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African catfish (*Clarias gariepinus*) visceral protease: its specific activity and molecular weight at different purification stages

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Abstract

The fish consumption has increased tremendously due to the dramatic increase in world population. Catfish, the most cultivated species worldwide, showed an increasing trend in global production from year to year. Hence, loads of fish by-products (FBP) were generated especially viscera, materials rich in digestive enzyme notably protease. FBP is often thrown away without any attempt of recovery leading to serious environmental pollution and disposal problems. However, the application of certain protease in either food or non-food industry has become a controversial issue with regards to religious belief (porcine-related product or unslaughtered animals without religious compliance) and health concerns (Bovine Spongiform Encephalopathy (BSE) diseases). Therefore, in this study, protease was extracted and purified from the visceral waste of African catfish (*Clarias gariepinus*). This study aimed to identify the specific activity and molecular weight of the purified protease at different purification stages. The crude protease was extracted and purified using 60% ammonium sulfate precipitation and dialysis. The study showed that the fresh viscera contained 5.9% protein. The specific activity of the protease indicated a gradual increase as it was further purified up to the dialysis stage (608.70 U/mg) from the crude extract (263.82 U/mg) by 2.3 folds with half of the visceral protease managed to be recovered (61.43%). In SDS PAGE analysis, purified protease from the dialysis process portrayed unique features such as dimer with an apparent low molecular weight of 15 kDa and 16 kDa. It was obvious that the utilization of the visceral waste from a halal source such as catfish for the extraction of a beneficial protease would be a win-win situation in both environment and industry players from all sectors.

1. Introduction

African catfish (*Clarias gariepinus*) or better known as 'ikan keli Afrika' among Malaysian, belongs to the family *Clariidae*. This omnivorous feeding habit fish is highly resistant to environmental stress and diseases (Adetuyi *et al.,* 2014; Schram *et al.,* 2014). In the Malaysia aquaculture industry, African catfish give a significant impact because of its rapid growth rate, easy cultivation and good market potential (Amiza and Kang, 2013). It is often sold live in the market at a low price (Abdi *et al.,* 2011). For the past two decades, this fish has grown massively surpassing red tilapia in 2008 which was previously most cultured and thus, becoming the highest produced finfish cultured in Malaysia both fresh or brackish water (Dauda *et al.,* 2018). There has been a direct relationship between fish production and the generation of FBP. Fish consumption trend per capita from 1960 to 2015 has increased tremendously from 9.0 to 20.2 kg, respectively, to an average rate of approximately 1.5% per year (FAO, 2018), hence, generates loads of FBP. Improper disposing of FBP creates serious pollution and often discarded without any recovery. More than half (>60%) of the total fish biomass have been disposed of as FBP such as muscle-trimmings (15–20%), skin and fins (1–3%), bones (9–15%), heads (9–12%), viscera (12–18%) and scales (5%) implicating that another 40% was used for human consumption (Martínez-Alvarez *et al.*, 2015; Zamora-Sillero *et al.*, 2018).

Viscera is very rich in digestives enzymes, especially proteases (Shahidi and Kamil, 2001; FAO, 2018). For instance, acidic and alkaline proteases were reported to be present in 25-20 kg/fish farmed giant catfish viscera (Vannabun *et al.,* 2014). Fish proteases possess the ability to enhance catalytic activities at relatively low concentrations and exhibit high activity over a wide

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range of pH and temperature conditions (Wangkheirakpam *et al.,* 2019). About half of the worldwide industrial enzyme market (approximately 60-65%) has been dominated by proteases (Kumari *et al.,* 2015). Proteases actively involved in the manufacturing of food (as a meat tenderizer, as juice clarification, in cheese-making and processing aids in baked goods, beer and wine) and non-food products (detergent, leather product and silver recovery from the use of x-ray films) (Choudhary, 2013; Gurumallesh *et al.,* 2019; Singh *et al.,* 2019). Besides, fish viscera proteases can be enjoyed by Muslims, Jews and Hindus, for example, gastric enzymes as possible rennet substitutes in halal, kosher and vegetarian products (Shahidi and Kamil, 2001; Sanromán and Deive, 2017).

Purification of enzymes typically involved salt or organic compounds precipitation, chromatography or column isolation. Though proteases from various sources have been extensively studied, those from fish have not been thoroughly explored (Murthy *et al.,* 2018). There have been relatively several attempts to extract protease from fish viscera such as protease from silver catfish (*Pangasius sutchi*) (Ismail and Jaafar, 2018), Nile tilapia (*Oreochromis niloticus*) (Chaijaroen and Thongruang, 2016), bolti fish (*Tilapia nilotica*) (El-Beltagy *et al.,* 2005), hybrid catfish (*Clarias macrocephalus x Clarias gariepinus*) (Klomklao *et al.,* 2011), farmed giant catfish (*Pangasianodon gigas*) (Vannabun *et al.,* 2014), Bogue (*Boops boops*) *(Barkia et al.,* 2010) and Indian major carp (*Labeo rohita*) (Geethanjali and Subash, 2013). Therefore, this research is a pioneering study of the African catfish visceral protease that aims to identify its specific activity and molecular weight at different purification stages.

2. Materials and methods

2.1 Sample preparation

Fresh African catfish (*Clarius gariepinus*) viscera was a gift from a fish breeder from Meru, Selangor Darul Ehsan. Solvents and chemicals used in this experiment were of analytical grade and obtained from Evergreen Engineering and Resources and FC-Bios Sdn. Bhd., Malaysia. The fresh viscera were packed in a polyethylene bag and placed in the Coleman® handheld box containing ice packs at 4°C and transported to the research laboratory immediately about 13 km distance within 30 min. At the laboratory, the viscera were washed with potable water, weighed and stored in an airtight glass bottle at -20°C until further used and analyzed.

2.2 Determination of moisture, protein and fat content of the viscera

Moisture, protein and fat content were assayed as described by AOAC (2000). Each analysis was carried out in triplicates. The protein content of the viscera was determined by the Kjeldahl method and calculated as Nitrogen (N) x 6.25. The moisture analysis was conducted by weighing five grams of minced fresh catfish viscera which was then placed in a preweighed aluminium dish and dried in an oven at a set temperature of 105°C. The sample was cooled by transferring the aluminium dish to a desiccator before reweighing until a constant weight was achieved. Moisture content was calculated as follows:

$$Moisture\ Content\ (\%) = \frac{(Weight\ of\ wet\ sample + aluminium\ dish) - (Weight\ of\ dry\ sample + aluminium\ dish)}{(Weight\ of\ wet\ sample + aluminium\ dish) - (Weight\ of\ aluminium\ dish)} \times 100$$

Total lipid content was determined by the Soxhlet extraction method. Approximately five grams of minced dried samples from the moisture analysis was used and extracted with 170 mL of petroleum ether (boiling point: 40/60) over 8 hrs of extraction period. The excess water and petroleum ether residue in the extracted oils was evaporated using a rotary evaporator (Heidolph, Germany) at a temperature of 45°C. The evaporated sample was then dried in an oven at 105°C for 1 hr.

2.3 Preparation of crude protease extraction

Crude protease from fresh viscera was extracted according to Bougatef *et al.* (2007) with slight modification. The extraction was conducted at temperatures not exceeding 4°C within 24 hrs. The viscera (50 g) were homogenized in a homogenizer (T25 Digital Ultra Turrax Homogenizer, Germany) for 5 mins in 100 mL extraction buffer A (10 mM Tris-HCl pH 8.0, containing 1 mM $CaCl_2$) at 4°C at the 1:2 (w/v) viscera to buffer ratio. The mixture was centrifuged (Eppendorf 5415 R Refrigerated Centrifuge, Germany) at 10,000 rpm for 10 mins at 4°C. Then, the supernatant was collected whereas the pellet was discarded. The supernatant was used as the crude protease and stored at -20°C within a month.

2.4 Purification of the protease

All the purification steps were conducted as soon as possible after Method 2.3 at temperatures not exceeding 4°C. The sample at each purification stage was stored at -20°C within a month prior to analysis.

Ammonium sulfate precipitation. Ammonium sulfate salt was mixed with the crude protease extract up to 60% (w/v) saturation (Ismail and Jaafar, 2018). Then, the mixture was subjected to centrifugation (Eppendorf 5415 R Refrigerated Centrifuge, Germany) at 5,000 rpm for 20

min. and the pellet was collected. The pellet was then, suspended in 10 mL buffer A (10 mM Tris-HCl pH 8.0, containing 1 mM $CaCl_2$) at a 2:1 ratio of pellet to buffer volume.

Dialysis. Desalting was performed by the dialysis process according to Rengasamy *et al.* (2016) with minor modification. The dialysis visking tube with molecular weight cutoff (MWCO) 12-16 kDa was first treated by soaking it in buffer A for 30 min. This was followed by rinsing the tube with deionized water and then packing the pellet solution in the tube. The tube was then soaked in a beaker containing 2 L of buffer A. The dialysis process was conducted for 24 hrs at 4°C as follows: the dialysis tube containing pellet solution was soaked in buffer A and stirred with a magnetic stirrer for 2 hrs. After 2 hrs, the buffer was renewed for another three times with 2 hrs interval and the last buffer was left overnight for 18 hrs. After overnight, the salinity of the sample reached 1%. The protease solution was collected and stored at -20°C.

2.5 Protein concentration

Protein concentration was determined by Bradford (Bradford, 1976) with slight modifications. Bovine serum albumin (BSA) range 0-2 mg/mL was used as the standard and measured at 595 nm (Thermo Fisher Scientific Spectrophotometer Helios Zeta, USA) to estimate the protein concentration of the samples.

Protein concentration, proteolytic activity, and molecular weight distribution for crude protease, ammonium sulfate purified protease and desalted protease were analyzed.

2.6 Proteolytic activity assay

Proteolytic activity was assayed by the casein Folin-Ciocalteau method (Oda and Murao, 1974). Approximately, 5 mL of 0.65% (w/v) casein solution in 25 mM Tris-HCl buffer (pH 8) was used as a substrate. Initially, the substrate was heated for 5 mins at 37°C. Then, the crude protease solution (1 mL) was mixed with the substrate and incubated for 10 mins at 37°C. Trichloroacetic acid (TCA) solution of 110 mM (5 mL) was used to stop the reaction and let it settled for 30 mins at 37°C. Then, the sample was subjected to filtration twice using the same Whatman No. 1 filter paper. The supernatant (2 mL) was mixed with 5 mL of 0.55 M Na_2CO_3 and 1 mL of 1 N Folin-Ciocalteu reagent. The solution was kept in an incubator at 37°C for 30 mins for colour development and absorbance was measured at 660 nm (Thermo Fisher Scientific Spectrophotometer Helios Zeta, USA). A standard curve was generated using solutions of 0.2 mg/mL tyrosine. One unit of protease activity is defined as the amount of enzyme required for liberating 1 µmol of tyrosine per minute from casein. The protease total activity was determined according to Ismail and Jaafar (2018) by the following formula:

$$\text{Protease Total Activity (U)} = \frac{E_a - E_b}{E_s} \times \text{concentration of standard tyrosine} \times \frac{V_r}{T_r} \times DF$$

Where E_a is the absorbance of the sample, E_b is the absorbance of the blank, E_s is the absorbance of standard (tyrosine), V_r is the reaction volume, T_r is the reaction time and DF is a dilution factor.

2.7 Protease specific activity

The specific activity, purification fold and percent recovery of the protease were calculated according to El-Beltagy *et al.* (2005) by the following formula:

$$Specific\ acitvity\ (U/mg) = \frac{Enzyme\ activity\ (U/mL)}{Total\ protein\ (mg/mL)}$$

$$Purification\ fold = \frac{Specific\ activity\ of\ purified\ protease}{Specific\ activity\ of\ crude\ protease}$$

$$\%Recovery = \frac{Total\ activity}{Total\ activity\ of\ crude\ protease} \times 100$$

2.9 Determination of molecular weight

The molecular weight of the protein from raw catfish viscera, crude protease extract, purified visceral proteases in i) ammonium sulfate precipitation and ii) dialysis process was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 4% stacking gel and 12% resolving gel. The samples of 10 µL (0.05 g/mL) were prepared and mixed with 2.5 µL NuPage sample buffer and 7.5 µL distilled water. The mixture was heated at 70°C for 10 mins. A low molecular weight marker (Seeblue plus2 Prestained Standard) ranging from 10 to 220 kDa was used in this experiment. Approximately 10 µL of the prepared sample was loaded into each well of the gel. Electrophoresis was performed for 1 hr at a constant current of 100 mA/gel. After electrophoresis, the staining of the gel was carried out in the Coomassie Brilliant Blue solution (NuPAGETris-Acetate Gel protocol). The protein patterns were visualized after destaining the gel until a clear background was achieved.

2.10 Statistical analysis

All measurements were carried out in triplicate. Statistical analysis was carried out using Minitab 17 at 95% confidence level ($p < 0.05$). Differences in proteolytic activities were compared across the purification method using one way ANOVA. Significantly different means were elucidated using Duncan Multiple Range Test.

3. Results and discussion

3.1 Determination of moisture, protein and fat content of the viscera

It is important to assess the chemical composition of food concerning human nutritional health and to identify its potential development and application of food materials in a food system (Roslan *et al.,* 2014). In fish, the chemical composition of its portion is influenced by factors such as species, sex, season, food, area and age (Haliloğlu *et al.,* 2004; Çelik *et al.,* 2005; Kiessling *et al.,* 2005; Rasoarahona *et al.,* 2005; Abdi *et al.,* 2011). Finding in this study showed the chemical analysis of African catfish viscera (Table 1) which contained 5.91% protein, 57.61% fat and 45.73% moisture content. Previous studies stipulated that the protein content of the fish viscera falls within the range from 1 to 77%. The result corresponds with Abdi *et al.* (2011) whereby the Asian catfish (*Pangasius bocourti*) viscera was reported to have less protein (1.00%) and more fat (93.32%). According to them, since lipids storage organ varied between species and also inactive fishes like the catfish family, hence, the fish tend to store more lipids in the liver compared to muscle. This is also evident in Asian catfish (*Pangasius bocourti*) viscera as mentioned by Thammapat *et al.* (2010) whereby its viscera contain a higher amount of fat (93.32%) and lower protein (<2%) and moisture content (<10%) compared to other portions. Their viewpoint was that the lipid and protein content of cultured fish is dependent on an exercise or movement of fish muscle as well as feeding. Moreover, the finding of this study was also in agreement with Bhaskar *et al.* (2008) as they reported that *Catla catla* contained low protein (8.52%) with high moisture (76.25%) and fat content (12.46%). It seems that the percentage of crude protein was dependent on the number of fats in the raw material. Raw materials containing a high amount of fats, on the other hand, show a low percentage of crude protein as seen in Table 1. Therefore, the finding indicates that the protein content in African catfish viscera was within the aforementioned range. The presence of protein in the viscera is deduced to be essentially possible to extract enzymes in it with a considerable amount of protein content available.

Table 1. Chemical analysis of the fresh viscera of African catfish (*Clarias gariepinus*)

Parameter (%)	Fresh Viscera
Moisture	45.73±1.63
Fat	57.61±1.69
Protein	5.91±0.66

Values were expressed as mean±standard deviation of three trials.

Unlike this study, (Adetuyi *et al.,* 2014) documented the proximate analysis of African catfish viscera having a considerably high amount of protein (16.40%) and moisture content (64.81%) than its fat content (3.00%). Relatively low protein (P) and high fat (F) content was observed in African catfish of this study compared to silver catfish (P: 13.79%) (Ismail and Jaafar, 2018), tilapia (P: 14.62%, F: 10.75%) (Shirahigue *et al.,* 2016), rainbow trout (P: 15%, F: 13%) (Taheri *et al.,* 2013), skipjack tuna (P: 55.37%, F: 36.57%) (Klomklao and Benjakul, 2017), black pomfret (P: 14.4%, F: 3.9%) (Jai Ganesh *et al.,* 2011), sardine (P: 77.65%, F: 4.9%) (Dumay *et al.,* 2006), cuttlefish and sardine (both having P: 15%, F: <5%) (Kechaou *et al.,* 2009) yellowfin tuna (P: 69.66%, F: 5.08%) (Ovissipour *et al.,* 2012), Alaska Pollock, Pacific cod and pink salmon (ranged from P: 13%-15.3% protein content, F: 2.0%-19.0%) (Bechtel, 2003).

3.2 Purification efficiencies of African catfish visceral protease

The protease activity of fish viscera depends on the genetic capability of different species (Sabtecha *et al.,* 2014). Different sources of enzyme portrayed different enzymatic activity along with the methods used for extraction and further purification (Rao *et al.,* 1998). Protease from the viscera of African catfish (*Clarias gariepinus*) was extracted and purified by the two steps procedure as described earlier (ammonium sulfate precipitation and dialysis process). The result of the purification procedure is summarized in Table 2 and act as an indicator of purification *efficiencies*. Based on the result, there was a gradual increase in the specific activity of dialysate (608.70 U/mg) from the crude extract (263.82 U/mg) by 2.3 folds. About half of the visceral protease was managed to be recovered (61.43%) up until the dialysis process. According to Geethanjali and Subash (2013), fat in the viscera of *Labeo rohita* which contained nearly 45% fat not only makes the homogenization difficult but the extract itself contained an enormous quantity of finely dispersed lipid which complicates the subsequent purification steps. Hence, in this study, the presence of high-fat content (57.61%) in the African catfish does cause interference and hindered the purification process. However, as the crude protease was further purified until the dialysis process, removal of finely dispersed lipid from one stage to another leaves out enzyme of interest (protease) in the mixture medium. At the last stage of purification, the protease percent recovery still shows a high percentage despite having deduction in number from the crude extract. About half of the protease managed to be recovered at the dialysis process, thus, indicated that the methods of purification adopted here were effective in purifying and recovering protease from the viscera of African catfish.

Table 2. The proteolytic activity of protease from the viscera of African catfish (*Clarias gariepinus*) at each purification stage

Purification stage	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Crude protease extract	1.357±0.002[a]	356.69±7.92[a]	263.82±5.86[c]	1	100
Ammonium sulfate (60%)	0.974±0.004[b]	272.51±5.87[b]	280.64±6.05[b]	1.064	76.4
Dialysis	0.358±0.003[c]	219.13±4.13[c]	608.70±11.48[a]	2.307	61.43

Values expressed on mean±standard deviation. Values with different superscripts within the same column indicate a significant difference at $p < 0.05$. Purification fold and percent recovery was calculated based on the mean of specific activity and mean of total activity, respectively.

There is an increasing trend of specific activity and purification fold whereas the decreasing trend in total activity and percent recovery throughout the purification steps. The crude protease has wide specificity due to the presence of various proteinases and peptidase isozymes (Murthy *et al.,* 2018). Thus, the crude extract showed higher total activity as seen in Table 1 compared to other purification stages. As the crude extract was further purified by ammonium sulfate precipitation, the protease total activity decreases. This showed that ammonium sulfate precipitation served as a step to remove other proteins present in the crude extract (Khangembam *et al.,* 2012) and capable of leaving out the protein of interest in the sample. This proves that the purification process able to reduce impurity in the samples as mentioned by Nurhayati *et al.* (2013). Klomklao *et al.* (2011) discussed the enzyme purification of a hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) viscera. They obtained a 2.6 fold increase of enzyme activity from the crude until ammonium sulfate precipitation with a specific activity of 0.47 U/mg and the total activity of 403.6 U/mL. Differences in fish enzyme activity are because of the species variation, age as well as the quantity and diet composition (Péres *et al.,* 1998). Bougatef *et al.* (2007) purified the crude extract from the viscera of sardine (*Sardina pilchardus*) using ammonium sulfate. The enzyme activity increased 3.58% fold with a specific activity of 3626.93 U/mg. As for Shalaby *et al.* (2016), they found out a similar trend where they obtained a high purification of 2.57 fold of mullet fish viscera from crude extract until the dialysis process with specific activity and the total activity of 0.244 U/mg and 1512 U/mL, respectively. According to El-Beltagy *et al.* (2005), they noticed that the dialysis process was able to increase the purification by 4.33 fold from alkaline protease crude extract (*Tilapia nilotica*) with a specific activity of 0.26 U/mg.

3.3 Molecular weight distribution

The electrophoretic pattern of the protein in fresh viscera sample, crude protease and protease purified by means of 60% ammonium sulfate precipitate and dialysate was analyzed using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). As shown in Figure 1, protease from the viscera of African catfish purified using dialysis showed apparent two prominent bands (column D) with a low molecular weight of 15 kDa and 16 kDa. This indicates that African catfish viscera protease was dimer comprising of two subunits and could produce low molecular weight peptides after undergoing several purifying processes.

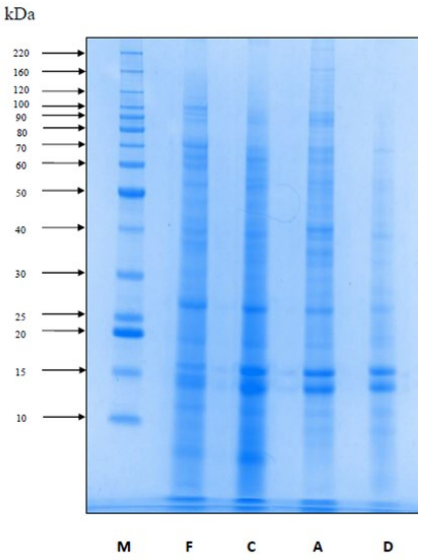

Figure 1. SDS PAGE of (M) protein marker (F) Fresh viscera of African catfish (C) crude protease extract of African catfish (A) African catfish visceral protease purified in 60% ammonium sulfate precipitation (D) African catfish visceral protease purified using dialysis process.

As mentioned earlier, crude protease has wide specificity due to the presence of various proteinases and peptidase isozymes (Murthy *et al.,* 2018). The same goes for fresh viscera resulting from the high fat content of the catfish itself. As a result, it was observed that both fresh viscera (column F) and crude extract (column C) showed a similar intensity of blue-stained with various protein bands. But, the intensity of the blue stained faded slightly and various protein bands started to disappear as the crude extract further purified using 60% ammonium sulfate precipitate (column A) and dialysis (column D). The disappearance of various bands indeed showed that these purification stages help in further removing other proteins present in the crude extract as well as salt, respectively. Three major prominent bands with different molecular weights of 27 kDa, 16 kDa and 15 kDa were spotted in all samples starting from fresh viscera until protease purified by means of dialysis.

Hybrid catfish showed the molecular weight of the trypsin of 24 kDa when the protease was further purified with gel filtration chromatography followed by ion-exchange chromatography (Klomklao *et al.,* 2011). Ismail and Jaafar (2018) obtained 35 kDa for silver catfish visceral protease. Nonetheless, Rajathi *et al.* (2011) found out that Indian mackerel depicted a single band (15 kDa) whereas salmon (20 kDa and 10 kDa) and seer fish (28 kDa and 15 kDa) were dimer comprising of two subunits. It can be said that most of the fish visceral proteases from different species having a molecular weight of around 10 to 35 kDa. Thus, this is in accordance with the present study.

4. Conclusion

In conclusion, African catfish visceral protease showed a gradual increase of specific activity protease up until the dialysis process (608.70 U/mg) from the crude extract (263.82 U/mg) by 2.3 folds with half of the visceral protease managed to be recovered (61.43%). The protease specific activity was tally and in accordance with the SDS PAGE result at each purification stage. The presence of various bands in SDS PAGE for fresh viscera and crude extract started to disappear until two bands were apparent (16 kDa and 15 kDa) at the last stage of purification (dialysis process). Major protein bands were spotted with a molecular weight of 27 kDa, 16 kDa and 15 kDa in crude extract until the dialysis process. Thus, the extraction and two steps purification process aforementioned effectively removes other various proteinases and peptides isozymes in the crude extract. As a result, the mixture becomes more purify and clearer (nearly homogenous) leaving out the enzyme of interest (protease) in the sample as seen in the SDS Page result. The present study, hence, able to utilize fish waste for the extraction of a beneficial enzyme namely protease thereby contributing to the reduction of pollution caused by the disposal of fish waste. Thus, further research on the characteristics of African catfish visceral protease is required as it may, under industrial conditions, be used as a source of halal protease with potential for industrial applications both food and non-food sector.

Conflict of interest

The authors declare no conflict of interest.

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