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Purification and Characterization of the Main Pepsinogen from the Shark, *Centroscymnus coelolepis*

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The main pepsinogen from the mucosa of the shark, *Centroscymnus coelolepis*, has been purified and characterized. This zymogen, the most abundant protein in terms of quantity and activity (yield 72%), is a homogeneous monomer of molecular weight 42±0.7 kDa, as determined by electrophoresis. The aspartyl proteinase nature of this enzyme was confirmed by the considerable inhibition by pepstatin. Its specificity as to the oxidized B-chain of bovine insulin was determined using electrospray ionization mass spectrometry (ESI-MS) coupled with reversed phase high pressure liquid chromatography (RP-HPLC). The 15-16 Leu-Tyr bond was rapidly cleaved in this substrate, followed by the 24-25 Phe-Phe, 25-26 Phe-Tyr, and 11-12 Leu-Val bonds.

Key words: characterization, pepsin, shark, specificity.

Pepsinogens are inactive precursors of pepsins. These zymogens are synthesized and secreted by the gastric mucosa, and converted into the corresponding pepsins under acidic conditions. They are known to be widely distributed in all vertebrates, *i.e.* both mammals and fishes, and to be responsible for the preliminary digestion of proteins. Sharks are carnivorous fish with an enormous digestive capacity (*1*), but their pepsins and pepsinogens have not been very well studied. In the literature, only some immunochemical studies (*2*), or structural information such as the N-terminal amino acid sequence of the main pepsinogen from shark *Squalus acanthias* (*3*) and the amino acid composition of dogfish *Mustelus canis* pepsinogens (*4*) could be found. Certain catalytic properties, such as the activity towards casein, of pepsins from the dogfish, *Scyliorhinus canicula* (*5*), have also been investigated.

In this paper we report basic characterization of the main pepsinogen isolated from the gastric mucosa of the shark, *Centroscymnus coelolepis*, and describe the catalytic specificity of this enzyme, in particular as to the oxidized B-chain of bovine insulin [a long-chain peptide most widely used to compare the specificities of proteinases (*6, 7*)].

MATERIALS AND METHODS

Purification of Pepsinogens from Shark Stomach—Sharks (*C. coelolepis*) were captured in the North Atlantic Ocean and shipped to Lorient in Brittany (France), where their stomachs were excised and stored at −70°C. The purification procedure was essentially based on a modification of that described by Tanji *et al.* (*8*) for the purification of tuna pepsinogens. In our protocol, instead of the gel filtration on Sephadex G-150, chromatography on poly-L-lysine/agarose (1.8 cm×40 cm; Sigma) was carried out. The gel was equilibrated with 0.02 M triethanolamine/HCl, pH 7.5, and eluted with a 0-1 M NaCl linear gradient in the same buffer. As the last step, the main pepsinogen (fraction 2) was fractionated further on a Mono Q HR5/5 column (Pharmacia) to increase the purification. The enzyme obtained at this step was used to perform all the following characterization. The purity of protein solutions was checked by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS/PAGE) according to Laemmli (*9*). A protein sample was incubated with pepstatin (pepstatin/protein at 3 : 1 mol/mol) before being processed and subjected to electrophoresis.

Assaying of Proteolytic Activity and Protein Concentration—Proteolytic activity was determined as described in Ref. *10*, *i.e.* a modification of Anson method (*11*), using a 2.5% solution of hemoglobin as the substrate at pH 2 and 37°C. One unit of enzyme activity was defined as the amount of enzyme necessary to cause an increase of 0.001 in absorbance at 280 nm per minute. Protein concentrations were determined by the method of Bradford (*12*) using crystalline bovine albumin as the standard.

Amino Acid Analysis—Electrophoretically pure freeze-dried enzyme (200 μg) was hydrolyzed in 6 M HCl for 24 h at 120°C. The amino acid composition was then determined by HPLC, with precolumn derivatization with *o*-phthalaldehyde (OPA) (*13, 14*). The derivatized products were separated on a Resolve column (C18 5 μ 3.9×150 mm; Waters), and detected as to fluorescence (excitation 335 nm and emission 420 nm). Cysteine was determined as cysteic acid after performic acid oxidation prior to hydrolysis,

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whereas tryptophan was determined after hydrolysis of the protein in methanesulfonic acid. The analysis and calculation were carried out automatically with a Baseline 8100 Station (Waters).

N-Terminal Sequence—Polyacrylamide gel electrophoresis (10%) according to Laemmli (*9*) in the presence of SDS of approximately 10 nmol of the protein was carried out using a vertical electrophoresis unit (Mini-Protean II, Bio-Rad). Proteins were then transferred from the gel to an Immobilon P membrane (Millipore) according to the tank transfer protocol described by Mansfield (*15*). The proteins transferred were then visualized with Coomassie Blue. After air drying, the protein band materials were subjected to automated Edman degradation with an Applied Biosystems Procize 492 protein sequencer.

Catalytic Activity—Inhibition experiments: The pepsinogen was activated as in Ref. *16* and diluted with 0.1 M phosphate buffer, pH 7, to obtain a final concentration of 500 U/ml after activation. Aliquots (200 μl) were then incubated for 10 h at 5°C with the following inhibitors: iodoacetamide, PMSF, 1,10 phenanthroline (9 μM) and pepstatin (9 μg/ml or 13 μM), prior to measurement of the residual activity. The inhibitory effect of pepstatin was also studied at different concentrations. Dialysis against EDTA (10 mM) and Chelex 100 resin was also performed, and the remaining enzymatic activity checked.

Specificity: In order to determine the splitting specificity of the enzyme as to a protein substrate, the oxidized B-chain of bovine insulin was used (*6, 17-19*). To a 1 ml solution of the oxidized B-chain of insulin (1 mg/ml, pH 1.3) was added 50 μl of a pepsinogen (activated) solution (200 U/ml). The mixture thus obtained was incubated at 37°C. After 5, 10, 20, 40, or 90 min, 200 μl of the solution was withdrawn, to which was added 5 μl of a 100 μg/ml pepstatin in DMSO solution. The hydrolysis products were then analyzed by HPLC coupled with ESI-MS (electrospray ionization mass spectrometry). In this experiment, peptides were separated on a Nucleosil column (C18 AB 5 μ 2.1 × 125 mm; Macherey-Nagel) using a 0-60% acetonitrile containing 0.1% TFA linear gradient over 35 min. Elution was performed at the flow rate of 0.2 ml/min and 40°C. The eluted peaks were detected as to the absorbance at 214 nm and analyzed by ESI-MS.

The mass spectrometer, API I Sciex (Thornhill, Ont., Canada), was a single-quadrupole mass spectrometer equipped with an atmospheric pressure ionization ion source. In the experiment, it was operated in the positive mode. Multiply charged peptide ions were generated by spraying the sample solution through a fused silica capillary of 75 μm ID, introduced into a stainless steel capillary held at high potential. The voltage of the sprayer was set between 5 and 5.5 kV. A coaxial air flow along the sprayer was provided to assist the liquid nebulization, and the nebulizer pressure was maintained in the range of 0.3-0.4 MPa. For the infusion experiment, the sample was delivered to the sprayer by means of a syringe infusion pump (Model 22; Harvard Apparatus, South Natick, MA, USA), whose liquid flow rate was set at 5-10 μl/min. For analyses by HPLC coupled with ESI-MS, 15% of the mobile phase was diverted to the mass spectrometer and 85% to the UV detector by means of a low dead volume connection permitting straightforward correlation of the total-ion current (TIC) trace with the UV trace. The interface between the sprayer and the mass analyzer was a 100 μm diameter conical orifice. The potential at the orifice was 80 V and a gas curtain formed by a continuous flow (0.8-1.2 liter/min) of nitrogen in the interface region served to break up any clusters. The instrument m/z scale was calibrated with ammonium adduct ions of polypropyleneglycol. All peptide mass spectra were obtained from the averaged signals of multiple scans. HPLC-ESI-MS experiments were performed while scanning m/z from 400 to 2,400 with a 0.33 Da step-size and a 0.5 ms dwell time. UV absorbance was detected simultaneously with the MS signal and registered with Tune 2.0 software Sciex. Molecular masses were determined from the measured m/z values for the protonated molecules. Data were acquired with an Apple Macintosh Quadra 900 and were processed using the software package, Mac Spec 3.2 Sciex. A reconstructed molecular mass profile was obtained by using a deconvolution algorithm (Mac Spec 3.2 Sciex).

RESULTS AND DISCUSSION

Purification of Shark Pepsinogen and Autolysis Effect—The results of purification are summarized in Table I. In this purification protocol, most of the impurities present were eliminated by poly-L-lysine/agarose chromatography. Proteolytic activity (94%) was retained on the column and eluted as one major peak at 0.5 M NaCl. Next, pepsinogens were separated into three protein components, namely fractions 1, 2, and 3, by Q-Sepharose chromatography. However, the preparation was not pure even after purification on a Mono Q column (Fig. 1A). When pepstatin was added to fraction 2 (enzyme : pepstatine = 1 : 3), it was possible to isolate an electrophoretically homogeneous purified protein by chromatography on Mono Q (Fig. 1B).

The molecular weight of the main pepsinogen was thus

TABLE I. **Purification of the main pepsinogen from the shark, *Centroscymnus coelolepis*.** One unit (U) of enzyme activity is defined as the amount of enzyme necessary to cause an increase of 0.001 in the absorbance at 280 nm per min under the assay conditions (37°C, pH 2).

Purification step	Total activity (U×10³)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Raw extract	231.0	1,650.0	140	100.0	1
$(NH_4)_2SO_4$	239.4	342.0	700	103.6	5
DEAE-cellulose	228.0	285.0	800	98.7	6
Poly-L-lysine	216.0	108.0	2,000	93.5	14
Q-Sepharose					
Fraction 1	14.9	4.9	3,040	6.4	
Fraction 2	170.6	39.0	4,375	73.9	31
Fraction 3	12.7	12.0	1,060	5.5	
Purification of pepsinogen from fraction 2 on Mono Q	167.2	33.0	5,068	72.4	36

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determined to be 42 ± 0.7 kDa, and the contaminants were found to be proteins of 37 ± 0.6 and 30.5 ± 0.5 kDa on SDS/PAGE according to Weber and Osborn (*20*). No multimeric form was observed when the enzyme was analyzed by electrophoresis on a native 10% polyacrylamide gel (without SDS) (*21*). This result was not surprising as animal pepsinogens were known to be monomeric and to have a typical molecular weight of about 40 kDa (*22*). After one year storage at −20°C, the intensities of the minor bands increased, indicating autodigestion. When the proteins were kept at room temperature (20°C) and pH 7, the protein band at 42 kDa disappeared whereas the intensity of the 37 kDa protein increased and other protein bands of lower molecular weight also became visible. This process was rapid (Fig. 2, A and B), only 8 min at ambient temperature and less than 5 min at 37°C being sufficient to observe the complete conversion of the 42 kDa protein into components of minor molecular weights. Under more acidic conditions, it was even faster. We always observed diffusion of proteins on electrophoresis and no clear protein band was visible if the sample was incubated at pH 2. However, this degradation process did not influence the enzyme activity very much. At pH 7 and 37°C, the enzyme lost half of its original activity in 50 min. At the same temperature, this half life time became 40 min at pH 6 and 20 min at pH 4. At pH 2, only 5 min was necessary. In any case, the activity loss became significant only after intense degradation, by which the proteins were so hydrolyzed that no clear band could be observed on electrophoresis. But the activated enzyme lost all its activity at pH 8. The autolysis of pepsinogens is well known, and has been studied between pH 2 and 5 thoroughly (*22*). The *Centroscymus* pepsin was very active on its own molecule and the conversion could be observed only at the limit of denaturation. In fact, in all the electrophoresis tests, if the enzyme was not inactivated by the addition of pepstatin, the sample preparation and electrophoresis time were sufficient to cause advanced degradation, and on the gel we only observed the diffusion of proteins or a peptide band to the migration front.

Amino Acid Composition and N-Terminal Sequence— Like the pepsinogens from other species, *C. coelolepis* pepsinogen has a high proportion of acidic residues (33 Asp + 46 Glu of a total of 351 residues). However, the relatively high basic/acidic ratio was found to be due to the high content in Arg. A similar result was obtained with pepsinogen from the dogfish, *M. canis*. On comparison with other pepsinogens, *C. coelolepis* pepsinogen was found to be poor in hydroxyl residues. The number of Trp residues present (14) was particularly high as this residue generally exists in very low proportions in proteins.

N-Terminal analysis gave the sequence, Leu-His-Arg-Val-Pro-Leu-His-Lys-Gly-Lys, for the 42 kDa protein. This sequence is in fact very well conserved in aspartyl proteinases, but some positions exhibit general variation for conservation of the hydrophobicity (Lys instead of Arg or Ile instead of Val). The two histidine residues in this sequence are therefore somewhat particular. One generally finds an Ile or Val residue (aliphatic) at each of these positions. In fact, the N-terminal sequence of the *S. acanthias* pepsinogen (*3*) was practically the same as that of the *C. coelolepis* one, but the two His of the latter were replaced by two Ile. The sequence most matching the new one was that of cat chymosin, in which the first histidine is replaced by a threonine and the second one is conserved. These two positions in bovine prochymosin were Thr and Tyr, both of which possess a hydroxyl function. Further investigations are necessary to elucidate the evolution of

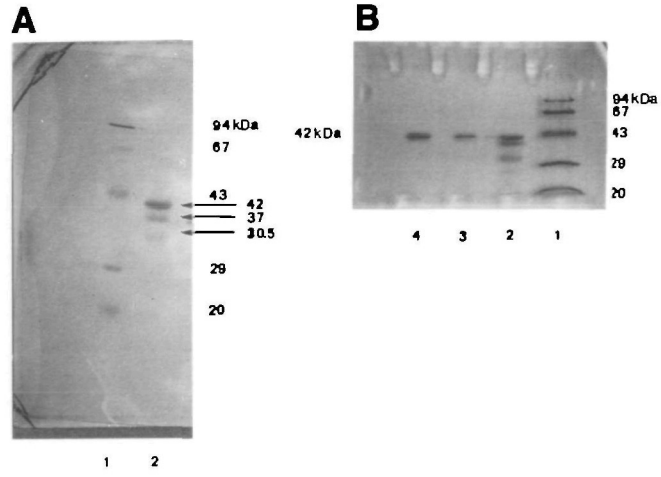

Fig. 1. **SDS/PAGE of *Centroscymnus coelolepis* pepsinogen.** A: Lane 1, markers; lane 2, shark pepsinogen after purification on a Mono Q column. B: Lane 1, markers; lane 2, shark pepsinogen after one year storage at −20°C; lanes 3 and 4, purification of shark pepsinogen on Mono Q in the presence of pepstatin in the sample (pepstatin:enzyme = 3 : 1 mol/mol) and in the eluent buffer (13 μM), respectively.

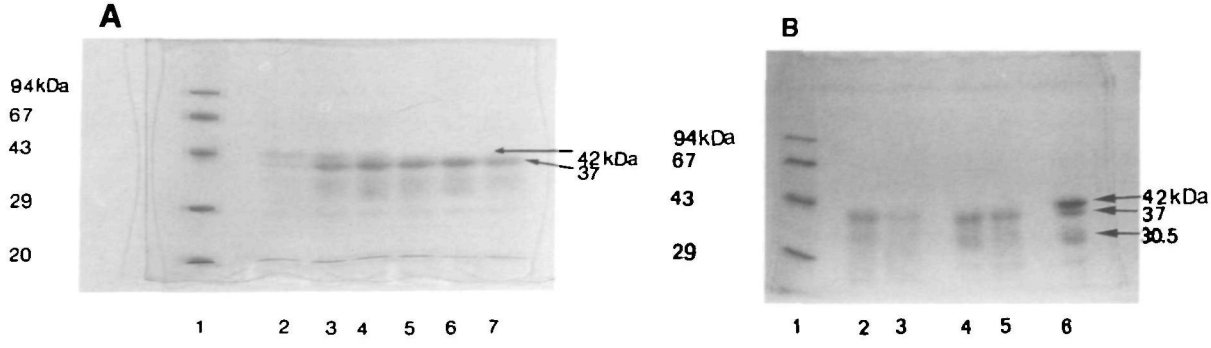

Fig. 2. **Autolysis of *Centroscymnus coelolepis* pepsinogen.** A: Lane 1, markers; lane 2, 2′, 20°C; lane 3, 5′, 20°C; lane 4, 7′, 20°C; lane 5, 8′, 20°C; lane 6, 9′, 20°C; lane 7, 10′, 20°C. B: Lane 1, marker; lane 2, 30′, 20°C; lane 3, 30′, 37°C; lane 4, 10′, 37°C; lane 5, 5′, 37°C; lane 6, shark pepsinogen after one year storage at −20°C.

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TABLE II. **Amino acid compositions of pepsinogens from *Centroscymnus coelolepis* and other species.**

Amino acid	Shark *Centroscymnus coelolepis* pepsinogen	Dogfish *Mustelus canis* pepsinogen A (*4*)	Tuna *Thynuus orientalis* pepsinogen (*8*)	Porcine pepsinogen A (*23*)	Bovine prochymosin (*24*)
Lys	15	14	11	10	6
His	4	7	4	3	5
Arg	12	14	8	4	8
Asp	33	44	34	44	37
Thr	15	23	23	26	24
Ser	32	43	37	46	35
Glu	46	39	42	28	39
Pro	20	19	13	19	16
Gly	35	40	35	35	32
Ala	12	18	22	19	17
1/2-Cys	8	7	6	6	6
Val	18	23	25	23	26
Met	5	7	7	4	8
Ile	20	22	22	25	22
Leu	25	28	18	33	29
Tyr	13	19	16	17	22
Phe	24	17	17	15	19
Trp	14	5	7	5	4
Total	351	389	347	362	373
M_r	41,500	41,500	37,800	41,000	40,777
Basic/acid	0.39	0.42	0.30	0.24	0.25
Glu/Ile	2.30	1.77	1.91	1.12	1.77
Phe/Ile	1.2	0.77	0.77	0.68	0.86
Trp/Ile	0.70	0.23	0.32	0.20	0.18

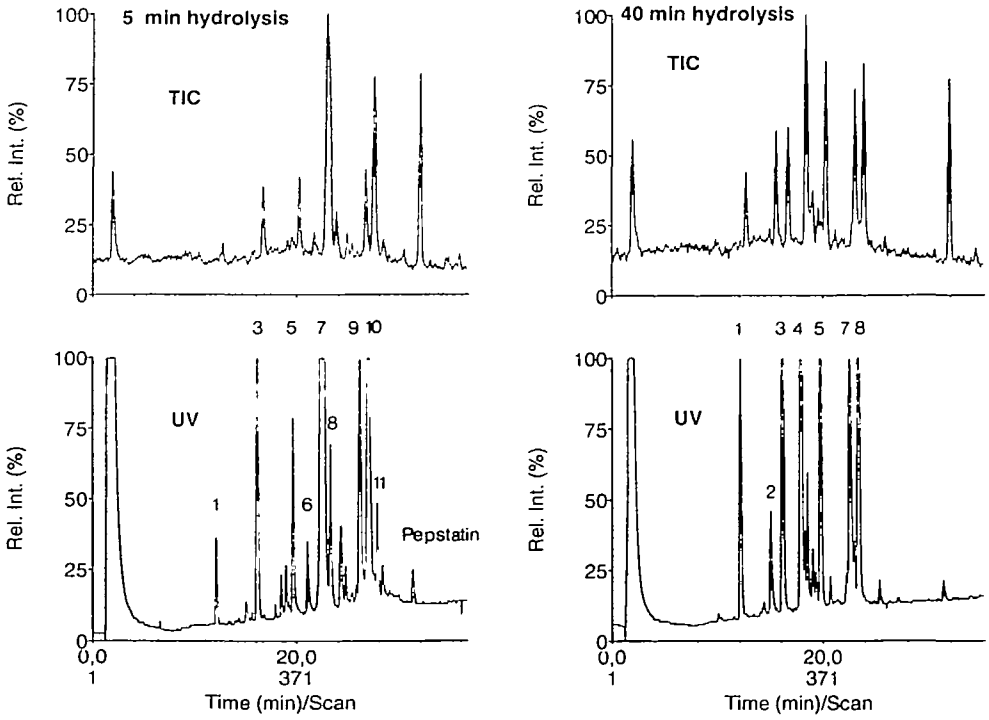

Fig. 3. **HPLC of insulin B hydrolysates.** The oxidized B-chain of bovine insulin (1 mg/ml) was incubated with *Centroscymnus coelolepis* pepsin (10 U/ml). Aliquots were withdrawn after 5 and 40 min, and then separated by HPLC on a Nucleosil C18 column. Peptides were detected as to UV 214 nm and by mass spectrometry (TIC).

this enzyme, as its activity towards casein was found to be similar to that of bovine chymosin in our preliminary tests (results not shown). As for the 37 kDa protein, its N-terminal sequence was found to be Glu-Ser-Thr-Glu-Pro-Met-Ile-Asn-Tyr-Leu-Asp-Met, which most matches those of cathepsins.

Catalytic Activity—For hemoglobin, the optimal pH for the hydrolytic activity of the enzyme was determined to be 1.5, which is slightly lower than the common value in the literature (*8, 23-25*). Approximately 50% of this optimal activity was lost at about pH 2.6 and very low activity was detected at pHs higher than 3. The temperature dependency of shark pepsinogen was a classic one with maximum activity at 55°C.

The aspartyl proteinase nature of the *C. coelolepis* pepsinogen was confirmed by the total inhibition by pepstatin. The influence of the inhibitors tested is shown in Table III. The effects of iodoacetamide and PMSF on the enzyme activity were almost insignificant, as those of EDTA and Chelex were. Further study on the inhibitory

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TABLE III. **Influence of inhibitors on *Centroscymnus coelolepis* pepsin.** The shark pepsin (0.1 mg/ml) was incubated with inhibitors (concentrations indicated in the table) for 10 h at 5°C. Activity towards hemoglobin was checked after the incubation.

Inhibitor (final conc.)	Remaining activity (%)
None	100.0
PMSF (9 μM)	97.4
Iodoacetamide (9 μM)	93.6
Pepstatin (13 μM)	0.0
1,10 phenanthroline (9 μM)	48.0
Dialysate against EDTA (10 mM)	95.0
Dialysate against Chelex 100 resin	100.0

TABLE IV. **Molecular masses and sequences of hydrolysate fragments obtained on RP-HPLC-ESI-MS.** The oxidized B-chain of bovine insulin (1 mg/ml) was incubated with *Centroscymnus coelolepis* pepsin (10 U/ml) at 37°C. The resulting solution was analyzed to determine the sensitive bonds.

Peak		Molecular masses (Da)		Fragment
No.	TR	Calculated	Theoretical	
1	12'00	578.2	578.67	26-30
2	14'80	430.4	430.51	12-15
3	15'90	725.4	725.85	25-30
4	17'70	1,302.2	1,302.44	1-11
5	19'20	1,090.8	1,091.22	16-24
6	21'40	1,636.2	1,635.86	17-30
7	22'40	1,715.2	1,714.93	1-15
		1,799.7	1,799.04	16-30
8	22'80	1,237.9	1,238.39	16-25
9	26'00	2,787.2	2,788.12	1-24
10	26'50	3,495.2	3,495.94	1-30 (Insulin)
11	27'30	2,934.7	2,935.30	1-25
	31'00	686.8	686.91	Pepstatin

effect of pepstatin at different concentrations showed that a pepstatin/pepsin ratio of 2.8 mol/mol gave total inhibition.

Substrate Specificity—To determine the substrate specificity of the shark pepsinogen, the oxidized B-chain of bovine insulin was used as a protein substrate. The hydrolysis products were analyzed by coupling liquid chromatography and mass spectrometry. Figure 3 shows the peptide profiles of the hydrolysates on detection as to TIC and by UV absorbance for 5 and 40 min hydrolysis times. These two profiles are qualitatively well correlated. The relative heights of some peaks are not identical since the measurements were based on different physical principles. Each peak was identified by determination of its m/z by ESI-MS, with prior knowledge of the amino acid sequence of the oxidized B-chain of bovine insulin. Table IV and Fig. 3 show the corresponding peptides for both hydrolysis times, where peaks are indicated by their retention times. Figure 4 shows the mass spectrum of the peak at the retention time of 22.4 min for the 5 min hydrolysis time (peak No. 7). Multiply charged ions were observed at m/z 858.7, 900.6, 1,716.0, and 1,799.2 Da, indicating the heterogeneity of this peak. Two series of ions were found: one at m/z 858.7 and 1,716.0 related to $[M+2H^+]^{2+}$ and $[M+H^+]^+$ charged ions, respectively, and corresponding to M_r 1,715; and the other at m/z 900.6 and 1,799.2 giving a mass of 1,798.2 Da. Analysis of the time course of hydrolysis showed that the 15-16 Leu-Tyr bond was split extremely rapidly. The two fragments resulting from this cleavage (1-15 and 16-30) were observed as one peak with the UV detector (peak 7), but could be distinguished by mass spectrometry (Fig. 7).

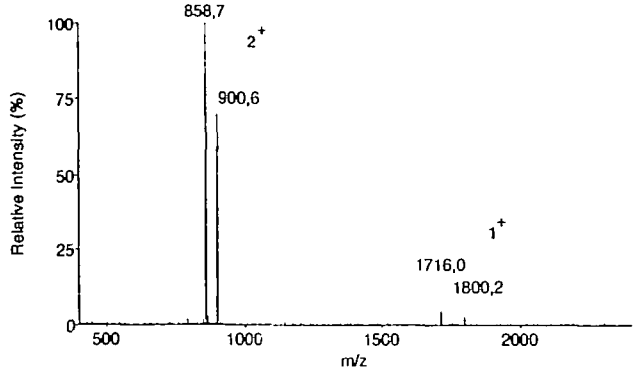

Fig. 4. **Mass spectrum of peak No. 7.** Two molecular weights were determined on mass spectrometry, corresponding to fragments 1-15 and 16-30.

When the hydrolysates were analyzed with a FPLC (Fast Protein Liquid Chromatograph) equipped with a PepRPC column (Pharmacia), these two peaks were well separated (Fig. 5), peak I corresponding to fragments 1-15 and peak II to fragments 16-30. At the concentration of 10 U/ml, the enzyme converted all of 1 mg insulin (in 1 ml) in less than 10 min. The Phe-Phe (24-25) and Phe-Tyr (25-26) bonds were also very sensitive: they were cleaved at the very beginning of the reaction but less rapidly than the Leu-Tyr one. These cleavages resulted in numerous fragments: peaks 1 (26-30), 3 (25-30), 5 (16-24), 7 (1-15 and 16-30), 8 (16-25), 9 (1-24), and 11 (1-25). Fragments 16-30 after the first cleavage remained a very good substrate for the enzyme and was digested completely within 40 min. Fragments 1-15 was more resistant to the enzyme, but was also cleaved at the Leu-Val (11-12) bond at a very lower speed. Cleavage of the Tyr-Leu (16-17) band was also observed at the beginning of the reaction (peak 6).

The specificity profile of shark pepsin as to the oxidized B-chain of insulin was found to be a typical one of an aspartyl proteinase with a preference for hydrophobic amino acids. This characteristic was confirmed by the strong inhibition of pepstatin of its activity. Shark pepsin was thus close to bovine cathepsin D (*26*) in the principal cleavages of the oxidized B-chain of bovine insulin (Leu-Tyr, Phe-Phe, and Phe-Tyr bonds, with a preference for the Leu-Tyr one in the case of shark pepsin), but different in the secondary cleavages. The cleavage of the Tyr-Leu (16-17) bond was somewhat particular: it was observed at the very beginning of the reaction, but no significant level was observed afterwards. When the Leu-Tyr(15-16) bond was split, the Tyr-Leu one probably became much less sensitive because of the lack of side chain recognition.

It should be noted that shark pepsin showed no activity towards small peptides: Ac-Phe-TyrI_2 is a common substrate for pepsin-like enzymes, but it was inert as to the action of shark pepsin. The Leu-Tyr bond of the oxidized B-chain of insulin was very well recognized, but dipeptide CBz-Leu-Tyr was totally inert. Fragments derived from the oxidized B-chain of insulin were not split any longer if they contained less than 6 amino acids. However, more study on small substrates is necessary to confirm this characteristic and to investigate the influence of the side chain recognition on its activity, which is indispensable for future applications.

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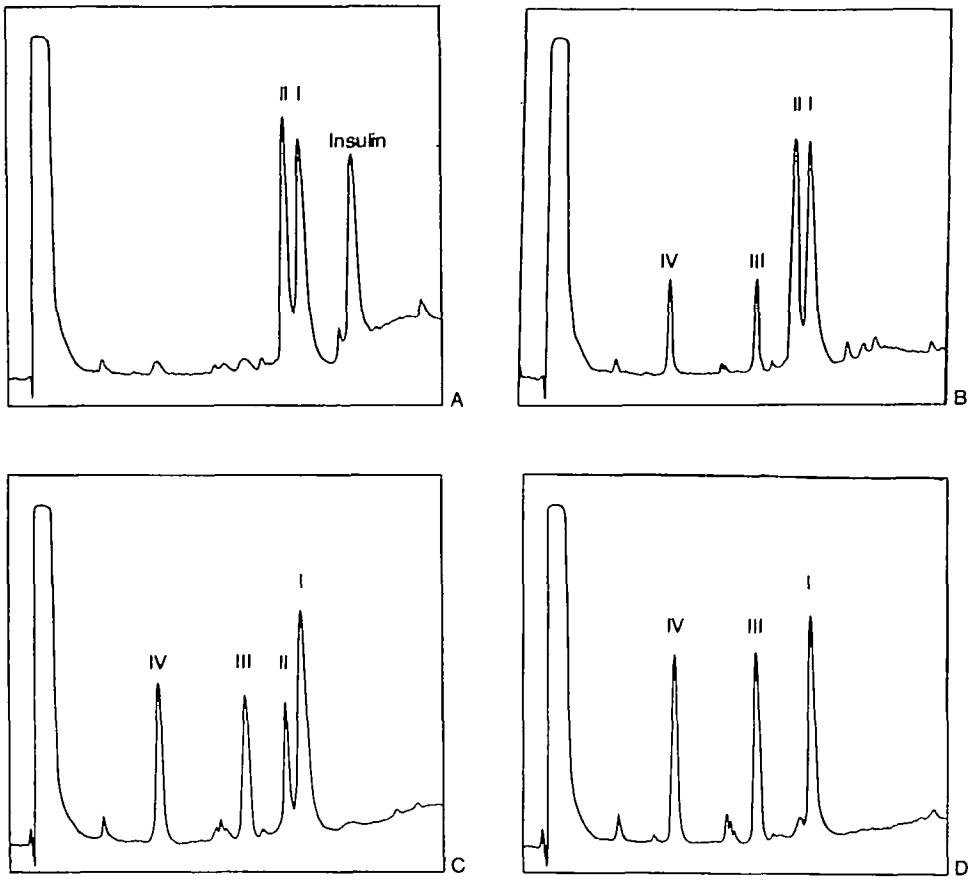

Fig. 5. **FPLC of insulin hydrolysates.** A: After 5 min; B: after 10 min; C: after 20 min; D: after 40 min. Oxidized B insulin (1 mg/ml) was incubated with *Centroscymnus coelolepis* pepsin (10 U/ml). Aliquots were withdrawn after 5, 10, 20, and 40 min, and then separated by FPLC on a PepRPC HR5/5 column. Peptides were detected as to UV 214 nm. Peak I: fragments 1-15. Peak II: fragments 16-30. Peak III: fragments 16-24 and 16-25. Peak IV: fragments 24-30 and 25-30.

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