Veterinary Parasitology 198 (2013) 111–115

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Effect of citric acid on the acidification of artificial pepsin solution for metacercariae isolation from fish

Min-Ki Kim[a], Kyoung-Ho Pyo[a], Young-Sang Hwang[a], Hyang Sook Chun[b], Ki Hwan Park[b], Seong-Hee Ko[c], Jong-Yil Chai[a], Eun-Hee Shin[a,d,*]

[a] Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, South Korea
[b] Department of Food Science & Technology, Chung-Ang University, Ansung 456-756, South Korea
[c] Department of Pharmacology, Seoul National University College of Medicine, Seoul 110-799, South Korea
[d] Seoul National University Bundang Hospital, Gyeonggi-do 463-707, South Korea

ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form 2 August 2013
Accepted 5 August 2013

Keywords:
Artificial digestive solution
Pepsin
Citric acid
HCl
Metacercariae
Fish

ABSTRACT

Artificial digestive solution based on pepsin is essential for collecting metacercariae from fish. To promote the enzymatic reactivity of pepsin, the pH of the solution has to be adjusted to pH 1.0–2.0. Hydrochloride (HCl) is usually used for this purpose, but the use of HCl raises safety concerns. The aim of this work was to address the usefulness of citric acid as an alternative for HCl for the acidification of pepsin solution, and to examine its potential to damage metacercariae during in vitro digestion as compared with HCl. Changes in pH after adding 1–9% of citric acid (m/v) to pepsin solution were compared to a 1% HCl (v/v) addition. Digestion of fish muscle was evaluated by measuring released protein concentrations by spectrophotometry. In addition, survival rates of metacercariae in pepsin solution were determined at different citric acid concentrations and were compared that of with 1% HCl. The present study shows that addition of citric acid reduced the pH of pepsin solutions to the required level. Addition of more than 5% of citric acid resulted in the effective digestion of fish muscle over 3 h in vitro, and 5% citric acid was less lethal to metacercariae than 1% HCl in pepsin solution. Pepsin solution containing 5% citric acid had digestive capacity superior to pepsin solution containing 1% HCl after 3 h incubation with released protein concentrations of 12.0 ng/ml for 5% citric acid and 9.6 ng/ml for 1% HCl. Accordingly, the present study suggests that the addition of 5% citric acid to pepsin solution is a good alternative to 1% HCl in infection studies because citric acid is a stable at room temperature and has a good safety profile. In addition, we suggest that the use of citric acid enables the preparation of commercial digestive solutions for the detection of microorganisms in fish and other vertebrate muscle tissue.

1. Introduction

The detection of foodborne parasites in fish and meat requires the digestion of host muscle protein because of the invasive natures of these parasites (Shin et al., 2006; Gamble and Murrell, 1998; Lysne et al., 1995). Artificial digestion using proteolytic enzymes has been designed for this purpose (Shin et al., 2006; Gamble and Murrell, 1998; Lysne et al., 1995; McDaniel, 1966; Prociv, 1989).

* Corresponding author at: 103 Daehak-ro, Jongno-gu, Seoul 110-799, South Korea. Tel.: +82 2 740 8344; fax: +82 2 765 6142.
E-mail addresses: ehshin@snu.ac.kr, ehshin1130@gmail.com (E.-H. Shin).

0304-4017
http://dx.doi.org/10.1016/j.vetpar.2013.08.004

Of the proteolytic enzymes, pepsin is an acidic protease that degrades food proteins into peptides in the stomach (Malik et al., 2005). After parasite-infected food samples have been digested using an artificial digestive solution based on pepsin, it is much easier to isolate and identify individual parasites (Shin et al., 2006; Gamble and Murrell, 1998; Lysne et al., 1995; McDaniel, 1966). Trypsin, another proteolytic enzyme, is mainly used for in vitro excystment of encysted metacercariae in parasite research (McDaniel, 1966). In general, the preparation of artificial digestive solution (ADS) using pepsin requires an acidic buffer with hydrochloric acid (HCl) to promote enzyme activity. Pepsin shows maximal enzymatic activity at pH levels from 1.0 to 2.0, and 0.5 to 2% HCl (v/v) is usually added pepsin ADS to reduce the pH to within the optimal range (Shin et al., 2006; Gamble and Murrell, 1998; Lysne et al., 1995; McDaniel, 1966; Prociv, 1989; Fan et al., 2002). However, HCl must be handled with considerable caution and presents safety issues for laboratory personnel. For example, exposure to low concentrations of hydrochloric acid can cause erythema, irritation, inflammation, pain, and ulceration of skin (Bull and Chapd, 2011). During in vitro digestion, host tissues must be soaked in acidified pepsin solution for several hours. However, the toxicity of HCl for parasites has not been examined. As an alternative for the acidification in ADS, we considered citric acid as a safe alternative for preparing artificial pepsin solution because it is widely available and is used commercially to make edible gelatin, sausage casings as a food additive, and other biological assay systems (Malik et al., 2005; Zhang et al., 2007). The U.S. Food and Drug Administration lists citric acid as a multipurpose generally recognized-as-safe (GRAS) food substance (Chuda et al., 1999). However, citric acid has not been considered for the acidification of artificial pepsin solution for parasite isolation or in terms of user safety, ease of use, or parasite damage. To facilitate its effective use, proper considerations must be given to the amount of citric acid required for optimal preparation of pepsin-containing ADS. Accordingly, we compared the efficacy between HCl- and citric acid-based digestive solutions on parasite survival, and sought to determine the minimum concentrations of citric acid required for acceptable enzymatic activities given suitable digestion times.

2. Materials and methods

2.1. Acidification of pepsin solution using citric acid

To examine changes in pH at given concentrations of citric acid (Shinyo Pure Chem, Osaka, Japan) in 1% pepsin solution, pH values at different concentrations of citric acid in ADS were measured using an Orion 3-star Benchtop pH Meter (Thermo Scientific, Delaware, USA). The concentration of pepsin (MP Biomedicals, Ohio, USA) used in the present study was 1% (w/v) and the concentration of HCl was 1% (v/v). Citric acid was added at 8 concentrations from 1% to 20% (w/v) (1, 3, 5, 7, 9, 13, 15, and 20%).

2.2. Digestive capacity of ADS containing different concentrations of citric acid

The digestive capacity of ADSs containing varying concentrations of citric acid were compared with ADS containing 1% HCl. The fish meat used in the study (mullet) were purchased from a market (Noryangjin, Seoul). Tissue samples were prepared by slicing the fish meat to produce 2 cm^2 (about 20 g), and these were placed in 50 ml conical tubes with 40 ml of the ADS samples. The tubes were incubated at 37 °C in a shaking incubator for 1–2 h. To determine digestive capacity of each solution, the concentrations of proteins released from digested samples were measured using a Nanodrop 2000 spectrometer (Thermo Scientific, Wilmington, Delaware, USA) at 280 nm.

2.3. Collection of Metagonimus yokogawai metacercariae

To investigate the effect of citric acid on parasite survival, *M. yokogawai*, a fish-borne intestinal trematode parasite, was collected from sweetfish, *Plecoglossus altivelis*, captured from a stream in an endemic area in Gyeongsangbuk-do (Pyo et al., 2013). The sweetfish were finely ground, mixed with ADSs, incubated at 37 °C for 1–2 h, filtered through a mesh (pore size 1 mm × 1 mm), and washed with 0.85% saline repeatedly until clear. The sediment was carefully observed under a stereomicroscope. *Metagonimus* metacercariae were identified morphologically and collected (Pyo et al., 2013).

2.4. Survival rates of parasites digested with HCl- or citric acid-based ADSs

The metacercariae of *M. yokogawai* were examined for the effects of ADSs on parasite survival. Metacercariae were incubated at 37 °C with each ADS, and surviving metacercariae were counted under an optical microscope (CHS-213E; Olympus, Tokyo) at 2 h intervals during the 8 h incubation period. The survivability of metacercariae was confirmed by their morphological characteristics. A metacercaria was considered dead if it did not move for 5 min at 25–37 °C with loss of the body wall integrity and faintness of the excretory bladder with few excretory granules.

3. Results

3.1. Concentration of citric acid required for the acidification of ADS

The conventional composition of ADS contains 0.5–1% of pepsin and 0.8–1% of HCl with a pH of 1.5–2.0. The addition of citric acid instead of HCl acidified the pepsin solution (Fig. 1). The pH of the pepsin solution itself was 3.76, and the addition of 1% HCl decreased the pH to less than 2.0. The addition of 1% citric acid decreased the pH to 2.44, and the addition of citric acid at concentrations greater than 5% decreased the pH to less than 2.0. ADSs containing citric acid between 5% and 20% resulted in pH values between 1.5 and 2.0 (Fig. 1). These findings show