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Purification and characteristics of cod muscle cathepsin

By Günther Siebert, Albert Schmitt and
Renate von Malortie

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1) G. Siebert, A. Schmitt and G. Träxler, in this Journal 332, 160 (1963).

2) G. Siebert, Experientia (Basel) 14, 65 (1958).

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2) G. Siebert, Experientia (Basel) 14, 65 (1958).

specificity, and on the basis of studies about the active site, we tried to make a comparison with the digestive proteinases which had been examined extensively. The following report deals with such a cathepsin from cod muscle, purified 3,000 times.

METHOD

Enzyme extract and activity tests: For the preparation of the raw extract, the frozen cod muscle* is cut up into small cubes, which are further reduced in size in the meat grinder, and then homogenized for some minutes in the "Ultra-Turrax" with a 1% KCl-solution in the ratio 1 : 2.5; after having it allowed to sit for 30 minutes, it is centrifuged until clear at 4,500 x g (raw extract).

The activity tests with urea denaturized hemoglobin, and the degradation tests with specif. substrates and dipeptides were conducted as stated earlier; the same applies to investigations with metals and SH-reagents.

Purification: All the chromatographic purification steps were carried out at +3 to 5°. In desalting on "Sephadex" G-25 the ratio of the column to the volume put in is around 5 : 1, for the gel filtration on "Sephadex" G-200 at 10 : 1. Hydroxyl-apatite was produced according to Tiselius[3]; for the chromatography on carboxymethyl-cellulose the interchanger, pretreated according to Peterson and Sober,[4] was used. Details about the purification are described under results.

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* See footnote on preceding page (Translator's Note) under * .

3) A. Tiselius, S. Hjertén and Ö. Levin, Arch. Biochem. Biophysics 65, 132 (1956).

4) E.A. Peterson and H.A. Sober, Methods in Enzymol. 5, 3 (1962).

The titration of the free SH-groups of the enzyme protein was carried out according to the principle stated by Boyer[5]: Reference cuvette and measuring cuvette (1 cm path of light) contain 1 ml 1 m acetate buffer each, pH 4.6, 0.5 ml water and
p.21 1 ml of the enzyme solution (250 γ /ml). In intervals of 10 min. 5 µl of a 0.002m solution of p-chlormercuri-benzoate are added in the cuvette, well mixed, and the extinction is measured against the measuring/reference cuvette at 250 mµ . In a 2nd mixture the extinction increase caused by the reagent alone is ascertained under the same conditions, but without any enzyme. The activity tests were conducted according to Anson.[6] The method was standardized with reduced glutathione.

The photo-oxydation was carried out as follows, similarly to the experiments made by Schwert and his coworkers[7]: A 10-ml-glass beaker contains 300 γ enzyme in 4 ml 0.05 m tris-buffer, pH 7.0 besides 50 γ methylene blue, and is then kept in a thermostat at 25°. At a distance of 8 cm there is a 100-watt-lamp above the surface of the enzyme solution. At various times parts of 0.5 ml are taken out, freed of colouring matter, on "Sephadex" G-25, with 0.05 m acetate buffer, pH 5.0, and subjected to the activity definition. An identical mixture with exporsure to light, without any addition of methylene blue, serves for control purposes.

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5) P. D. Boyer, J. Amer. chem. Soc. 76, 4331 (1954).

6) M. L. Anson, J. gen. Physiol. 22, 79 (1938).

7) D. B. Millar and G. W. Schwert, J. biol. Chemistry 238, 3249 (1963).

The specificity definition on insulin B was carried out in the way described earlier[1]. Insulin B was presented according to Fittkau[8].* After completion of the degradation of the model protein (follow-up through ninhydrin analyses), the mixture of the cleavage products was filtrated over a column with "Sephadex" G-25 (1 x 90 cm) in 0.1 n acetic acid, the UV-absorption was measured at 276 and 230 μm in the 3-ml-fractions, and aliquots of the individual fractions were chromatographed unidimensionally on silica gel G with phenol/water 8 : 2. Fractions of the same composition were united and dried. The thus pre-separated degradation products were segregated into the individual components and identified by thin layer chromatography as in the degradation through cod spleen cathepsin.[1] The degradation of cleavage peptides with carboxypeptidase A was carried out according to Fraenkel-Conrat[9], and the identification of the C-permanent amino acids was, in turn, made by thin-layer chromatography.

RESULTS

Purification of the Enzyme

1. Acid denaturation: For the purpose of determining the optimum pH-value for the acid denaturation of enzymatically inactive accompanying proteins, samples of the raw extract with

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8) S. Fittkau, Naturwissenschaften, 50, 522 (1963).

* The authors wish to thank Professor Schmidt-Thomé, Farbwerke Hoechst, for so generously supplying crist. insulin.

9) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, Methods biochem. Analysis 2, 359 (1955).

2n acetic acid were set on the glass electrode for different pH-values ranging from pH 3.8 to 5.4, heated to 35° for 10 min., centrifuged until clear after having cooled off, and then the protein content and the activity of the solution were determined. It follows from Illustration 1 that the optimal purification effect of this procedure (2½ - 3-fold concentration at 70 - 75% yield) is given at pH 4.6. The enzyme solution thus obtained can be stored in the refrigerator over-night without any noteworthy loss in activity.

Ammonium sulphate fractionation: The protein fraction resulting between 30 and 65% saturation is absorbed by 1/10 of the initial volume (water).

p.22

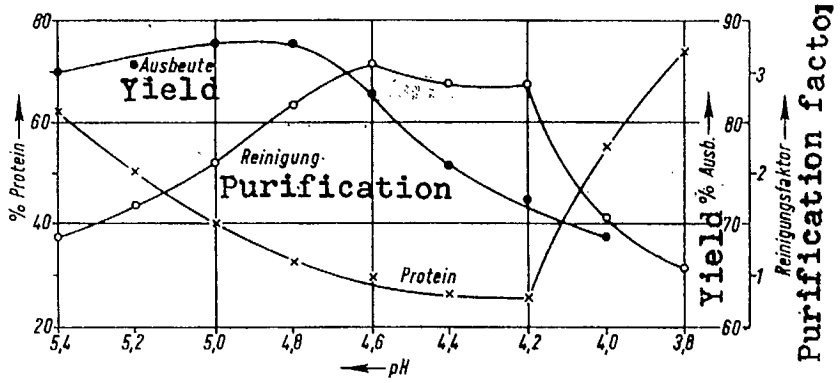

Abb. 1. Ermittlung des optimalen pH-Wertes für die Säuredenaturierung enzymatisch inaktiver Fremdproteine.

Illustration 1. Definition of the optimal pH-value for the acid denaturation of enzymatically inactive foreign proteins.

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2. Acid denaturation: The optimal pH-value, calculated as above, is now at pH 4.1. The dissolved sediment of the salt precipitation is set at pH 4.1, with 2n acetic acid, heated at 35° for 10 min., cooled off and centrifuged until clear.

In order to free it from any remaining ammonium sulphate, the enzyme solution is filtrated over a column with"Sephadex"G-25. The saltfree protein fractions (check with Nessler-reagent) are united and dried frozen. The enzyme preparation thus obtained can be kept in the refrigerator for several weeks, by excluding humidity, without any loss in activity. The concentration, in relation to the raw extract, is now around 30-fold, and the yield about 50%.

Acetone fractionation: The material dried frozen is dissolved in 1% NaCl (5 mg protein/ml) and subjected to acetone fractionation at - 5°; the protein fraction obtained between 45 and 70 Vol.-% acetone is added to 0.1m phosphate buffer, pH 6.0, and centrifuged until clear (purified 3 to 4 times).

Gel filtration on "Sephadex" G-200: The enzyme solution is filtrated in 0.1 m phosphate buffer, pH 6.0, over "Sephadex" G-200; the fractions of highest specif. activity were united. The purifying effect of the gel filtration follows from Illustration 2 and Table 1.

Chromatography on hydroxyl apatite: The enzyme fraction obtained on "Sephadex" G-200 through gel filtration is transferred to a column of hydroxyl apatite, set at pH 6.0, with 0.1m phosphate buffer. The proteins which are not fixed are wahed out with the same buffer, and the bound proteins are eluted by a gradual buffer gradient (0.1 - 0.5m). The purifying effect of this process is apparent in Illustration 3: A lesser

portion of the catheptic activity appears in the break-through peak, and a larger part at elution with 0.4m phosphate-buffer. On the average, this enzyme fraction shows a 3- to 4-fold purification at a yield of about 30%. Repeated chromatography on

p.23 hydroxyl apatite shows no further increase in the specif. activitity. The activity peak emerging first from the column has 1/4 of the specif. activity of the second peak; the first component is rejected.

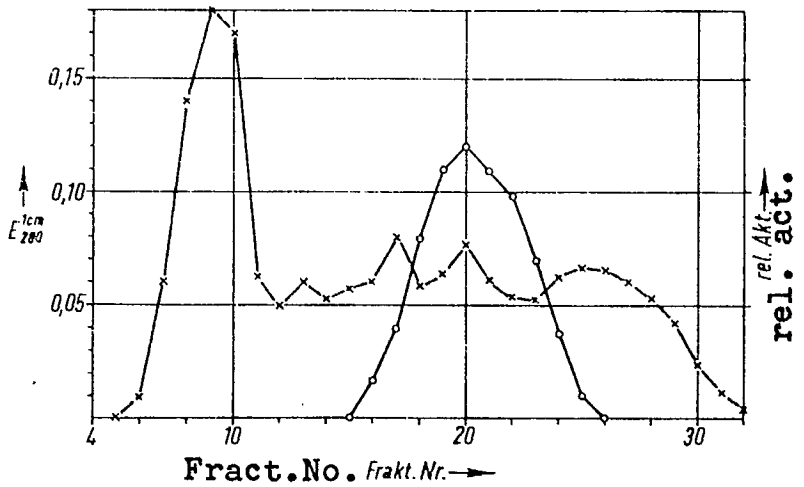

Abb. 2. Gelfiltration von teilgereinigtem Dorschmuskel-Kathepsin an Sephadex G-200 (2 × 35 cm). ○: Aktivität (μMol Tyrosin pro Stde. und ml); ×: Protein (aus E_{280}). Fraktionen zu 5 ml.

Illustration 2. Gel filtration of cod muscle cathepsin partly purified on "Sephadex" G-200 (2 x 35 cm). o : Activity (μ mol tyrosine per hour and ml); x : protein (from E_{280}). Fractions at 5 ml.

Chromatography on CM-cellulose: The enzyme fraction (main peak) is desalted on "Sephadex" G-25, and set at the same time at pH 5.5, on 0.05 m acetate buffer. In the chromatography on CM cellulose, the enzyme is bound by the interchanger , and can be obtained again by gradual elution with a NaCl-gradient between 0.2 and 0.4m NaCl. For the purpose of desalting, the enzyme is filtrated over "Sephadex" G-25.

The total result of the individual purification processes is an about 3,000 concentration of the enzyme at a yield of about 3% (Table 1). At this stage of purification, further purification attempts (repeated salt fractionation, CM-cellulose chromatography) were unsuccessful. The enzyme solution, desalted on CM cellulose after chromatography, keeps frozen for several weeks without any noteworthy loss; the keeping capacity apparently is unlimited after lyophilisation.

Experiments for the purpose of characterizing the active site.

Metal ions: In Table 2 the influence of the metal ions examined and that of the complex-former ethylene-tetraacetate (EDTA)

p.24

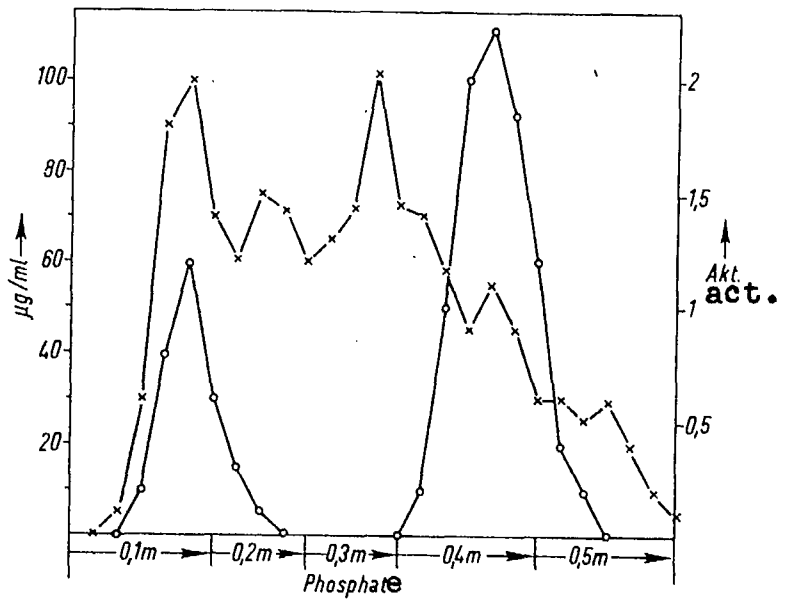

Abb. 3. Chromatographie von teilgereinigtem Dorschmuskel-Kathepsin an Hydroxylapatit. Puffergradient: 0,1—0,5*m* Phosphatpuffer, pH 6,0. Rechte Ordinate und ○: Aktivität (*µ*Mol Tyrosin pro Stde. und ml): Linke Ordinate und ×: Protein nach Lowry[10].

Illustration 3. Chromatography of cod muscle cathepsin, partly purified, on hydroxylapatite. Buffer gradient: 0.1 - 0.5m phosphate buffer, pH 6.0. Right ordinate and o : Activity (μ mol tyrosine per hour and ml): Left ordinate and x : protein according to Lowry.[10]

[10] O.H. Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J.biol. Chemistry 193, 265 (1951).

Table 1. Purification of Cod Muscle Cathepsin

Purification process	Volume (ml)	mg Protein/ml	mg Prot./Fract.	$\frac{E_{280}}{E_{260}}$	Total* Activity	Specif** Activity	Purification	% Yield
1. Raw extract.	1330	15.5	20700	0.71	665	0.032	---	100
2. 1st acid denaturation: pH4.6; 10 min. 35°. . .	1260	4.4	5550	0.92	490	0.088	2.8	73
3. Ammonium sulphate fractionation: 30 - 65%. . . .	140	10	1400	0.95	335	0.24	7.4	50
4. 2nd acid denaturation: pH 4.1; 10 min. 35°. . .	150	1.8	270	1.5	265	0.98	30	40
5. Acetone precipitation: Sediment 45 - 70 Vol.-% . .	15	2.6	39	1.5	150	3.9	120	22
6. Gel filtration on Sephadex G-200: 0.1m phosphate buffer, pH 6.0	30	0.25	7.5	1.5	100	13	400	15
7. Adsorption on hydroxyl apatite: Elution between 0.4 and 0.5m buffer.	6	0.14	0.83	1.6	40	48	1500	6
8. CM-cellulose chromatography: Elution between 0.2 and 0.4m NaCl	5	0.042	0.21	1.6	20	95	3000	3

* μ mol tyrosine per hour and fraction.

** μ mol tyrosine per hour and mg protein

p.25 with reference to the activities of metalfree controls, are compiled. Except for the case of Hg-ions in rather high concentration (1×10^{-2}m) no significant changes in activity can be noted. Even the addition of EDTA has no influence on the enzyme activity.

Table 2. The infuence of metal ions on the activity of purified cod muscle cathepsin.

Admixture	Final concentration (mol/l)		
Zusatz	Endkonzentration [Mol/l]		
	1×10^{-2}	1×10^{-3}	1×10^{-4}
ohne. without	100	100	100
EDTA	100	98	100
$Mg^{2\oplus}$	100	102	94
$Ca^{2\oplus}$	100	96	96
$Mn^{2\oplus}$	—	96	99
$Zn^{2\oplus}$	—	90	96
$Fe^{2\oplus}$	100	100	100
$Co^{2\oplus}$	—	96	87
$Ni^{2\oplus}$	—	108	93
$Hg^{2\oplus}$	72	91	96
$Cu^{2\oplus}$	95	96	100

Sulfhydryl Groups: After 15 min. pre-incubation of the purified enzyme, the activities listed in Table 3 were observed, after application of the appropriate sulfhyldryl reagents. An inhibition can only be observed with p-chlormercuri-benzoate at a concentration of 1×10^{-3}m. All other SH-reagents are not having any noteworthy effect on enzyme activity.

For the purpose of determining the free SH-groups, the purified cod muscle cathepsin was titrated according to the spectral-photometric method with p-chlormercuri-benzoate

p.26 according to Boyer[5]. The results of this investigation are presented in Illustration 4. The straight line A corresponds to

Table 3. The influence of sulfhydryl reagents. Numerical values referred to reagentfree controls = 100% activity.

Admixture | Final concentration (mol/l)

Zusatz	Endkonzentration [Mol/l] 1×10^{-3}	1×10^{-4}	1×10^{-5}
ohne without	100	100	100
Cystein*	95	104	98
Cyanid	100	100	103
p-Chlormercuri-benzoat	55	97	102
o-Jodoso-benzoat	87	102	100
Jodacetamid	105	105	102
N-Äthyl-maleinimid	97	102	94

* In diesem Falle wurde statt der Folin-Reaktion der Zuwachs der UV-Absorption bei 280 mμ gemessen.

* In this case the accrual of the UVpabsorption at 280 mμ was measured instead of the Folin reaction.

the extinction increase, caused by the reagent alone; the broken line B illustrates the titration of the purified muscle cathepsin. The extinction increase, caused by the formation of mercaptide, amounts to $\Delta E = 0.03$. A calculation, related to a molecular weight of 45,000 - 55,000*, results in a molar absorption coefficient $\varepsilon_{\text{mercaptide}} = 15{,}000$. In relation to the molar absorption coefficient of the mercaptid bond for glutathion (ε_M= 7,500) this results in two free SH-groups per enzyme molecule.

* On the basis of the behaviour of the enzyme protein at gel filtration on Sephadex G-200.[11]

11 P. Andrews, Biochem. J. 91, 222 (1964).

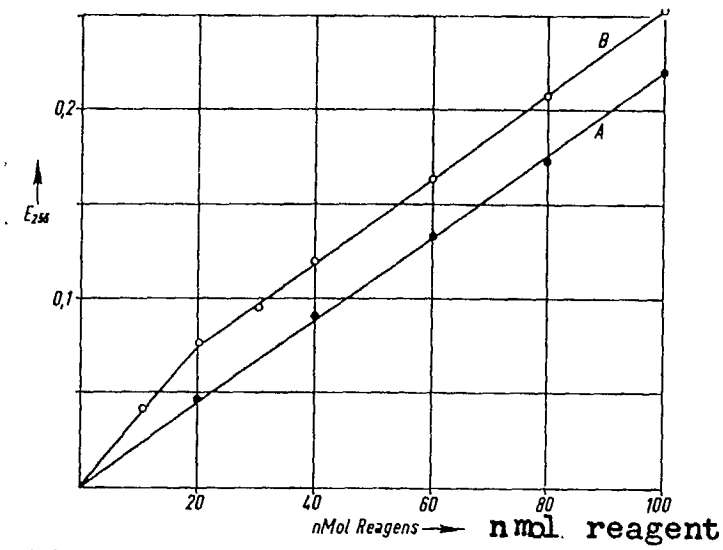

Abb. 4. Titration freier SH-Gruppen mit *p*-Chlormercuri-benzoat.

Illustration 4. Titration of free SH-groups with p-chlormercuri-benzoate.

p.27

Imidazole groups: In the photo-oxidation of proteins in the presence of methylene blue, primarily histidine residue is destroyed.[12] The decrease in activity attending photo-oxidation of an enzyme would therefore seem to indicate the participation of imidazole groups in the catalytic mechanism. The results of the photo-oxidation of the purified cod muscle cathepsin are represented in Illustration 5. Within 25 minutes the mixture, having been exposed to light in the presence of methylene blue, losses 50% of its activity. This finding would seem to substantiate perhaps the presence of catalytically essential histidine residues in muscle cathepsin.

[12] L. Weil, S. James and A.R. Buchert, Arch. Biochem. Biophysics 46, 266 (1953).

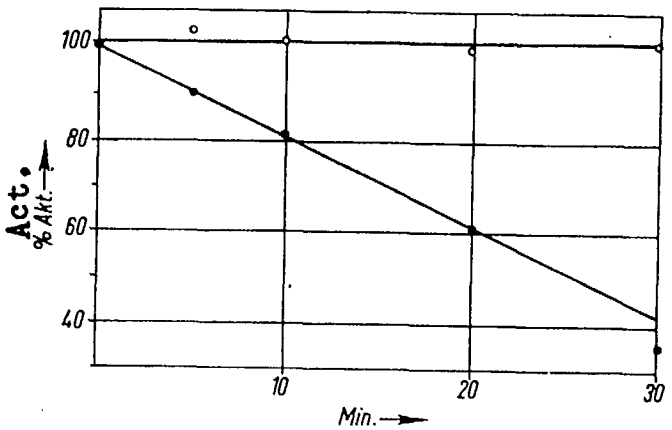

Abb. 5. Photooxydation von gereinigtem Dorschmuskel-Kathepsin. Mit (●) und ohne (○) Methylenblau (Kontrolle).

Illustration 5. Photo-oxidation of purified cod muscle cathepsin. With (●) and without (o) methylene blue (control).

Serine: The presence of catalytically essential serine residue was tested by pre-incubation of purified enzyme in the presence of different concentrations diisopropyl fluorophosphate and subsequent definition of activity. In relation to a diisopropyl-fluorophosphate-free comparative mixture, at reagent concentrations of 1×10^{-3}, 1×10^{-4} and 1×10^{-5} mol/l, no activity decrease of the cathepsin as against the hemoglobin solution as substrate was noted (Table 4); under the same conditions, depending on the reagent concentration and the time of the action, trypsin shows the inhibition as reported in scientific literature.[13]

Degradation of proteins

pH-optimum: For the purpose of determining the pH optimum, samples of the partly purified enzyme (1,000 times) were subjected to the activity tests with hemoglobin solutions of varying

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13 E.F. Jansen, and A.K. Balls, J. biol. Chemistry, 194, 721 (1952)

pH values (4 - 7.8).

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p.28

Table 4. Influence of diisopropyl-fluorophosphate on the activity of purified cod muscle cathepsin and trypsin (Boehringer, crist. 3 times): 25 γ enzyme/ml, 0.05m acetate buffer, pH 5.5 at 25°. Activity tests according to Anson[6] at pH 4.6 (cathepsin) and 7.5 (trypsin). Diisopropyl-fluorophosphate in propanol-(2), properly thinned, controls with pure propanol-(2).

Conc. (Mol/l) % residual activity after hours pre-incubation

Konz. [Mol/l]	% Restaktivität nach Stdn. Vorinkubation					
	1	2,5	4	1	2,5	4
	Trypsin			Cathepsin / Kathepsin		
1×10^{-3}	15	11	4	98	92	108
1×10^{-4}	60	42	27	92	98	99
1×10^{-5}	82	71	65	98	87	99

The results of this investigation are presented in Illustration 6. Cod muscle cathepsin develops its maximal proteolytic activity at pH 4.6; 50% of the activity were noted at pH 4.1 and 5.6.

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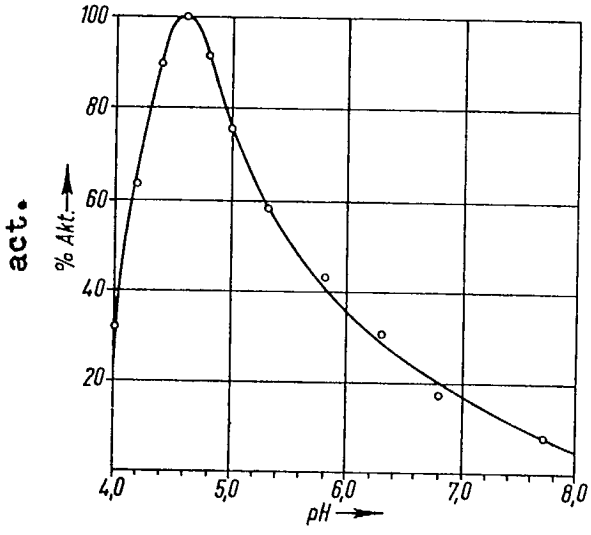

Illustration 6. pH-optimum of cod muscle cathepsin as against urea-denaturized 3%-hemoglobin solution as substrate.

Lack of degradation of peptide substrates: Samples of the raw extract and the purified enzyme were incubated with specif. substrates of defined proteinases and peptidases, and the activity was ascertained as compared to these substrates. The results of this investigation are compiled in Table 5. The specif. substrates for pepsin (cathepsin A), trypsin (cathepsin B), chymotrypsin (cathepsin C), as well as for leucinaminopeptidase are neither
p.29 degraded by raw extracts nor by the purified enzyme. The raw extract of cod muscle shows relatively high dipeptidase activities; they are removed in the case of the purified enzyme, within the range of sensitivity stated for the method of definition.

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Table 5. Degradation of specific substrates by raw extract and the purified cod muscle cathepsin.

Substrate	Specific for	Activity* Raw extract	Purified enzyme
Substrat	spezifisch für	Aktivität* Rohextrakt	gereinigtes Enzym
N-Benzyloxycarbonyl-glutamyl-L-tyrosin . . .	Kathepsin A Pepsin	< 0,01	< 0,01
N-Benzoyl-L-argininamid .	Kathepsin B Trypsin	< 0,05	< 0,05
Glycyl-L-phenylalaninamid	Kathepsin C Chymotrypsin	< 0,05	< 0,05
L-Leucinamid	Leucinaminopeptidase	—	< 0,05
Glycyl-L-leucin	Dipeptidasen	0,75	< 0,005
Glycyl-L-methionin		0,73	< 0,005
L-Valyl-glycin		0,2	< 0,005
L-Seryl-glycin		0,2	< 0,005
L-Leucyl-glycin		0,2	< 0,005

* µMol je Min. und mg Protein.

* µ mol per min. and mg protein.

Various protein substrates: In addition to hemoglobin solutions denaturized in different ways, myogen- and actomyosin-fractions from fish musculature* were also used as substrates.

* Translator's Note: Please see footnote on next page.

Substrate concentrations (1%-solution in 0.1 m acetate buffer), pH value (5.4) and enzyme concentration (50γ enzyme/ml test mixture) are identical in the various incubation mixtures, the results thus being directly comparable (Table 6). As has been described for proteases elsewhere[14] the activity increases as compared with hemoglobin solutions, to the extent that the degree of denaturation increases; in the case of the muscle proteins (myogen, actomyosin), which are at least partially denaturized under the test conditions applied, the velocity of the enzymatic degradation is lesser.

Kinetics of protein degradation: the chronological sequence of the hemoglobin degradation has been followed up according to three different procedures of analyses: 1. Increase of amino groups through ninhydrin analyses according to Moore and Stein;[15] 2. Increase of acid-soluble tyrosine with phenol-

p.30 reagent;[16] 3. Definition of the free amino acids according to van Slyke's[17] carboxyl-N-method. The results of these experiments

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* The authors wish to thank Dr. J.W. Jebsen, Fiskeridirektoratets Kjemisk-Tekniske Forskningsinstitutt, Bergen, Norway, for supplying myogen and actomyosin from cod musculature.

14 P. Desnuelle in P.D. Boyer, H. Lardy and K. Myrbäck, The Enzymes, 2nd ed., Vol. IV, p. 93, Academic Press, New York 1960.

15 S. Moore and W.H. Stein, J. biol. Chemistry 211, 907 (1954).

16 O. Folin and V. Ciocalteu, J. biol. Chemistry 73, 627 (1927).

17 D. van Slyke, D.A. McFadyen and P. Hamilton, J. biol. Chemistry 141, 671 (1941).

are presented in Illustration 7. The results of the ninhydrin-analyses and the carboxyl-N-titrations are given in leucine equivalents (μmol leucine/ml filtrate), and can be compared directly with the values for acid-soluble tyrosine (μ mol tyrosine/ml filtrate).

Aliquots of the samples freed of albumin were subjected to total hydrolysis with 6 n HCl, and the additional amino groups now freed were also determined according to Moore and Stein.[15] From the relation of the amino groups set free before and after the total hydrolysis, conclusions are drawn concerning the average chain length of the cleavage products.

It follows from Illustration 7 that the increase in ninhydrin-positive material indicates a gradual linear rise (Line II).

Table 6. Degradation of various proteins through purified cod muscle cathepsin

Protein sample	Activity (μmol tyrosine/ hours and mg protein)
Hemoglobin (dissolved directly in acetate buffer).	31
Hemoglobin (denaturized with alkali).	48
Hemoglobin (denaturized with alkali plus urea)..	60
Moygen (suspended in acetate buffer).	12
Actomyosin (dissolved in acetate buffer).	8

The increase in free amino groups after total hydrolysis of the specimens with HCl is also linear (Line I), i.e. over a period of 6 hous, degradation products of constant mean chain length

obviously develop. The increase in free amino acids also has a linear direction (Line III). After correction by the amount of the free amino acids formed, it is calculated that, in relation to leucine equivalents set free, there is a mean minimum chain length of 4.5 amino acid residues per acid-soluble peptide set free. The relatively high proportion of free amino acids developing is noteworthy. A comparison of Line I and III shows that around 10% of the entire material having become acid-soluble falls to the share of free amino acids. As curve IV in Illustration 7 shows, the increase in degradation products containing tyrosine slightly drops after 4 hours of incubation. At the beginning of the degradation, 7 leucine equivalents correspond to one tyrosine equivalent; after 6 hours the ratio tyrosine/leucine is reduced to below 1 : 10.

The degradation of the B-chain of insulin: The course of the degradation of the B-chain was also followed by ninhydrin analyses according to Moore and Stein; as can be seen in Illustration 8, in the case of an enzyme : substrate ratio of 1 : 50 the degradation is nearly complete after a few hours.

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Proteolytic Specificity

B-chain of Insulin: The B chain was incubated for 5 hours under the conditions set out in Illustration 8, the enzymatic degradation was interrupted by a brief heating process, and the degradation products formed were pre-separated by gel filtration on Sephadex G-25 (Illustration 9). The fractions showing the same

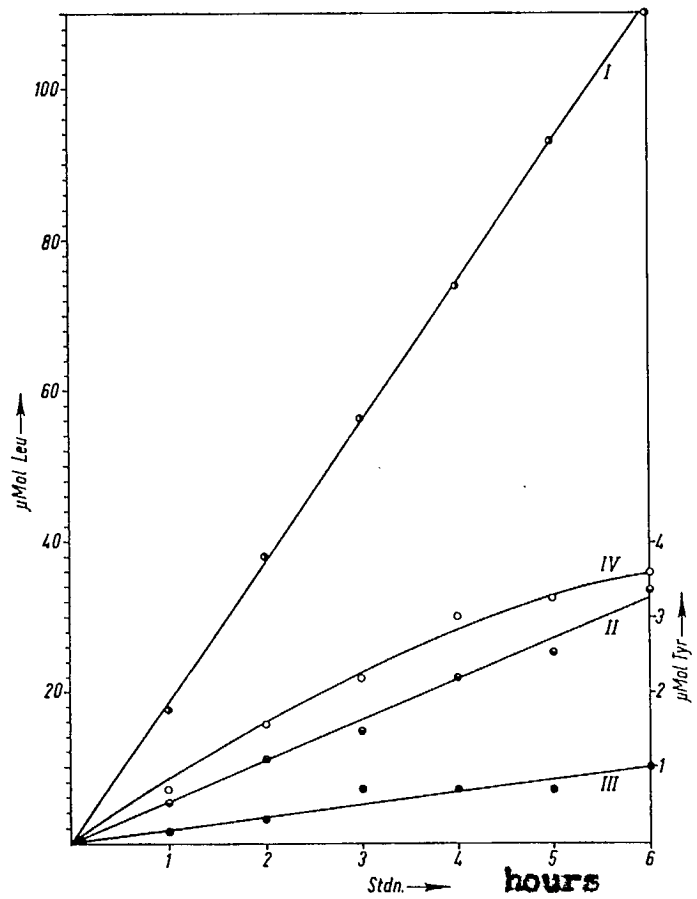

Abb. 7. Verfolgung der Hämoglobin-Hydrolyse durch Messung der Freisetzung von Tyrosin, Peptiden und Aminosäuren (Maßeinheiten siehe Text). Enzym:Substrat-Verhältnis wie 1:600. I: Freie Aminogruppen nach Totalhydrolyse; II: Ninhydrinpositives Material; III: freie Aminosäuren; IV: Tyrosin enthaltende Abbauprodukte.

Illustration 7. Observation of hemoglobin hydrolysis by measuring the tyrosine, peptides and amino acids set free (see Text for measuring units). Enzyme: Substrate ratio like 1 : 600. Free amino groups after total hydrolysis; II; Ninhydrin-positive material; III: free amino acids; IV: Degradation products containing tyrosine.

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at uni-dimensional thin-layer chromatography composition/were united (A, B, C, D, E), and further severed two-dimensionally after freeze-drying, as described earlier.[1] The

p.32 identification of the chromatographically uniform degradation products was made by ascertaining the amino acid composition, dinitrophenylation of amino end-constant amino acids and, if required,

also by degradation with carboxypeptidase[9]. The results of this investigation are summarized in Table 7. A total of 12 components were isolated and identified, including four free amino acids.

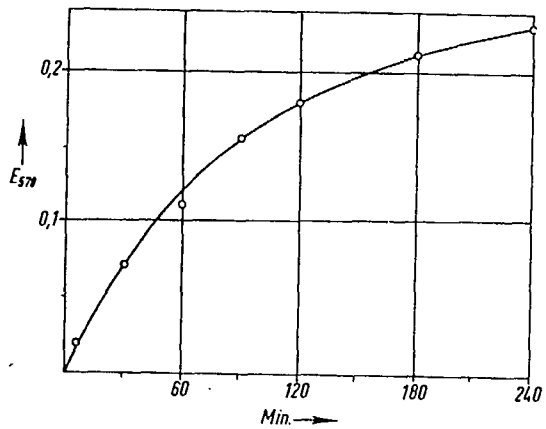

Abb. 8. Verlauf der Hydrolyse der B-Kette von Insulin durch gereinigtes Dorschmuskel-Kathepsin bei pH 4,6; Ninhydrin-Analysen nach Moore und Stein[15].

Illustration 8. Sequence of hydrolysis of the B-chain of insulin by purified cod muscle cathepsin at pH4.6; ninhydrin analyses according to Moore and Stein.[15]

The specificity of the enzyme as correlated to the B-chain of insulin follows from the degradation products recorded in Table 7. Illustration 10 lists the indentified insulin fragments according to their origin from the B-chain. The free alanine present can only originate from position 14, since no degradation product occurs with lysine but without alanine; thus two cleavage sites, the bonds between 13/14 and 14/15 are defined. The cleavage of the bond 13/14 is also secured by the peptide Phe_1-Glu_{13}. The free phenyl alanine occurring can only originate from position 25, since no fragment with amino-end-constant valine from position 2 (Bond 1/2),

or carboxyl-end-constant glycine (Bond 23/24) was found. The free leucine comes from position 15 (follows from free Ala_{14} and Tyr_{16}) as well as also from 17 (from lacking overlapping of degradation products at bond 16/17 and the occurrence of peptide Val_{18}-$Phe_{24(25)}$). The free tyrosine originates with certainty from position 16 (for lacking overlapping of the degradation products at bond 15/16 and 16/17), possibly also from position 26; Tyr_{26} is recognized with certainty by dinitrophenylation in fragment C_1, and in fragment C_5 by carboxypeptidase degradation. Whether Tyr_{26} also supplies free tyrosine depends on the uncertainty as to whether the

p.34 degradation products B_2 and B_3 end with Phe_{24} or Phe_{25}. From the identified amino acids and peptides the following bonds can be determined as cleavage sites: Glu_{13}/Ala_{14}, Ala_{14}/Leu_{15}, Leu_{15}/Tyr_{16}, Tyr_{16}/Leu_{17}, Leu_{17}/Val_{18}, Phe_{24}/Phe_{25}, Phe_{25}/Tyr_{26}, Tyr_{26}/Thr_{27}. The only cleavage sites where no overlapping occurs (Illustration 10) are the bonds between Leu_{15}/Tyr_{16} and Tyr_{16}/Leu_{17}; we should thus like to regard these as the main cleavage sites.

Cleavage products from beef hemoglobin: After 6 hours incubation the mixture freed of albumin (Illustration 7) was dinitrophenylated, the DNP* products formed were hydrolyzed with

p.35 6 n HCl, and the free DNP amino acids were identified by way of thin layer chromatography. Evaluated by the colour intensity in the chromatogram, the DNP amino acids found may be classified as follows; the +-signs are a rough measure of the colour intensity

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* DNP: Dinitrophenyl.

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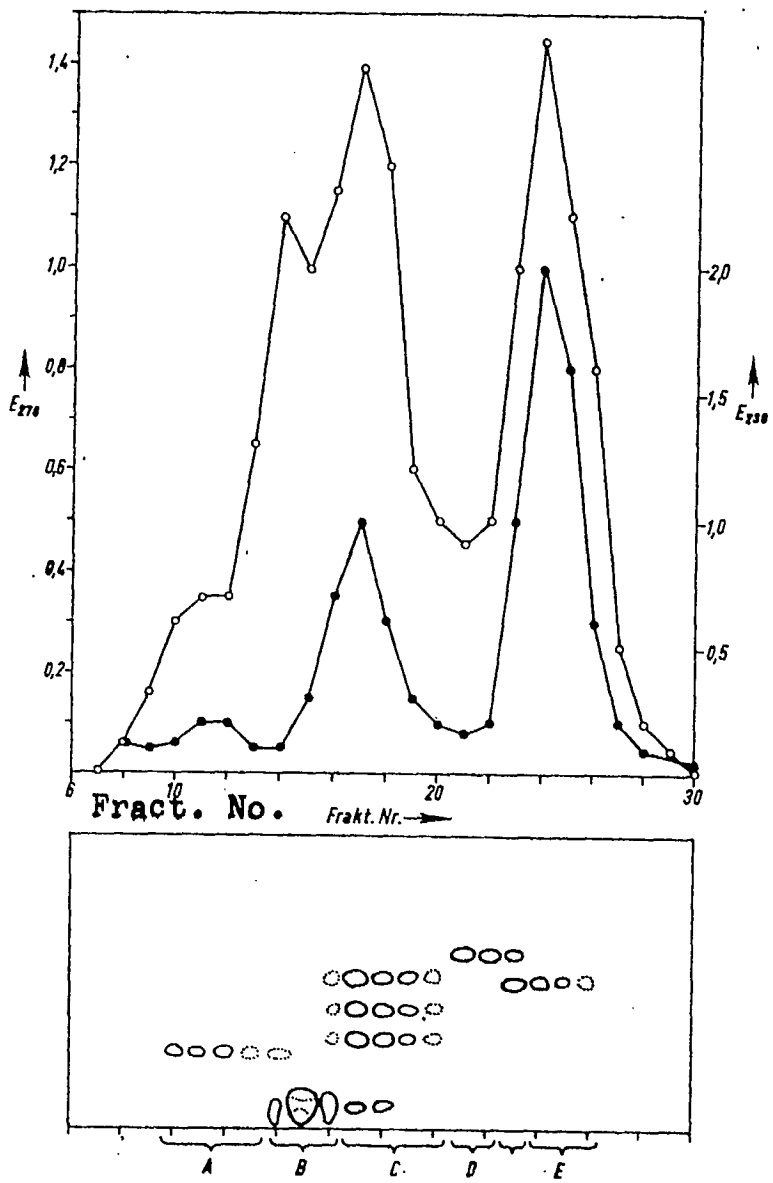

Abb. 9. Gelfiltration der Abbauprodukte der B-Kette von Insulin an Sephadex G-25 und Dünnschicht-Chromatographie der einzelnen Fraktionen an Kieselgel G mit Phenol/Wasser. Linke Ordinate und ○: E_{276}; rechte Ordinate und ●: E_{230}. Fraktionen zu 3 ml.

Illustration 9. Gel filtration of the degradation products of the B-chain of insulin on Sephadex G-25 and thin layer chromatography of the individual fractions on silica gel G with phenol/water. Left ordinate and o : E_{276}; right ordinate and ● : E_{230}. Fractions at 3 ml.

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continued

Table 7. Degradation products of the B-chain of insulin after hydrolysis through cod muscle cathepsin.

Fraktion von Sephadex G-25	Komponenten	Aminosäurezusammensetzung	N-ständige Aminosäure	C-ständige Aminosäure	Sequenz in der B-Kette
A	A_1	Phe, Leu, Val, Glu, Gly, Ser, Asp, His, $CysSO_3H$	Phe	—	1—13
	A_2	Phe, Leu, Val, Ala, Glu, Gly, Asp, Ser, His, $CysSO_3H$	Phe	Leu	1—15
	A_3	Phe, Leu, Tyr, Val, Ala, Glu, Gly, Ser, Asp, His, $CysSO_3H$, Arg, Lys, Thr, Pro	—	—	1—30 (B-Kette) chain
B	B_1	Phe, Leu, Val, Ala, Glu, Gly, Ser, Asp, $CysSO_3H$, His	Phe	Leu	1—15 = A_2
	B_2	Phe, Val, Glu, Gly, $CysSO_3H$, Arg	Val	—	18—24 (25)
	B_3	Phe, Leu, Val, Glu, Gly, $CysSO_3H$, Arg	Leu	—	17—24 (25)
C	C_1	Tyr, Ala, Thr, Pro, Lys	Tyr	—	26—30
	C_2	Leu	—	—	15 u. 17
	C_3	Ala, Thr, Pro, Lys	Thr	—	27—30
	C_4	Ala	—	—	14
	C_5	Phe, Leu, Tyr, Val, Glu, Gly, $CysSO_3H$, Arg	Leu	Tyr	17—26
	C_6	Phe, Val, Glu, Gly, $CysSO_3H$, Arg	Val	—	18—24 (25) = B_2
D		Phe	—	—	25
E		Tyr	—	—	16 (26)

p.35
cont'd

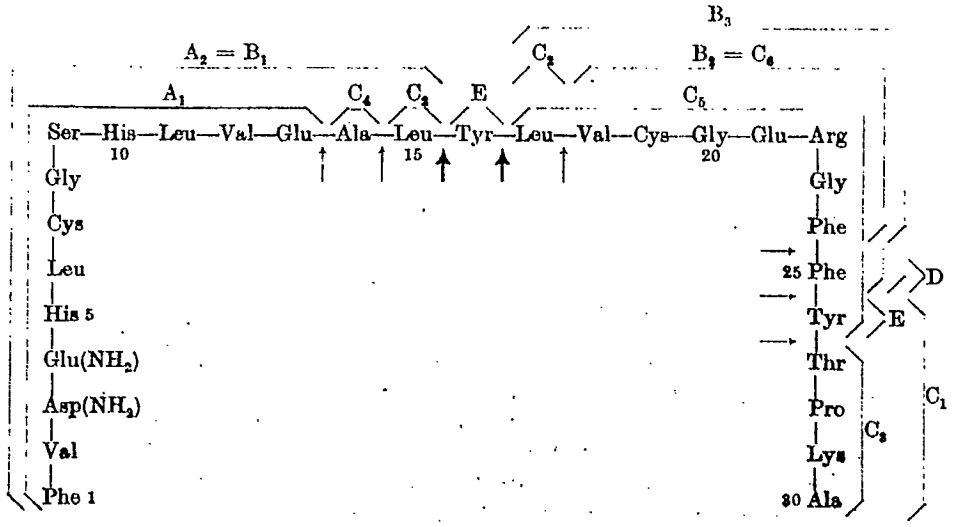

Abb. 10. B-Kette von Insulin; identifizierte Abbauprodukte und daraus resultierende Spaltorte (die Stärke der Pfeile ist ein ungefähres Maß der Hydrolyse).

Illustration 10. B-chain of insulin; identified degradation products and cleavage sites resulting therefrom (the thickness of the arrows is an approximate measure of hydrolysis).

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of the spots.

DNP-Leucin	+++++	DNP-Glutaminsäure	(+)
DNP-Valin	+++	DNP-Asparaginsäure	(+)
DNP-Phenylalanin	++	DNP-Threonin	(+)
DNP-Tyrosin	++	DNP-Serin	(+)
DNP-Alanin	+		

The nature of the amino acids affected in dinitrophenylation suggests the following conclusion concerning the specificity of cod muscle cathepsin: Primarily those peptide bonds are affected whose N-terminal amino acids are of a hydrophobe character. This finding agrees with the cleavage sites observed on the B-chain of insulin.

DISCUSSION

After about 3,000-fold purification, no further

concentration of the enzyme can be achieved, not even by varying the conditions underlying the experiment. In the course of repeated chromatogrphy on hydroxylapatite, and CM-cellulose respectively (relatively sharp activity peaks), the chromatographic behaviour of the enzyme protein does not suggest any greater impurities by enzymatically inactive foreign proteins. The enzyme
p.36 now has 1/3 of the specif. acitivity of the purified cod muscle cathepsin[1], or about 1/5th of the activities of digestive proteinases as compared to urea denaturized hemoglobin.[6] The foreign activities (peptidases) contained in the raw extract are excluded in the purified enzyme; any interference with the definition of the specificity on the B-chain of insulin through secondary degradation of cleavage products is thus eliminated.

It clearly follows from experiments with both heavy metal ions and SH-reagents that muscle cathepsin cannot contain any catalytically essential SH groups on the active site. The activity-restricting effect of the $Hg^{2\oplus}$ ions and of p-chlormercuribenzoate at relatively high concentrations (1×10^{-2} or 1×10^{-3} mol/l) should not be interpreted as an interference at the catalytically active site of the enzyme, since lower reagent concentrations have no effect. The titration of the enzyme with p-chlormercuri benzoate shows two free SH groups, it is true, but in accordance with the above findings they cannot be catalytically effective; inhibition actually only occurs with reagent quantities (Table 3) which are more than 100 times higher than required for the complete conversion of the SH groups (Ill. 4).

Obviously, cod muscle cathepsin does not require any metal ion for its activity either: the admixture of EDTA in varying concentrations (1×10^{-2}, 1×10^{-3} and 1×10^{-4} mol/l) does not have any influence on the enzyme activity; in the series of the bi-valent metal ions used no definite activation is observable, in any of the cases.

Due to the fact that there is no inhibition possible with diisopropyl-fluorophosphate, it has to be concluded that the cod muscle cathepsin cannot contain any serine as a component of the enzymatically active site. By this fact it differs from many other proteases, such as chymotrypsin, trypsin, thrombin or subtilisin, in the case of which the enzyme activity is blocked through an esterification of the serine residues.[18,14]

At the present time, only one positive statement can be made concerning the structure of the active site: The results of photooxydation (Illustration 5) make the participation of imidazol groups in the catalytic mechanism appear very likely.

The cod muscle cathepsin does not show any identical specificity as compared with any of the known proteinases in relation to the B chain of insulin. In comparison with the crystallized digestive proteinases the muscle cathepsin shows some similarities with chymotrypsin, but also many differences:

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18 B. Hagihara in P.D. Boyer, H. Lardy and K. Myrbäck, The Enzymes, 2nd ed., Vol. IV, p. 193, Academic Press, New York 1960.

On the B chain the enzyme attacks all bonds which are also split by chymotrypsin; over and above, the muscle enzyme also attacks four further bonds which are not hydrolized by chymotrypsin (13/14, 14/15, 17/18, 24/25). Specific substrates which are often used (Table 5) for determining the activities of di-

p.37 gestive proteinases, are not at all affected by the muscle cathepsin. Thus the enzyme is also distinguished from cathepsins A, B and C, which can be distinguished by the degradation of the specif. substrates named.[19] Moreover, the muscle cathepsin can be compared only under certain conditions with other cathepsins (lung cathepsin[20], suprarenal cathepsin[21]) whose proteolytic specificities on the B chain of insulin has been determined: The enzyme splits some bonds characteristic for these cathepsins (13/14, 15/16, 24/25 and 25/26) and also shows, further more, new characteristics of specificity. The muscle cathepsin also differs quite markedly from the cathepsin of cod spleen[1] by the absence of cleavage of the bond Val-Asp (2/3) which is the main cleavage site in the case of spleen cathepsin.

Additional indications concerning the proteolytic specificity of the enzyme were obtained by experimenting with the degradation of beef hemoglobin. The fact that those peptides whose N-terminal amino acids are of a hydrophobe character (Leu, Val, Phe, Tyr) are primarily set free agrees with the findings

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19 H.H. Tallan, N.E. Jones and J.S. Fruton, J.biol.Chemistry, 194, 793 (1952).

20 A.M. Dannenberg and E.L. Smith, J. biol. Chemistry 215,45,55(1955)

21 T.A.A. Dopheide and P.E.E. Todd, Biochim. biophysica Acta (Amsterdam) 86, 130 (1964).

om the B chain of insulin. Due to the fact that amino acids are set free even at the inception of hydrolysis, next to relatively low molecular cleavage peptides it would seem to be apparent that there is an intensive proteolysis of hydrophobe peptide spheres.

As has been shown earlier[1], the specif. substrates introduced by Bergmann and Fruton[22] are not sufficient for classifying the catheptic enzymes. Only some of the cathepsins examined so far are at all able (Cathepsin A, B and C)[19] to degrade such synthetic substrates. The muscle cathepsin examined here is not active either in relation to specif. substrates (Table 5). Thus a classification of the cathepsins can only be made via the degradation of appropriate model proteins. In the degradation of the B chain of insulin the cathepsins[1, 20, 21, 23], however, examined so far, show no extremely different characteristics which would permit a classification of enzymes of shaprly differing specificities. On the contrary, the feature the cathepsins have in common is a type of cleavage of peptide bonds more or less favoured in whose structure the hydrophobe amino acids are participating. The only characteristics for classification for the catheptic nature of a proteinase are the intracellular occurrence and the optimum effectiveness in the slightly acid sphere. The presence of catalytically essential SH-groups in cathepsins, postulated earlier,[24]

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[22] M. Bergmann, Advances in Enzymol. 2, 49 (1942).

[23] E.M. Press, R.R. Porter and J. Cebra, Biochem. J. 84, 501 (1960).

[24] A. Schäffner, Methoden der Fermentforsch. (Methods of ferment research). 2, 2069 (1941).

is not a characteristic of general validity for this group of enzymes, after the findings concerning muscle cathepsin. Russian
p.38 authors have also described the lack of catalytically active SH groups in a purified cathepsin from beef brain.[25]

The ubiquitous occurrence of the cathepsins and the frequent and abundant presence of these enzymes in many tissues raise the question of their possible physiological significance. Their participation in tissue autolysis is quite probable. The relatively high cathepsin activity in fish musculature suggests, in addition, a special function of the muscle cahepsin which is related with the physiological peculiarities of fish: Some fishes perform a considerable rebuilding of protein from the musculature in the direction of the reproductive organs, prior to spawning time. Before spawning time, e.g. in salmon, the weight increases about 10 to 20 times, while at the same time the lateral muscles lose about 40% of their weight. According to what is known about the new formation of proteins, the muscle protein, which serves as a source of amino acid in this process, has to be degraded completely before the proteins of the reproductive organs can be formed. The only proteinase of relatively high activity found in fish musculature for this degradation is the muscle cathepsin. The cathepsin activity of fish musculature is 5 to 10 times higher[2] than that of comparable mammalian tissue; the same also

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25 K.F. Firfarova, A.D. Morozkin and V.N. Orekhovich, Biokhimiya (Moskau) 29, 673 (1964).

applies to the activities of dipeptidases[26] and transaminases[27]. It may thus be possible that these peculiarities in the life cycle of fish are causing the abundance of proteolytic enzymes in the muscle tissue. Consequently, fish muscle cathepsin would not only function in emergencies in connection with autolyses but would have a vital task in the protein make-up of these animals.

We wish to express our gratitude for the grant (AIF-J506) from the Bundeswirtschaftsministerium (Federal Department of Eommerce and Trade) in support of these experiments.

SUMMARY*

1. The main catheptic component of cod muscle was purified 3,000 times. The enzyme fraction does not cleave dipeptides or synthetic peptide derivates of the Bergmann-Fruton series.

2. The pH optimum was 4.6. The activity is not influenced by metal ions. The enzyme contains - with an approximate molecular weight of 50,000 - two SH groups per mol which, however, are inactive catalytically. Serine also does not participate in the active site (no inhibition by diisopropylfluorophosphate) whereas histidine is involved in the catalytic process (photooxydation).

3. Cod muscle cathepsin, which acts like an endopeptidase,

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26 G. Siebert and A. Schmitt, FAO Symposium, "Significance of Fundamental Research in the Utilisation of Fish", Husum, May 1964.

27 G. Siebert, A. Schmitt and I. Bottke, Arch. Fischereiwiss. 15, 233 (1965).

* Translator's Note: The original text ~~also~~ gives a translation into English of the Summary in German, which is copied here.

splits several proteins. The products are peptides with an average chain length of 4.5 residues and free amino acids.

4. The degradation of the B chain of insulin leads to the recognition of two major cleavage sites (15/16 and 16/17); this is in agreement with analyses of cleavage products from hemoglobin. Therefore, cod muscle cathepsin is different from cod spleen cathepsin, and despite some similarities, it is also profoundly different in proteolytic specifity from chymotrypsin and pepsin.

5. A hypothesis is advanced which may explain the biological significance of cathepsin from cod muscle.

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