의학석사 학위논문

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

물고기에서 피낭유충 분리를 위한 인공 펩신 용액의 산성화에 대한 구연산의 효능

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

by

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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. 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.

Keywords: Artificial digestive solution, Pepsin, Citric acid, HCl, Metacercariae, Fish

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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 (1-3). Artificial digestion using proteolytic enzymes has been designed for this purpose (1-5). Of the proteolytic enzymes, pepsin is an acidic protease that degrades food proteins into peptides in the stomach (6). 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 (1-4). Trypsin, another proteolytic enzyme, is mainly used for in vitro excystment of encysted metacercariae in parasite research (4). 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 (1-5, 7). 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 (8). 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 (6, 9). The U.S. Food and Drug Administration lists citric acid as a multipurpose generally recognized-as-safe (GRAS) food substance (10). 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.

MATERIALS AND METHODS

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. 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 cm2 (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.

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 (11). 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 (11).

4. Survival rates of parasite digested with HCl- or citric acid-based ADS

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.

RESULTS

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 (Figure 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 (Figure 1). These findings show that citric acid concentration needs to be greater than 5% to achieve maximal pepsin activity.

2. Comparison of the digestive capacities of HCl- and citric acid containing ADSs

The digestion of mullet tissue (20 g) was assessed using a nanodrop2000 spectrometer at 280 nm. After 1 h of incubation, the protein concentration for mullet tissue treated with HCl-based ADS was 5.77 ± 1.21 (mg/ml) (Figure 2). After 2 h of incubation, the protein concentration increased to 7.77 ± 1.12, which was similar to that of ADSs containing more than 7% citric acid. After 3 h of incubation however, the digestive activity of ADSs containing >5% citric acid was superior to HCl-based ADS. The protein concentration in HCl-based ADS after 3 h of incubation was 9.62 ± 2.84, but those of 5%, 7%, and

9% citric acid-based ADSs were 12.05 ± 3.23, 14.20 ± 4.66, and 15.80 ± 2.05, respectively. After 4 h of incubation, the protein concentration in HCl-based ADS was 12.03 ± 2.78, which was lower than those of ADSs containing more than 3% citric acid. Given that fish samples are digested in HCl-based ADS for 1–3 h to collect metacercariae in laboratory setting, citric acid at >5% appear to be a useful alternative. Metacercariae in ADS containing >13% citric acid floated onto the surface of the solution during incubation, which could have been caused by the higher buoyancy of these solutions. For this reason, ADSs containing >13% citric acid were excluded from survival rate studies.

3. Influence of HCl- and citric acid-based ADSs on metacercaria survival after digestion

To investigate the influences of HCl and citric acid on metacercaria survival, the metacercariae of *M. yokogawai* were subjected to each ADSs, and the survival rates were examined (Figure 3). Each eighty metacercariae were incubated in HCl- or citric acid-based ADSs for 8 h at room temperature, and the number of living metacercariae was counted under a stereomicroscope at 1 h intervals for 8 h. At 1 h, all metacercariae survived in each ADSs solution. After 2 h of incubation, 2.8% of metacercariae in 1% HCl-based ADS died and floated onto the surface, whereas all metacercariae in citric acid-based ADS survived (Figure 3). At 4 h, dead metacercariae were found in ADS containing 1% HCl ADS, in 3% citric acid ADS, and in pepsin solution. The percentages of dead metacercariae in pepsin only, 1% HCl, and

3% citric acid were 2.0%, 5.8%, and 2.3%, respectively. At 5 h after incubation, less than 3% of metacercariae incubated in citric acid-based ADSs had died. In these experimental conditions, more metacercariae died in 1% HCl-based ADS than in 1–9% citric acid-based ADSs.

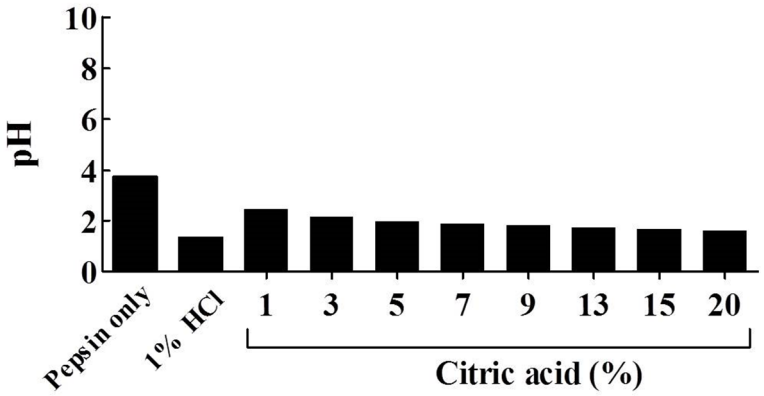

Figure 1. pH depended on the percentage of citric acid present in the pepsin solution.

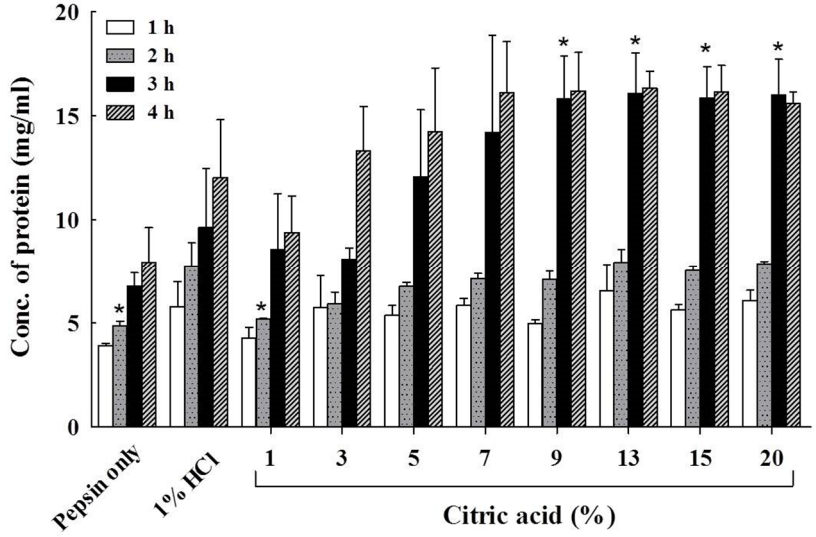

Figure 2. Concentrations of proteins released from fish muscle digested in ADS solutions. Protein concentrations were measured at 1 h intervals during an incubation period of 4 h. Asterisk signifies a statistical differences ($P < 0.05$) between 1% HCl-based ADS and different concentration of citric acid contained in ADSs at the same incubation time.

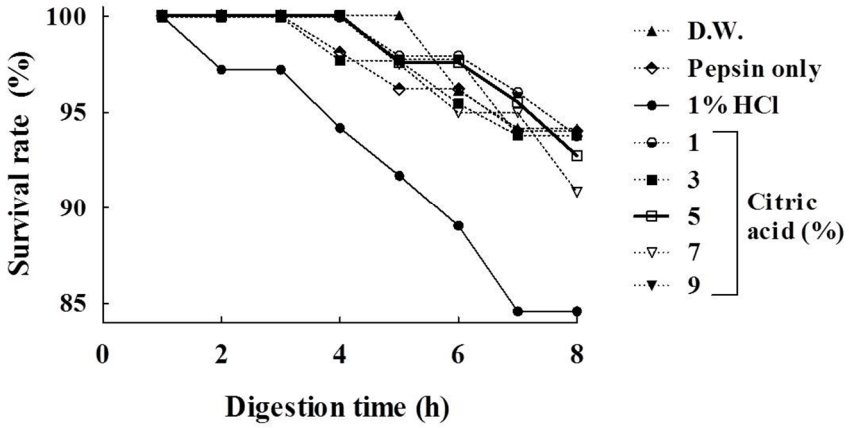

Figure 3. Survival rates of *Metagonimus yokogawai* metacercariae after incubation in 1% HCl-based and 1, 3, 5, 7, or 9% citric acid-based ADSs.

DISCUSSION

Traditionally, metacercariae in fish had been detected using the compression method, whereby the fish flesh is compressed between two glass microscopic slides (12). This method is inaccurate, however, and isolation of metacercariae is not always possible. Thus, acidified pepsin based dissolution was devised to digest protein in vitro for detection and isolation of metacercariae. This artificial digestion method has been used by many researchers undertaking epidemiologic, immunologic, or chemotherapeutic studies related to parasitic diseases, and the prevailing opinion is that artificial digestive solution (ADS) provides a reliable way to isolate parasites from vertebrate muscle tissue (1-3, 7, 11). In particular, pepsin–HCl solution is frequently used to harvest the metacercariae of trematodes from fish, amphibian, and reptile hosts (1, 3, 12, 13). Sometimes, the isolation of a parasite from fish is needed for further experiments such as investigation of parasite survival rates or immune responses against parasites in host models. Accordingly, it is important to determine whether parasites are adversely affected by the in vitro digestive process. Although the pepsin/HCl ADS solution has been used for a considerable time, no study has been undertaken to optimize the solution for research purposes. Pepsin is used to mimic the gastric digestion of fish muscle, and requires an acidic buffer for enzymatic activity. Although HCl is present naturally in gastric acid, and its chloride ion is an essential electrolyte in all body fluids and is responsible for maintaining acid/base balance, the impact examination of HCl on parasites is also needed

because parasites are exposed to HCl for a considerable time in the in vitro digestion of tissues (14). Second, it needs to investigate whether the HCl to be replaced by a safer ingredient including citric acid which it decrease pH (10). Because of safety issues associated with handing and transportation, there is a need to replace HCl with another 'safe' acid, which should ideally have a greater digestive effect and less impact on parasites. As an alternative of HCl, citric acid was proposed as a buffer agent in ADS because it is already used for the in vitro digestion of enhanced green fluorescent protein (EGFP) in pepsin and pepsinogen fluorometric assays and also used for isolation of pepsin-soluble collagen (6, 9). For ADSs used in parasitic experiments however, the use of citric acid has not been considered with respect to the concentrations required, the degree of digestion of host flesh, and above all, to parasite viability after in vitro digestion.

In the present study, we found that the use of citric acid enables safe and easy preparation of ADS without loss of digestive capacity. In fact, ADS containing citric acid had higher digestive capacity than HCl-based ADS. In particular, 5% citric acid ADS was superior to 1% HCl ADS with a digestion time of 3 h. Because citric acid is safe enough to be used in the food industry (15), our results provide a reason enough to replace HCl with citric acid. Another important advantage of using citric acid is that it does not harm metacercariae to the same extent as ADS containing HCl does; more metacercariae survived in citric acid-based ADS than in HCl-based ADS. This implies that, after pepsin digestion, metacercariae are in better condition for further experiments. In addition, citric acid can be easily stored and

transported to remote areas by anyone without incurring the risk of serious injury. Compared to HCl, the use of citric acid does increase the cost of preparations, which might be a concern when extensive epidemiological surveys are undertaken. Nevertheless, this cost is justifiable if survivability of metacercariae is essential to an experiment. The present study provides an alternate acid buffer for pepsin-based ADS. Our results indicate that citric acid is a better alternative in the preparation of acidic pepsin solutions from the viewpoints of user safety and parasite survivability.

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국문 초록

펩신을 기반으로 하는 인공소화액은 물고기로부터 피낭유충을 수집할 때 필수적인 것이다. 펩신의 효소 반응성을 촉진시키기 위해서 용액의 pH는 1.0 – 2.0으로 조정되어야 한다. 염산이 이러한 목적으로 주로 쓰이고 있지만, 염산의 사용은 안전성에 대한 우려를 일으킨다. 이 실험에서는 펩신 용액의 산성화에 사용되는 염산의 대체물로써 구연산의 유용성을 알아보고, 인공소화 중 피낭유충을 훼손시키는 정도를 염산과 비교 시험하고자 했다. 펩신 용액에 1–9%의 구연산을 넣은 후와 1%의 염산을 넣은 후의 pH 변화를 비교하였다. 각 용액에 담긴 물고기 근육으로부터 녹아 나온 단백질의 농도를 분광 측정법으로 측정하여 소화력을 평가하였다. 또한, 1% 염산과 비교하여 여러 가지 다른 구연산 농도의 펩신 용액 안에서 피낭유충의 생존율을 확인하였다. 구연산은 펩신 용액의 pH 를 필요수준까지 감소시키는 것을 확인하였다. 5% 이상의 구연산을 첨가한 경우 3 시간 이후에는 물고기 근육을 효과적으로 소화시켰고, 펩신 용액에 1% 염산이 들어갔을 때 보다 구연산이 들어간 경우 피낭유충에 덜 치명적인 것으로 확인되었다. 물고기 근육을 3 시간 동안 소화시켰을 때 5% 구연산이 들어있는 펩신 용액은 단백질 농도 12.0ng/ml 로 나왔으며 1% 염산이 들어있는 용액에서 나타난 9.6 ng/ml 보다 더 우

수한 소화능력을 나타내었다. 펩신 용액에 5% 구연산은 안정적이고 안전한 특성이 있기 때문에 감염에 대한 연구에서 1% 염산을 대신 할 수 있는 좋은 대체물질로 보여진다.

주요어 : 인공소화액, 펩신, 구연산, 염산, 피낭유충, 물고기
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