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Potential of carrot concentrated protein as a natural cryoprotectant in fish surimi during frozen storage

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Abstract
Surimi has become the intermediate material for variety of sea food analogues, which have a growing consumer demand in India. Surimi is stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is water-washed for removal of sarcoplasmic proteins, blood, inorganic salt, and some lipid and other undesirable materials like pigments so as to enhance the gel forming ability of the washed proteins. Usually, surimi is blended with chemical cryoprotectants like sucrose 4%, sorbitol 4%, phosphate 0.3% etc. before storing at -18 to -20 °C to extend self-life of frozen surimi by preventing deleterious changes in myofibrillar proteins caused by freezing, frozen storage and thawing and then used in the production of fabricated fish food products. Although cryoprotectants such as sucrose, sorbitol and phosphates have been used in surimi industry since long time but the problem of sweetness and high calorific content are of growing concern as these could raise blood glucose level leading to the problems especially for those suffering from defective glucose metabolism, such as person with hypoglycemia or diabetes mellitus. Many studies showed that sorbitol and phosphate have adverse effect on human health like celiac disease, gastrointestinal diseases and diabetes, etc. which made various researchers to search for alternative natural cryoprotectants. Carrot (*Daucas carota*) is a main vegetable crop grown throughout India. It is reported that carrot roots have anti-freeze protein and natural source of antioxidant and carotenes, mainly beta-carotene and sufficient phenolic compounds. It is proved that the carrot concentrated protein (CCP) contains 18% carrot antifreeze protein which is a leucine-rich repeat protein with the molecular weight of 36.8 k Da and possess strong anti-crystallization ability, provide a softer texture and thermal hysteresis and simultaneously prevent oxidation. Furthermore, supplementation of CCP to food product did not show any adverse effect on food quality

Keywords: Surimi, cryoprotectant, carrot (*Daucas carota*) concentrated protein, human health

Introduction
Presently, a challenging task before the food processing industries is preserving the food at lower temperature with minimum quality losses so as to serve the products almost similar to the original one. Most important quality change during frozen storage includes changes in textural properties leading to damaging texture of food due to ice crystals formation during freezing, re-crystallization, and drip loss during thawing. Ultimately such changes exert a deleterious effect on the nutritional profile resulting a low grade food. Therefore, it has become a challenge to the food processor to control or inhibit ice re-crystallization and decreasing freezing point to avoid ice crystal formation in order to increase shelf-life of food.
Surimi is an important intermediate product containing stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is water-washed for removal of sarcoplasmic protein, blood, inorganic salt, and some lipid and other undesirable materials like pigments so as to enhance the gel forming ability of the washed proteins (Venugopal, 2006) [103]. Otherwise these solubles interfere with the storage and rheological characteristics (Lee and park, 1998) [45]. Usually, surimi is blended with cryoprotectants before storing at -18 to -20 ^{0}C and then used in the production of fabricated fish food products. It has become the intermediate material for variety of sea food analogues, which have a growing consumer demand in India.
Cryoprotectants are used to extend the shelf-life of frozen foods by preventing deleterious changes in myofibrillar proteins caused by freezing, frozen storage and thawing (MacDonald *et al*. 1996) [49]. At present, in food industry especially in fish surimi industry, chemical preservatives (cryoprotectants) which are being utilized at commercial level includes sucrose, sorbitol, phosphate etc. in different proportion. Chemical cryoprotectants function by forming hydrogen bonds with biological molecules (protein in surimi) and replaces the water molecules

surrounding it, leading to retain the native physiological structure and function of the biological molecules.

Although cryoprotectants such as sucrose, sorbitol and phosphates have been used in surimi industry since long time (Pigott, 1986) [78] but the problem of sweetness and high calorific content are of growing concern (Park and Morrissey, 2000) [70] as these could raise blood glucose level leading to the problems specially for those suffering from defective glucose metabolism, such as person with hypoglycemia or diabetes mellitus. Many studies showed that sorbitol and phosphate have adverse effect on human health like celiac disease, gastrointestinal diseases and diabetes, etc. which made various researchers to search for alternative natural cryoprotectants. Sucrose can contribute to development of metabolic syndrome (Aguilera *et al.* 2004) [1]. It was reported that sucrose (particularly prevalent in processed foods) causes health hazards, including obesity and tooth decay (Moynihan *et al.* 2004) [55].

Carrot (*Daucas carota*) is a main vegetable crop grown throughout India. It is used as vegetable and salad, and is very beneficial to human from the viewpoint of nutrients. It was reported that carrot roots have a sufficient anti-freeze protein. A novel cold-induced AFP was identified and isolated from carrot (Smallwood *et al*. 1999) [92].

It is proved that the carrot concentrated protein (CCP) contains 18% carrot antifreeze protein (Zhang *et al.* 2007) [103]. Meyer *et al*. (1999) [54] studied that carrot (*Daucas carota*) antifreeze protein (*Dc* AFP) is a leucine-rich repeat protein with the molecular weight of 36.8 kDa. It has been reported that CCP possess strong anti-crystallization ability, provide a softer texture and thermal hysteresis (Zhang *et al.* 2007) [103]. However, no study about direct application of *Dc*AFP in fish could be found. Furthermore, supplementation of CCP to food product did not show any adverse effect on food quality and brought a pleasant aroma like *Michelia alba* by *trans*-caryophyllene (Zhang *et al.* 2007) [103]. He also concluded that *Dc*AFP can be used as a natural cryoprotectant in food preservation.

Surimi

Surimi is stabilized myofibrillar proteins from fish muscle. It is mechanically deboned fish flesh that has been washed with water, and blended with cryoprotectants to provide a better frozen shelf life (Park *et al*. 1997) [73]. Washing the minced meat is a critical step in the production of surimi, which not only removes fat, pigments, amines and sarcoplasmic proteins but also concentrates myofibrillar proteins with consequential increase in actomyosin concentrations (Toyoda *et al.* 1992) [97]. Higher levels of actomyosin result in more cross-linking and therefore greater gel strength, which explains why surimi has a more elastic texture than unwashed minced fish meat (Okada, 1964) [66]. The mincing of fish flesh and washing of mince can improve taste, and addition of different kinds of additives that enable the elimination of unwanted flavours and odours studied by various authors (Hoke *et al.* 2000; Negbenebor *et al.* 1999) [35, 58]. The concentrated myofibrillar proteins are salt-solubilized and then heated to form a surimi hydrogel (Numakura *et al.* 1990; Chen and Huang, 2008) [65, 20]. Gelation is an aggregation of proteins forming a three-dimensional network in which water is entrapped (Hermansson, 1979; Pomeranz, 1991) [34, 78].

Surimi possesses some functional properties such as gel-forming ability, water-holding capacity (Somjit *et al.* 2005) [93], and is used as intermediate foodstuff with a long shelf-life and high potential for various texturized products which include crabsticks, crab legs, crab meat, young eel, scallops, and others (Benjakul *et al*. 2003; Carvajal *et al.* 2005; Blanco *et al*. 2006) [6, 16, 10]. Both myosin and actomyosin have dominant roles in surimi gelation and show species specificities with regard to gelation properties (Shimizu *et al*. 1983; Numakura *et al*. 1985) [87, 64]. Generally, myosin alone forms excellent gels. Actin has a synergistic or antagonistic effect on myosin gelation, depending upon the myosin/actin ratio in the gelling system (Grabowska and Sikorski, 1976; Matsumoto, 1979) [32, 53]. Differences in cross-linking of myosin head chain (MHC) contribute to the differences in gel forming ability among the muscles of various fish (Benjakul *et al.* 2001) [7].

Quality deterioration of surimi during frozen storage

The processing and preservation of fresh fish are of utmost importance since fish is highly susceptible to deterioration immediately after harvest and also to prevent economic losses (Okonta and Ekelemu, 2005) [68]. Although microbial growth and almost all chemical reactions can be temporarily slowed by low temperature, but freezing and frozen storage may be responsible for many chemical and physical changes in fish/surimi, which can affect the functional and sensory properties of the products (Krivchenia and Fennema, 1988) [42]. These changes are mainly caused by alterations in fish myofibrillar proteins during frozen storage as a result of the formation of intermolecular cross-linkages, and consequently the aggregation and denaturation of actomyosin (Connell, 1959; Buttkus, 1970; Jiang and Lee, 1985) [23, 14, 37]. During freezing, the decrease in the amount of liquid water available to the proteins, as well as the increase in electrolyte concentration and mechanical damage of muscle structures, caused by ice crystal growth are considered to be the main causes of protein denaturation in frozen fish as suggested by Fennema *et al.* (1973) [26]; Sikorski *et al.* (1976) [32]; Shenouda (1980).

Although, freshness of fish is generally considered as the most important factor determining the gel-forming ability of surimi, but time and temperature of storage of the fish between capture and processing can affect the final surimi quality (Park and Morrissey, 2000) [70]. Lower gel quality is generally associated with extended storage times in ice. However, the rate of loss of gel forming ability appears to vary among species (Benjakul *et al*. 2002) [8]

Denaturation of fish protein due to freezing results lowering down of myosin ATPase activity leading to a reduced shelf life of the frozen fish/surimi (Benjakul *et al.* 2005; Benjakul and Sutthipan, 2009) [9, 5]. Loss of gel-forming ability in surimi during frozen storage was attributed to denaturation and aggregation of myofibrillar protein (Sikorski *et al.* 1976; Suzuki, 1981) [32, 92]. Several reports are there regarding gradual decrease of gel-forming ability of surimi with increase in frozen storage period (Jiang *et al.* 1985; Scott *et al.* 1988; MacDonald *et al*. 1992) [37, 84, 50]. Factors responsible of deleterious biochemical changes during frozen storage include ice crystal formation, formaldehyde formation and effect of lipid oxidation products on proteins leading to aggregation (Sikorski *et al.* 1976; Chen *et al.* 1989) [32, 19]. Formaldehyde level in fish muscle has been used as an index of frozen storage deterioration. Myofibrillar proteins that react with formaldehyde become denatured and the formation of protein aggregate occurred. The formaldehyde formation was associated with the decrease in gel-forming ability of hoki during frozen storage at -20 ^{0}C studied by MacDonald *et al*. (1992) [50]. Sikorski (1980) [87] reported regarding production

of formaldehyde during frozen storage and its role in favouring denaturation of myofibrillar proteins in several species of fish. In addition, freezing results in concentration of solutes and restructuring of water molecules bound to proteins and cause alterations in protein structure, and can expose reactive groups for interaction to form aggregates (De Man, 1980; [24] Martens *et al.* 1982). In a dehydrated state, protein-water interactions in tissues are disrupted, and protein molecules are exposed to an organic environment that is less polar than water. These changes result in increased exposure of hydrophobic side chains and, therefore, changes in protein conformation (Privalov *et al*. 1986; Franks, 1995) [79, 28]. The retention of functional properties, particularly gel-forming ability and water-holding capacity (WHC), is important for manufacturing fish-based texturised products.

Cryoprotectants and its mode of action

Cryoprotectants are known to lower the denaturation and/or aggregation of myofibrillar protein during frozen storage, thereby maintaining functional properties, such as gel-forming ability, water holding capacity and solubility of protein and simultaneously slow down the rate of ice crystal growth and alter crystal shapes (Sych *et al*. 1990; Alvarez *et al*. 2010) [93, 2]. The deleterious changes in myofibrillar proteins caused by freezing, frozen storage and thawing could be prevented by using compatible cryoprotective compounds resulting extension of shelf-life of frozen foods (MacDonald *et al*. 1996) [49]. Systematic studies on many cryoprotective substances, such as amino acids, carboxylic acids revealed that these compounds have some common structural principles with respect to the capacity to prevent freeze denaturation of actomyosin (Noguchi and Matsumoto, 1975) [60]. The following requirements for exhibiting cryoprotective effect for fish muscle proteins have been proposed:

1. The molecule must have one of the essential groups:-COOH, -OH or -OPO3H2 and more than one of the supplementary groups:-COOH, -OH, -SH, -NH2, -SO3H, and/or -OP3H2.
2. The location of the essential and supplementary groups on the molecule must be oriented in a particular way.
3. The molecule must be relatively small.

The primary function of an ideal cryoprotectant is to prevent unfolding of the protein molecules (Carpenter and Crowe, 1988) [15]. Proteins stabilize by cryoprotectants through their interaction with the surrounding water (MacDonald, 1992; Park, 1994) [50, 68]. The spatial structure of cryoprotectants, i.e. the configuration of hydroxyl groups in various stereoisomers is thought to account for their cryoprotective effect (Noguchi *et al*. 1976) [61]. Carpenter and Crowe (1988) [15] suggested that the cryoprotection of proteins in solution was accounted for the exclusion of the solutes from the protein surface. The only mechanism common to a wide variety of compounds such as sugars, polyols, amino acids, methylamine, and inorganic salts with cryoprotective effect on proteins, is the exclusion of the solute from the surface of the protein. The core of protein molecules is composed of hydrophobic amino acids, which have a tendency to bury within the protein structure. In addition, large fractions of the protein surface are likewise hydrophobic given that the surface is occupied with atoms that do not have the ability to form hydrogen bonds (Bull and Bresse, 1968) [13]. Therefore, exclusion of hydrophilic compounds from the surface of the protein, thereby stabilizing the native protein structure.

During freezing, as the temperature decreases, the strength of the intramolecular hydrophobic interactions, which stabilize the native protein structure, also decrease (Privalov, 1990) [78]. Gekko and Morikawa (1981) [29] revealed that water preferentially hydrates the surface of the protein through hydrogen bonds as well as dipole-dipole, and ion-dipole interactions. Presence of water on the protein surface can induce the hydrophobic regions in protein interior to remain buried within the structure. In addition, as pure water freezes into ice, the solute concentration, as well as solute surface tension increase and counter the effect of weakened intramolecular hydrophobic interactions. In this way, the native conformation of the protein is maintained in the presence of cryoprotectant during freezing and frozen storage (MacDonald and Lanier, 1997) [47]. Another mechanism for high molecular weight cryoprotectants has been suggested. Glasses (amorphous structure) formed by high molecular weight polymers at higher temperatures than low molecular weight compounds (MacDonald, 1992) [50]. As the concentration of cryoprotective polymers in solution is increased, the Tg (glass transition temperature) occurs at temperatures higher than the normal freezing point. Levine and Slade (1988) [45] proposed that the formation of ice crystals is shut down as water is immobilized, and due to the glassy state of the solution immobilizes the proteins and retain their native state.

Addition of cryoprotectants was felt since long time in order to protect the beneficial functionality of fish surimi protein during frozen storage (MacDonald and Lanier, 1994; Kijowski and Richardson, 1996) [48, 38]. Various cryoprotectants, such as sucrose, sorbitol, and polyphosphates, have been used since long time as synthetic cryoprotectants during frozen storage of surimi due to their easy availability and low cost (Lee, 1984; Okada, 1985) [43, 67]. But due to some undesirable implication of chemical cryoprotectant compounds, alternative cryoprotectants with reduced sweetness have gained increasing attention. Ruttanapornvareesakul *et al*. (2006) [82] reported that protein hydrolysate from shrimp head containing short chain peptides could inhibit denaturation of fish myofibrillar protein. Moreover, protein hydrolysates from various sources were also used as a cryoprotectant in fish products (Cheung *et al*. 2009; Somjit *et al*. 2005) [21, 93]

Antifreeze protein and its use as natural cryoprotectants in food

Many plant derived product such as root preparations, vegetable preparations, spices or extracts have been used for centuries for the preservation and extension of the shelf life of foods (Chattopadhyay and Bhattacharyya, 2007) [18]. Recently, interest has evolved in identifying other cryoprotectants with reduced sweetness and less inducer of Maillard browning reaction for use in surimi (Noguchi and Matsumoto, 1975; Noguchi *et al*. 1975; Park and Lanier, 1987; Park *et al*. 1988; Sych, 1990) [60, 62, 70, 71, 39]. Antifreeze proteins (AFPs) are found in polar organisms such as fish, plants, fungi, and insects and are characterized by their ability to cause non-colligative depression of the freezing point (Davies and Hew, 1990). A more generally applicable name "ice structuring proteins (ISP)" has been proposed, given that all AFPs influence ice crystal growth by controlling the size, morphology, and aggregation of ice crystals but do not prevent freezing (Clark *et al*. 2002) [22]. AFPs bind to small ice crystals to inhibit growth and re-crystallization of ice that would otherwise be fatal. This feature enables the use of AFPs in cryogenic preservation of cells, tissues, as well as food products (Fletcher *et al*. 1999; Venkatesh and

Dayananda, 2008) [87, 97]. AFPs may inhibit recrystallization during frozen storage, transport, and thawing, thus preserving food texture by reducing cellular damage and minimizing the loss of nutrients by reducing drip (Knight *et al*. 1984; Mueller *et al*. 1991) [39, 55]. AFPs have also been shown to inhibit the activity of bacterial ice-nucleating proteins (Parody *et al*. 1988). AFPs have been applied in ice cream (Regand and Goff, 2006) [80], frozen meat (Payne *et al*. 1994) [75], pre-slaughter lamb (Payne and Young, 1995) [74], and frozen dough (Zhang *et al*. 2007) [103].

In case of frozen meat, the large ice crystals formed during frozen storage results in drip and loss of nutrients during thawing (Payne *et al*. 1994; Payne and Young, 1995) [75, 74]. Soaking bovine and ovine muscle in a solution containing 1 mg/mL of AFP (Payne *et al*. 1994) [75], or injection of antifreeze glycoprotein (AFGP) before slaughter of lambs (Payne and Young, 1995) [74], reduced drip loss and ice crystal size. Antifreeze activity has now been reported in more than 27 species of higher plants (Griffith *et al*. 1992; Urrutia *et al*. 1992; Zamecnik and Janacek, 1992) [33, 95, 99], as well as in more primitive plants such as ferns and mosses. Antifreeze activity is exhibited only when the plants are acclimated to low temperatures (Griffith *et al*. 1992; Urrutia *et al*. 1992; Marentes *et al*. 1993) [33, 95, 51]. Concentrated antifreeze protein from carrots (*Daucus carota*) has been shown to improve the leavening fermentation capacity of frozen dough (Zhang *et al*. 2007) [103], resulting in maintenance of loaf volume and improving the softness of the dough during frozen storage (Zhang *et al*. 2007) [103].

Carrot (*Daucus carota*) as cryoprotective agent

Composition of carrot

The moisture content of carrot varies from 86 to 89% (Anon, 1952; Howard *et al*. 1962; Gill and Kataria, 1974; Gopalan *et al*. 1991) [3, 36, 30, 31]. Carrot is a good source of carbohydrates and minerals like Ca, P, Fe and Mg. Gopalan *et al*. (1991) [31] have reported the chemical constituents of carrot as moisture (86%), protein (0.9%), fat (0.2%), carbohydrate (10.6%), crude fiber (1.2%), total ash (1.1%), Ca (80 mg/100 g), Fe (2.2 mg/100 g) and p (53 mg/100 g). The crude fiber in carrot roots consist of 71.7, 13.0 and 15.2% cellulose, hemicllulose and lignin respectively (Kochar and Sharma, 1992) [40]. The cellulose content in four carrot varieties varied from 35 to 48% (Robertson *et al*. 1979) [81]. The average nitrate and nitrite content in fresh carrot have been found as 40 mg and 0.41 mg per 100 g respectively (Bose and Som, 1986; [12] Miedzobrodzka *et al*. 1992). The taste of carrots is mainly due to the presence of glutamic acid and the buffering action of free amino acids.

Carotenoids are important micronutrients for human health (Castermiller and West, 1998) [17]. The total carotenoids content in the edible portion of carrot roots range from 6,000 to 54,800 µg/100 g (Simon and Wolff, 1987) [89]. The main physiological function of carotenoids is as precursor of vitamin A (Nocolle *et al*. 2003) [58, 59]. In the past decades, carotenoids such as β-carotene have attracted considerable attention because of their possible protective effect against some types of cancers (Bast *et al*. 1996; Santos *et al*. 1996; Van, 1996) [4, 83, 96]. The presence of high concentration of antioxidant carotenoids especially β-carotene may account for the biological and medicinal properties of carrots. Carrots have been reported to have diuretic, N-balancing properties and are effective in the elimination of uric acid (Anon, 1952) [3]. Phenolics or polyphenols have received considerable attention because of their physiological functions, including antioxidant, anti-mutagenic and antitumor activities. They have been reported to be a potential contender to combat free radicals, which are harmful to human body and foods systems (Nagai *et al*. 2003) [56].

Carrot Concentrated Protein (CCP)

This has been reported by many researchers that carrot concentrated protein (CCP) contain antifreeze protein, having molecular weight 36.8kDa (Meyer *et al*. 1999; Smallwood *et al*. 1999; Zhang *et al*. 2007) [54, 92, 103]. It has been reported that carrot antifreeze protein has strong anti-recrystallization ability (Zhang *et al*. 2007) [103] and other cryoprotective properties. Some researcher used CCP and reported some significant result. Zhang *et al*. (2007) [103] used CCP in frozen dough to increase the shelf-life. In addition, CCP prolongs fermentation times and deteriorates the texture of bread (Zhang *et al*. 2007 and 2008) [103, 104]. Most of the works on the use of CCP have been reported to improve the texture property of dough as the dough was found to be softer and steadier due to low freezable water content during frozen storage (Zhang *et al*. 2007 and 2008) [103, 104]. Some studies have been done on transgenic plants, results clearly prove that carrot antifreeze protein can protect tomato plants at chilling temperature, the effect being probably through the stabilization of membrane system in transgenic plants (Kumar *et al*. 2014) [42]. Zhang *et al*. (2008) [104] studied the effects of carrot concentrated protein (CCP) containing 15.4% w/w carrot (*Daucus carota*) AFP on texture properties of frozen dough and volatile compounds of crumb. CCP supplementation lowered the freezable water content of the dough, resulting in some beneficial effects including holding loaf volume steady and making the dough softer and steadier during frozen storage. In a more recent study, Carrot's antifreeze protein (*Ca*AFP) extraction and effects of *Ca*AFPs on thermo-physical properties, texture properties, cooking properties and microstructure of frozen white salted noodles were studied (Ding *et al*. 2014) [25]. They also reported that the NMR measurement showed that the addition of *Ca*AFPs could shift water from mobile state to less mobile state.

Boonsupthip and Lee (2003) [11] were the first to demonstrate the potential usefulness of a type III AFP in preservation of the gel-forming properties of fish muscle under frozen and chilled conditions. As a result, even after frozen temperature abuse, AFP still provided better protection than conventional cryoprotectants. They also reported that antifreeze protein (AFP) remarkably preserved Ca^{2+}ATPase activity of actomyosin during frozen and chilled storage. Under frozen conditions, AFP helped to retain the Ca^{2+}ATPase activity of actomyosin much higher than that of conventional cryoprotectants (sucrose-sorbitol mixture).

Payne *et al*. (1994) [75] suggested that the ice recrystallization inhibition property of AFPs can be utilized in frozen meat and fish. In their study, small pieces of bovine meat were soaked in a concentrated AFP solution and drained to dry. AFP was shown to help maintain the ice crystal size in the frozen meat. Payne and Young (1995) [74] reported that the addition of AFP into the meat prior to freezing could reduce frozen storage damages. The lethal freezing temperature of rainbow trout was found to be lowered in direct proportion to the amount of type I AFP injected into the fish (Fletcher *et al*. 1986). Fish related study also showed that fish flesh containing antifreeze protein had a significantly lower drip loss on thawing and was found juicier in sensory trials than that of a species devoid of antifreeze proteins (Payne *et al*. 1994) [75].

Conclusion

Partial or complete replacement of conventional synthetic cryoprotectant by CCP during frozen storage of surimi would increase its acceptability even for the diabetic and consumers concerned in presence of chemicals in food. On the basis of lipid oxidation and textural properties, CCP could be replaced up to 100% of the conventional cryoprotectant (sucrose, sorbitol and phosphate). The study would be indicate the positive role of CCP in preventing deleterious effects of sub-zero temperature on surimi during storage. However there is no study available on fish or fish products along with CCP.

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