

Enzymatic hydrolysis as an approach to produce alternative protein from cephalopods ink powder: a short review

Ayu Shazwani, Z. and *Rabeta, M.S.

Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang

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Abstract

Cephalopoda is a class of Mollusca that lives in marine environments, well-known as the most distinctive features of their ink especially squid and cuttlefish species. The various valuable constituents in their ink have been used over millennia for practical and commercial purposes. The current world population of 7.6 billion is expected to reach 8.6 billion in 2030, 9.8 billion in 2050, and 11.2 billion in 2100. Thus, the amount of food, types of food in demand, and their relative contribution to diets will all change. We may face a reduction in protein sources worldwide. Humans need protein in their daily diet to maintain good health. To date, information on the benefits of squid and cuttlefish ink hydrolysates in Malaysia as an alternative protein is lacking. This study aims to review this gap and propose the potential of squid and cuttlefish ink hydrolysates as an alternative protein.

1. Introduction

Cephalopoda belong to the Phylum Mollusca includes animals with soft-bodied, and bilaterally symmetrical, Nautiloidea (nautilus) and Coleoidea are two major groups that represent cephalopods in which squids, cuttlefish, nautilus and octopus are included (Nair *et al.*, 2011; Derby, 2014). The number of species of cephalopods is relatively low (about 700), but they are widely distributed in different marine oceanic habits (Derby, 2014). Cephalopods have become valuable commercial fishery resource worldwide as Thailand recorded the largest exporter, followed by other Southeast Asian countries (Hoque *et al.*, 2010). For a long history, especially Greek and Egyptians have been used cephalopod meat, even in the broader application of foods (Sundaram, 2009). Squid and cuttlefish have become an important fishery product in Southeast Asian countries and are exported worldwide (Food and Agriculture Organization of the United Nations, 2018).

Visceral organs included ink sac are the major by-products discarded and generated during the processing of cuttlefish and squid, consequently, offer an enormous crisis of ecological and environmental pollution (Vate and Benjakul, 2013). Numerous researchers demonstrated interest in comparative studies of melanogenesis in squid ink (Wang *et al.*, 2019). Melanogenesis is the reaction of melanin pigment

produced dark color of squid and cuttlefish ink which occurred in the presence of melanogenic enzyme such as tyrosinase, dopachrome rearranging enzyme and peroxidase (Jiménez-Cervantes *et al.*, 1994; Ahamed *et al.*, 2018). The darker color of black pigment in squid ink is affected by the number of enzymes presents that allow more melanin (Palumbo, 2003). Besides blackened by melanin, it is rich with proteins, lipids, glycosaminoglycans, enzymes, various minerals and metals such as copper, cadmium and lead (Mimura *et al.*, 1982; Zhong *et al.*, 2009). The presence of large amounts of amino acids revealed from the crude melanin of squid ink included glycine, glutamic acid and aspartic acid, as well as a trace of sulfur, contained amino acids through the chemical analysis process (Mimura *et al.*, 1982).

Among of these by-products, it has been utilized as a source of bioactive compounds that have been used in a vast scale as alternative medicine and therapeutic properties (McConnell *et al.*, 1994; Nair *et al.*, 2011; Vate and Benjakul, 2013). According to Sasaki *et al.* (1997), cephalopods ink is a by-product that commonly utilized as a food additive for application in food processing. Cephalopod ink historically has been used to promote human health and has been contributed in traditional medicine application for Eastern culture in China while in Western for ancient Greece and Rome (Zhong *et al.*, 2009; Nair *et al.*, 2011; Derby, 2014). Traditionally in Japan, squid ink is used to preserve food

*Corresponding author.
Email: rabeta@usm.my

products, as 'Ika-shiokara', a cured cuttlefish meat extended the shelf-life when treated with cuttlefish ink as believed exhibits antiseptic (Takai *et al.*, 1993; Sasaki, 1997). In Malaysia, especially in eastern peninsular, squid ink is purposely mixed in dishes to rise the flavor (Ahamed *et al.*, 2018). The squid ink has been affected the growing performance of broiler chicken, besides having an antioxidant and immunity functions (Liu *et al.*, 2011). Cephalopods ink is found to consist of natural antioxidant value in melanin and melanin-free components (Derby, 2014). Hence, the utilization of these by-products helps in lessening pollution threats, disposal problem and indirectly benefit the processing industry (McConnell *et al.*, 1993; Lian, 2005). It is well-documented of cephalopods waste materials are multifunctional marine sources that have been revealed their various beneficial properties. In this review, future cephalopods ink was analysed to be considered as an alternative protein.

2. Constituents of Cephalopods ink

Cephalopod species, such as squid and cuttlefish, release a dark cloudy ink from their sacs as an adaptive response when confronted with danger and physical threats (Lei *et al.*, 2007; Liu, 2011). Squid and cuttlefish defend themselves by camouflaging to match their surroundings, escaping from enemies, and giving a signal to others (Lucero *et al.*, 1994; Derby, 2014; Nicomrat and Tharajak, 2015). The ink sac lumen stores the ink gland, which immediately secretes ink to the hindgut via a duct alongside the anus (Derby, 2014). In addition, their muscular walls and sphincters are highly specialized to control the ejection of ink from the sac. While, glutamate, nitric oxide and cGMP help in regulated of ink production by activating the pathway to increase the tyrosinase and production of melanin pigments (Palumbo *et al.*, 1997; Palumbo *et al.*, 2000; Derby, 2014).

Researchers discovered that squid ink mainly consists of suspensions of melanin granules and protein polysaccharide complexes which are located at the bottom of the sac and are created in the mature cells of the ink gland (Liu *et al.*, 2011; Nair *et al.*, 2011). Variant melanogenic enzymes, such as tyrosinases, peroxidases, and dopachrome-rearranging enzymes, are found in ink glands (Palumbo *et al.*, 1998; Derby, 2014). Peroxidase in the later stages of melanin biosynthesis and promotes the formation of eumelanin polymers from monomers. All factors participating in melanin formation, such as hydrogen peroxide, enzymes, starting products, and intermediates, are present during melanogenesis (d'Ischia *et al.*, 1991).

3. Enzymatic hydrolysis

Squid and cuttlefish ink, which are waste products in the processing industry, may be used to prepare protein hydrolysates through hydrolysis by different enzymes. Kechaou *et al.* (2009) reported the enzyme and substrate used are affected the total amino acid obtained. The enzymatic method seems the best at recovering bioactive materials compared to chemical or physical methods and microbial fermentation (Wang *et al.*, 2019). Particularly, the application of hydrolysate helps in developing a new functional food where it allows the production of small peptides and amino acids that enhanced the flavor properties (Su *et al.*, 2012). Lian *et al.* (2015) discovered squid by-products expressed the highest level of protein for the generation of peptides and free amino acids through proteolytic hydrolysis. The enzymatic hydrolysis process of by-products is a widely applied for improvement of protein properties (Baek and Cadwallader, 1995; Liaset *et al.*, 2000; Nielsang *et al.*, 2005; Jae-Young *et al.*, 2007; González-félix *et al.*, 2014).

In addition, enzymatic hydrolysis is an important role for digestibility activity (Panyam and Kilara, 1996). Fish protein hydrolysates have been well studied in terms of production, biochemical, and functional properties (Kristinsson and Rasco, 2000). Quaglia and Orban (1990) supported the enzymatic treatment improved the functional characteristics under controlled condition. It is believed to increase levels of essential amino acids and well-define the functional as well as the nutritional value of hydrolysates, regardless of the protease used (Kechaou *et al.*, 2009). The crude ink extracts from cephalopods species have been working on its various functional values such as amino acid composition, solubility, stability, emulsifying and many other properties (Zayas, 1997a; Zayas, 1997b; Ian and Ee, 2005; Kechaou *et al.*, 2009; Derby, 2014).

4. Physicochemical properties of cephalopod ink

4.1 Chemical composition

Crude ink extracts of squid (*Loligo duvauceli*) reported consisting high amount of protein, followed by the mineral, moisture and fat content (Fatimah Zaharah and Rabeta, 2017). Okuzumi and Fujii (2000) revealed the squid is considered high protein as the main constituent is 80% of myofibrillar protein, 12-20% of myoplasmic protein and 2-3% of myostroma protein, whereas mineral content differed depending on maturity, biological stage, habitat, species also affected by the concentration of the mineral in seawater. Liu *et al.* (2011) reported cuttlefish (*Sepiella maindroni*) ink is an intermixture, and its primary components are protein

polysaccharide complexes that consist of 16 amino acids, high aspartic acid content, and nine fatty acids (approximately 1.34%) constituted by 43.4% and 56.6% of unsaturated and saturated ones, respectively. Calcium is the most common mineral element found in cephalopods ink (Takaya *et al.*, 1994; Chen, 2000; Zheng *et al.*, 2002; Liu *et al.*, 2011). The minerals present in melanin substances is generally implicated in the maintenance of normal metabolic and physiological functions and are important components of enzymes and structural proteins (Neifar *et al.*, 2009).

Neifar *et al.* (2009) worked on cuttlefish (*Sepia officinalis*) specimens found out the melanin is composed of carbohydrates polymer and proteins and concluded the crude ink is higher crude protein. *Sepia* melanin is an oligomeric structure that contains 75% 5,6-dihydroxyindole 2-carboxylic acid (DHICA) derived units and 20% 5,6-dihydroxyindole (DHI) derived units, which are mostly degraded to pyrrole-2,3-dicarboxylic acid and pyrrole-2,3,5-tricarboxylic acid, respectively. The peptide fragments found in melanin are called melano-proteins (Knicker *et al.*, 1995; Pezzella *et al.*, 1997). The glycosaminoglycans in melanin include polysaccharides (Chen *et al.*, 2010), tyrosinase (Naraoka *et al.*, 2003) and angiotensin-converting enzyme inhibitor (Kim *et al.*, 2003) and exhibit strong free radical protection activity (Menon and Haberman, 1977).

The Boreo Pacific gonate (*Gonatopsis borealis*) squid ink discovered to contain high amounts of protein compared to other squid species, such as neon flying (*Ommastreohes bartrami*) and boreal club-hook (*Onychoteuthis borealijaponica*). All three inks also contain high amounts of carbohydrate and have been traced for large amounts of homarine and glycine betaine, rich of taurine and hydroxyproline and contain the least amount of trimethylamine oxide (Shirai *et al.*, 1997). The presence of high molecular weight sugars is reported in squid ink (Matsue *et al.*, 1995). Derby (2014) reported cephalopod ink is rich in dissolved free amino acids. Six species of cephalopods, including squid (*Doryteuthis (Loligo) pealeii*, *Lolliguncula brevis*), octopus (*Octopus vulgaris*, *Octopus bimaculoides*) and cuttlefish (*Sepia officinalis*), contain total dissolved free amino acids ranging from 0.5 mM to 132mM. The ink from five out of these six species contains 50% or more taurine and glutamate ranging from 3% to 7%. Approximately 0% to 2% of the total dissolved free amino acids contain 0 mM to 2.9 mM tyrosinase (Derby *et al.*, 2007).

Mimura *et al.* (1982) revealed crude melanin of squids (*Ommastrephes bartrami*) known as a melanoprotein, composed a large amount of melanin

pigment, protein and carbohydrate and detected several amounts of amino acids such as glycine, aspartic acid and glutamic acid. The documented evident through the major species of cuttlefish which are *Sepia pharaonis*, *S. prabahari* and *S. ramanias* found the presence of protein quite lower compared to other crustacean is commonly ranged between 12.59% to 16.47%. The constituted of ash and carbohydrates, particularly is less than 3%, whereas fat content stated for cuttlefish species is ranged between 0.19% to 0.21% (Ganesan *et al.*, 2017). Lopez-Gonzalez *et al.* (2003), also reported the similar results of proteins, minerals, fats and carbohydrates in the dried squid ink sample. The squid (*Loligo duvauceli*) ink found out protein is higher followed by carbohydrate are estimated by Folin-Ciocalteu reagent and Anthrone method, respectively (Nisha and Suja, 2018). The presence of functional properties such as the composition of amino acids, and environmental factors includes temperature, pH and ions affected the ability of protein as a food ingredient (Geirsdottir *et al.*, 2011). Various species of cephalopods ink has been studying their proximate analysis, which is represented in Table 1. Protein is a prominent and significant compound discovered due to the higher percentage followed by the amount of fat, carbohydrate, and ash in different kinds of ink sample. Protein hydrolysate with lower fat content helps in improving the stability of products (Nilsang *et al.*, 2005). In terms of protein content, several researchers have been reported the similar results different fish and degrees of hydrolysate (Kristinsson and Rasco, 2000; Gbogouri *et al.*, 2004).

4.2 Solubility

Good solubility is one of the most important functional properties of protein hydrolysates (Sila *et al.*, 2014), which directly affected the properties of emulsification and foaming (Kristinsson and Rasco, 2000). Ahamed *et al.* (2018) have been performed water solubility index (WSI) of Indian squid (*Loligo duvauceli*) and cuttlefish (*Sepia latimanus*) ink powders and the results showed 17.12% and 10.51%, respectively which are considered high. Jeyasanta and Patterson (2020) also reported squid (*Loligo duvauceli*) ink powder showed a WSI of 16.8%. A high WSI value indicates good dispersibility of protein molecules, resulting in fine dispersion and formation of colloidal systems (Zayas, 1997a). Protein solubility is affected by pH, solvent type, ionic strength, temperature, and processing conditions. This parameter increases when the electrostatic repulsion between molecules is higher than the hydrophobic interaction (Zayas, 1997b).

In terms of pH factor, hydrolysates showed low solubility at the isoelectric points in weak acidic

condition. These solubility variations attributed to both peptides net charges, pH moves away from the pI as increases, in the other hand, the surface hydrophobicity encourages aggregation via hydrophobic interaction (Sorgentini and Wagner, 2002). The improvement of protein solubility by enzymatic hydrolysis could be an effective alternative as promotes the soluble peptide release (Sila *et al.*, 2014). Klompong *et al.* (2007) studied the protein degradation to smaller peptides contributes more soluble products. Jridi *et al.* (2014) suggested the solubility increase in protein hydrolysates due to the enzymatic reaction release of smaller polypeptide units thus reduce the molecular size of peptides. Moreover, lower of molecular size peptides produces through enzymatic treatment showed more hydrophilic and solvated in aqueous solution (Li *et al.*, 2013). The ability of smaller peptides to form hydrogen bonds with water is presumed to have proportionally more polar residues and consequently increase solubility (Gbogouri *et al.*, 2004).

4.3 Emulsification

Kinsella (1976) reported protein helps in forming and stabilizing food emulsion. Cuttlefish (*Sepia latimanus*) and squid (*Loligo duvauceli*) are reported higher of emulsifying activity and stability. The emulsion activity showed the highest mean value of 15.38 and 11.88, whereas emulsion stability showed higher mean value of 56.56% and 39.09% for cuttlefish and squid inks, respectively. The value is different due to the difference in their protein contents, hence cuttlefish and squid ink presented good emulsifying properties (Ahamed *et al.*, 2018). The emulsion stability value is based on the electrostatic repulsion between protein molecules. This result discovered these inks showed good stability against flocculation and droplet coalescence. Decreasing particle size is dependent on the attractive forces acting between the droplets, whereas the particle size slightly affected the steric repulsion range (Qian and McClements, 2011).

Protein hydrolysates are surface active and promote oil-in-water emulsions as comprised of hydrophilic and hydrophobic components (Wilding *et al.*, 1984). The emulsifying properties of hydrolysate related to the peptide effectiveness in lessening interfacial tension between hydrophobic and hydrolytic elements in food (dos Santos *et al.*, 2011). Many factors accounted for the value of Emulsion stability index (ESI) and one of the reasons might be due to different pH levels during hydrolysis (Baharuddin *et al.*, 2016). Increasing of negative charge peptides present in highly alkaline condition contributes to more unfolding polypeptides. As more exposure of hydrophilic and hydrophobic peptide

residue, more interactions at the oil-water interface (Taheri *et al.*, 2013).

4.4 Other properties of cephalopods ink

Indian squid (*Loligo duvauceli*) and cuttlefish (*Sepia latimanus*) ink have been evaluated the essential part of the powder flow evaluation, which is the bulk and tapped density and found out both possess of higher bulk and tapped density (Ahamed *et al.*, 2018). The powder bulking property directly related to several factors such as preparation, treatment and storage of the sample (World Health Organization, 2012). Raftani Amiri *et al.* (2016) revealed the foaming activity index (FAI) and foaming stability index (FSI) of cuttlefish (*Sepia pharaonis*) hydrolysates showed cuttlefish protein hydrolysates (CPHs) muscle prepared by protamex (CPHP) had higher FAI and FSI than alcalase (CPHA), while the foaming properties reduced at the acidic condition which is pH 4. The higher of FAI and FSI obtained, it is considered to have the ability as foaming agent that can be used in food applications. The pH indicates major effect towards foam stability as it might be decreased at acidic pH due to the repulsion of peptides (Klompong *et al.*, 2007; Elavarasan *et al.*, 2014).

Ahamed *et al.* (2018), also studied the properties of water activity (a_w) of squid (*Loligo duvauceli*) and cuttlefish (*Sepia latimanus*) ink powder and the results are relatively low which below 0.2. The pathogen growth is stated over 0.98 and the food products have lower a_w are considered safe (Lund *et al.*, 2000). The a_w for dry food in the range 0 to 1 and moist food is 0.99, whereas the optimum a_w promotes the stability and life span of products under ambient storage. The criteria of squid and cuttlefish ink mostly satisfy the industrial needs, where it has presented lower a_w , higher tapped density, good oil absorption capacity, foaming ability, water solubility and emulsifying ability (Ahamed *et al.*, 2018). Thus, all these factors contribute to rising interest among researchers in recent years.

5. Conclusion

Among the marine waste materials, cephalopod ink is one of the potential sources of natural bioactive product because of its fewer side effects compared to synthetic sources. Several studies proved that the information on functional characteristics in various modes of application satisfies the needs of the industry. This review summarizes the potential cephalopods ink, particularly its component, physical and functional properties as an alternative protein. Cephalopod ink is biochemically rich in proteins, minerals, lipids and carbohydrates, however, information on this material

especially its functional characteristics remains scarce. Although squid ink has been studied for many years, especially in pharmacology, their wastes are still discarded and generated. Thus, understanding their valuable origin and providing an opportunity for further utilization might be a meaningful work that may lead to the reuse and reduction of marine waste.

Conflict of Interest

The authors declare no conflict of interest.

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