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Short communication

Sustainable efficient way for opioid peptide LVV-h7 preparation from enzymatic proteolysis in a microfluidic-based reaction-extraction process with solvent recycling

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ABSTRACT

LVV-h7 (LVVYPWTQFR) is a bioactive peptide that can be obtained from blood as waste of food industry, more precisely from hemoglobin hydrolysis by pepsin. This opioid peptide belongs to the hemorphins family and have strong physiological effects that bring its use in pharmaceutics and various therapeutic treatments attractive, in particular for substituting its costly chemically synthetized analogous. Hemoglobin hydrolysis by pepsin generates a huge variety of peptides among whose LVV-h7 can be purified by liquid–liquid extraction (LLE). Herein, selective preparation of this peptide is proposed by a microfluidic-based continuous reaction-separation process. Hemoglobin hydrolysis in microreactor was firstly coupled to LVV-h7 LLE in octan-1-ol and then coupled to LVV-h7 back LLE in acidic water. This continuous process allowed to prepare pure LVV-h7, as confirmed by liquid chromatography and mass spectrometry. The microfluidic circuit also allowed octan-1-ol recycling in a closed loop, making this method more sustainable than similar biphasic batch process.

1. Introduction

Since several years, it has been demonstrated that wastes of agricultural and food processing could be considered as valuable resources [1]. Nowadays, recycling of such products is increasingly regarded as an important procedure that can be conducted at industrial scale [2]. Among these products, animal blood (or cruor) is underutilized regarding to its abundance and its potential as source of active peptides. Indeed, hemoglobin represents a good protein source that can be treated to improve protein functional property in food [3], or to obtain bioactive peptides [3–5], which can be shown to have effects on human health [6]. By using proteolytic enzymes such as pepsin or trypsin [3], a biocatalysis-based treatment of bovine or porcine hemoglobin liberates amino acids sequences with various physiological effects and activities such as antimicrobial [7], opioid [8], antihypertensive [8], or antioxidant [9]. Among them, the opioid and antihypertensive effects of hemoglobin-derived hemorphins are notable [8]. The opioid peptide LVV-h7 (LVVYPWTQRF) that is released during bovine hemoglobin hydrolysis by pepsin confers interesting analgesic effects to be exploit in pharmaceutics and therapeutic uses instead of chemically synthetized drugs [10]. In many cases, the hemorphin LVV-h7 has been successfully used in ACE (Angiotensin-converting enzyme) inhibition [8], which confers to this peptide a blood pressure regulation capacity. In addition, this peptide shows a high potential in cancer therapeutics due to its cell cytotoxic and antiproliferative effects [11], but also in Alzheimer's disease therapeutic treatment [12]. Indeed, a role in learning and memory has also been reported, which hyphenates the strong impact of LVV-h7 on various physiological processes.

Purification of the peptide LVV-h7 can be realized through its strong hydrophobic properties that allows its solubilization in organic solvents, *i.e.* that selective liquid–liquid extraction (LLE) can be performed. Classical batch LLE is one of the most used method to enrich or purify a wide range of compounds. Purifying this intermediate peptide strongly depends on its appearance-disappearance kinetics during hemoglobin hydrolysis by pepsin [13]. Several approaches have thus been tried to develop extraction-based process. Froidevaux et al. proposed a biphasic reactor in order to purify this peptide in the course of hemoglobin hydrolysis [14,15].

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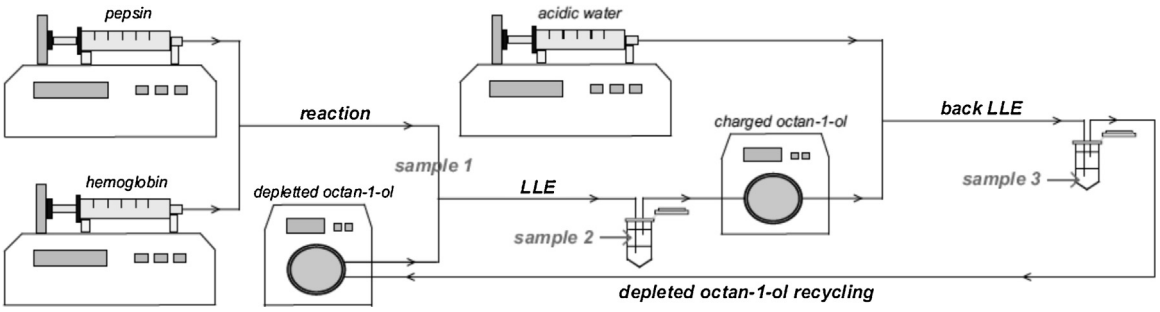

Fig. 1. Experimental protocol for continuous preparation of LVV-h7 from pepsic hydrolysis of bovine hemoglobin. Sample 1 refers to the hydrolysate after 15 s reaction, sample 2 corresponds to the octan-1-ol phase after LLE and sample 3 is the aqueous phase containing the peptide LVV-h7 recovered after both LLE and back LLE.

However, the main disadvantages were the slow extraction kinetics that was diffusion-dependent (avoiding emulsion and immobilized pepsin deactivation by the solvent) and the elevated volumetric ratio between organic phase (butan-2-ol:octan-1-ol, 80:20, v:v) and aqueous phase, which consumes a high solvent volume. Moreover, recycling of a biological waste as bovine blood reasonably implies the development of a sustainable process Consequently, LLE remain a powerful first choice method due to the high selectivity that can be reached to LVV-h7 target molecules. Nevertheless, classical LLE suffers from some difficulties like strong emulsification, automation and continuous processing difficulties, use of large amounts of organic solvents (costs, safety risks, environmental considerations), introduction of impurities and amount of time that is non negligible [16].

Intensification of (bio) chemical processes aiming at the effective use of raw materials and energy increasingly may involve miniaturization of chemical reactors and platforms and the use of continuous-flow processes [21]. Microstructured devices can serve as efficient tools to perform continuous-flow processes with high performances or specific benefits compared to batch processes, with the advantages of easy scale up, improved sustainability with reduced waste at low energy consumption but also easy process control and automation with inherent reactor safety [17]. Thus, performing LLE in microstructured devices can be attractive. As example, microfluidics offers designs that allow using three-phase laminar flow to provide efficient back extraction by double liquid–liquid interface area [18]. Microfluidics brings a huge surface-to-volume ratio, the specific interfacial area of multiphase systems can be considered in the range of 5000–30,000 $m^2\ m^{-3}$ [19]. Enhancement of mass-transfer performances at microscale combined with a huge specific interfacial area between two immiscible fluids could allow efficient extraction-based process as well as impressively reducing its time in comparison to batch. In addition, microfluidics not only allows fast extraction of a solute from a complex mixture but also theoretically allows a continuous reaction-extraction process to be performed in a continuous-flow laminar reactor [20]. However, obtaining a stable liquid–liquid interface between two immiscible fluids as well as separating phases remains challenging even if some interesting approaches have been reported [20].

In this work, we rely on the existed strategy of laminar flow LLE but promote this technology in a different manner by applying it to (*i*) perform a continuous-flow reaction-separation process for LVV-h7 preparation in one-step and (*ii*) drastically minimize the solvent volume. Previously, we have shown that LVV-h7 optimally appears after around 15 s during hemoglobin hydrolysis by pepsin in microfluidic reactor [21]. Herein, our process distinguishes previous work by the coupling of: (*i*) hemoglobin hydrolysis by pepsin in microreactor, (*ii*) LVV-h7 selective extraction by octan-1-ol and (*iii*) LVV-h7 back extraction from octan-1-ol in acidic water. Since the boiling point of octan-1-ol is very high (~192 °C) and an adsorption-desorption process involves a discontinuous step, direct coupling of back extraction has been adopted to allow pure LVV-h7 recovery in water. Recycling of the depleted solvent after back extraction was performed in a closed circuit to obtain a low organic phase/aqueous phase volumetric ratio.

2. Experimental protocol

2.1. Reaction

Fig. 1 describes the experimental protocol, according to our previous work [21]. Syringes containing denatured hemoglobin (2% w/v) and pepsin (2.5% w/w) were thus placed in the same syringe pump (New Era Pump Systems, Farmingdale, NY, USA) fixed at a flow-rate of 1 $\mu L\ min^{-1}$. A T-connector was used to put in contact the flow of enzyme and substrate in a capillary (150 μm I.D. × 75 μm O.D.) with an adjusted length to allow 15 s reactions. The hydrolysate after 15 s reaction is denoted as sample 1 (Fig. 1).

2.2. Extraction

Another T-connector was used for the coupling of the last capillary to the LLE capillary. Octan-1-ol was chosen as solvent due to the high selectivity of polar alcohol solvent for LVV-h7. Octan-1-ol was pumped by a peristaltic pump at a flow-rate of 20 $\mu L\ min^{-1}$. The biphasic flow was recovered in a 1.5 mL tube after 15 s residence time of the octan-1-ol flow.

2.3. Back extraction

As the density of octan-1-ol is much lower than that of water, a capillary was immersed in organic phase to pump the solvent. By using another T-connector, octan-1-ol was connected to acidic water (pH 3.0) in another capillary (150 μm I.D. × 75 μm O.D.) with a flow rate of 20 $\mu L\ min^{-1}$ to perform back LLE and recovering LVV-h7 in acidic water (sample 3, Fig. 1). Moreover, this module was suited to LVV-h7 recovery in one-step in comparison to other methods. Indeed, octan-1-ol boiling point is very high and the coupling of a selective adsorption process involves a desorption step.

2.4. Depleted solvent recycling

Recycling of depleted octan-1-ol phase is described in Fig. 1. This recycling module allowed keeping a fixed volume of solvent to be processed continuously. The limitation of such a recycling system lies in the need to employ efficient pumping systems to allow a large interval of flow-rates. In this study, we were limited to a minimum of 20 $\mu L\ min^{-1}$ by the peristaltic pump. Nevertheless, using such an off-chip system facilitates phase separation when the fluids behavior within microchannels have not been studied or