EFFECT OF TEMPERATURE AND DIGESTIVE ENZYMES ON ENCAPSULATED *Arthrospira platensis* EXTRACT WITH THROMBOLYTIC ACTIVITY

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ABSTRACT

With the aging of the population and the growing number of individuals predisposed to cardiovascular diseases, it is necessary to develop new functional dietary supplements aimed at cardiovascular health, which are still incipient. *Arthrospira platensis* has been shown to be a promising source of fibrinolytic enzyme. The objective is to evaluate the effect of temperature and gastrointestinal enzymes in vitro on the *A. platensis* extract containing fibrinolytic enzymes microencapsulated. Microcapsules subjected to simulated digestion of saliva, gastric and intestinal fluids were evaluated for protein concentration and fibrinolytic activity in each phase. Additionally, the microcapsules were subjected to a temperature of 10 - 80 ºC for 4 years and enzyme activity was evaluated every 1 hour. Microcapsules were resistant to gastrointestinal digestion, obtaining higher and faster release of the enzyme in the intestinal stage. Microcapsules were resistant to temperatures of up to 50ºC for 4 hours, withstanding a temperature of 80ºC for 1 hour, however, it has high stability at temperatures of 10ºC. Thus, these microcapsules can be a promising dietary supplement to prevent cardiovascular diseases.

Keywords: Microalgae. Enzyme. Microencapsulation. Fibrinolytic. Stability.

1 INTRODUCTION

Cardiovascular diseases (CVDs) are the main cause of death worldwide and about 32% of all global obits are related to CVDs[1]. In special, thrombosis is a CVD characterized by the excessive and pathologic formation of blood clots, which lead to the obstruction of arterial and venous vessels, causing severe clinical complications, such as heart strokes, heart attacks, and pulmonary problems[2].

Currently, the first-choice treatment for thrombosis is based on the use of anticoagulants, such as unfractionated heparin or low-molecular-weight heparin, followed by warfarin (vitamin K antagonist), which acts by avoiding clot formation[3]. However, these drugs are often reported to cause adverse reactions, including excessive anticoagulation, bleeding, and thrombocytopenia[4]. Additionally, these conventional treatments also have some limitations such as low fibrin specificity and low cost-effectiveness[5].

Then, fibrinolytic enzymes have been recently investigated due to their advantages, e.g., low-cost production, possibility of large-scale production, and high specificity for fibrin. Fibrinolytic enzymes such as Reteplase, Tenecteplase, and Alteplase are already allowed by the Food and Drug Administration and are commercially available[6]; however, they are associated with some side effects, such as bleeding events, cerebral edema, and cerebral vascular accident[7]. On the other hand, fibrinolytic enzymes from other sources have shown interesting *in vitro* results; however, further studies are necessary to make their use feasible in thrombolytic therapy.

In this context, the fibrinolytic enzyme obtained from *Arthrospira platensis* exhibited high fibrinolytic activity and showed optimum pH and temperature at 6.0 and 40 ºC, respectively. Additionally, the enzyme was stable in the pH range of 6.0 to 10.0 for 24h, and thermostability below 50 ºC[8]. Although promising fibrinolytic activity, the *in vivo* oral administration could affect enzyme absorption due to factors such as gastrointestinal fluids, different pH ranges, and enzyme activities[9]. In this sense, microencapsulation technology can protect the bioactive compounds and act as barriers to control release, solubility, and bioavailability[10]. However, to achieve desired performance, it is essential to investigate the physicochemical properties of microcapsules. Therefore, this study aimed to evaluate the effects of temperature on the enzyme activity of microcapsules, as well as its stability after gastrointestinal simulation.

2 MATERIAL & METHODS

Arthrospira platensis cultivation and cell extract preparation

Arthrospira platensis (UTEX, 1926) was cultivated in modified Schlösser medium (1982); without sodium nitrate (NaNO3) and supplemented with 0.2% corn steep liquor (Corn Products Brazil industrial ingredients). After cultivation, biomass was suspended in sodium phosphate buffer pH 7.0 to 20 mM at 50 mg/ml and homogenized by constant stirring for 40 minutes in an ice bath to obtain the cell extract[8].

Arthrospira platensis extract microencapsulation

Sodium alginate (1% w/w) was mixed with the lyophilized cell extract at 1% w/v and added at 2% calcium chloride solution by dropped using peristaltic pump. The contact between both solutions form the microcapsule. Microcapsules were ruptured by adding a sodium citrate solution.

In vitro gastrointestinal digestion of microcapsule

Digestion with simulated saliva, gastric and intestinal fluids was performed in triplicate. To simulate digestion, 1g of microcapsule was resuspended in simulating salivary fluid (SSF) for 2 minutes at pH 7 in the oral phase. In the gastric phase, it was added of 0.6% (w/v) pepsin and lipase (Sigma-Aldrich, St. Louis, USA) and further incubated for 2 h, pH 2.0, at 37 °C. The pH was adjusted to 2.0 by adding HCl solutions. After 120 min of incubation, sample was diluted 1:1 (vol/vol) with simulated intestinal fluid (SIF), bile salts (Merck, Darmstadt, Germany) in PBS buffer pH 8 and 0.1% (w/v) pancreatin (Sigma-Aldrich, St. Louis, USA) and incubated at pH 7 for an additional 2 h. The entire digestive process was carried out in a shake at 37°C at 100 rpm. The enzyme was inactivated by boiling at 80°C for 5 min. The microcapsule was harvested in each step by centrifugation at 14.560 × g for 5 min at 4 °C (Hermle Labortechnik, Wehingen, Germany) and then washed twice with sterile PBS buffer pH 7.4 to determination of protein total and fibrinolytic activity.

Effect of temperature on microencapsulated fibrinolytic enzyme

The optimal temperature was evaluated by enzyme activity assay at different temperatures (10 - 80 ºC), using fibrin as substrate. The thermal stability was assessed by incubating the microcapsules in sodium phosphate (pH 7.0) at different temperatures for 4 hours. For this, aliquots were collected every 1 hour to test the residual fibrinolytic activity.

Determination of protein concentration and fibrinolytic activity

Determination of Protein concentration was determined in triplicate according to Smith et al. (1985) using BCA Protein Assay Kit. Fibrinolytic activity was determined using fibrin as a substrate which is formed from 0.72% w/v fibrinogen from bovine plasma (dissolved in 0.15 M Tris-HCl buffer, pH 7.5) by the action of 20 U·mL−1 thrombin from bovine plasma (dissolved in 0.2 M sodium phosphate buffer, pH 7.0) to form a fibrin clot. Then, samples with fibrinolytic enzymes were added and incubated for 1 h at 37ºC. After stopping the reaction by the addition of 0.2 M trichloroacetic acid followed by centrifugation (8,000 × g for 10 min at 4 °C), the supernatant was collected and measured at 275 nm. One fibrin degradation unit (U) of enzyme activity was defined as the amount of enzyme able to cause a 0.01 absorbance increase per minute

Statistical analysis

The study was carried out in duplicate and analyzed by one-way analysis of variance (ANOVA). For all statistical analyses, p-values less than 0.05 were considered significant according to the Tukey's test.

3 RESULTS & DISCUSSION

Effect of temperature on fibrinolytic activity and stability

The effects of temperature on fibrinolytic activity have been investigated at temperature values from 10 to 80 ºC (Figure 1). The optimal activity was found at 70 ºC and decreased fastly at 80 ºC with relative activity around 20%, after 1 hour of incubation (Figure 1A). Differently, the purified free enzyme has optimum temperature of 40 ºC, denaturing completely at temperature above 60 ºC[8]. This increase can be associated with the microcapsules action as a protection of the amino acids on the enzyme's surface, as well as at the active site, avoiding the enzyme denaturation process, even at high temperatures[11]. This is a promising result since it can allow microencapsulated enzymes to resist industrial application at high temperature conditions. Furthermore, the microcapsules were stable at temperatures between 50 and 70 °C with significant relative activities (> 40%), after 2 hours of incubation. Additionally, after 4 hours of incubation, the microcapsules exhibited relative activity above 20% at a temperature range of 40 to 60 ºC. Barros et al., (2020) reported that the purified free enzyme was stable at temperatures under 50 ºC, but denatured at 60 ºC, after 3 hours of incubation. These results indicate the microencapsulation can act as barriers protecting the enzyme from adverse conditions (i.e., high temperatures). This provides a significant advantage, as they can be applied in different industrial processes.

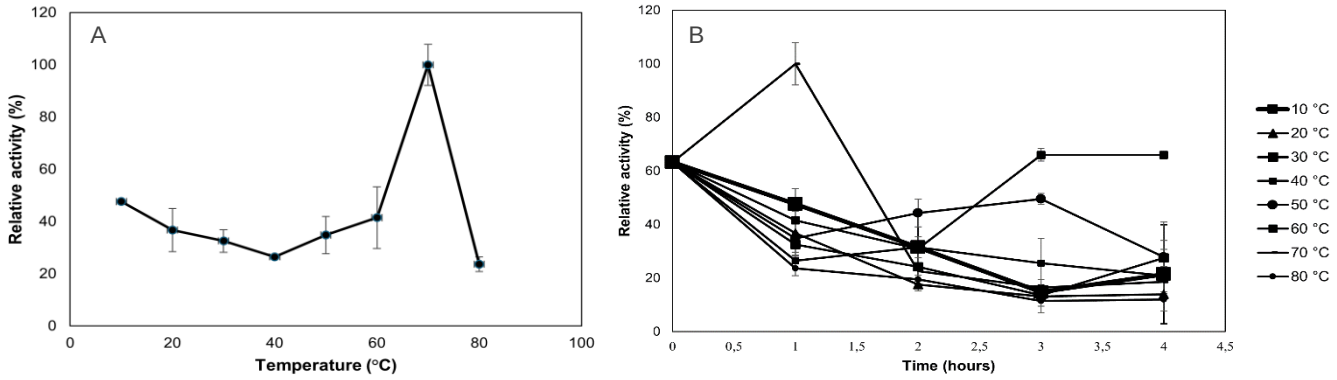

Figure 1 Effects of optimal temperature (A) and stability (B) of microcapsules containing the fibrinolytic extract of *Arthrospira platensis*

Effect of simulated digestion in vitro on *A. platensis* microencapsulated

Protein concentration and fibrinolytic activity values released in each phase of the gastrointestinal system are described in Table 1. After the microencapsulation process, 0.84 ± 0.22 mg/ml of protein and 92.14 ± 4.31 U/mg were observed after breaking the microcapsules by adding sodium citrate. Microcapsules were able to resist oral phase, which there was no change in the color of the microcapsules, remaining green and was observed the release of 0.78 ± 0.24 mg/mL of protein and a fibrinolytic activity of 71.61 ± 3.86 U/mg after 30 min of addition sodium citrate. In the gastric phase, a change in color from green to brown was observed, however the microcapsules resisted gastric fluids with acidic pH and containing the enzyme pepsin, with release of 1.24 ± 0.60 mg/mL of protein and thrombolytic activity of 51.69 ± 6.23 U/mg after 30 min of addition sodium citrate. In the intestinal phase, the microcapsules continued to be brown in color and released 0.40 ± 0.25 mg/mL of protein and thrombolytic activity of 774.19 ± 63.68 U/mg after 2 min of addition sodium citrate, showing a highest release of fibrinolytic enzyme and in short time was in this phase. Alginate is characterized by being able to resist acidic environments and disintegrates under slightly basic conditions. This makes them an ideal encapsulating material to protect against digestion in the stomach and allow controlled release in the small intestine[12,13], where nutrients are absorbed. Other advantages of this carrier material are that it is recognized as safe (GRAS), being non-toxic, non-antigenic, satisfactorily biocompatible and sufficiently biodegradable[14].

Table 1 Protein concentration and fibrinolytic enzyme activity of the cell extract, after microencapsulation and in the gastrointestinal phases.

Item	Protein concentration (mg/ml)	Fibrinolytic activity (U/mg)
Cell extract	4,64 ± 1,18^{a}	255,38 ± 23,13^{b}
Microcapsules	0,84 ± 0,22^{c}	92,14 ± 4,31^{c}
Oral phase	0,78 ± 0,24^{c}	71,61 ± 3,86^{d}
Gastric phase	1,24 ± 0,60^{b}	51,69 ± 6,23^{e}
Intestinal phase	0,40 ± 0,25^{c}	774,19 ± 63,68^{a}

Values with the same superscript are not significantly different according to the Tukey test ($p< 0.05$). Lowercase letter compares among treatments

4 CONCLUSION

The search for thrombolytic bioactive compounds derived from natural sources has emerged as an alternative to synthetic drugs and their disadvantages. The photosynthetic prokaryote *A. platensis* has demonstrated that its extract contains an enzyme with high fibrinolytic activity. However, these bioactive compounds can be affected by extrinsic factors, such as temperature, and physiological processes, such as gastrointestinal digestion, resulting in the loss of their biological functions. Encapsulation has proven to be a promising solution to this issue, maintaining enzymatic stability and activity under high temperatures and protecting the enzyme during the gastrointestinal journey, thereby increasing its bioavailability and stability. Alginate has stood out as an excellent carrier material, resisting pH changes, enzymatic actions, and controlling the release of the bioactive compound.

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