeasts [7–10] or plant proteins [7,10–12] have then shown promising capacity to improve cell growth and recombinant protein productivity. Yeast extract (YE) corresponds to the water soluble fraction of molecules yielded after full yeast autolysis. It is a complex mixture mainly composed of free amino acids, peptides, carbohydrates, nucleic acids, vitamins and minerals [13]. Protein hydrolysates are, in most of cases, produced from enzymatic proteolysis and can also be considered as complex mixtures of various unknown peptides. However, such a complexity can be problematic for different phases of biological production processes such as medium feeding [14], downstream process or end product release [15,16]. Therefore, the characterization, especially via physicochemical properties, of groups of YE compounds which impact animal cell culture, is currently a great challenge.

Due to the high number of compounds in YE or protein hydrolysates, the separation of each molecule and the evaluation of its activity on cells would be far too much time and cost consuming. Today, the only method available to better identify the active molecules is (*i*) to implement mixture fractionation processes, (*ii*) to assay the effect of the obtained fractions and (*iii*) to discuss on the basis of their composition. Even if this procedure cannot lead to an acute identification of single molecules involved

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in the bioactivity, this allows the characterization of their main physicochemical properties. This knowledge is also important to accurately choose appropriate separation process to simplify and to improve the initial mixture. Following this strategy, authors showed that low molecular size fractions of YE [8] or rapeseed and soy protein hydrolysates [17,18], obtained by membrane ultrafiltration, could replace the raw mixture without altering the cell performances. Other works confirmed the influence of the molecule size by using size exclusion chromatography (SEC), suggesting that active molecules could be short oligopeptides [5,19,20]. In fact, several authors pointed out the key role of peptides from protein hydrolysates to improve animal cell growth [21–23]. More recently, anion exchange chromatography (AEC) was used to evaluate soy peptone impact on CHO cell culture [20]. The results pointed out the potential role of the electrical charge of molecules in animal cell growth promoting effect. However, there is still a lack of extensive study investigating the role of molecule size or electrical charge. Moreover, the influence of molecule hydrophobicity, which could also act on the molecule activity, was rarely discussed.

The aim of the present study was to better characterize the physicochemical properties of molecules, contained in complex YE mixtures and which improve CHO cell cultures, by using various chromatographic strategies. A first step was to quantify YE influence on CHO cell growth and to analyze its main components. Then, the YE was fractionated with one-step AEC, SEC or hydrophobic interaction (HIC) preparative chromatographies. This led to fractions composed of molecules sharing a common physicochemical property, such as molecular size, electrical charge or hydrophobicity. The fractionation impact on fraction composition and activity was discussed. Lastly, a three-step fractionation process based on three sequential chromatographies was performed. The influence of the obtained fractions was evaluated on cell growth and IgG production and interesting insights on the properties of the YE active molecules were highlighted.

2. Materials and methods

2.1. Yeast extract preparation

The commercial yeast extract used in this study was a soluble fraction of yeast autolysis (Bio Springer, Maisons-Alfort, France). It was diafiltrated with deionized water through a nanofiltration membrane (Nanomax 50, Millipore, Bedford, MA) using a tangential filtration system (ProScale, Millipore). The transmembrane pressure was set to 6.5×10^5 Pa and the temperature was regulated at 25 °C. The YE diafiltration was monitored by measuring the conductivity in the permeate compartment, using a WTW LF96 conductivity meter. The operation was stopped when the conductivity became stable. Eight diavolumes were necessary to reach this condition. The product obtained in the retentate was called nYE and used for further studies.

2.2. Preparative chromatographic processes

2.2.1. Anion exchange chromatography

Anion exchange chromatography was carried out on Q Sepharose fast flow (GE Healthwcare Life Science) columns equilibrated in Trizma 20 mM at pH 8 (equilibration buffer). Laboratory-scale elutions were first implemented in a 1.6 × 10 cm column in order to determine the maximal loading capacity of the column and the NaCl solution stepwise required to elute fractions containing an equivalent content of biomolecules (data not shown). These conditions were then scaled-up to a 10 cm × 10 cm column according to the standards [24], to recover sufficient amount of matter in each fraction for characterization and activity assays. 1.2 g of nYE dissolved in equilibration buffer at 20 g/L was applied onto the column and the stepwise elution program reported in Table 1 was followed. The fractions A.1, A.2 and A.3, corresponding to the elution with 0, 0.1 and 1 M NaCl respectively in the equilibration buffer, were collected, desalted, concentrated by nanofiltration and finally freeze-dried for further analyses and cell culture assays.

2.2.2. Hydrophobic interaction chromatography

A lab-scale column elution (1.6 cm × 10 cm) was first implemented in order to determine the maximal loading capacity of the column and the required ammonia sulphate solution stepwise (data not shown). These conditions were scaled-up in a 5 cm × 10 cm column. Thus, hydrophobic interaction chromatography was carried out by using a Phenyl Sepharose fast flow (GE Healthwcare Life Science) preparative column equilibrated in phosphate 50 mM and $(NH_4)_2SO_4$ 3 M at pH 7.2 (equilibration buffer). 0.3 g of nYE dissolved in the equilibration buffer at 20 g/L was applied onto the column and the stepwise elution program reported in Table 1 was used. The fractions H.1 and H.2, corresponding to the elution either with equilibration buffer or with phosphate 50 mM, were collected and treated as those stemmed from AEC.

2.2.3. Size exclusion chromatography

Size exclusion chromatography was carried out in a Sephadex G-15 fast flow (GE Healthwcare Life Science) preparative column (5 cm × 75 cm) equilibrated with 6 mL/min deionized water, after development of a lab-scale elution (1.6 cm × 75 cm column). The maximal loading capacity of the column and the harvest times to recover similar quantities of raw material were determined (data not shown). 0.45 g of nYE dissolved in equilibration buffer at 30 g/L was applied onto the column. Then, the isocratic elution program reported in Table 1 was followed and three fractions called S.1, S.2 and S.3 were collected and freeze-dried.

2.2.4. Three-step chromatography process

A sequential fractionation protocol was achieved with successive AEC, HIC and SEC as described in Fig. 1. Chromatographies were performed as described for one-step operating conditions (Table 1). Eighteen runs of AEC were needed to get a sufficient amount of matter for further fractionations and bioactivity assays. Ten HIC runs were necessary to fractionate each AEC-fractions. Finally, each sub fraction was fractionated by a single SEC step.

2.3. nYE fraction characterization

2.3.1. Molecular size distribution

The molecular size distribution of the peptides contained either in nYE or in one-step chromatographic fractions was set out by analytical SE high performance liquid chromatography (SE-HPLC) using a Superdex peptide column coupled to an UV detector. Elution conditions are reported in Table 2. The column was calibrated with standard peptides of molar mass 238.2, 303.4, 437.5 and 658.9 g/mol (Sigma–Aldrich Co., St. Louis, USA) and 811.1, 1151.5, 1332.6 and 2106.9 g/mol (LCPM – CNRS, Nancy, France).

2.3.2. Total and free amino acids

The total amino acid composition of freeze-dried samples was determined after peptide hydrolysis in HCl 6 N at 110 °C for 24 h. The solutions were then cooled at room temperature, adjusted to pH 4.5 with NaOH 4 N and filtered through a membrane of 0.22 μm pore size. Acid hydrolysis resulted in the deamination of the amide groups present in asparagine and glutamine to yield aspartic acid, glutamic acid and ammonia. Amino acids were derivatized with 9-fluoroenylmethyl chloroformate and o-phthalaldehyde and analyzed by reverse phase HPLC according to the conditions described in Table 2. The amino acid concentrations were calculated from calibration curves performed with an amino acid kit (Sigma–Aldrich Co., St. Louis, USA).

2.3.3. Carbohydrates

The carbohydrate composition of freeze-dried samples was determined after hydrolysis in HCl 2 N at 104 °C for 4 h. The solutions were diluted 25 times in deionized water and filtered through a membrane of 0.22 μm pore size. Then, monosaccharides were analyzed by ion exchange HPLC according to the

Table 1
Operating conditions of nYE fractionation by using three types of preparative chromatography.

Separation method	Column	Eluent	Elution mode	Flow rate (mL/min)	Harvested volume	Fraction
AEC	Q Sepharose H 10 cm, Φ 10 cm	A: Trizma 20 mM, pH 8	0–1179 mL: 100% A	40	394–1807 mL	A.1
		B: Trizma 20 mM + 1 M NaCl, pH 8	1572–2751 mL: 90% A, 10% B		1807–3378 mL	A.2
			2751–5109 mL: 100% B		3378–4321 mL	A.3
HIC	Phenyl Sepharose H 10 cm, Φ 5 cm	A: Phosphate 50 mM, $(NH_4)_2SO_4$ 3 M, pH 7.2	0–590 mL: 100% A	20	99–590 mL	H.1
		B: Phosphate 50 mM, pH 7.2	590–1080 mL: 100% B		590–1080 mL	H.2
SEC	G-15 Sephadex H 75 cm, Φ 5 cm	Deionized water	0–850 mL: Isocratic	6	527–560 mL	S.1
					560–618 mL	S.2
					618–824 mL	S.3