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ENZYMATIC UPGRADING OF FISH AND CRUSTACEAN PRODUCTS

By

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ABSTRACT

Fish wastes constitute an important source of biologically active molecules possessing peculiar properties and practical application promises in various areas (agriculture, medicine, chemistry, biotechnology). Several enzymes from crustacean and fish wastes for which sizable stocks of viscera are actually available have been considered. These enzyme activities are namely an aminopeptidase from tuna, a carboxypeptidase from dogfish, a specific crustacean protease extracted from the crab Carcinus maenas and from the cultivated shrimp Penaeus monodon and pepsins from several fish species. A second utilization of these wastes aims to generate biologically active substances (immunostimulants, gastro-intestinal peptides etc.) through the hydrolysis of marine fish or invertebrates and to use them for enhancing growth and disease resistance of animals or as therapeutical molecules.

INTRODUCTION :

Less than 50% of the total catches of the fish industry are used for human consumption. The remains are either discarded at sea or processed to animal feed. These wastes constitute however an important source of biologically active molecules possessing peculiar properties and practical application promises in various areas (agriculture, medicine, chemistry, biotechnology). Extracts, hydrolysates and enzymes from sea water fish species and marine invertebrates have revealed interesting characteristics (1-3). Most important is the activitytemperature relationship : high activities are obtained at 5-12°C for enzymes of marine origin, instead of 30-35°C for classical systems. Hence, these enzymes are suitable for gentle processing at low temperature [4].

On the other hand, hydrolysates and extracts from fish or marine invertebrates viscera have been shown to contain biologically active factors. Of special interest is the occurrence, in fish hydrolysates, of small gastro-intestinal peptides such as gastrins and cholecytokinins (CCK) [5,6] that stimulate the secretion of digestive enzymes and the production of hormones controlling satiety. This observation may reveal a possibility of both economical and environmentally compatible improvements in aquaculture.

Several other classes of molecules can be generated by enzymic digestion of fish proteins, peptides with opioïd-like activity are found in several types fish hydrolysates [7]. Fish protein hydrolysates are also known as possible substitute of usual protein hydrolysatest based substrates for microbial cultures [8].

A first research project has been mainly directed on the enzyme activities already present in fish and crustacean wastes and on their potential use in various processes of economical significance. The project was particularly aimed to the studying of the specific properties which render then proper to be included in new industrial processes, such as the production of soluble peptides, production of peptones, development of flavours, etc. With respect to these objectives, several enzymes from crustacean and fish (tuna, mackrel, sardine, blue shark) wastes for which sizable stocks of viscera are actually available have been considered. These enzyme activities are namely an aminopeptidase from tuna, a carboxypeptidase form dogfish and pepsins from several fish species.

A second part of the work project aimed to explore the possibility of obtaining biologically active peptides from extracts or hydrolysates of marine food processing waste with the ability to be used for enhancing growth and disease resistance of animals, as attractants or flavourants in feed and food and possibly as pharmacological tools. This presentation is restricted to gastrin-CCK-like peptides and to caicitonin and calcitonin - gene - related peptide immuno - related molecules [9,10] from partially digested proteins in fish and shrimp hydrolysates.

MATERIAL AND METHODS

Enzyme extracts : Procedures for enzyme extraction and activity measurements have been previously described [3,12-15].

Proteolysis : A major element of the waste from the fishing industry is the gastrointestinal tract of fish, which contains a variety of proteases together with lipases and carbohydrases required for digestion of food. The enzymes have been either extracted and purified or used as endogenous sources of catalysts for the production of silage form fish waste (Atlantic cod, sardine, tuna). Adjusting hydrolysis conditions to allow the action of, for example, trypsins, cotrypsins, cathepsins, collagenases, elastases, etc. results in different hydrolysis patterns and the production of different groups of peptides. Peptides resulting from the hydrolysis are filtered on 10 kDA [16] cutt-off membranes and directly conserved or concentrated under vacuum at 50°C {16]. Hydrolysates are characterised by their molecular weight distribution and amino acid composition.

Radioimmunoassays. Gastrin radioimmunoassays (RIA) are performed using a rabbit antiserum, synthetic 1251-gastrin as a tracer and synthetic gastrins as standards (kit GASK-PR from CIS bointernational) [6]. Immunoreactive calcitonin and CGRP (calcitonin related gene product) were measured according to previously described assays [9,11] using ^{125}I-labelled peptides. (Amersham) Receptor binding ability of immunoreactive molecules was developped using rat kidney membranes and rat liver membranes for clacitonin and CGRP, respectively [17].

RESULTS

Proteolytic enzymes. Enzyme activities subjected to this study belonged to the group of esterases ; endoproteases (trypsin, chymotrypsi, elastatse collagenase), exoproteases (aminopeptidases, carboxypeptidases) [13-16]. Chitinases and lipases which are likely to be present in the same sources of material have been also studied as co-products. A pepsin activity was purified from blue shark stomach with the main objective of using methods manageable from pilot to industrial stage. Elastase activity was detected in pancreatic extracts of tropical tuna (Thunnus albacora) autocatalytically activated. The purification yielded an enzyme suitable for enzymatic hydrolysis of insoluble elastine. It is worth to indicate that this enzyme presents a practical interest for the preparation of elastine hydrolysates used in cosmetics [14].

Several pepsins from fish (capelin, bonito, sardine, Atlantic cod, Polar cod, dogfish) have been purified and characterized. Notwithstanding differences from mamalian homologous enzymes in several of their molecular features and physical properties these pepsins share the common properties of being active at acidic pH and of initiating the milk clotting process as a consequence of limited proteolysis of casein .

A pepsin isolated from the gastric mucosa of the dogfish *Scyliorhinus canicula* can be characterized by a set of catalytic properties similar to those of calf chymosin (Table1).

The K_{cat} and K_m values for synthetic hexapeptides mimiking the environment of the Phe_{105} Met_{106} peptide bond of casein put the dogfish pepsin intoa class ($K_{cat} / K_m < 100\ mM^{-1}/s$) that also includes chymosin and a series of fungal proteases. Other mammalian pepsins appear far less efficient ($K_{cat} / K_m < 100\ mM^{-1}/s$) [13,20] Electrophoretic patterns of casein and casein subfractions after proteolysis by dogfish pepsin or calf chymosin show that that both enzymes hydrolyse the κ casein subfraction with the production of κ paracasein peptide. αs1 and β subfractions hydrolysis is stronger with the fish enzyme than with chymosin. It is concluded that despite a broader specificity, the activity spectrum of dogfish enzyme is in many respects similar to that of chymosin. However this broader specificity of dogfish pepsin has various implications for the use of this enzyme in cheese-making. It concerns particularly the developpement of bitterness during the cheese ripening process and thus restricts the use of this enzyme to "cooked" cheeses.

Fish hydolysates. The size distribution profile of a sardine hydrolysate on Sephadex G50 shows three separate peaks for an approximate molecular weight under 3000 Da (Fig1). The elution profile of cod head hydrolysate begin just after the dead volume (30 000 Da) and shows fractions with higher molecular weight than other hydrolysates.

Hydrolysates subjected to gastrin radioimmunoassays (Fig.2) show that the cod head fraction do not seem to display any secretagogue activity. Conversely, the parallelism between the results of shrimp and sardine hydrolysates and the line of control indicate explicitely the presence in these hydolysates of gastrin immunologically related material.

In the CGRP radioimmunoassay (Fig.3, left panel), each extract interacted in a similar manner as unlabeled CGRP. In the CT assay (Fig.3, right panel), no displacement of the ^{125}I-CT binding to its antibody was observed with the stomach hydrolysate. In the CGRP radioimmnoassay, shrimp and sardine hydrolysates, (Fig.4, left panel) interacted similarly to unlabeled CGRP.

The immunoreactive positive molecules were further characterized using a radioreceptorassay specific for CGRP or CT using rat liver and kidney membranes, respectively. None of the hydrolysates interacted with the CT radioreceptorassay. In the CGRP radioreceptorassay (Fig.5), only hydrolysates from cod viscera and from the two batches of sardine hydrolysates were able to displace the labelled CGRP bound to its receptors similarly to that measured with the unlabeled hormone.

DISCUSSION

The functional properties of fish enzymes are increasingly used in various industrial processes. As far as proteases, which are mainly present in fish viscera are concerned, the choice is extremely broad as fishes represent the most important and the most diversified group of vertebrates

(more than 20,000 species of finfishe and approximatively 7,000 species of sharks and rays). In many cases, fish enzymes offer several advantages over enzymes from "classical" sources. Although possessing catalytic functions very similar to that of other proteases from bacteria, plants, mammals currently used in industry, enzymes from marine origin can be distinguished by their optimal conditions of activity : they operate in salty solutions and for the enzymes obtained from the major industrial fish species, display their optimum activity at low temperatures. Conversely these enzymes exhibit a low stability at medium or high temperatures (25°C to 50 °C) and therefore are easily inactivated.

Enzymes such tuna amiopeptidases can be used for reducing bitterness in various alimentary products. Fish proteases can also be used for deskining squids or eliminating the membranes protecting fish eggs, thus sparing teduious manual treatments or destructive mechanical operations. Similarly peeling of shrimps at an industrial level is greatly facilitated by a prior enzyme treatment.

Here, a fish pepsin was used for initiating milk-clotting, the first step in any process of cheese making. Although slightly different from the classical chymosins and from other replacement catalysts, fish pepsin appears to satisfy largely to the requisite qualities specificity and activity. Side hydrolytic effects on other peptide bonds than the Phe_{105} - Met_{106} bond can be considered as minor if one takes in account the important sparing of energy during the enzymatic process.

There is no general rule to determine the best hydrolysate with the most important biological activity. Each raw material, each enzyme and each condition of hydrolysis will give a different final product. But the elution profile on exclusion column and the result of biological tests could give a good idea of the potential value of the hydrolysates and could be good parameters to follow and to optimize the hydrolysis conditions such as pH, temperature and the amount of enzyme.

Differences between hydrolysates from several origins and obtained with the use of different kinds of enzymes are observed. The specificity of proteases is also an important parameter and has an influence on the final product [2,4]. The choice of the enzyme permits to change the size of peptides in the FPH and to improve the biological composition of the hydrolysate. Autolysis of the raw material gives a wide range in the size of peptides of the final product because of the presence of different endogenous enzymes. Gastrin and cholescystokinins are small peptides (between 4 and30 amino acids) in comparison with growth factors which are larger molecules. Conversely, a less hydrolysed fraction will show growth factor activity. This indicates that extensive hydrolysis is not an advantage when specific types of activities are investigated [18].

Calcitonin and / or CGRP immunorelated molecules are present in fish and shrimp

hydrolysates. Whereas cod hydrolysate interacted only in the CGRP radioimmunoassay, shrimp and sardine hydrolysates were able to displace the labelled hormone binding in both radioimmunoassays. Thus, these extracts, i.e.the cod viscera extracts and the two sardine hydrolysates do contain immunologically and biologically CGRP related molecules and the peptidic fragments obtained from these hydrolysates are likely to possess the structural determinants necessary for interacting in the CGRP radioreceptorassay.

In summary, we have demonstrated that several fish and shellfish hydrolysates do contain molecules that are biologically related to physiologically active peptides. The presence of gastrin-CCK peptides is of uppermost interest for the formulation of diets for animal feeding. The isolation of molecules biologically related to CGRP may represent an important challenge in the production of new therapeutic agents. Its potential role in the control of pain and inflammation, type II diabetes and in conditions with intractable hypotension such as septic shock syndrome.

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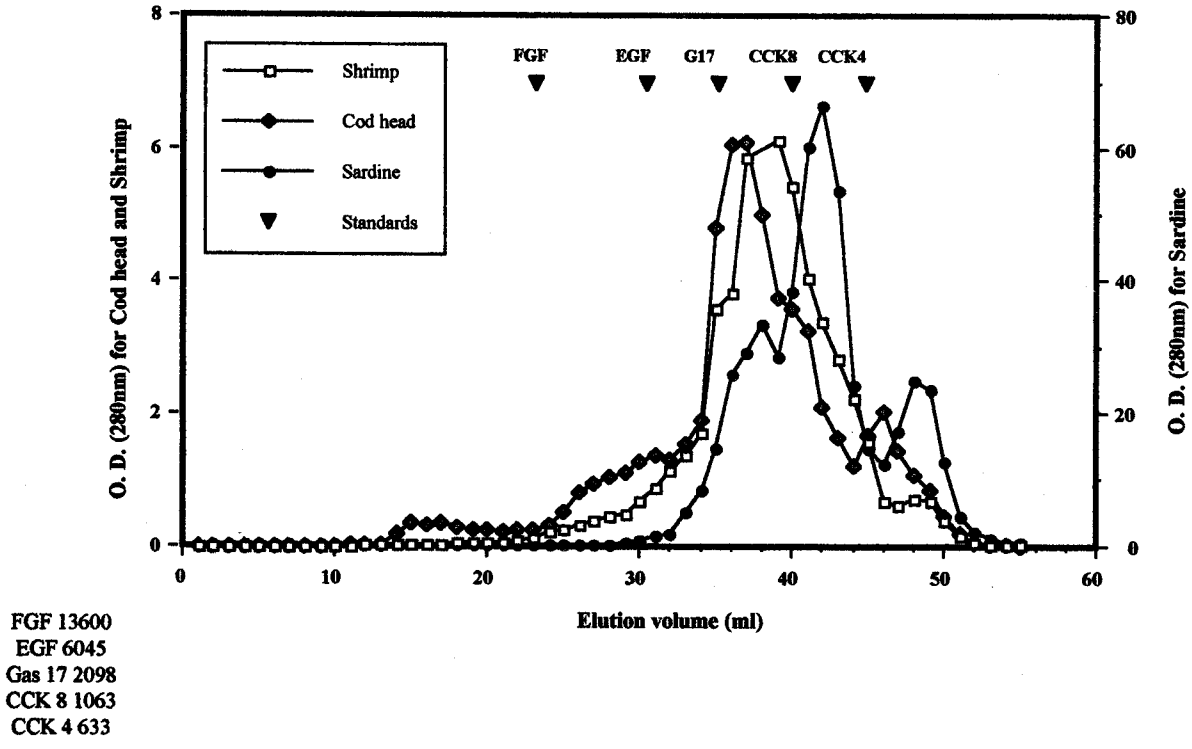

FGF 13600
EGF 6045
Gas 17 2098
CCK 8 1063
CCK 4 633

Fig. 1 : Gel filtration on Sephadex G50 of shrimp waste, cod head and sardine hydrolysate fraction. Markers : Fibroblast Growth Factor : 13600 Da, Epidermal Growth factor : 6045 Da, Gastrin 17 : 2098 Da, CCk8 : 1063 Da and CCK4 : 633 Da.

Table 1. Kinetic parameters of cleavage of Leu-Ser-Phe (NO_2)-Nle-la-Leu-OMe

Enzyme	Substrate range (M)	K_m (mM)	K_{cat} (per sec)	K_{cat}/K_m (per mM/sec)
Dogfish pepsin II	0.007-0.35	0.30	5.83	19.45
Chymosin A	0.08-0.50	0.96	24.4	25.4
Chymosin B	0.08-0.50	0.87	22.2	25.5
Crystalline chymosin	0.10-0.79	0.35	8.7	25.2
Mucor miehei protease	0.10-0.40	0.135	5.6	41.5
Mucor pusillus protease	0.10-0.36	0.089	2.7	30.3
Bovine pepsin A	0.011-0.290	0.021	83.3	4020
Bovine gastricin	0.024-0.263	0.051	67.0	1314

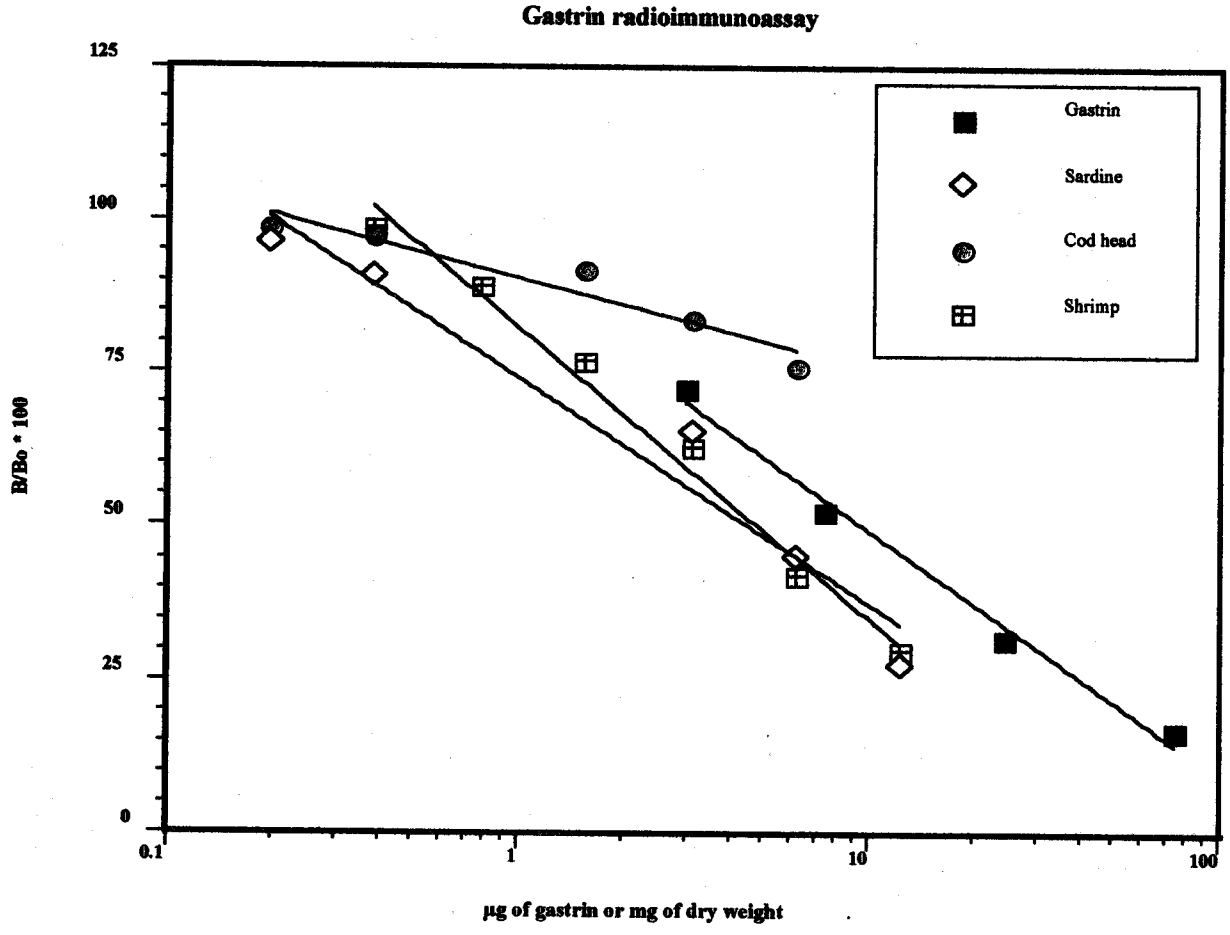

Fig. 2 : Shrimp waste, sardine and cod head hydrolysates subjected to a gastrin radioimmunoassy. Each value represents the mean of two samples.

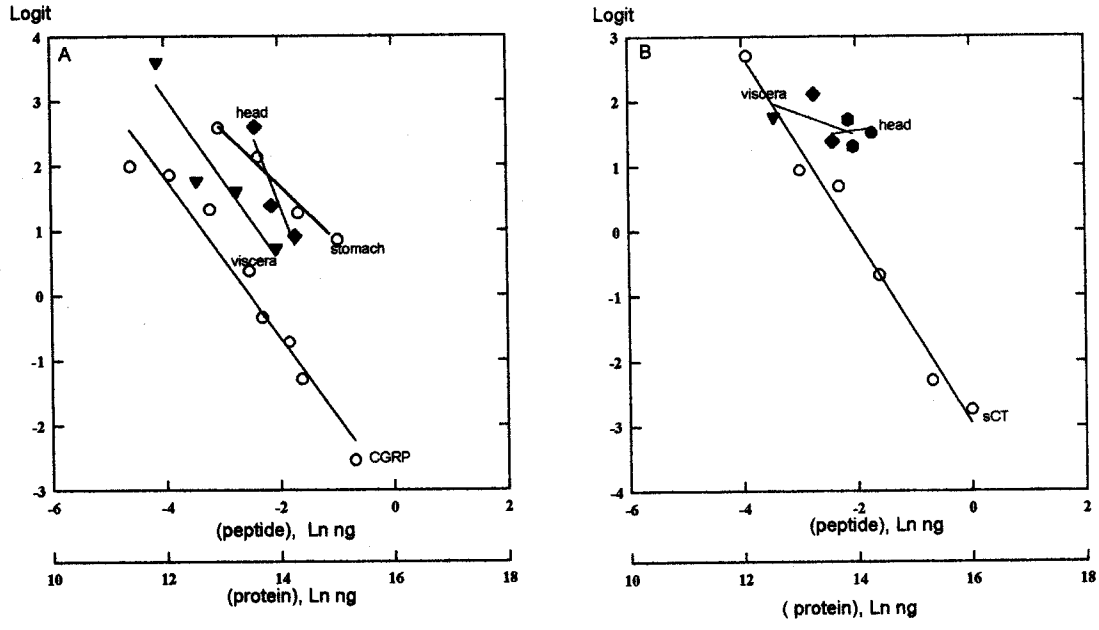

Fig. 3 : Effect of increasing concentrations of cod hydrolysate on the binding of ^{125}I labelled-CGRP (left panel) and salmon CT (right panel) to their respective antibodies. The logit transformation of the percentage of initial CGRP binding (B/Box100) was poltted against the logarithm of the unlabeled CGRP or protein extract concentration.

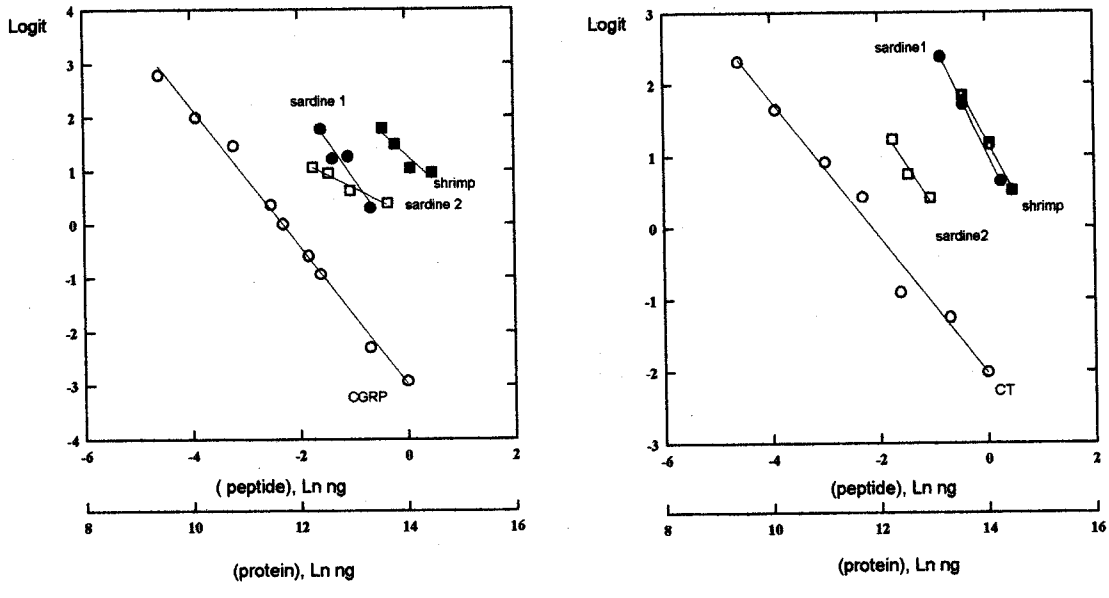

Fig. 4 : Effect of increasing concentrationn of sardine and shrimp hydrolysate extracts on the binding of ^{125}I CGRP (left panel) and salmon CT (right panel) to their respective antibodies. Data were analysed as described in the legend to Fig. 3.

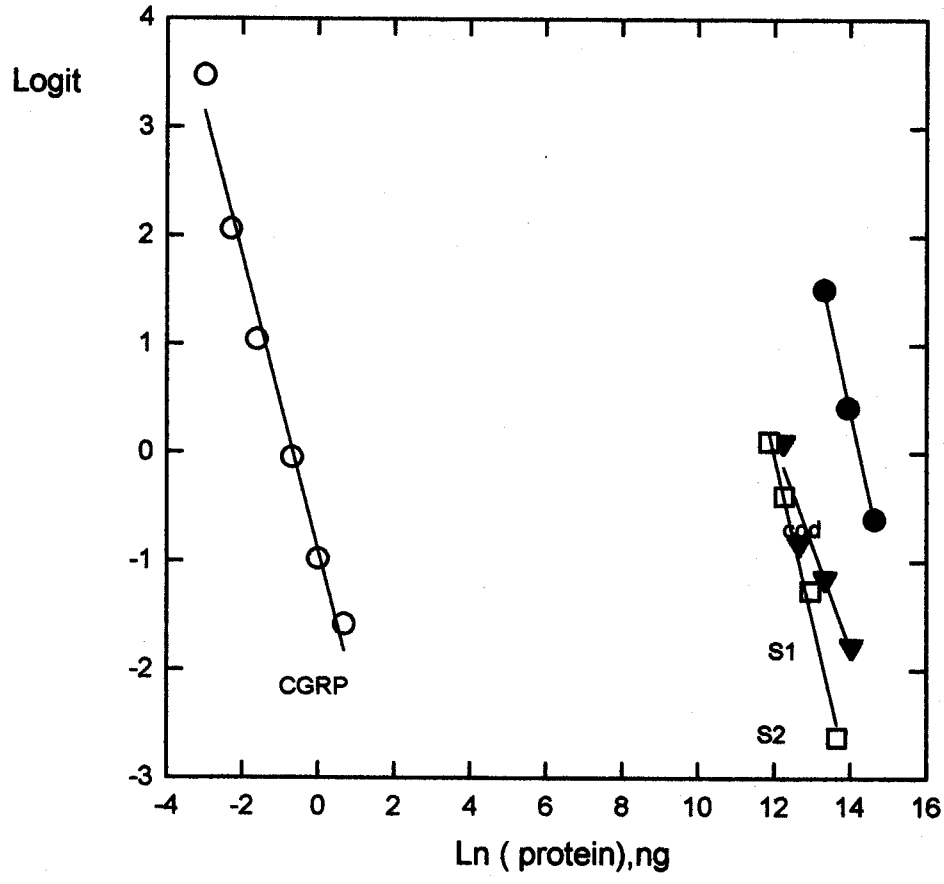

Fig. 5 : Effect of increasing concentrations of cod and sardine hydrolysates on the ^{125}I-CGRP binding to its specific receptors. Data were analysed as described in the legend of Fig 3.