M-I3

QUELQUES DONNÉES SUR L'ACTIVITÉ HYALURONIDASIQUE CHEZ LE POISSON ENGRAULIS ENCRASSICHOLUS PONTICUS DE LA MER NOIRE

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SUMMARY :

The highest hyaluronidasic activity in viscera and gonads of Engraulis encrassicholus ponticus occurs during the maturation period of gonads. The enzyme is active within a large pH domain, ranging between 3.6 and 8.0, and showing its highest activity between pH 3.6 and 5.6. The hyaluronidase from E. encrassicholus ponticus presents four optimal pH : 3.6, 4.4, 5.0 and 5.4, and two optimal temperatures : 40°C and 60°C. At the same time the enzyme shows a high degree of thermostability.

L'hyaluronidase est une glucosaminidase (DORFMAN, I955) qui catalyse l'hydrolyse des groupements réducteurs de la partie N-acétylglucosaminique de l'acide hyaluronique, ainsi que l'hydrolyse de l'acide chondroïtinsulfurique présent dans les cartilages. Dans nos recherches préliminaires nous avons étudié l'activité hyaluronidasique et la détermination des paramètres optimaux d'activité enzymatique chez Engraulis encrassicholus ponticus.

On a effectué les analyses des extraits protéiques en tampon CH_3COOH/ CH_3COONa à un pH de 4,3,à partir des viscères, y compris les gonades, au cours de la période de maturation, quand l'activité hyaluronidasique est maximale. L'activité enzymatique et la concentration protéique ont été déterminées conformément aux méthodes décrites dans nos travaux antérieurs (ROSOIU et coll., I985).

Les résultats obtenus ont mis en évidence les faits suivants :

- On a décelé une activité hyaluronidasique durant la période de maturation des gonades, dans les viscères y compris les gonades, jusqu'à 10-12 UI/mg protéine.
- La vitesse de réaction catalysée par l'hyaluronidase augmente en fonction de la diminution de la concentration protéique dans le milieu de réaction. On n'a pas pu mettre en évidence un domaine de proportionnalité.
- L'hyaluronidase présente une activité dans l'intervalle de pH compris entre 3,6 et 8,0, ayant un maximum dans l'intervalle 3,6 - 5,6. Les pH optimaux d'action sont compris entre 3.6 et 5.4.
- L'activité maximale de l'hyaluronidase a lieu à des températures de 40° C et 60°C, manifestant, en même temps, un haut degré de thermostabilité.
- La vitesse de réaction catalysée par l'hyaluronidase augmente selon une courbe hyperbolique jusqu'à 125 ug hyaluronate de sodium dans le milieu de réaction, après quoi suit une inhibition à de plus grandes concentrations de substrat.
- La vitesse de réaction catalysée par l'hyaluronidase est directement proportionnelle seulement au premier stade de la réaction, ensuite la fonction devient hyperbolique, ce qui est dû probablement au changement de l'ordre de réaction.

DORFMAN A., I955 - In "Methods in Enzymology", edited by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, vol.I.

ROSOIU N., SERBAN M., VOINESCU I., I985 - Cercetări marine (Recherches marines), 18 (in press).

M-I4

PROTHYMOSIN α IN TROUT. ISOLATION AND TISSUE DISTRIBUTION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND A RADIOIMMUNOASSAY

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Thymosin alpha 1, a 28 amino acid residues peptide has shown promise as an immune enhancer. Two other related peptides, thymosin alpha 11 and des (25-28)-thymosin alpha 1 were also isolated from calf thymus extracts. By heat inactivation of endogenous proteases and a radioimmunoassay against synthetic thymosin alpha 1 (1), the native polypeptide prothymosin alpha (2) was isolated from rat thymus and spleen and in lower amounts from nonlymphoid tissues (3). The elucidation of the primary structures (4) revealed that the above 3 peptides were fragments of different lengths of the NH_2-terminus of the 113 amino acid residues prothymosin alpha. Comparison of the primary structures of rat and human prothymosin alpha revealed for the latter only one deletion at the NH_2-terminus and 4 substitutions and one deletion at the COOH-terminus (5). Prothymosin alpha has been found 10-20 times more effective than thymosin alpha 1 in protecting mice against opportunistic infections.

In order to study the molecular evolution of prothymosin alpha in vertebrates, the isolation and tissue distribution of prothymosin alpha in fish (trout) was undertaken. A new radioimmunoassay (RIA) was developed using rabbit antibodies against synthetic calf thymosin alpha 1 recognizing the epitope 1-10 at the NH_2-terminus and of alpha thymosins and thymosin alpha 1 (1-10) Tyr (^{125}I) as tracer. The useful range of the RIA was 0.3-10 pmoles (1-32 ng) of thymosin alpha 1 (Fig. 1). Trout tissues were immediately dropped in liquid nitrogen after sacrifice and the frozen tissues pulverized. The powder was dispersed in boiling water for the rapid inactivation of endogenous proteases. The suspension was homogenized and after centrifugation the supernatant was mixed with equal volume of buffer A (1M HCOOH/0.2 M pyridine, pH 2.8) and kept at -10°C. A volume corresponding to 7 gr of tissue was thawed, centrifuged and the supernatant lyophylized, redisolved in 1 ml of buffer A and applied on a Sephacryl S-200 column equilibrated with the same buffer (Fig. 2). The fractions containing the immunocrossreacting material were pooled and lyophylized. Finally the sample was applied on a reverse phase high performance liquid chromatography (RP-HPLC) C18 column (Fig. 3).

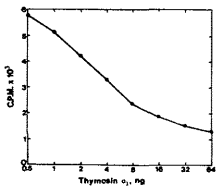
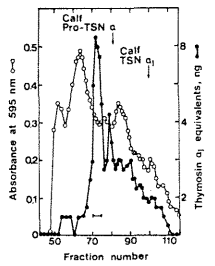


Fig 1. Displacement curve for thymosin alpha 1.

In the gel filtration experiment of the trout spleen extract, the major peak of crossreacting material was observed at an elution volume corresponding to an apparent molecular weight of 55,000, higher than that of calf prothymosin alpha.This may reflect a higher molecular weight and/or a difference in the shape of the molecule of trout prothymosin alpha.No thymosin alpha 1-like material was detected by the RIA. Trout prothymosin alpha was finally isolated by RP-HPLC (Fig. 3) and appeared as a single symmetrical peak at the position of the second major protein peak detected at 220 mm (0.16 AUF) and at a later elution time than calf thymosin alpha 1 and calf prothymosin alpha. The subsequent determination of the sequence of this isolated polypeptide will show the degree of homology of fish and mammalian prothymosin alpha.

Alpha-thymosinswere measured in spleen, liver, testes and ovaries extracts with the RIA. The quantities expressed in ng of thymosin alpha equivalents/g of fresh tissue were 873, 270, 131 and 42 respectively. In gel filtration experiments of liver and testes extracts (not shown) the crossreacting material was characterised as prothymosin alpha-like material, according to the elution volume. The higher concentration found in spleen as compared to non-lymphoid tissues is in agreement with related measurements in mammalian species (3). By the same RIA the amount of prothymosin alpha in calf thymus was 7,238 ng of thymosin alpha 1 equivalents/gr of tissue. The much lower amount of prothymosin alpha detected by the RIA in fish tissues as compared to the mammalian ones may reflect structural differences mainly in the epitope sequence, expected by the phylogenetic distance of fish and mammalian species.



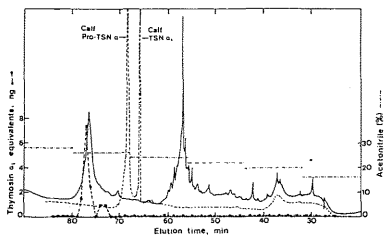


Fig. 2 (left). Separation on a Sephacryl S-200 column (90x1.5 cm) of peptides from boiled trout spleen tissue. The flow rate was 6.71 ml/h and each fraction was 1.34 ml. For the radioimmunoassay and the protein assay aliquots of 300 µl and 100 µl respectively were used from each fraction. For the subsequent purification by HPLC, the fractions indicated by the horizontal bar were pooled and each third of the material separately chromatographed. Arrows indicate fractions corresponding to the elution volumes for calf prothymosin alpha (Mr=35,000) and thymosin alpha 1 (Mr=15,000) both appearing as oligomers.

Fig. 3 (right). Separation of immunocrossreacting peptide(s) on RP-HPLC C18 column. Elution was with a step-wise gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions (0.6ml) were collected and 50 µl aliquots taken for the radioimmunoassays. In the experiment shown fractions 76-78 were pooled and upon rechromatography showed a single peak. An HPLC run of pure calf prothymosin alpha and synthetic calf thymosin alpha 1 is superimposed for comparison (---).

In conclusion a mammalian-like prothymosin alpha has been isolated from trout spleen and found in lesser amounts in other trout tissues. This calls for the examination of the participation of prothymosin alpha in the fish cellular immunity, an already reported role for this polypeptide in mammals.

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