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Effects of α-Amylase, Protease and Lipase on Haruan (*Channa striatus*) Mucus Extract Antinociceptive Activity in Mice

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Abstract: This study was attempted to preliminarily establish the basic chemical properties of the bioactive compound responsible for *Channa striatus* (Haruan) mucus extract antinociceptive activity using the acetic acid-induced abdominal constriction test in mice. Pre-treatment of extract with enzymes were carried out at the temperature of 40°C for 60 min. The extract, pre-treated with distilled water (DH_2O), was found to show a significant and concentration-dependent antinociceptive activity with the 25 and 50% concentration extracts showing insignificant activity when compared together. The extracts, at the concentrations of 50 and 100%, were pre-treated with 10% concentration α-amylase and lipase and 0.1% concentration protease, respectively. The extracts, pre-treated with α-amylase or protease, were found to produce a concentration-dependent activity, which are, however, not significant when compared to the extracts pre-treated with DH_2O (positive control). Interestingly, pre-treatment of the extracts with lipase enhanced the activity significantly ($p<0.001$). However, this activity did not follow the concentration-dependent pattern. The failure of α-amylase and protease to influence the extracts antinociception seems to suggest that the bioactive compound is neither a simple carbohydrate nor protein. Meanwhile, the ability of lipase to enhance the extracts activity, especially of the 50% concentration extract, lead to suggestions that the lipid may act as a carrier of the bioactive compound or the bioactive compound itself is a lipid-based compound. Finally, the bioactive compound is suggested to be a short chain macromolecule, which is resistant to the effect of the three respective enzymes. However, further studies need to be carried out to clarify this finding before the real chemical properties of the bioactive compound can be ruled out.

Key words: Haruan (*Channa striatus*), mucus extract, antinociceptive, abdominal constriction test, enzymes

INTRODUCTION

Channa striatus, also known locally as haruan, is a snakehead fish indigenous to many tropical and subtropical countries including Malaysia[1]. It is a fresh water, air-breather and carnivorous fish widely consumed in Malaysia and other Southeast Asian countries due to its high source of protein and also for its putative effects on wound healing[2]. It has been used for a long time due to its medicinal benefits and is belief to promotes wound healing and alleviates post-operative pain and discomfort[3]. The whole fish is consumed either as dry-fried, grilled or boiled as in porridge, as the dietary medicine for wound healing[3].

The mucus layer covering the surface of fresh water fishes is the final barrier between the fish and its environment. It consists of about 95% water and the major organic components in mucus are glycoproteins[4,5], which typically have high sialic acid content[5,6]. The mucus glycoproteins are important in the gel-forming properties and the viscous nature of the mucus substance[4,5]. The mucus secreted by *C. striatus*, which make them very slippery and therefore difficult to catch, is believed to be part of its defense mechanism. This statement is based on the observations that when *C. striatus* is under stressful conditions, such as when it is under attack or when it burrows through mud, it secretes a lot of mucus. The finding by Mat Jais *et al.*[7] that the *C. striatus* mucus exerted an antinociceptive activity seems to support the above connotation because when the fish is under stress or injury as mentioned above it will secretes the mucus as an adaptive mechanism to deal with its stress or injury.

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Based on the preliminary report made by Mat Jais *et al.*[7], it was decided to establish the basic chemical properties of the bioactive compound which is responsible for *C. striatus* mucus extract antinociceptive activity by pre-treating the mucus with three generally used enzymes, namely α-amylase, protease and lipase.

MATERIALS AND METHODS

Experimental animals: Balb-C mice (25-30 g; 5-7 weeks old), obtained from the Institute of Medical Research (IMR, Kuala Lumpur, Malaysia), were used in this study. The animals were and kept under room temperature (37±2°C; 70-80% humidity; 12 h light/darkness cycle) in the Animal Holding Unit, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia for at least 48 h before use. Food and water are supplied *ad libitum*. At all times the mice were handled in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals[8]. All experiments were conducted between 09.30 and 18.30 h to minimize the effects of environmental changes.

Chemicals: The enzymes, α-amylase (BDH), lipase (Sigma) and protease (Sigma), were used in this study. Each enzyme was prepared as 10% solution by dissolving 1 g of it in 10 mL of DH_2O except for protease. Protease was prepared as 0.1% solution by dissolving 0.01 g of the enzyme in 10 mL of DH_2O.

Animal material: Throughout the study, 6-month-old (400-500 g) *C. striatus* were used. They were cultured from Pontian, Malaysia and transferred to UPM and acclimatized for at least 3 days at the Fish Hatching Unit, Universiti Putra Malaysia prior to experiments.

Preparation of *C. striatus* Mucus Extract (CsME): Preparation of *C. striatus* Mucus Extract (CsME) was carried out according to the method described by Mat Jais *et al.*[7]. Precleaned live fish was weighed and placed into an enclosed plastic bag (15x30 cm) with approximately an equal volume of distilled water at room temperature. The *C. striatus* fish were subjected to hypothermic stress in order to stimulate mucus production in which the plastic bag, containing the live fish, was sealed and then transferred to a freezer at - 20°C for 24 h. At the end of the 24 h, the bag was removed from the freezer and left to thaw for about 4 h at room temperature. At the end of 4 h, the fish was removed and the remaining mucus extract was collected and centrifuged (1000 rpm; 30 min). The supernatant, containing the mucus and considered as stock supernatant (100% concentration), was collected and stored at 4°C until use. The mucus extract was also prepared in another two concentrations (25 and 50%) by diluting the stock supernatant with distilled water (DH_2O). The concentration of the diluted mucus extract (v:v) refers to the volume of full strength stock supernatant in the preparation.

Pretreatment of CsME with the respective enzymes: Two milliliter of the respective enzyme solution was then mixed with 2.0 mL DH_2O, 50 or 100% concentration CsME extract and kept in water bath (40°C) for 10 min before use. Each enzyme-treated DH_2O, 50 or 100% concentration extract was then left to cool at room temperature before use.

Experimental procedure: One hundred and thirty mice were used in this study. Forty mice were treated with DH_2O or CsMEs (25, 50 and 100% concentration), which were earlier pre-treated with DH_2O. The remaining 90 mice will be treated with DH_2O or CsMEs that were earlier pre-treated with α-amylase-, protease- or lipase. The animals pre-treated with DH_2O followed by either DH_2O or CsME were used as a negative and positive control group, respectively. In all cases, the DH_2O or CsME were administered subcutaneously (sc) in the volume of 10 mL kg^{-1} of mice.

Antinociceptive assays: The acetic acid-induced abdominal constriction test was used as described by Dambisya and Lee[9] to determine the basic chemical properties of the bioactive compound responsible for *C. striatus* antinociceptive activity. The acetic acid (0.6%; v/v) (J.T. Baker, USA), used to induce pain in mice peritoneal cavity, was administered intraperitoneally (ip) in a volume of 10 mL kg^{-1}, 30 min after the administration of DH_2O or CsMEs pre-treated with DH_2O or the enzymes, respectively. The abdominal constrictions resulting from IP injection of acetic acid consisting of a contraction of the abdominal together with a stretching of at least one of the hind limbs[10]. The number of abdominal constrictions was counted cumulatively over a period of 25 and 5 min after the acetic acid administration. Antinociception was calculated as the percentage inhibition of abdominal constrictions (percentage of inhibitory level) using the formula below:

$$\frac{\text{Saline control group mean-test group mean}}{\text{Saline control group mean}} \times 100\%$$

Statistical analysis: The Jandel Scientific Sigma Stat's (Version 2.00) statistical programs were used to analyze

and compare the data and all values were presented as Mean±SEM. Data from the control and CsMEs treated animals were compared by Analysis of Variance (ANOVA) followed by Tukey Test, with $p<0.05$ as the limit of significance.

RESULTS

Pre-treatment of CsME with DH_2O: The CsME pre-treated with DH_2O was found to produce a concentration-dependent antinociceptive activity, which is significant ($p<0.001$) when compared against the negative control group (30.14±1.70). The number of abdominal constrictions was found to decrease with the increased in the concentration of mucus extract. The data obtained for the 25, 50 and 100% concentration extracts were 13.80±1.20, 11.20±1.50 and 8.20±0.60, respectively (Fig. 1). Due to the insignificant data obtained for 25 and 50% concentration extracts, the 50% concentration extract together with the 100% concentration extract were chosen for further study.

Pre-treatment of CsME with α-amylase: There is a demonstrable concentration-response pattern in the antinociceptive activity of CsME pre-treated with α-amylase in which there is an increased in the inhibitory levels with increased in the concentration of the extract. The 50 and 100% concentration extracts were found to give the number of abdominal constrictions of 11.5±1.2 and 7.2±0.7, respectively. The data were found to be significant ($p<0.001$) against the negative control group. However, when compared with the positive control data at their respective concentration, both extracts failed to produced any significant effect ($p<0.05$) (Fig. 2).

Pre-treatment of CsME with protease: Pre-treatment of CsME at the respective concentration with protease was also found to show a demonstrable concentration-response pattern. This activity, which is significant ($p<0.001$) when compared with the negative control group, was found to be insignificant ($p<0.05$) when compared against the positive control group. The antinociceptive activity of the 50 and 100% concentration haruan extracts, which were 12.3±1.1 and 7.4±0.8, was also found to be insignificant ($p<0.05$) when compared against the α-amylase pre-treated extract groups at their respective concentration (Fig. 3).

Pre-treatment of CsME with lipase: Pre-treatment of CsME with lipase, however, was found to improve its antinociceptive activity at both concentrations, respectively. This activity, however, did not follow the concentration-response pattern as observed in α-amylase and protease-pre-treated extracts. Both concentrations of extract were found to produce significant ($p<0.001$)

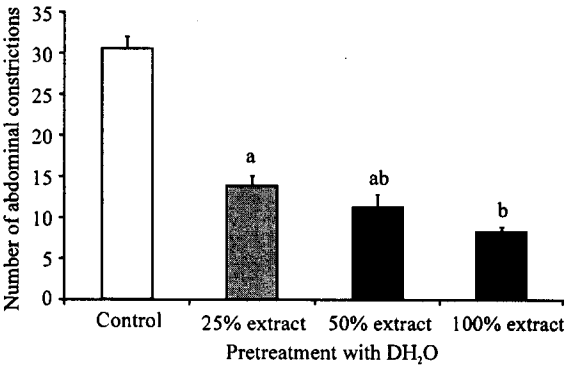

Fig. 1: The antinociceptive activity of haruan mucus extracts pre-treated with DH_2O. [a,b]Significant against control group

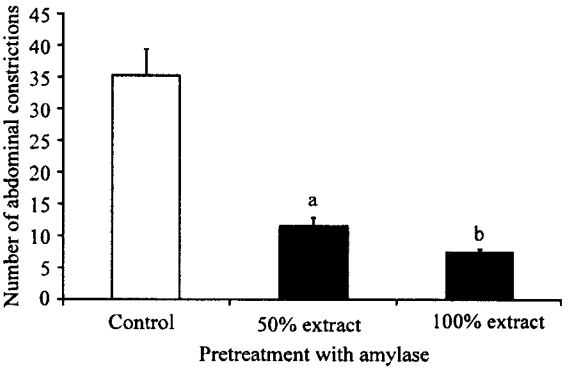

Fig. 2: The antinociceptive activity of haruan mucus extracts pre-treated with α-amylase (10% concentration). [a,b]Significant against control group

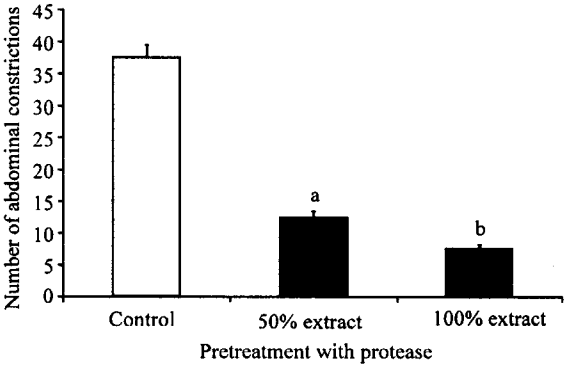

Fig. 3: The antinociceptive activity of haruan mucus extracts pre-treated with protease (1% concentration).[a,b]Significant against control group

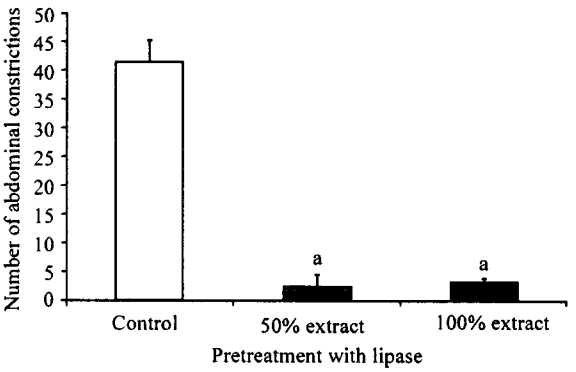

Fig. 4: The antinociceptive activity of haruan mucus extracts pre-treated with lipase (10% concentration). [a]Significant against control group

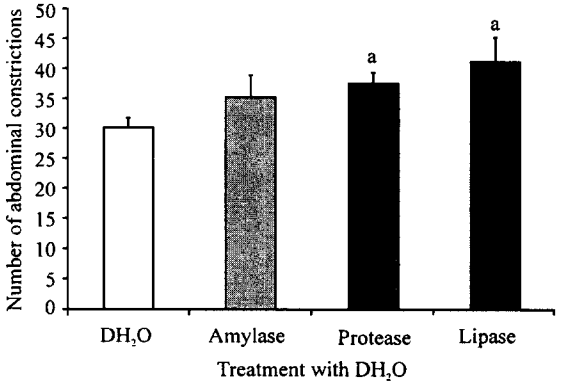

Fig. 5: Comparison on the number of abdominal constrictions after pre-treatment of DH_2O with α-amylase, protease and lipase. [a]Significant against control group

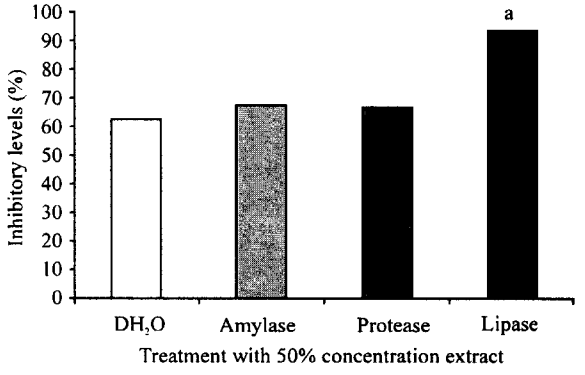

Fig. 6: Comparison on the effects of α-amylase, protease and lipase on 50% concentration extract antinociception. [a]Significant against control group

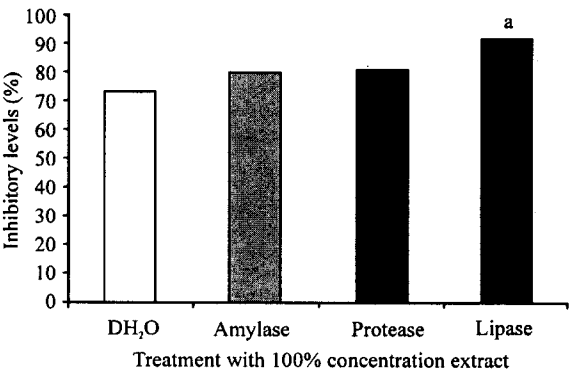

Fig. 7: Comparison on the effects of α-amylase, protease and lipase on 100% concentration extract antinociception. [a]Significant against control group

antinociceptive activity when compared against the negative and positive control groups, respectively. Furthermore, only the 50% concentration extract pre-treated with lipase was found to show a significant ($p<0.001$) activity when compared with the α-amylase and protease-pre-treated extracts at the same dose. The 50% concentration mucus extract was found to give a marked reduction in number of abdominal constrictions (2.6±1.8) as compared to the 100% concentration extract which produced a 4.9±2.4 number of abdominal constrictions (Fig. 4).

Comparison of the effects of α-amylase, protease and lipase on CsME at the same concentration: Comparison was also made on the effects of α-amylase, protease and lipase on DH_2O and CsME of the same concentration, respectively. Protease and lipase were found to exhibit significant hyperalgesic activity (Fig. 5). In the 50% concentration extracts, lipase was found to cause significant ($p<0.001$) increased in the percentage of inhibitory level of abdominal constrictions when compared to the α-amylase and protease-treated groups. However, α-amylase and protease were found to produce insignificant percentage of inhibitory level of abdominal constrictions when compared together. The inhibitory levels for the 50% concentration extracts pre-treated with lipase, α-amylase and protease were 93.7, 67.2 and 67.1%, respectively (Fig. 6). For the 100% concentration extracts, the percentage of inhibitory level between the respective enzyme pre-treated groups were found to be insignificant when compared together. The percentage of inhibitory level for the 100% concentration extracts pre-treated with lipase, α-amylase and protease were 79.4, 80.3 and 88.1%, respectively (Fig. 7).

DISCUSSION

The *C. striatus* mucus and fillet extracts have been found to exhibit a concentration-dependent antinociceptive activity as assessed by abdominal constriction test[7]. This activity is, however, not demonstrable in the tail-flick test on its own. Both extracts were also found to enhance the morphine antinociception in both abdominal constriction and tail-flick tests. The abdominal constriction test, which is very sensitive and can detect antinociceptive effects of compounds/dose levels that may be inactive in the tail-flick assay[11,12], is thought to involve, in part, local peritoneal receptors while the tail-flick response is essentially a spinal reflex[13]. These differences in sensitivity of the two tests and the mechanisms involved may explain the apparent lack of effect of these extracts in the tail-flick test[7]. Furthermore, this seems to suggest a peripheral mechanism involvement in *C. striatus* extract antinociceptive activity and, due to this fact, the tail-flick test is not used in this studies.

The results showed that the antinociceptive activity of CsME was not destroyed when treated α-amylase, protease or lipase, respectively. The extract was found to exhibit a concentration-dependent antinociceptive activity when pre-treated with α-amylase and protease and these activities were insignificant when compared to extract pre-treated with DH_2O. This seems to suggest that the bioactive compound responsible for the above-mentioned activity is not a pure and simple carbohydrate or protein, respectively. This suggestion is due to the fact that α-amylase and protease, which are responsible for the breakdown and destruction of carbohydrate and protein molecules[14], did not affect the concentration-dependent antinociceptive response of the extract. Based on this finding, the bioactive compound in CsME is suggested to be of a conjugated form, which is a combination of carbohydrate and protein. Furthermore, the failures of those enzymes to diminish the extract antinociception lead us to suggest that the bioactive compounds are a stable and short chain macromolecule, which is resistant to the effect of those enzymes. This suggestion seems to be concomitant with the finding by Dambisya *et al.*[15] who reported that the bioactive compound is resistant to extreme temperature and pH. It is generally known that a short chain macromolecule is resistant to the effects of extreme temperature and pH.

On the other hand, pre-treatment with lipase, in the case of 50% concentration extract, was found to increase the antinociceptive activity of the extract. This finding seems to suggest the important physiological role of lipid in the antinociceptive mechanisms of CsME. This is due to the fact that in the presence of lipase, which is known to digest lipid molecules[14], the 50% concentrations extract activity was found to improve significantly when compared against the α-amylase and protease pre-treated groups. The 100% concentration extract activity was also found to improve after pre-treatment with lipase, an activity which is not significant when compared against the α-amylase and protease pre-treated groups. Lipid compound, in this study, is suggested to be part of the bioactive compound in which it might act as a bioactive compound by itself or as a carrier for the respective compound. The former is suggested because the lipid metabolite, released by lipase from the parent compound, is believed to be in a more active form than the parent compound. On the other hand, the later is suggested because the lipid breakdown is believed to release a lipid-free compound, which is more active than the lipid-bound compound. These compounds, which were thought to act directly at the peritoneal cavity receptors, are believed to be responsible for the marked antinociceptive activity as observed in this study.

Protease, in this study, was prepared in a concentration of 0.1%, instead of 10% as what was the case for α-amylase and lipase, because it was found to cause death of the mice after its IP administration in the concentration of 10, 5 and 1%. The reason for this is not known but it is suggested that the protease might form a toxic compound or exhibit a toxic effects that lead to death of the mice. The protease is also suggested to cause breakdown of the cell protein in the gastrointestinal tract and other part that came into contact with it.

Finally, it was concluded that the basic chemical properties of the bioactive compound responsible for CsME antinociception to be of a short chain macromolecule, which is neither a pure and simple carbohydrate nor protein. This macromolecule, which has been previously reported to be resistant under extreme temperature or pH, was also found to be resistant against the effect of various enzymes used. Furthermore, this bioactive compound is believed to take a form of an active lipid metabolite or might use lipid as its carrier, to cross certain lipid-based barrier such as on the blood and blood vessel cell walls, in order to reach the target receptors in the peritoneal cavity. Thus, the bioactive compound is believed to be in a conjugated form, such as glycolipid, lipoprotein or glycolipoprotein. However, further studies need to be carried out before the final conclusion can be made on the chemical properties of the respective bioactive compound.

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