Intensive Ion Exchange Process for Soluble Proteins Recovery from Industrial Alfalfa Green Juice

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Juice expressed from alfalfa (*Medicago sativa*) has a high protein content. Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase) is the main soluble protein of alfalfa juice with potential applications in many fields, such as human nutrition, pharmaceuticals, environmental... In order to recover Rubisco, an ion-exchange process in expanded bed mode (EBA) was studied. The expanded bed allows the treatment of crude extracts, and charged and viscous solutions. The anionic resin Q Hyper Z, especially developed for protein separation in such a process, was used.
Juices at different Rubisco concentrations and ionic strengths were treated. Breakthrough curves and dynamic capacity at 10% ($Q_{10\%}$) were determined for each experiment. Results showed that the working capacities are significant, even when $Q_{10\%}$ decreases for highly concentrated solutions. Finally, optimal elution conditions were analyzed. The application of 0.5 M NaCl in fixed bed mode at very small throughput produced high concentrated fractions with good purity.

1. Introduction

In biorefinery and food processing industry, alfalfa (*Medicago Sativa*) is treated by two-step process that consists on mechanical compression and drying, usually called dewatering in France (Hamm, 2001). This process results in solid fraction (press cake) usable for animal feeds and liquid fraction (green juice) containing suspended matter and soluble compounds (e.g. vitamins, amino acids, leaf proteins...). Both fractions have an economic value. Many studies took a particular interest in peptides and proteins recovering from green juice since they can be beneficially used in the food, chemical or pharmaceutical industries. Indeed, leaf proteins have been recognized by FAO as a potential and effective source of high quality proteins for human consumption due to their abundance of source, nutritive value, and free of animal cholesterol (Chen and Qiu, 2003). In addition, leaf proteins have interesting properties for food and pharmaceuticals systems. Several researches deal with leaf proteins concentrate (LPC) production from alfalfa juice. Most of processes developed consist on fractionation of the green juice by heat coagulation, pH adjustment, precipitation with solvents, centrifugation and combination of some of these techniques leading to a "whole juice LPC", a green or white LPC (Kohler and Knuckles, 1977; Pirie, 1969; Telek, 1983). White fraction LPCs from alfalfa have been shown to have a good balance of amino acids which are not significantly different from those of casein (Bickoff et al., 1975). However, evaluation of various preparations of

alfalfa LPC has shown that the heat coagulated LPC had low solubility over a wide pH range (Lamsal et al., 2007). Therefore the application of such heat-coagulated LPCs in food industry still limited. The main soluble protein of alfalfa juice is Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase). It comprises up to 65% of the total soluble proteins in leaf extracts of all photosynthetic leaves (Ellis, 1979). Due to its natural abundance its physicochemical properties, Rubisco could be obtained in good yield at a high purity. Rubisco is one of the largest proteins in nature with a molecular weight of about 560 kDa. It is a hexadecamer protein, comprising eight large subunits (56 kDa each) and eight small subunits (14 kDa each) (Douillard and Mathan, 1994).

Recovery of proteins from crude plant extract such alfalfa green juice is usually performed through a combination of different unit operations which consist in different separation units like solid-liquid separation, concentration, purification...These steps combined to coagulation methods are expensive and may represent an important increase in leaf protein concentrate production costs. In addition to the cost, the required time of such processes is very important which contributes to the alteration of the target protein (hydrolyse, aggregation...). Expanded bed adsorption (EBA) has been proven as an efficient technology to capture target proteins directly from feedstocks and crude extracts (Hjorth, 1997; Hamilton et al., 2000; Hubbuch et al., 2005). Compared to the classic process, the EBA combines solid-liquid separation with an adsorption step in a single unit operation with the advantages of increased overall yield and reduced operation time, as well as lower cost for capital investment and consumables (Xia et al., 2007). The aim of the current investigation is to evaluate the use of EBA for the recovery of soluble proteins, particularly Rubisco from industrial alfalfa juice by using an ion exchanger. The effect of initial Rubisco concentration on the dynamic capacity was studied. Operational parameters such as elution flow rate were used in order to understand process mechanisms and limitations. Finally, optimal conditions of Rubisco recovery are proposed.

2. Materials and Methods

2.1 Raw material preparation

Industrial juice resulting from pressing of *Medicago Sativa* was provided by Luzerne R&D (ARD, France) as a spry-dried green powder (96.4%). Juice was prepared by solubilisation of this powder in ultra purified water to obtain a 10% dry matter (crude juice). Then it was centrifuged at 4°C for 10 minutes and at 5000 g to remove large size cell debris. Even when literature considers EBA able to treat solutions charged with solids, in a first approach, particles have been eliminated in this work. Crude juice was used directly or diluted 6 (juice 1) or 10 (juice 2) times. All juices obtained were analysed by a specific HPLC SEC method (Kerfai et al., 2011) to determine their initial Rubisco content. Characteristics of prepared juices are summarised in Table 1.

Table 1: Characteristics of Alfalfa juices treated by EBA process

	Crude juice	Juice 1	Juice 2
Conductivity (mS.cm^{-1})	16.9	2.1	1.2
Rubisco content (g.L^{-1})	13,35	2.20	1.13

2.2 Ion exchanger

Q Hyper Z anion exchanger was provided by Pall Corporation (PALL BioSepra, Cergy, France). This adsorbent composed of hydrogel-filled porous zirconium oxide particles was used for expanded bed adsorption application. It is characterised by a particle size distribution going from 40 to 105 μm, a dry density of 3.1 mg.mL^{-1}.

2.3 Experimental set-up and loading protocols

The expanded bed system used in this work consisted of a Streamline C-25 column (25 mm i.d.) (GE Healthcare, Uppsala, Sweden) packed with 49 ml (10 cm settled bed height) of Q Hyper Z, connected to a peristaltic pump and a UV detector. The ion exchanger was equilibrated with 100 mM phosphate buffer, pH 5.8, during 1 h in expanded mode at flow velocities of 305 cm h^{-1} at which bed height is close to 20.5 cm. When the bed was stabilised at 20.5 cm, alfalfa juice was applied to the column at a superficial velocity of 318 cm.h^{-1}. The breakthrough was monitored by sampling effluent and protein concentration measurement (C) by HPLC SEC, like in feed solutions (Kerfai et al., 2011). The experiment was carried out for the three juices presented in Table 1.

In order to determine the dynamic capacity (Q$_{10\%}$) the normalized effluent concentration (C/C$_0$) was plotted versus the amount of juice loaded. Q$_{10\%}$ was calculated as follows:

$$Q_{10\%} = \frac{C_0\left(V_{10\%} - V_p\right) - \int_0^{V_{10\%}} C \cdot dv}{m_R} \qquad (1)$$

where V$_p$ is the porous volume of the system (ml) and V$_{10\%}$(ml) represents the volume of solution treated when outlet concentration is C/C$_0$ = 0.1.

2.4 Elution

After application of the total juice volume, the column was washed with ultra purified water until the conductivity of the effluent reached the conductivity of water. Washing was performed in expanded mode. Elution was then achieved by using 0.5 M NaCl. Two elution protocols were tested: in expanded mode at a superficial velocity of 160 cm.h^{-1} and, in fixed bed mode at a superficial velocity of 24 cm.h^{-1}. In the last case, the upward flow was stopped, the particles were settled in the column, and the adaptor was moved down to the settled bed surface. Fractions of 10 mL were collected during elution by using a fraction collector. The analysis of eluted fractions by HPLC SEC method allowed determining concentration and purity of the target protein. Subsequently, cleaning-in-place of the adsorbent bed was performed using 1M NaCl and distilled water.

3. Results and Discussion

3.1 Dynamic capacity determination

Rubisco breakthrough curves obtained for each juice are showed in Figure 1. As can be observed, for the experimental conditions applied, the saturation was not achieved in columns, and only dynamic capacity can be analyzed.
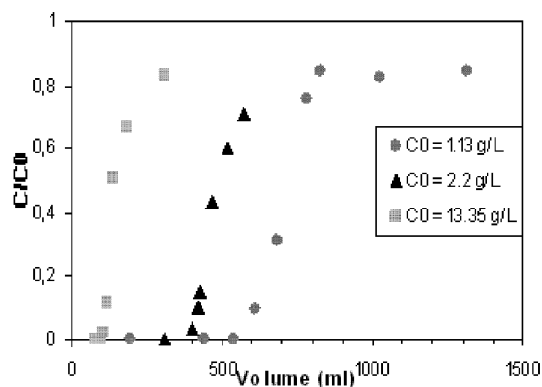
Figure 1: Experimental breakthrough curves for Rubisco obtained for a Q Hyper Z column in expanded bed mode at constant superficial velocity of 318 cm.h^{-1}. Feed solutions were the juices indicated in Table 1.

The dynamic capacity for each experimental condition was determined when the Rubisco concentration in the column effluent reached 10% of the initial concentration. Table 2 summarizes $Q_{10\%}$ obtained for each experiment. It can be observed that $Q_{10\%}$ decreased to 5.4 mg $_{Rubisco}$/g $_{Q\ Hyper\ Z}$ for highly concentrated juice. The high conductivity of this juice could explain this result. Thus, it is recommended to reduce the salinity of alfalfa juice before its treatment. The total amount of fixed Rubisco was determined for each breakthrough curve (Table 2). This parameter can not be used to analyze experiments because the curves are not completed. Anyway mass balances must be verified when compared with elution experiments.

Table 2: $Q_{10\%}$ and elution yield obtained for different experimental conditions

	Crude juice	Juice 1	Juice 2
$Q_{10\%}$ (mg$_{Rubisco}$/g$_{Q\ Hyper\ Z}$)	5.4 (±0.55)	8.0 (±0.80)	6.5 (±0.65)
Rubisco binding (g)	1.31 (±0.13)	1.11 (±0.11)	1.15 (±0.11)
Recovered Rubisco (g)	1.34 (±0.13)	1.16 (±0.11)	0.32 (±0.03)
Elution yield (%)	100	100	27.8

3.2 Rubisco recovery

Elution was carried out in fixed bed mode after loading experiments performed with crude juice and juice 1. For the juice 2, elution was performed in expanded bed mode. Eluted fractions were analyzed by a specific Rubisco HPLC SEC method. Figure 2 shows chromatograms obtained for fractions (4 to 9) during experiment performed with juice 1. The first fractions eluted correspond to purified Rubisco since only the characteristic Rubisco peak was detected, without any contaminant.

Determination of Rubisco concentration from HPLC SEC chromatograms allowed plotting elution curves (Figure 3). The amount of Rubisco eluted for each loading experiment was then calculated by integration of the elution curves. As summarized in Table 2, elution yield of Rubisco in expanded bed mode was 27.8% of fixed proteins

while 100% of fixed Rubisco was eluted in fixed bed mode. Elution fractions obtained in fixed bed showed Rubisco concentrations up to 60 g.L^{-1}.

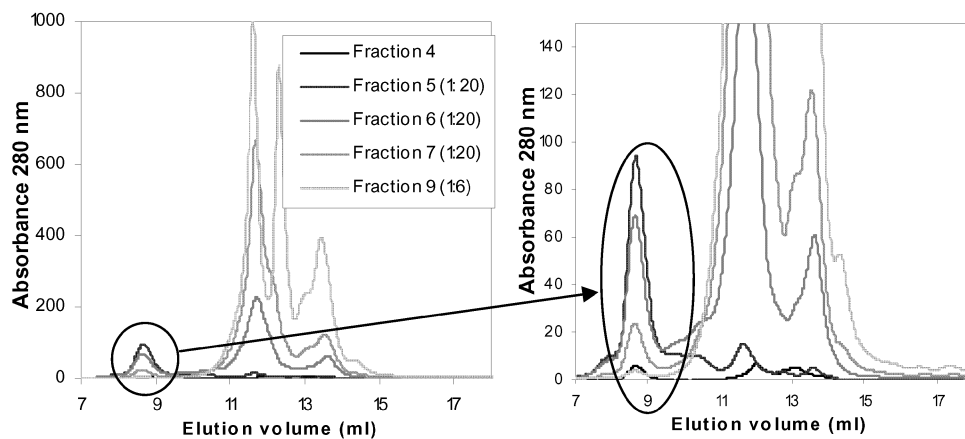*Figure 2: HPCL SEC chromatograms obtained for fractions 4 to 9 eluted in fixed bed mode at 24 cm.h^{-1} with 0.5 M NaCl. Column has been previously loaded by application of EBA process to juice 1.*
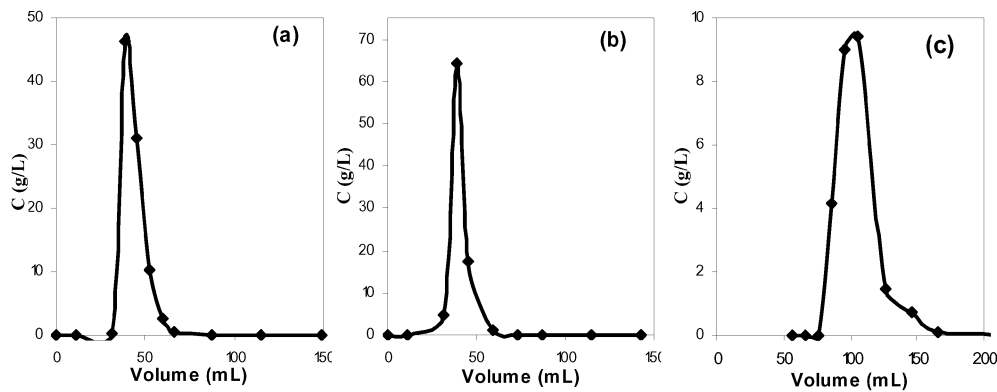

Figure 3: Elution curves of Rubisco performed with 0.5 M NaCl. (a)Fixed bed mode elution at 24 cm.h-1 from loading experiment performed with juice 1, (b) perfomed with crude juice, (c) Elution curve in expanded bed mode at 160 cm.h^{-1} for juice 2.

4. Conclusion

This study showed that recovery of Rubisco from centrifuged alfalfa juice by expanded bed ion exchange process using Q Hyper Z can be an interesting process. Fractions obtained have a high concentration in Rubisco (up to 60 g.L^{-1}, concentration factor from 5 to 21) and good purity. Nevertheless, a dilution step of the juice before EBA application can be interesting in order to reduce its ionic strength and increase the dynamic capacity of the adsorbent.

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