THE SYNTHESIS OF FREEZING-POINT-DEPRESSING PROTEIN OF THE WINTER FLOUNDER PSEUDOPLEURONECTUS AMERICANUS IN XENOPUS LAEVIS OOCYTES*

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SUMMARY

The serum of the winter flounder Pseudopleuronectus americanus contains a freezing-point-depressing protein of a molecular weight approximately 10,000 with 60% alanine in its composition. When injected into Xenopus oocyte, a 6-10 S, poly A-rich RNA preparation isolated from the fish liver polysomes stimulated 3-4 fold the incorporation of [^{3}H] alanine into 10% trichloroacetic acid-soluble, non-dialysable proteins. Analysis of the protein fractions showed a translation product similar in molecular weight and electrophoretic mobility to flounder freezing-point-depressing protein. These observations indicated that the 6-10 S RNA from the flounder contained mRNA for the synthesis of flounder's freezing-point-depressing protein.

INTRODUCTION

The freezing-point-depressing proteins (FPDPs) or commonly known as the "anti-freeze" proteins are a unique class of serum proteins found in some cold-water fish. These proteins prevent the fish from freezing by inhibiting the formation of ice lattice in the serum and are essential for their survival in the freezing environment (1). The FPDPs of an antarctic fish, Trematomus borchgrevinki have been isolated and characterized by DeVries and coworkers (2,3). Eight distinct glycoproteins ranging from 2,600 to 33,700 in molecular weight have been isolated. All of these proteins are composed of repeating unit of tripeptide, Ala Ala Thr. A disacharide of N-acetyl-galactosamine and

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Abbreviations - FPDP (freezing-point-depressing protein). SDS (sodium dodecyl sulfate).

galactose is linked to Thr. FPDPs are found also in the winter flounder Pseudopleuronectus americanus which resides in the Northern Atlantic coast of America and Canada in the winter (4,5). In this communication, we report the identification of the FPDP in the winter flounder and the synthesis of flounder FPDP in the Xenopus oocytes, which had been injected with a preparation of 6-10 S RNA obtained from the liver polysomes of this fish.

MATERIALS AND METHODS

The identification of flounder's FPDP

Freshly prepared concentrated flounder's serum was applied directly on a Sephadex G75 column in 0.05 M NH_4HCO_3. Freezing-point-depressing activities were monitored using an Advanced Digimatic Osmometer (Model 3D, Advanced Instruments Ltd., Needham Heights, Mass. 02194).

The isolation of FPDP mRNA

Liver polysomes were isolated from the fish caught in the winter, The liver was homogenized in buffer NS (10mM Tris, 100mM KCl, 40mM NaCl, 5mM $MgCl_2$, 6mM β-Mercaptoethanol pH 7.6, heparin 1mg/ml and 0.25M sucrose). The homogenate was first centrifuged at 6,000 x g for 10 min. The supernatants were treated with Triton X-100 to a final concentration of 1%. The polysomes released were pelleted through 1M sucrose in buffer N (buffer N S without sucrose) at 38,000 rpm for 3 hr in a spinco No. 40 rotor. A poly A-rich RNA fraction was isolated from the polysomes using the method described by Lee et al (6). This RNA preparation was further fractionated on a 10-20% sucrose gradient in 0.01 M Tris-HCl pH 7.6 in a S W 27 rotor at 25,000 rpm for 48 hr. RNA fractions corresponding to 6-10S were pooled, precipitated with ethanol, dialysed extensively in distilled water and lyophilized.

Injection of oocytes and the identification of product

Oocytes of Xenopus laevis were injected with the RNA fraction according to the method described by Gurdon et al (7). Routinely 25 oocytes were injected and incubated in the presence of [^{3}H]-alanine (50μci; 30-50 Ci/mmole) for 40 hr. at 19°C followed by three successive washed at 1/2 hour interval with buffer containing non-radioactive alanine (1mg/ml). The oocytes were then homogenized in 10% trichloracetic acid. The acid-soluble materials were then dialysed in visking tubing (18/32, Union Carbide). These materials were analysed in sodium dodecyl sulfate (SDS) disc gel (8) and in urea-acrylamide disc gel electrophoresis (15% cross-linked, 4M urea at pH 9.2 in tris-glycine buffer). The gel was cut into 1.5 mm sections. Radioactivity in each section was determined by liquid scientillation counting after oxidation with 0.4 ml of ammoniacal 30% H_2O_2.

RESULTS AND DISCUSSIONS

Sephadex G75 chromatography was used to identify the number and size of the freezing-point-depressing proteins in the flounder's serum. In this chromatographic system, most of the serum proteins eluted off in the void volume and have little "anti-freeze" activities. Two distinct "anti-freeze"

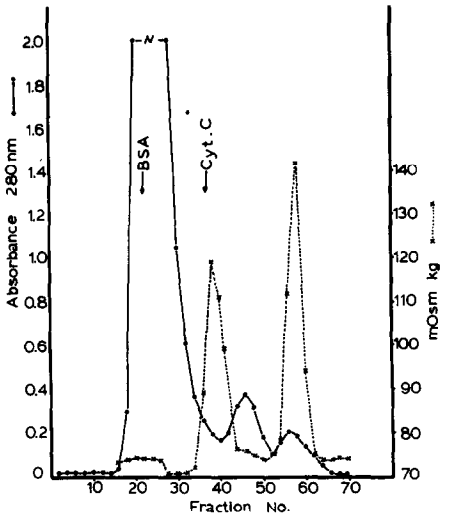


Fig. 1. The identification of flounder FPDP on sephadex G75 chromatography. Freshly prepared concentrated serum (2.5 ml) were applied on the column (1.5 x 86 cm) and 70 fractions (2.5 ml each) were collected. The "anti-freeze" activities of each fraction was monitored with an advanced osmometer.

activities were detected in the column (Fig. 1). However, the slow-running peak corresponded to the inorganic salts present in the concentrated, non-dialysed serum. The winter flounder appears to contain only one major anti-freeze protein having a molecular weight of approximately 10,000 when compared to the cytochrome C marker. This is different from the FPDP of the antarctic fish which were shown to have at least 8 components of different molecular weights (2).

It is known that flounder FPDP is soluble in 10% CCl_3COOH and non-dialysable (4). We have purified the FPDP using this simple procedure. When this acid-soluble, non-dialysable preparation was chromatographed under the same condition, one major component was eluted in the same position as the intact FPDP shown in Fig. 1 with similar activities (5). The amino acid composition of this component was similar to that published by Raymond et al (9) using ion exchange chromatography (Table I). This protein does not contain any obvious repeating unit in the sequence as in the antarctic fish (10). One distinct

Table I

Amino acid composition of the freezing-point-depressing protein of the winter flounder Pseudopleuronectus americanus*

	(a)	(b)
Asx	7	7
Thr	6	6
Ser	2	2
Glx	1	1
Ala	32	38
Leu	3	3-4
Lys	1.5	2
Arg	1	1

(a) Ref. 9
(b) Ref. 5

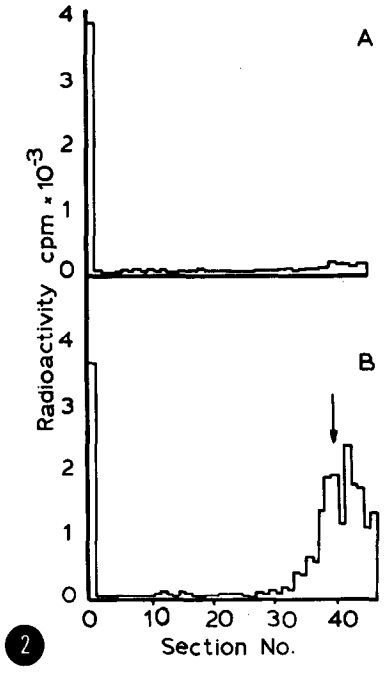
* Sample for amino acid analysis were hydrolyzed *in vacco* in constant boiling HCl at 110°C for 18 hours. Analysis was performed on a Beckman-spinco model 121 amino acid analyzer. The number of residues was expressed in molar ratio.

Table II. The incorporation of [^{3}H]-alanine into 10% CCl_3COOH-soluble, nondialysable proteins from RNA-injected and control oocytes.

		cpm/20ul
Expt. 1	Control	338
	RNA-injected	1414
Expt. 2	Control	379
	RNA-injected	1909

feature of the composition is the content of alanine, which account for 60% of the total number of residues. In addition, the flounder FPDP appears to contain little or no carbohydrate.

This procedure was utilized to identify the translation product of a 6-10 S, poly A-rich RNA fraction derived from the fish liver polysomes in the oocytes system. The incorporation of [^{3}H]-alanine into 10% CCl_3COOH-soluble, non-dialysable protein fractions was consistently 3-4 fold higher in the RNA-injected oocytes than the control oocytes (Table II). SDS disc gel electrophoresis of this protein fraction obtained from the RNA-injected oocytes revealed the presence of a translation product having a molecular weight of approximately 10,000 (Fig. 2b). In addition, its mobility was similar to that of FPDP in



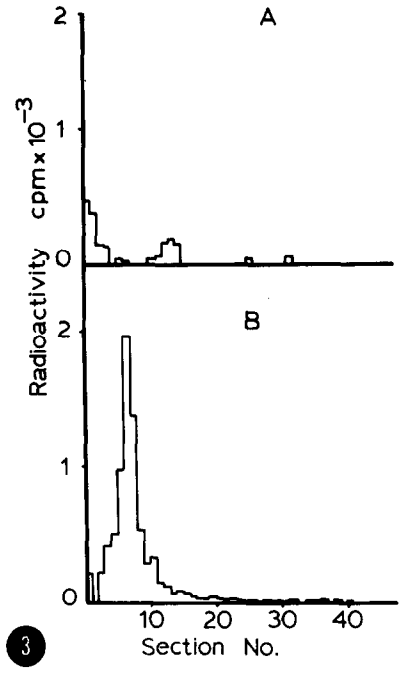


Fig. 2. SDS disc gel electrophoresis of control (A) and RNA-injected (B) oocytes. 10% CCl_3COOH-soluble, non-dialysable fractions prepared from the oocytes were analysed on 10% acrylamide SDS disc gels. The molecular weight of the peak indicated by the arrow gives a value of approximately 10,000 daltons.

Fig. 3. 4M Urea-tris-glycine disc gel electrophoresis of control (A) and RNA-injected (B) oocytes. 10% CCl_3COOH-soluble, non-dialysable fractions prepared from these oocytes were analysed. The mobility of the flounder FPDP coincided with the radioactivity on cochromatography in (B).

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urea, tris-glycine disc gel electrophoresis (Fig. 3b). The control, however, did not contain this product when analysed on both separation systems (Fig. 2a,3a). These observations indicate that the poly A-rich 6-10 S RNA isolated from the liver polysomes of the flounder in the winter exhibited mRNA activity for the synthesis of FPDP when injected into Xenopus oocytes.

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