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BIOCHEMICAL ANALYSIS AND PHYSICOCHEMICAL STABILITY OF A PARTIALLY PURIFIED LECTIN FROM HILSHA EGGS

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Abstract

Context: Hilsha is one of the most popular fish in Bangladesh. Biochemical analysis of Hilsha egg revealed that it is quite a healthful food. A specific class of proteins called lectins is partially purified from this source.

Objectives: To carry the nutritional analysis of Hilsha eggs and to isolate sugar-specific lectins from the animal source by applying effective purification techniques.

Materials and Methods: The moisture, ash, protein, lipid, polysaccharide, free sugar, cholesterol, calcium, phosphorus and iron contents were determined by the conventional methods and the lectins were isolated by using the affinity chromatographic technique. The effect of temperature, pH and metal ions were observed by performing the hemagglutination assay.

Results: The moisture, ash, protein, lipid, polysaccharide, free sugar, cholesterol, calcium, phosphorus and iron contents were 34-, 2.4-, 32.59-, 28.35-, 0.036-, 0.059-, 0.40-, 0.178-, 0.193- and 0.136%, respectively. The partially purified crude protein by affinity chromatography from Hilsha eggs agglutinated rabbit erythrocytes and the hemagglutinating activity is inhibited by 25mM Raffinose followed by 100 mM of glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and D-galactosamine-HCl each. In the presence of the chelating agent EDTA, the lectin lost its activity completely. Thermal and pH inactivation assay of the lectin indicates that the activity is highest at 30 to 40^0 C and at the pH 5 to 9, respectively.

Conclusion: Hilsha fish eggs can be recommended as a nutritious food and as well as can be regarded as a source of animal lectins, a group of sugar-binding proteins.

Key words: Nutritional analysis, Hemagglutination assay, Temperature, pH, *Tenualosa ilisha*.

Introduction

Hilsa fish (*Tenualosa ilisha*) is a member of the family Clupeidae, order Clupeiformes. Locally known as 'Ilish', the fish has been designated as the national fish of Bangladesh. Its unique taste and nutritional value have made it the most popular fish not only in Bangladesh but also in many parts of the world.

The Hilsa is a highly fecund fish. A large-sized female can produce up to 2 million eggs. Lipids are the most abundant dry constituent of most fish eggs in addition to proteins and play a major role as membrane constituent and energy reserves in developing fish embryos. The major lipid classes found in fish eggs are phospolipids and triacylglycerols. Phospholipids can be present in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol forms. The Cholesterol content in fish eggs is also high (Mukhopadhyay and Ghosh 2007). The lipids are rich in polyunsaturated fatty acids (PUFAs). Among PUFAs, arachidonic acid (20:4 n-6 AA), Eicosapentaenoic acid (20:5 n-3 EPA) and docosahexaenoic acid

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(22:6 n-3 DHA) are present. Palmitic acid (16:0) is the most abundant saturated fatty acids present in fish eggs. Fish eggs are rich in PUFAs along with high lipid content in body tissue that makes it not only tasty but also very much essential for human health and membrane development.

Lectins are a group of sugar-binding proteins that recognize specific carbohydrate structures and thereby agglutinate cells by binding to cell-surface glycoproteins and glycocpnjugates (Lis and Sharon 1998). Lectins are found in all types of living organisms, either in soluble or in membrane-bound form. They are usually structurally complex molecules with one or more carbohydrate-recognition domains (Dod and Drickamer 2001). Till now, most of the lectins are purified from plant sources and much smaller numbers from animal sources. A number of egg lectins have been isolated from various fish families such as Salmonidae, Clupeidae, and Cyprinidae (Tateno *et al.* 1998, Galliano *et al.* 2003). The present state of knowledge permits us to organize the known animal lectins into several categories depending on sequence similarity and common characteristics such as sugar binding specificity, conserved carbohydrate recognition domains, and ion requirements. So far no such information is available whether or not Hilsha egg contains any such lectin. In order to obtain information regarding the nature of protein present in Hilsha eggs, the present investigation has undertaken. In this paper we are reporting the nutritional analysis of Hilsha eggs and a study relating to the physico-chemical effect on Hilsha egg lectin activity.

Materials and Methods

Materials: For the experimental purpose, Hilsha fishes (*Tenualosa ilisha*) were collected from Shaheb Bazar, Rajshahi metropolitan area. The fishes were mainly caught from the Meghna River near Chandpur. Mature eggs were collected from those fishes and was used for experimental purposes. The eggs were not overripe as the overripe eggs have higher lipid content than the ripe eggs.

Nutritional Analysis: The pH was determined by using a pH meter. The moisture content was determined by the conventional procedure. Ash content was determined by following the method of AOAC (1980). Lipid content of Hilsha eggs was determined by the method of Bligh and Dyer (1959). Protein content was determined with Micro-Kjeldahl apparatus. The starch and free sugar contents were determined by the Anthrone method as described by Jayaraman (1988) and Morse (1949), respectively. Calcium content was determined by the titrimetric method (Sten 1978) while phosphorus content was determined by the method of Boltz (1958). Iron content was determined spectrophotometrically by the thiocyanate method (Vogel 1978).

Crude protein Preparation: Fifty gram of Hilsha eggs was homogenized with 300 ml 50 mM of tris-HCl buffer containing 0.15 M NaCl at 4^0C and centrifuged at 8,000 rpm for 30 minutes. Clear supernatant was used as crude protein extract.

Binding ability of Hilsha egg lectin to affinity column: An affinity column material was prepared by attaching N-acetyl-D-glucosamine and Rhamnose to the Sepharose 4B gel as a matrix by using epicholorohydrine as a cross linker. The column matrix is activated following the usual method and then de-aerated properly. Then the matrix was poured on the column. After washing the column by 10 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, the crude protein was applied to the column. Then the column was washed by the same buffer and the lectin was eluted by using the same buffer containing 0.6 M N-acetyl-D-glucosamine. Finally the eluted fraction was dialyzed against distilled water and TBS and was then subjected for hemmaglutination activity.

Hemagglutination assay: The hemagglutination assay was performed in 96-well microtiter U-bottomed plates. A final volume of 100 µl containing 50 µl of 2% suspension of albino rat erythrocytes (previously washed with 0.15 M NaCl) and 50 µl of two-fold serial diluted lectin solutions were used for the assay. After gentle shaking, the plate was kept at room temperature for 30 minutes. Then the agglutination titer of the maximum dilution giving the positive agglutination was recorded.

Hemagglutination Inhibition: Inhibition of hemagglutinating activity was examined by adding a serial dilution of sugars like D-glucose, D-galactose, D-Mannose, D-arabinose, D-rhamnose, D-raffinose, Methyl-α-D-galactopyranoside and N-acetyl-D glucosamine to the incubation mixture. The hemmaglutination activity was checked after 30 minutes.

Temperature and pH on hemagglutination activity: To examine the thermo stability, crude protein was incubated at 30^0-80^0C for 30 minutes. After heat treatment, the protein solutions were centrifuged at 6,000 rpm, 4^0C for 10 minutes, and the clear supernatant was used for detecting the hemagglutination titers against 2% rabbit erythrocyte. Among the techniques used to establish the conditions for reversible denaturation, the lowering of pH has become quite common (Itano and Singer 1958). The pH stability was determined by incubating the crude protein solutions against different buffers (pH value ranging from 3 to 11) containing 0.15 M NaCl for 6 hours at room temperature. The protein solutions were again dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl for 12 hours. The following buffers were used: 50 mM sodium citrate (pH 3.0-5.0), 50 mM sodium phosphate (pH 6.0- 7.0), 50 mM Tris-HCl (pH 8.0), and 50 mM glycin-NaOH (pH 9-11).

Metal ions on hemagglutination activity: To determine the dependency of hemagglutination activity on the divalent cations, the protein was incubated with 0.1 M EDTA for 2 hours at room temperature. The protein solution was dialyzed against 20 mM TBS buffer (pH 7.8) in presence and absence of Ca^{2+} for 12 hours at 4^0C and then subjected to the hemagglutination assay.

Results and Discussion

Nutritional analysis: The pH value of Hilsha eggs was found to be in the basic side of the scale. The moisture content of Hilsha fish egg was recorded as 34% while the ash content was found to be 2.5%. Protein and lipid content of Hilsha eggs were found to be 32.59 g and 28.35 g per 100 g. The amount of polysaccharide and free sugar present were 36.0 mg and 59.0 mg per 100 g of egg respectively. The cholesterol content was determined to be 400.0 mg per 100 g of eggs. So, Hilsha egg is a moderate source of cholesterol and the data conforms that the cholesterol content of most fish egg lies in the range of 250 to 650 mg/100 g, which is considerably higher than that of fish fillet and much lower than that of bird egg yolk (900-1600 mg/100 g) (Iwasaki and Harada 1983). The total calcium, phosphorus and iron content of Hilsha egg were recorded as 178.0 mg, 193.0 mg and 136.0 mg per 100 g of eggs respectively. All the results of the nutritional analysis are shown in Table-1.

Binding ability of Hilsha egg lectin to the affinity column: The crude protein extract was found to be bound to the affinity column and the bound fraction was eluted from the column using 10 mM Tris-HCl buffer (pH 8.0) buffer containing 0.6 M N-acetyl-D-glucosamine sugar. The eluted bound fraction gave strong agglunating activity against rabbit erythrocytes.

Hemagglutination and Hemagglutination inhibition assay: Hemagglutination activity (2^{-6}) of the crude protein extract revealed that Hilsha eggs might contain the lectin proteins. The carbohydrate-binding specificity was evaluated by inhibiting the agglutination of 2% rabbit erythrocytes using different sugars. The Hemagglutination activity of the bound fraction was inhibited by 25 mM Raffinose followed by 100 mM of glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and D-galactosamine-HCl as shown in Table - 2. This result indicates that Hilsha egg contains a lectin that may be specific for galactose and glucose. Further study is needed to confirm the sugar specificity specifically for purifying the lectin(s).

Effect of denaturants and divalent ions on Hilsha Egg lectin activity: To examine the effect of divalent cation, Ca^{2+} as well as chelating agent EDTA were added to the lectin solution separately, and the result showed that the lectin activity was completely abolished in the presence of 100 mM EDTA (Table-3). In presence of

Ca^{2+} the hemmaglutinating activity of the lectin enhanced significantly indicating that Ca^{2+} is vital for the hemagglutination of the Hilsha egg lectin that was released entirely from the lectin after the treatment with EDTA. The inhibitory effect of EDTA on the hemagglunating activity of other lectins such as TM (Tora-Mame) lectin has also been reported (Itoh *et al*. 1980).

Effect of Temperature and pH on hemagglutination activity: Thermal inactivation of the crude lectins was investigated by incubating the protein solution at different temperatures for 30 minutes and assaying the agglutination activity. The obtained result indicates that the lectins retained its activity up to 100% at 40^0C but when the temperature was increased to 50^0C, the lectin lost its activity to 88%. It lost its activity almost completely at 70^0C as shown in Fig. 1. The lectin was found to be stable in the pH value of 5 to 9. Below and above this pH ranges the lectin drastically lost its activity (Fig. 2).

Effect of metal ions on hemagglutination activity: Many lectins have been reported to be metalloproteins (Goldstein and Hayes 1978) and a part of the metal is necessary for the hemagglutination activity (Tunis 1965), polysaccharide precipitation (Paulova *et al*. 1971) and lymphocyte transformartion (Takahashi *et al*. 1971). A new lectin is purified in South Korea from the hard roe of Skipjack Tuna (*Katsuwonus pelamis*) (Jung *et al*., 2003). From the present data it can be suggested that the hemagglutinating activity of this lectin is influenced by the ca^{2+} and that optimum activity was observed at 40°C temperature and at a pH range of 6.0-8.5.

In the case of Hilsha egg lectins, calcium (Ca^{2+}) may be present in small amounts. This possibility might be supported from the observation that the hemagglutinating activity increased to about 40 % by the addition of Ca^{2+} to the lectins (Table - 3).

Table 1. Nutritional Analysis of Hilsha eggs

Studied Parameters	Results
pH	8.6
Moisture (%)	34.00 ± 0.04
Ash (%)	2.50 ± 0.03
Protein (g %)	32.59 ± 0.18
Lipid (g %)	28.35 ± 0.12
Cholesterol (g %)	0.40 ± 0.03
Polysaccharide (g %)	0.036 ± 0.05
Free sugar (g %)	0.059 ± 0.04
Calcium (mg %)	178.00 ± 0.04
Phosphorus (mg %)	193.00 ± 0.04
Iron (mg %)	136.00 ± 1.08

Table 2. Inhibition of hemagglutination activity of Hilsha egg lectin by different sugars

Sugar	MIC (mM)
Raffinose	25
Glucose	100
N-acetyl-D-glucosamine	100
N-acetyl-D-galactosamine	100
D-galactosamine-HCl	100
D-mannose	-
L-Fucose	-
L-Rhamnose	-

Table 3. Effects of Ca^{2+} and EDTA on the hemagglutination activities of Hilsha Egg lectin

Salts Added	Concentrations (mM)	Relative Hemagglutinating activity (%)
None	-	100
EDTA	100	-
$CaCl_2$	100	140

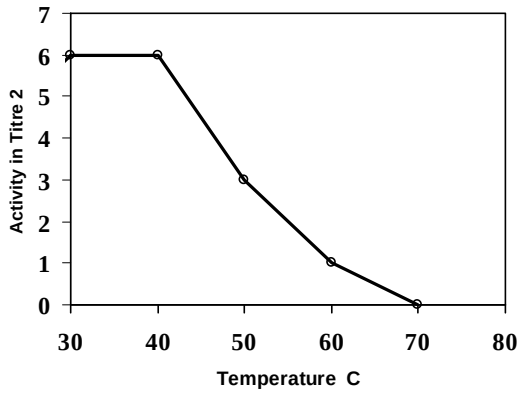

Fig. 1. Effect of temperature on Hilsha egg lectin hemagglutination activity.

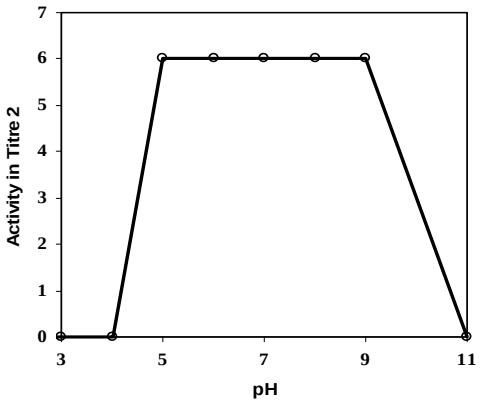

Fig. 2. Effect of pH on Hilsha fish egg lectin hemagglutination activity.

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