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Use of a Commercial Protease and Yeasts To Obtain CGRP-like Molecules from Saithe Protein

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Different bioactive molecules, such as CGRP-like peptides, can be found in fish protein hydrolysates. Calcitonin gene-related peptide (CGRP) is a neuropeptide known to act as a potent arterial and venous vasodilator in humans. This study focuses on the industrial obtaining of CGRP-like molecules from saithe (*Pollachius virens*) byproduct, derived from the filleting process. Protein from *P. virens* was primarily hydrolyzed with Alcalase and later treated with *Saccharomyces cerevisiae* live cells. Treatment with *Saccharomyces* doubled the quantity of bioactive molecules obtained. The CGRP-like molecules were partially purified by chromatography, and the immunoreactive material was further analyzed for its CGRP-like bioactivity, using a specific radioreceptor assay. The concentration of CGRP-like molecules increased over 100-fold after purification. The bioactive molecules were able to induce cyclic AMP stimulation in rat liver membranes. Finally, partial sequencing of the bioactive peptide was performed, showing some homology with α-actin and myosin of several fish species.

KEYWORDS: Functional foods; *Pollachius virens*; CGRP; protein hydrolysate; *Saccharomyces cerevisiae*; saithe

INTRODUCTION

The use of proteolytic enzymes provides the possibility of controlling the cleavage degree of protein, thus enabling the production of hydrolysates with different molecular structures and different bioactive properties, which could have therapeutic or nutritional interest. In this field, we reported the presence of CGRP-like molecules in several fish protein hydrolysates (*1, 2*). CGRP (calcitonin gene-related peptide) is a 37-residue neuropeptide, which was first described in 1982 (*3*). This peptide is one of the most potent arterial and venous vasodilators found (*4*), as been reported in vitro (*5, 6*) and in vivo (*7*). CGRP is most often found in the central nervous system, where it acts as a neurotransmitter, and in the cardiovascular system, where it acts as a potent vasodilator (*8*). Its capacity to increase heartbeat rate and force of contraction, as well as its ability to decrease food intake, have also been widely described (*9*−*11*).

In addition to enzymatic hydrolysis, the use of microbials provides a natural technology applicable for the production of bioactive peptides. The potential of this approach is already well demonstrated by the presence of bioactive peptides in fermented dairy products (*12, 13*). Thus, antihypertensive, immunomodulatory, antioxidative, and antimicrobial peptides have been identified in fermented milk hydrolysates. The fermentation process usually involves natural or added microorganisms (starter cultures) and anaerobic conditions. During their growth, microorganisms hydrolyze sugars and proteins available in their surrounding medium, resulting in peptides with different amino acid sequences and bioactive properties. The degree of proteolysis is highly dependent on the bacterial species involved and the physical conditions of fermentation (*14*). In many studies, proteolytic starter cultures have also been used together with digestive enzymes, also being effective in the generation of short functional peptides (*15*). In the seafood area, fermentation has been used to improve the sensory and hygienic qualities of the end product (*16*), but rarely to obtain bioactive peptides. Most of the works published on this subject are about fermentation of fish sauces with halophilic bacteria. Ichimura et al. (*17*) found angiotensin I-converting enzyme (ACE) inhibitory activity in fermented anchovy sauce, as well as insulin secretion-stimulating activity in cultured insulinoma cells. The ACE inhibitory activity of fermented fish sauces made from salmon, sardine, or anchovy has also been reported by Okamoto et al. (*18*). Shan et al. (*19*) reported the presence of ACE inhibitory peptides in Arabesque greenling surimi fermented with *Lactobacillus delbrueckii*. Furthermore, the use of yeasts could be an interesting alternative for producing protein hydrolysates. Neklyudov et al. (*20, 21*) reported the use of enzymic extracts of *Saccharomyces* spp. for protein hydrolysis. Yeasts contain

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considerable amounts of hydrolytic enzymes, which can certainly be used after lysis for the production of protein hydrolysates with low and medium degrees of protein conversion. Protein hydrolysis would be improved if a yeast lysate is combined with exogenous enzymes. The use of yeast live cells after hydrolysis with proteases could also be an interesting alternative for producing protein hydrolysates.

As previous studies (data not shown) have shown that CGRP-like molecules can be obtained from industrial hydrolysis of saithe (*Pollachius virens*) byproduct derived from filleting, we primarily examined the effect of subsequent incubation with *Saccharomyces cerevisiae* live cells in aerobic conditions on the level of CGRP-like molecules in the hydrolysate. The hydrolysate was partially purified, and the immunoreactive material was further analyzed for its CGRP-like bioactivity using a specific radioreceptor assay. Furthermore, its capacity to induce a cAMP stimulation in rat liver membranes was also used to test its CGRP-like biological activity. Finally, partial sequencing of the bioactive peptide was performed to obtain the sequence of these active peptides.

MATERIALS AND METHODS

Chemicals. Human CGRP (hCGRP) was obtained from Bachem (Weil am Rhein, Germany). Labeled hormones (specific activity = 2000 Ci/mmol) were from GE Healthcare (Chalf-ont St. Giles, U.K.). The anti-human CGRP antibody was a generous gift from Dr. A. Jullienne from Paris, France. Male Wistar rats were obtained from Janvier breeding (Le Genest sur Isle, France). The enzyme inhibitor h-αCGRP$_{8-37}$ was obtained from Sigma Chemical (St. Louis, MO). Bovine serum albumin (Sigma Chemical) was heat-inactivated before radioreceptor assays. Other chemicals were of reagent grade.

Protein Hydrolysate. Protein hydrolysate of saithe was prepared by the enterprise COPALIS (Boulogne-Sur-Mer, France). Protein from saithe muscle (*P. virens*) was used as raw material. Alcalase 2,4 L (2.4 AU/g) from Novozymes (Bagsvaerd, Denmark) at optimum pH (8) and temperature (60 °C) was used for 60 min for protein hydrolysis. The quantity of Alcalase 2,4 L used was 1.7 g/100 g of protein. The enzyme was inactivated by boiling at 80 °C for 10 min, and the hydrolysate was sieved to remove traces of bone. The hydrolysis value (DH) of the process was 16−17%. The enterprise Fermensys SA (Dury, France) further treated the hydrolysate with a culture of *S. cerevisiae* for 24 h (30 °C, 400 rpm, 5 L of oxygen/min). The liquid was clarified by using a centrifuge to remove the insoluble residues and the biomasses (15000*g*, 20 min). The protein hydrolysate was then dried with a freeze-dryer.

Radioimmunoassay (RIA). Immunoreactive CGRP was measured following a previously described assay for hCGRP (*22*): in brief, an anti-CGRP antiserum at a final dilution of 1/200,000 was incubated with serial dilutions of synthetic human CGRP or saithe hydrolysate for 18 h at 30 °C. Then, ^{125}I-labeled hCGRP was added and incubation continued for 24 h at 4 °C. Bound and free hormones were separated by charcoal−dextran precipitation. Control (specific antibody omitted) tubes were incubated in each assay. The hydrolysate was assayed in triplicate at multiple dilutions. Results were expressed as the percentage of initial binding, $100 \times B/B_0$, where B_0 represents the binding of labeled peptide in the absence of unlabeled hormone. Linearization of the standard curves was achieved by plotting logit $[\ln(B/B_0)/[1 - (B/B_0)]]$ as a function of ln hormone or protein concentration. Only the straight lines that presented a slope similar to that obtained with the standard were considered to be positive, that is, containing CGRP-like molecules. The detection limit for the radioimmunoassay was 10 pg of immunoreactive peptides per tube.

Liver Membrane Preparation. Liver membranes were prepared using male Wistar rats according to the method of Neville until step 11 (*23*). Proteins were quantified via the Lowry method, using BSA as the standard (*24*).

Radioreceptor Assay (RRA). Receptor binding ability of immunoreactive molecules was developed using rat liver membranes and ^{125}I-labeled hCGRP. Incubations, in a 400 μL final volume, were performed at 22 °C for 1 h (*25*). At the end of the incubation, bound and free ligands were separated by centrifugation using a microcentrifuge of Beckman Coulter, Inc. (Fullerton, CA). Each batch was tested at least with four increasing protein concentrations, and only straight lines presenting slopes similar to that obtained with the standard hormone (10−100 pg/tube) were considered to be positive. The quantity of CGRP-like molecules was calculated and expressed as nanograms per microgram of protein. Receptor binding ability of each purified fraction (ED_{50}) was also determined and expressed as the quantity of protein (milligrams) that induced a 50% inhibition of the initial binding to rat liver membranes. The experiment was performed in triplicate.

Adenylate Cyclase Activity. The adenylate cyclase activity in rat liver membranes was determined by measuring the synthesis of cyclic AMP (cAMP) from non-radioactive ATP as previously described (*25*). The assay was initiated by the addition of membranes (12 μg) to preheated assay tubes (4 min at 30 °C) with sample or hCGRP. The tubes were then incubated for 30 min at 30 °C with Hepes−Tris buffer (25 mM, pH 7.4) containing 1 mM isobutylmethylxanthine (IBMX), 1.3 mM EGTA, 5 mM $MgCl_2$, 0.4 mg/mL BSA, 1 mM ATP, and 100 μM GTP. The buffer also contained 10 mM phosphocreatine and 0.8 mg/mL creatine phosphokinase as a regenerating system. After incubation, the reaction was terminated by the addition of 200 μL of Tris−EDTA buffer (pH 7.4), and the tubes were placed in a boiling water bath for 4 min. cAMP was quantified on 50 μL aliquots using the radioreceptor assay kit from GE Healthcare. The adenylate cyclase activity was expressed as picomoles of cAMP synthesized by 1 mg of membrane protein during 30 min of incubation. To demonstrate the specificity of the CGRP-like molecule action, the adenylate cyclase activity was also measured as described above, but in the presence of 0.5 μM h-αCGRP$_{8-37}$, a specific antagonist of CGRP-action (*26*). The experiment was performed in quadruplicate.

Purification of Saithe Hydrolysate. Saithe hydrolysate was prepurified by gel exclusion chromatography on an HW 40 Toyopearl column (2.5 × 33.5 cm) using 0.2 M ammonium acetate (pH 5) as eluant. The flow rate was 22 mL/h. The column was calibrated with the following molecular mass markers: aprotinin (6000 Da), hCGRP (3750 Da), and bacitracin (1411 Da). Aliquots were analyzed for CGRP immunoreactivity. Immunoreactive fractions were then analyzed using CGRP radioreceptor assay. Immunoreactive and biologically active fractions were subjected to reverse-phase HPLC on a Prosphere 300 C18 column (5 μm, 150 × 4.6 mm, Alltech Associates, Deerfield, IL), using a linear gradient of 10−60% acetonitrile in 0.1% TFA. The flow rate was 0.9 mL/min, and fractions were collected every minute. Optical density was measured at 214 nm. Immunoreactive fractions were subjected to radioreceptor assay, and positive fractions were then repurified using the same column and a linear gradient of 10−20% acetonitrile in 0.1% TFA.

Protein Determination. The protein concentration of each analyzed fraction was quantified using the bicinchoninic acid protein assay reagent from Interchim (Montluçon, France) using BSA as the standard (*27*).

Sequence Determination. The active fraction (70 μL) was diluted with 30 μL of 70% acetonitrile−0.1% TFA and further purified by reverse phase HPLC, primarily using a C18 column (1.2 mm diameter) with a 2−70% acetonitrile linear gradient in the presence of 0.1% TFA for 55 min. The flow rate was 60 μL/min, and detection was set to 214 nm. Peptides in the predominant peak were then separated on a Vydac C-18, 218 TP-52 column (2.5 × 250 mm, Vydac Corp., Hesperia, CA), using a linear gradient of 0−15% acetonitrile in 9 mM sodium acetate (pH 5.5) for 80 min. The flow rate was 80 μL/min. Detection was set to 214 nm. The main peak fractions were screened by automated protein sequencing using an Applied Biosystem precise sequencer. The database of the National Center for Biotechnology International (NCBI) was used for searching similarities of the sequences with others of known proteins.

Statistical Analysis. Variance analyses (ANOVA) were performed to determine the existence of significant differences between slopes (Statgraphics plus software). A value of $p < 0.01$ was considered to be statistically significant. ANOVA was also used to compare the

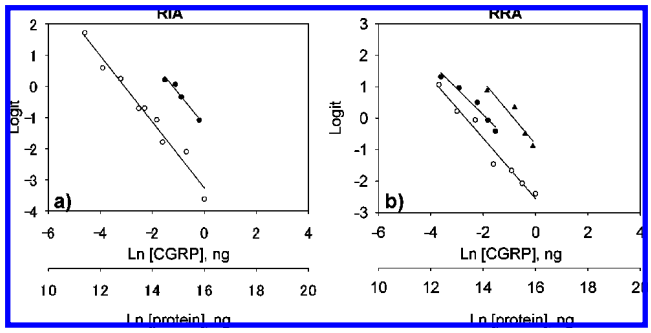

Figure 1. Radioimmunoassay and radioreceptor assay of saithe hydrolysate. (**a**) RIA: effect of increasing concentrations of saithe protein hydrolyzed with Alcalase plus incubation with *Saccharomyces cerevisiae* (●) and also of unlabeled hCGRP (○) on the binding of ^{125}I-CGRP to its antibody. Correlation coefficients for the different lines were $y = -1.06x - 3.26$ ($R^2 = 0.97$) and $y = -1.03x + 15.16$ ($R^2 = 0.96$) for unlabeled HCGRP and saithe hydrolysate, respectively. (**b**) RRA: effect of increasing concentrations of saithe hydrolysates on the binding of ^{125}I-CGRP to its receptors in rat liver membranes. Correlation coefficients for the different lines were $y = -0.96x - 2.55$ ($R^2 = 0.97$) for unlabeled HCGRP (○), $y = -0.99x + 15.05$ ($R^2 = 0.90$) for saithe protein hydrolyzed with Alcalase (▲), and $y = -0.82x + 11.65$ ($R^2 = 0.98$) for saithe protein hydrolyzed with Alcalase plus incubation with *S. cerevisiae* (●).

Table 1. Purification of CGRP-like Molecules Obtained from Saithe Hydrolysate

	proteins, μg	CGRP-like immunoreactivity, pg	specific activity,[a] pg/μg of protein	purification factor[b]	ED_{50},[c] mg of protein
crude extract	10070	550	0.055 ± 0.01		1.38
gel exclusion	1700	173	0.102 ± 0.02	1.85	1.19
HPLC 1 (10−60)	13	85	6.546 ± 0.48	119.02	0.12
HPLC 2 (10−20)	11	270	23.985 ± 2.87	436.1	0.06

[a] The specific activity represents the quantity of CGRP-like molecules (pg) per microgram of protein. [b] The purification factor is the ratio of the specific activity obtained at each purification step to that of the crude extract. [c] Results of ED_{50} express the quantity of proteins (mg) inducing a 50% inhibition of the CGRP-labeled binding.

adenylate cyclase activities obtained in the different experimental conditions ($p < 0.05$).

RESULTS

CGRP Radioimmunoassay and Radioreceptor Assay of Crude Extract. Saithe hydrolysate was resuspended in distilled water at a concentration of 0.5 g/mL and analyzed for the presence of CGRP-like molecules. By RIA, it was shown that the extract included immunoreactive molecules, due to the absence of significant differences between slopes of the straight lines obtained (**Figure 1a**). A further RRA demonstrated the ability of the CGRP immunorelated peptides to interact with the CGRP receptors in rat liver membranes (**Figure 1b**). The quantity of immunoreactive CGRP-like molecules in the hydrolysate (before incubation with *Saccharomyces* live cells) was 28.7 pg/mg of protein. The ED_{50} value, that is, the quantity of protein that produces an inhibition of 50% of the initial CGRP binding to their receptors, was obtained with 3.87 mg of protein. After incubation with *Saccharomyces* live cells, the quantity of CGRP-like molecules was increased to 55.28 pg/mg. The ED_{50} value was obtained with a lower quantity of protein (1.38 mg, **Table 1**).

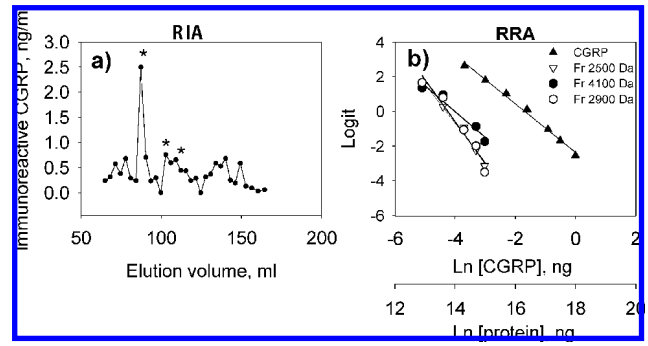

Figure 2. Gel exclusion chromatography of the siki hydrolysate. (**a**) RIA: CGRP immunoreactivity of fractions resulting from molecular sieving of saithe hydrolysate on a HW Toyopearl column. An asterisk (∗) indicates fractions used in the subsequent radioreceptor assay. (**b**) RRA: effect of increasing concentrations of the immunoreactive fractions on the ^{125}I-CGRP binding to its receptor in rat liver membranes. Correlation coefficients for the different lines were $y = -1.40x - 2.87$ ($R^2 = 0.99$, hCGRP), $y = -1.49x + 20.79$ ($R^2 = 0.95$, fraction 4100 Da), $y = -2.41x + 33.22$ ($R^2 = 0.96$, fraction 2900 Da), and $y = -2.24x + 30.74$ ($R^2 = 0.98$, fraction 2500 Da).

Molecular Sieving. The hydrolysate was subjected to gel filtration chromatography to partially purify the CGRP-like molecules. By this technique, the peptides included in the hydrolysate were separated according to their apparent molecular mass. The different fractions collected were further analyzed using CGRP-radioimmunoassay. A prominent peak, corresponding to the fraction eluted at 87 mL, was observed (**Figure 2a**). That fraction included molecules of around 4100 Da, slightly higher than that of the hCGRP (approximately 3500 Da). **Figure 2a** shows two other peaks, at 103 and 109 mL of elution. They contained a lesser quantity of immunoreactive material and corresponded to fractions including molecules of around 2900 and 2500 Da, respectively. Only the molecules included in the fraction eluted at 87 mL were able to interact with the corresponding receptors in rat liver membranes [slope (a) = −1.49], as the slopes corresponding with the other fractions ($a = -2.41$ and -2.24 for fractions 2900 and 2500 Da, respectively) were significantly different from that of hCGRP ($a = -1.40$, **Figure 2b**). The specific activity of the bioactive molecules in the fraction of 4100 Da was 2-fold higher after molecular sieving (**Table 1**). Similarly, the quantity of protein inducing a 50% inhibition of the CGRP binding to its receptors (ED_{50}, **Table 1**) decreased after exclusion chromatography (1.19 mg).

Adenylate Cyclase Assay. A wide variety of biological effects on various tissues, including the liver, has been reported for CGRP (*28*). The increase of cyclic adenosine monophosphate (cAMP) through protein G coupling to CGRP receptors is the main activation pathway of CGRP in cells. To demonstrate that the partially purified CGRP-like molecules from saithe hydrolysates were fully biologically active, we analyzed the effect of these molecules on the stimulation of cAMP production in rat liver membranes.

The activation of the CGRP receptors with the CGRP-like molecules produced the stimulation of the adenylate cyclase activity and the subsequent production of cAMP (**Figure 3a**). Increasing protein concentrations between 0.5 and 5 μg induced a dose-related stimulating activity. With 0.5 μg of protein, the stimulated adenylate cyclase activity represented a 282-fold increase over the control sample. However, the stimulation induced by 0.5 μg of protein was similar to that induced by 0.1 ng of hCGRP (**Table 2**). A quantity of protein of 0.5 μg included

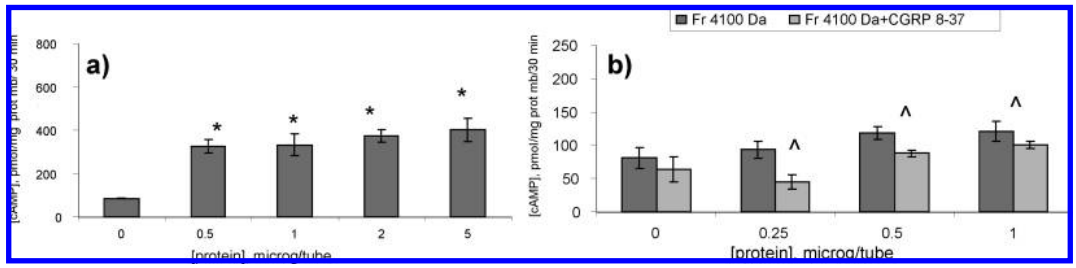

Figure 3. Adenylate cyclase activity of the purified molecules. (**a**) Effect of CGRP-immunorelated molecules obtained after gel filtration chromatography on the CGRP-stimulated adenylate cyclase activity in rat liver membranes. Each bar represents the mean ± standard deviation of one experiment performed in quadruplicate. (∗) $p < 0.05$ when compared to the basal level. (**b**) Inhibitory effect of 0.5 μM h-αCGRP$_{8-37}$ on the stimulation of the adenylate cyclase activity mediated by the CGRP-like molecules presented in the 4100 Da fraction. (^) $p < 0.05$ when compared in samples with or without h-αCGRP$_{8-37}$.

Table 2. Capacity To Stimulate cAMP Production of either the Partially Purified CGRP-like Molecules Included in the Hydrolysates or hCGRP

protein, μg/tube	partially purified CGRP-like molecules, pg/tube	cAMP, pmol/mg of protein
0	0	85 ± 1.1
0.5	0.06	326.0 ± 30.8
1	0.10	332.8 ± 49.6
2	0.20	373.2 ± 50.4
5	0.51	401.9 ± 54.3
	100 (pg of hCGRP/tube)	368.2 ± 57.5
	200 (pg of hCGRP/tube)	507.4 ± 8.42

0.05 pg of CGRP-like molecules (**Table 1**), which suggests the cAMP production mediated by the CGRP-like molecules was at least as intensive as the effect induced by the hCGRP (**Table 2**). However, although the reaction appears to be saturated at low concentrations of CGRP-like molecules, a higher quantity of cAMP was finally obtained when the reaction was mediated by hCGRP.

To demonstrate the specificity of this effect, the same experiments were performed in the presence and absence of the antagonist h-αCGRP$_{8-37}$ (**Figure 3b**). Concentrations between 0.25 and 1 μg of protein were used. The antagonist interfered with the binding between the CGRP-like molecules and the specific receptors in rat liver membranes, suppressing the hormone-induced adenylate cyclase activity. This effect was significant when 0.25 and 0.5 μg of protein were used; these represented inhibitions of cAMP production of 52 and 26%, respectively.

Purification by High-Performance Liquid Chromatography (HPLC). HPLC was used for purifying the CGRP-like molecules. First, a linear gradient with 10−60% acetonitrile−TFA was used for 60 min. The immunoreactive profile showed five main peaks (∗), at elution times of 19, 24, 27, 32, and 34 min (**Figure 4**), which were further analyzed using the CGRP radioreceptor assay. Only peptides eluted after 24 min were able to interact with the corresponding receptors in rat liver membranes, as shown in **Figure 5a**. Fractions 27, 32, and 34 did not show cross-reaction. The purification factor obtained was calculated as being close to 119, and the quantity of immunorelated molecules was around 6.5 pg/μg of protein (**Table 1**); that is, the concentration of CGRP-like molecules increased by >100 times after purification by HPLC, when compared to the value for the crude extract. The ED$_{50}$ was also lower (0.12 mg of protein), as shown in **Table 1**.

The fraction eluted after 24 min was subjected to a second chromatography. A linear gradient of 10−20% of acetonitrile−0.1% TFA was used for 60 min (**Figure 6**). Two immunoreactive peaks were observed, corresponding with

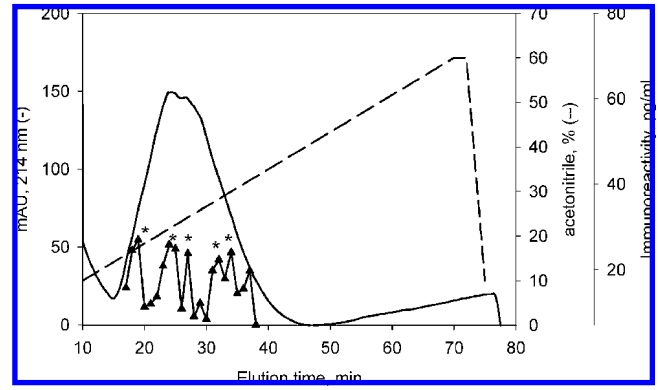

Figure 4. Elution profile on C18 protein/peptide HPLC column of the immunoreactive molecules obtained after molecular sieving. A linear gradient (10−60% acetonitrile in 0.1% TFA) was used during 60 min. CGRP-like molecules (▲) were determined on an aliquot from each fraction.

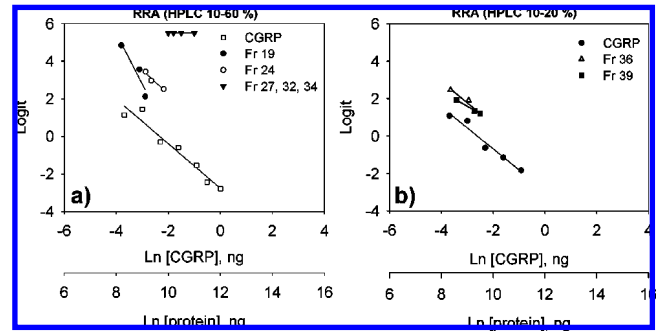

Figure 5. Radioreceptor assay of the immunoreactive fractions obtained after HPLC. (**a**) Effect of increasing concentrations of the immunoreactive fractions obtained after the first HPLC on the ^{125}I-CGRP binding to its specific receptors. Correlation coefficients for the different lines are $y = -1.19x - 2.77$ ($R^2 = 0.95$, hCGRP), $y = -2.71x + 27.14$ ($R^2 = 0.90$, fraction 19), $y = -1.29x + 15.13$ ($R^2 = 0.95$, fraction 24). (**b**) Effect of increasing concentrations of the immunoreactive fraction obtained after HPLC 10−20% acetonitrile in 0.1% TFA (fraction 24) on the ^{125}I-CGRP binding to its specific receptors. Correlation coefficients for the different lines are $y = -1.12x - 2.92$ ($R^2 = 0.96$), $y = -1.15x + 12.10$ ($R^2 = 0.91$), and $y = -0.82 + 8.93$ ($R^2 = 1$) for unlabeled hCGRP, fractions 36 and 39, respectively.

fractions eluted after 36 and 39 min (**Figure 6**). The quantities of immunoreactive molecules (**Table 3**) were similar in both fractions (approximately 30 pg/μg of protein). After RRA of these fractions (**Figure 5b**), a 50% inhibition of the initial binding of unlabeled hCGRP to its receptors was observed with only 0.04−0.06 mg of protein (**Table 3**). Both fractions were mixed together, and one aliquot was selected for further

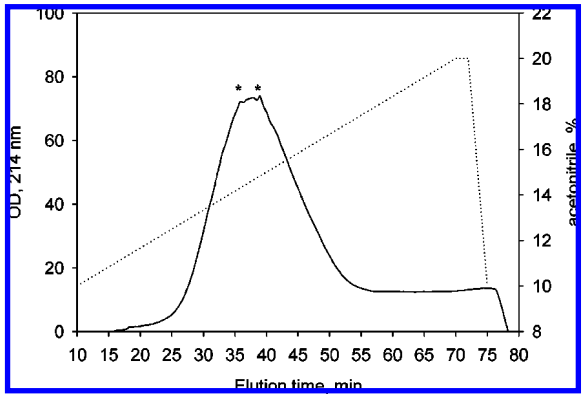

Figure 6. Elution profile after subjecting the CGRP-like molecules obtained from the first HPLC chromatography to a linear gradient of 10−20% acetonitrile−TFA for 60 min. An asterisk (∗) indicates fractions used for testing CGRP-RRA.

Table 3. Specific Activity and ED_{50} of the CGRP-like Molecules Included in Fractions Eluted at 36 and 39 mL after Injection of Semipurified Sample in HPLC[a]

fraction	specific activity, pg/μg of protein	ED_{50}, mg of protein
36	27.99 ± 3.24	0.04
39	31.24 ± 3.90	0.06

[a] A linear gradient of acetonitrile−0.1% TFA 10−20% was used for 60 min.

sequencing. The remaining aliquot was subjected to RRA, obtaining an average quantity of CGRP-like molecules of around 24 pg/μg of protein (**Table 1**). The final ED_{50} value was 23-fold lower than that obtained with the crude extract.

Sequence Determination. The obtained mixture was further purified by successive reverse phase HPLC. A 2−70% acetonitrile−0.1% TFA gradient was first used. One prominent peak eluted at 25−27% acetonitrile was observed (**Figure 7a**), which was then purified using a linear gradient of 0−15% acetonitrile in 9 mM sodium acetate (**Figure 7b**). Two major peaks were obtained, eluting at 9.5 and 11.7% acetonitrile. These two peptides were then sequenced using an Applied Biosystem sequencer. The two N-terminal sequences obtained from the main peaks (highlighted with symbols ^ and ∗) were VAPEE-HPT (∗) and PEDVI (^). From a search in the NCBI database, the first sequence showed homology with α-actin of several fish species, being rainbow trout (*Oncorhynchus mykiss*, accession no. AAM28592), red seabram (*Pagrus major*, accession no. BAF80059), rock cod (*Epinephelus coioides*, accession no. AAW29030), carp (*Cyprinus carpio*, accession no. AAR04426), and Nile tilapia (*Oreochromis niloticus*, accession no. ABN58897). This suggests that the bioactive peptide came from saithe actin muscle. Significant homology was found between the second sequence (PEDVI) and the myosin regulatory light chain of turbot (*Scophthalmus maximus*).

DISCUSSION

In this work, we have shown that it is possible to obtain CGRP-like molecules from fishery products by hydrolysis and subsequent treatment with *S. cerevisiae*. Obtaining CGRP-like molecules with promising therapeutic properties is extremely significant, as the possible use of CGRP as a therapeutic weapon is mainly limited by its short half-life in mammalian plasma and cost (*29*). Korhonen et al. (*30*) reported the use of protein hydrolysis for obtaining peptides with different activities, such as opioid, antimicrobial, or antihypertensive. In research of dairy products, lactic acid bacteria are also used to obtain certain

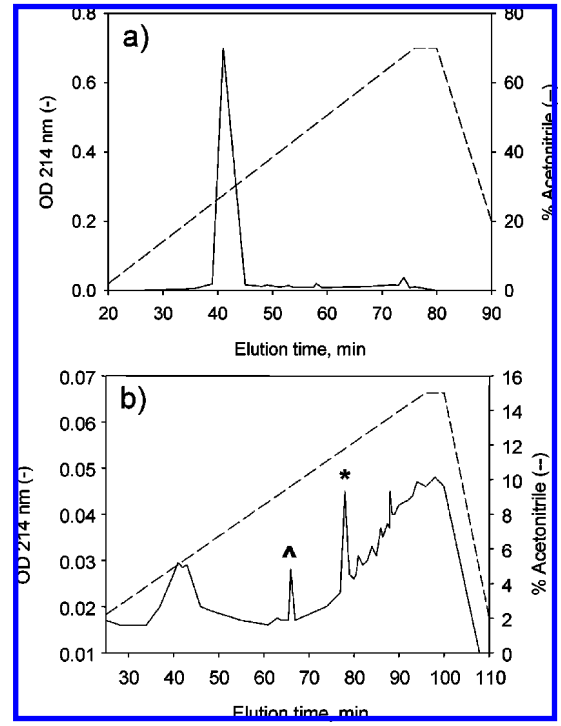

Figure 7. Elution patterns in HPLC fractionation of the active fraction. First, a linear gradient of 2−70% acetonitrile in the presence of 0.1% TFA was used (**a**). The predominant peak was then purified (**b**) using a Vydac column C18 218-TP52 and a linear gradient of 0−15% acetonitrile in 9 mM sodium acetate, pH 5.5. Predominant peaks (^ and ∗) were further sequenced using an Applied Biosystem sequencer.

bioactive molecules, such as ACE-inhibitory, opioid, or immunomodulatory peptides (*31*). However, the use of lactic acid bacteria is rare in the area of fishery products, halophilic bacteria mainly being used to obtain fish and shellfish sauces by fermentation. The process is of high interest for consumers, as it has some influence on the taste and flavor of the final product. The process could also be very useful for obtaining bioactive molecules, a theory supported widely, for which high concentrations of NaCl and long times of fermentation have been used. Recently, Je et al. (*32*) isolated an ACE inhibitory peptide derived from fermented blue mussel sauce. In this study, a treatment with yeasts improved the yield of production of CGRP-like molecules. Yeast extracts are more used than live cells for preparing protein hydrolysates. The extracts include a pool of proteolytic enzymes that allows the obtaining of protein hydrolysates with low and medium degrees of conversion. Neklyudov et al. (*20, 21*) used *Saccharomyces carlsbergensis* as a source of enzyme preparation for hydrolyzing blood proteins, obtaining a yield of blood hydrolysate of 11%. Despite cultures of *Saccharomyces* spp. not apparently being used for hydrolyzing fish protein, their use may be interesting because they can also hydrolyze protein. However, the yield obtained would be lower. Faid et al. (*33*) also used a culture of *S. cerevisiae* for making fish waste silage. They observed transformation of protein nitrogen to nonprotein nitrogen after 10 days of fermentation. Although we used conditions different from those reported by Faid et al. (*33*), we think it is also possible the yeast induced proteolysis on our saithe hydrolysate, improving production of CGRP-like molecules. Induction of small modifications in the bioactive peptides could not be dismissed. From 10070 μg of protein present in the crude extract, we obtained 11 μg of CGRP-like molecules after purification. These molecules were obtained with a purification factor of 436.

The apparent molecular mass of those molecules (about 4100 Da) is slightly higher than that of hCGRP (3500 Da), but they possess similar biological activities. The stimulation of adenylate cyclase activity is higher with lower doses of protein. Interestingly, the molecules appear to be more active than hCGRP, which could be very interesting for the pharmaceutical industry. However, the reaction is rapidly saturated, and a higher quantity of cAMP is produced by stimulation of adenylate cyclase activity, mediated by human CGRP. We have also reported the obtaining of CGRP-like molecules from the industrial hydrolysis of siki (*2*), shrimp, cod, and sardine protein (*1*). The CGRP-like molecules obtained after hydrolysis and subsequent fermentation of saithe protein are different from those obtained after the hydrolysis of siki protein. The molecular mass is different, as the bioactive molecules present in the saithe hydrolysates are bigger (4100 Da, in contrast to 1500 Da). Their roles, however, are very similar, as both could interact with the same receptors and induce cAMP production.

With regard to the partial sequence of the bioactive molecules, the main similarity of the sequences VAPEEHPT and PEDVI to α-actin and myosin of different fish species suggests that we could obtain a similar peptide by using the same production process and another source of fish muscle. This could be of interest, as nontarget fish species or different fish byproducts could be used to obtain those bioactive molecules. Although limited partial sequencing of the CGRP-like peptide did not show the same resemblance with the sequence of hCGRP, its structure could be important for binding with CGRP receptors. In this sense, a valine residue is highly conserved in CGRP of human and rat at the end of the α-helix (residue 23), whereas residues P (proline) and T (threonine) coincide with residues 25, 29, and 30, respectively, of rat amyline, a molecule with strong similarities to human and rat CGRP (*28, 34*). Besides the presumptive therapeutic effect of the CGRP-like molecules, it is worth mentioning that the sequence VAP, which was found within the sequence VAPEEHPT, could have, after protein digestion, potential antihypertensive activity by different pathways. The sequence VAP, related with ACE-inhibitory properties (*35*), can exert an antihypertensive effect. In addition, that inhibitory effect could hinder the formation of angiotensin II mediated by ACE, which would indirectly permit CGRP release in humans (*36*).

In summary, in this work we have demonstrated that it is possible to produce highly active CGRP-like molecules via fish protein hydrolysis and subsequent treatment with *S. cerevisiae* live cells. The production of protein hydrolysates including these types of CGRP-like molecules may potentially be very interesting for the food or pharmaceutical industry. Further studies could be based on the search of the effect in vivo of this hydrolysate.

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