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Extraction and identification of α-amylase inhibitor peptides from *Nephelium lappacheum* and *Nephelium mutabile* seed protein using gastro-digestive enzymes

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ABSTRACT

The potential of *N. lappacheum* and *N. mutabile* seed as a source of α-amylase inhibitor peptides was explored based on the local traditional practice of using the seed. Different gastro-digestive enzymes (i.e. pepsin or chymotrypsin) or a sequential digestion were used to extract the peptides. The effects of digestion time and enzyme to substrate (E:S) ratio on the α-amylase inhibitory activity were investigated. Results showed that chymotrypsin was effective in producing the inhibitor peptides from *rambutan* seed protein at E:S ratio 1:20 for 1 h, whereas pepsin was more effective for *pulasan* seed protein under the same condition. A total of 20 and 31 novel inhibitor peptides were identified, respectively. These peptides could bind with the subsites of α-amylase (i.e. Trp58, Trp59, Tyr62, Asp96, Arg195, Asp197, Glu233, His299, Asp300, and His305) and formed a sliding barrier that preventing the formation of enzyme/substrate intermediate leading to lower α-amylase activity.

1. Introduction

Native to Southeast Asia, *rambutan (Nephelium lappaceum* L.) and *pulasan* (*Nephelium mutabile*) belong to the same family (Sapindaceae) as the sub-tropical fruits lychee and longan [1]. These fruits are important commercial crops in Asian countries, where they are consumed fresh, canned, or processed, and appreciated for their refreshing flavour and exotic appearance [2]. The harvest of the *rambutan* and *pulasan* fruits was estimated to be half a million tons every year. In Malaysia, an individual *rambutan* tree aged 8 years old or older produced as much as 440 lbs (200 kg) for one season and only 132 lbs (60 kg) the next [3]. However, only the flesh of the fruit is consumed. As a result, large amounts of waste are produced from the peels and the seeds. Vast quantities of agricultural-food waste produced annually worldwide can cause a serious environmental impact. Nowadays, numerous investigations on waste utilization have been aimed at evaluation of the waste materials in possible value-added applications. In Malaysia, the dried *rambutan* rind has been used as a local medicine [4]. Other studies reported that high amount of ellagic acid, corilagin, and geraniin were found in the *rambutan* peel [5], while some amino acids may contribute to the bitter taste [6]. Some locals claimed that by consuming the powdered *rambutan* seed, the blood sugar level can be controlled. Based on the health practices by the folks in Gayo Lues, Acheh, it was found that the *rambutan* seeds contained a lot of benefits in the prevention of diabetes. Since *pulasan* is closely allied to *rambutan*, the potential that it possesses the same benefits was also investigated in this study.

In this case of study, bioactive peptides were the targeted components. Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence our health [7]. Such peptides are inactive within the sequence of the parent protein and they can be released in three ways: (a) through hydrolysis by digestive enzymes, (b) through hydrolysis by proteolytic microorganisms, and (c) through the action of proteolytic enzymes derived from microorganisms or plants. Many of the known bioactive peptides have been produced using gastro-digestive enzymes, usually pepsin and trypsin. Other digestive enzymes and different enzyme combinations of proteinases, including alcalase, chymotrypsin, pancreatin, pepsin, and thermolysin as well as enzymes from bacterial and fungal sources have also been utilized to generate bioactive peptides from various proteins [8,9]. Therefore, hydrolysis using gastro-digestive enzymes was suggested to be appropriate and beneficial in generating bioactive peptides.In this study, α-amylase was used as targeted inhibiting enzyme because previous evidence demonstrated that the inhibition of carbohydrate hydrolyzing enzymes, in particular, α-amylase, has led to a significantly reduced rise in postprandial blood glucose levels upon a meal by delaying starch hydrolysis [10]. The

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residues of α-amylase, in particular, Asp197, Glu233, and Asp300, were described to function as catalytic residues [11,12]. It was suggested that Asp197 acts as a nucleophile that attacks the substrate at the anomeric center of sugar to form a covalently bound reaction intermediate. The reducing end of the substrate is then cleaved off the sugar skeleton, followed by nucleophilic attack by a water molecule in order to break the covalent bond between Asp197 and the substrate. During the reaction, Glu233 and Asp300 either individually or collectively act as acid/base catalysts. In this case, secondary binding sites are likely involved in the binding and sliding of the substrate chain [13]. Although binding at the subsites alone is not able to induce hydrolysis, but the additional subsite interactions are essential to lead to a productive binding mode. A number of aromatic residues locates along the substrate-binding pocket have been reported to have a direct interactions with the substrate. In particular, aromatic residues such as Trp58, Trp59, Tyr151 and Phe256, are prominently located in the vicinity of the active site of α-amylase, have played a substantial role in substrate binding, enzyme activity and catalysis [14]. With the increased understanding of the role of each domain, it provides an excellent basis for the initiation of the design enzyme inhibitors. There were a number researches studied on α-amylase inhibitor peptides which were extracted from different sources, such as cumin seeds, pinto bean, royal jelly, common bean, and egg white albumin [15–19]. The researchers reported that the substrate analogs (i.e. Trp59, Trp58, Tyr62, His299, and His305) as well as the catalytic subsites (i.e. Asp197, Glu233, and Asp300) that located in active domain A and B, which act as proton receptor or donor in the catalytic function of α-amylase, were successfully bound by the peptides. Thus, substrate, such as starch or other carbohydrate polymers, was inhibited from being captured and hydrolyzed by α-amylase [15,16].

2. Materials and methods

2.1. Materials

Rambutan and *pulasan* were purchased from local markets in Penang, Malaysia. Enzymes involved in hydrolysis were: pepsin (P7125), and chymotrypsin (C4129) with activity of ≥400 units/mg and ≥40 units/mg, respectively. The enzyme used in the assay was α-amylase (A3176) with a declared activity of 26 U/mg. The enzymes and all other chemicals in this study were of analytical grade and purchased from Sigma-Aldrich.

2.2. Preparation of rambutan and pulasan seed protein

The *rambutan* and *pulasan* seeds were separately washed and air-dried. Defatted seeds were prepared by incubating the samples with n-Hexane at a ratio of 1:5 (w/v) for 30 min with a constant agitation speed of 200 rpm at room temperature. The samples were then centrifuged for 30 min at a speed of 2500*g* and the supernatant were discarded. The step was repeated 2 times until no oil residue was present on the filter paper. The defatted samples were then dried overnight at room temperature, followed by grinding to fine powders using a blender, and sieving (60 mesh).

Protein was extracted using phosphate buffer solution (pH 8.0 ± 0.1) at a sample-to-buffer ratio of 1:50 (w/v) and incubated at 80 °C with an agitation speed of 250 rpm. The extraction period for *rambutan* and *pulasan* was 2 and 3 h, respectively. The samples were then centrifuged (2500*g*) for 15 mins. The supernatant was collected and the pH was adjusted to 4.5 using 0.1 M HCl or NaOH. The resulting precipitate (protein sample) was then collected after centrifuged down (2500*g*), washed with ethanol and subsequently lyophilised. The yield of *rambutan* and *pulasan* seed protein were approximately 12 and 14 mg/g, respectively. These samples were stored at 4 °C prior to hydrolysis.

2.3. Release of peptides using gastro-digestive enzymes

The protein samples extracted in Section 2.2 were subjected to simulated *in vitro* digestion. In this study, three digestion conditions were used:

2.3.1. Hydrolysis using pepsin

Protein samples were added to buffer solution at pH 2.0 at a concentration of 10 mg/ml. Pepsin was then added at sample to substrate ratio of 1:20 or 1:40 (w/w). The samples were incubated at 37 °C with constant agitation at 55 rpm for 1 or 2 h. Subsequently, the digestion process was terminated by heating the samples at 95 °C for 30 min using a thermomixer. The protein hydrolysates were then membrane filtered at a 3 kDa molecular weight cutoff. The samples were lyophilized, and reconstitute with water prior to analysis.

2.3.2. Hydrolysis using chymotrypsin

The digestion was performed using chymotrypsin at pH 8.0 according to the procedure stated in Section 2.3.1.

2.3.3. Hydrolysis using pepsin followed by chymotrypsin (pepsin-chymotrypsin)

The digestion of samples was started using pepsin (Section 2.3.1), and then the pH of the sample was adjusted to pH 8.0 using 0.1 M NaOH prior to the addition of chymotrypsin for the subsequent hydrolysis process (Section 2.3.2).

2.4. Amylase inhibitory activity determination

The α-amylase inhibitory activity assay was carried out according to the method of Apostolidis et al. [20]. The sample (25 µl) was added with 25 µl of α–amylase solution (1 mg/ml), and incubated at 25 °C for 10 mins. After incubation, 25 µl of starch solution (20 mg/ml) in phosphate buffer, which was at a boiling temperature for 15 mins followed by cooling to room temperature, was added and incubation was resumed for 10 mins. A control sample using buffer instead of the sample, and a sample blank using buffer intead of the enzyme, were also prepared. The reaction was terminated by the addition of 50 µl of dinitrosalicylic acid (DNS) reagent, followed by heating at 95 °C for 5 mins, before cooling to room temperature. The mixtures were subsequently added with 750 µl of distilled water and centrifuged at 2650*g*. The supernatant (200 µl) were pipetted into respective wells and read at 540 nm using a spectrophotometer. The α-amylase inhibitory activity was expressed as percent inhibition as calculated using the Eq. (1):

$$\%\text{inhibition} = \frac{A_c - (A_s - A_b)}{A_c} x100\% \quad (1)$$

where A_b was the absorbance of sample blank, which is the mixture of starch solution and sample without the addition of α-amylase, whereas A_c was the absorbance of control, which is the mixture of starch solution and α-amylase without the addition of sample and A_s was the absorbance of sample, which is the mixture of sample, starch and α-amylase.

2.5. Identification of bioactive peptide

The peptide samples were first introduced into a liquid chromatography mass spectrometry (LCMS) LTQ Orbitrap system for peptide sequencing purposes [21]. The identified sequences with high average local confidence (> 60%) were chosen for Peptide Ranker [22] and Pepsite 2 [23] analysis. The novelty of the digested *rambutan* and *pulasan* seed peptide were validated using BIOPEP [24] and PeptideDB [25] databases, which were accessed at http://www.uwm.edu.pl/biochemia and http://www.peptides.be, respectively, on 16 December 2017. The structure-activity relationship (SAR) study was then carried out using Pepsite 2 and mammalian pancreatic α-amylase (PDB code: