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A NEUTRAL METALLOPROTEINASE FROM STREPTOMYCES RIMOSUS

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A neutral metalloproteinase was isolated from the culture filtrate of Streptomyces rimosus using ion exchange and gel chromatography. The pure enzyme was immobilized on CNBr-activated Sepharose 4B and some biochemical properties of the two enzymes were compared. The Michaelis constant did not change on immobilization but temperature optimum and pH optimum were lowered. Although pH stability was increased, the temperature stability was reduced. It is concluded that no diffusional limitations influence the enzyme kinetics. The observed changes in stability probably result from conformational changes of the immobilized metalloproteinase.

Our initial investigations have shown that Streptomyces rimosus, grown under conditions to produce oxytetracycline, synthesizes also proteolytic enzymes. Using various proteinase inhibitors we have determined that serine and metalloproteinases, active over a wide pH range, were excreted in the culture liquid (1). We have already purified and characterized an alkaline serine proteinase (2). In this paper we present the results of our work on a neutral metalloproteinase which we isolated from the waste culture liquid of S. rimosus after oxytetracycline production.

EXPERIMENTAL

S. rimosus K-OK-0.2 grown in a fermentor at 28 °C under conditions for oxytetracycline production was supplied by Krka. At the end of the fermentation the biomass was removed by filtration using a vacuum drum filter (IMMA, Italy) and the obtained culture filtrate was used as the source of enzyme.

Chemicals. Sephadex G-75 Superfine, CM-Sephadex C-50, CNBr-activated Sepharose 4B were from Pharmacia, Sweden. Phenylmetanesulphonylfluoride (PMSF), p-chloromercuribenzoate (PCMB), ethylenediamine tetraacetic acid (EDTA), N-benzoyl-L-tyrosine ethyl ester (BTEE) leucine-2-naphthylamide (LeuNA), t-Boc-Ala-p-NO₂ were from Sigma, USA. CM-cellulose and protein standards for molecular weight determination were purchased from Serva, FRG. Hemoglobin was prepared in our laboratory according to Anson (3). All other chemicals were of a reagent grade, obtained either from Merck, FRG, or BDH, England.

Enzyme assay. Activity toward protein substrates was measured according to the method of Anson (3). Esterolytic activity toward BTEE was measured by the method of Walsh (4). Protein content was determined by the Lowry method (5).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed in gels containing 15% acrylamide in a 0.34 M β-alanine buffer of pH 4.4, according to Davis (6). Gels were stained with Coomassie brilliant blue.

Isoelectric focusing. This method was performed on a Desaga apparatus (Desaga, FRG) as described by the manufacturer. Five percent polyacrylamide gels (9 x 16 cm) containing carrier Ampholines (LKB, Sweden) of a pH range 3-9 were used. The voltage was gradually increased from 100 to 1000 V during 90 min focusing time, while the current dropped from an initial 22 to 10 mA. The gels were washed with 10%

trichloroacetic acid (TCA) to remove Ampholines and were subsequently stained with Coomassie brilliant blue. A mixture of 8 standard proteins was run in parallel in the same gel. The pI values of the proteins were determined by measuring the pH in the gel using a microcombination electrode (Desaga, FRG).

Immobilization of metalloproteinase. Metalloproteinase was reacted with CNBr-Sephacrose, according to the manufacturer's instructions, and washed exhaustively with acetate buffer pH 4.0 and borate buffer pH 8.0. The amount of enzyme attached to the Sepharose was determined from the difference between the protein content of the starting solution and the combined filtrates after washing. The proteolytic activity of the insoluble enzyme was measured by the method of Anson (3). 1 ml of suspension of immobilized metalloproteinase was pipetted on a glass filter and the buffer removed with suction. The solid residue was transferred to a test tube and a substrate solution was added. After incubation, the reaction mixture was filtered through a glass filter into 4 ml of TCA and the amount of TCA soluble products determined as described (3).

Molecular weight determinations. The molecular weight was determined by the gel filtration method, according to Andrews (7), in a Sephadex G-75 column in the presence of standard proteins. It was also determined by sodium dodecyl sulphate (SDS) gel electrophoresis according to Weber and Osborn (8).

Metal ion content. The content of Zn^{++} and Ca^{++} in isolated metalloproteinase was determined by atomic absorption spectrometry using Varian atomic absorption spectrophotometer by the flame technique. The metal ions were removed from the enzyme by gel chromatography. The enzyme solution and 10 mM EDTA were incubated for 15 min and applied on the column of Sephadex G-100. It was eluted with 0.05 M Na-acetate buffer pH 6.0. The proteolytic activity and metal content, in fractions containing proteins, were measured.

pH optimum. The pH optimum determinations of soluble and immobilized enzyme were made with a 2% hemoglobin solution of an appropriate pH in a Johnson-Lindsay buffer.

pH stability. The stability of proteinase, in solutions of different pH, was tested by the preincubation of enzyme with buffer solutions of appropriate pH, for 2 hrs at 37 °C. Afterwards, the proteolytic activity toward hemoglobin, at pH 6.0, was determined.

Temperature optimum. The optimal temperature for the hydrolysis of hemoglobin by soluble and immobilized metalloproteinase was measured by incubating the enzyme and a previously warmed substrate at different temperatures. The thermal stability of the free and immobilized metalloproteinase was examined by warming in a 0.05 M acetate buffer pH 6.0 for 10, 20 and 30 min at various temperatures and subsequently assayed for proteolytic activity.

Effect of potential inhibitors. The following substances were tested: pepstatin, iodoacetamide, 1,10-phenantrolin, diisopropylphosphofluoridate (DFP) and Na-tripolyphosphate. Enzyme and effectors were preincubated at room temperature for 30 min prior to proteinase assay with hemoglobin.

RESULTS

Purification of the enzyme. Purification scheme of neutral metalloproteinase is shown in Fig. 1. Concentrated culture liquid, chromatographed on a CM-Sephadex C-50, was resolved in three main peaks after the application of a NaCl gradient (Fig. 2). The second protein peak, which contained the bulk of proteolytic activity, was chromatographed on a CM-cellulose at pH 8.0 and finally on a Sephadex G-75 Superfine. Fractions exhibiting proteolytic activity were pooled and concentrated. PAGE showed a single protein band. Isoelectric focusing confirmed the homogeneity of the enzyme which focused at pH 9.6 (Fig. 3A). Table I

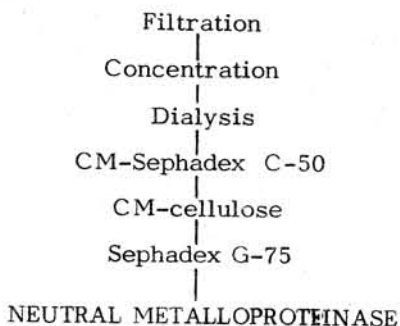


Fig. 1. Purification scheme of neutral metalloproteinase.

shows the results of a typical purification experiment. Neutral proteinase was purified 36-fold with 10% yield.

Properties of the soluble and immobilized metalloproteinase. Molecular weight of the enzyme as determined by gel chromatography was 25 700 and 35 000 by the SDS electrophoresis (Fig. 3B). By the atomic absorption method, the metal content was 1.2 μg Zn/mg enzyme

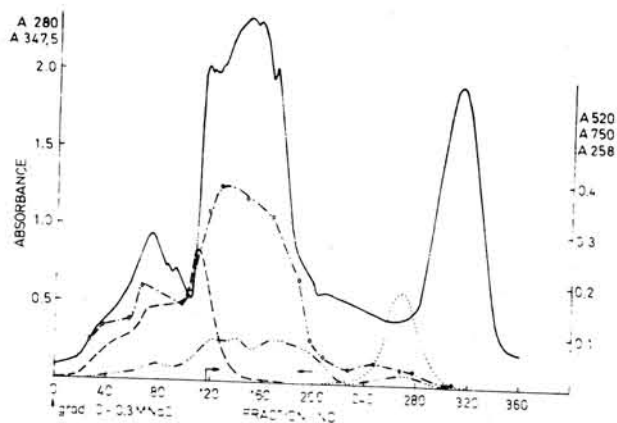


Fig. 2. CM-Sephadex C-50 ion exchange chromatography. Column was equilibrated with 0.01 M Na-acetate buffer pH 5.5. A linear gradient toward 0.3M NaCl was applied as indicated by an arrow. Flow rate was 40 ml/h, fractions of 10 ml were collected.
 — A_{280} (protein); activity toward: - - - - hemoglobin pH 7.5,
 ······ BTEE, - - - - LeuNA, ······ t-Boc-Ala-p-NO₂.

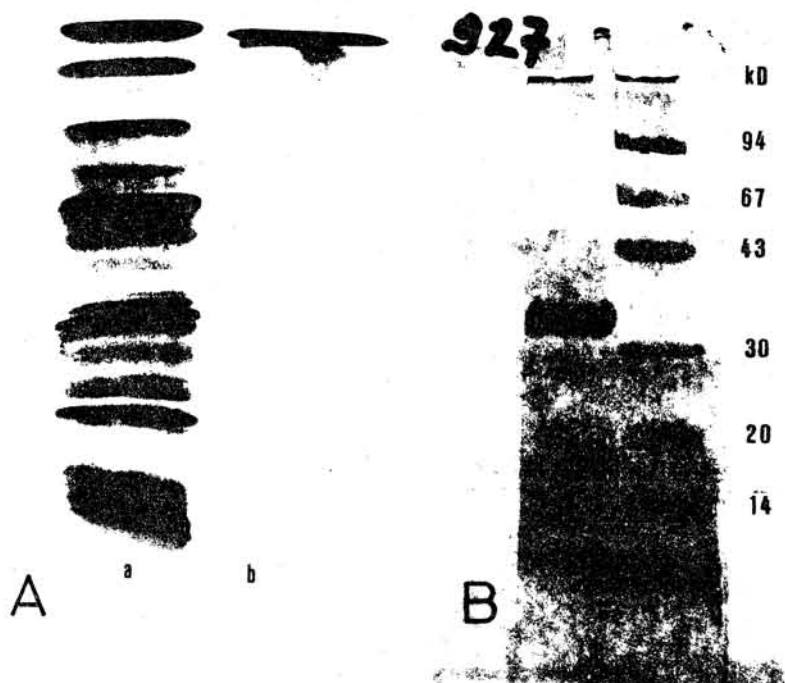


Fig. 3A. Isoelectric focusing of neutral metaloproteinase (b); protein standards (a).

Fig. 3B. SDS-PAGE of neutral metaloproteinase (a); protein standards (b).

and 6.1 $\mu\text{g Ca/mg}$ enzyme.

Binding of metaloproteinase to Sepharose 4B. Approximately 60% of the enzyme protein was bound to the carrier as determined from the difference between the protein content of the original solution and the combined filtrates and washing solutions.

pH optimum. Optimal pH for the hydrolysis of hemoglobin by the soluble enzyme was at pH 6.5. The pH dependence curve for immobilized enzyme showed the maximum at pH 6.0 with a shoulder at pH 9 (Fig. 4).

pH stability. Soluble metaloproteinase was stable within the pH region 5-8. The bound enzyme, however, had a much broader stability, being completely stable at pH 4 as well as at pH 10 (Fig. 5).

Table I.
Purification of neutral metalloproteinase

Purification step	Vol. (ml)	Protein (mg)	Sp. act. ($\frac{\mu\text{g Tyr}}{\text{mg}\cdot\text{min}}$)	Yield (%)	Purif. factor
Filtrate	8000	72 000	79	100	1
Concentrate	500	13 650	322	78	4
Dialysate	550	6 900	652	60	8
CM-Sephadex	800	1 280	1007	35	12
CM-cellulose	195	406	2015	14	26
Sephadex G-75	324	185	2086	9	36

Temperature optimum and thermal stability. The optimal temperature for hemoglobin hydrolysis was at 55 °C. At 80 °C the enzyme still retained a half of its maximal activity. Insoluble metalloproteinase exhibited approximately the same temperature optimum, but at higher temperatures the activity dropped faster in comparison with soluble enzyme. When these data are plotted in an Arrhenius diagram, a break in the curve for the immobilized enzyme can be observed (Fig. 6). Thermal sta-

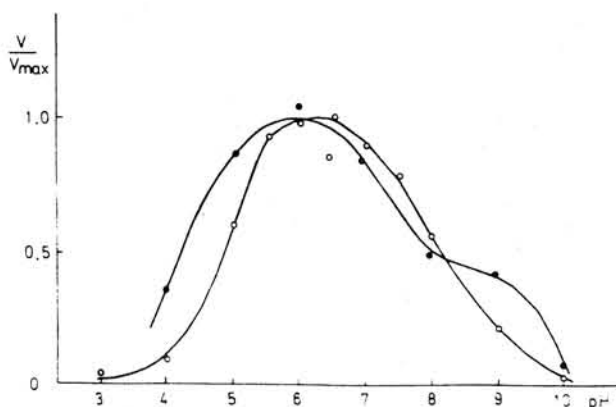


Fig. 4. pH dependence of proteolytic activity toward hemoglobin for soluble (-o-o-) and immobilized (-●-●-) enzyme.

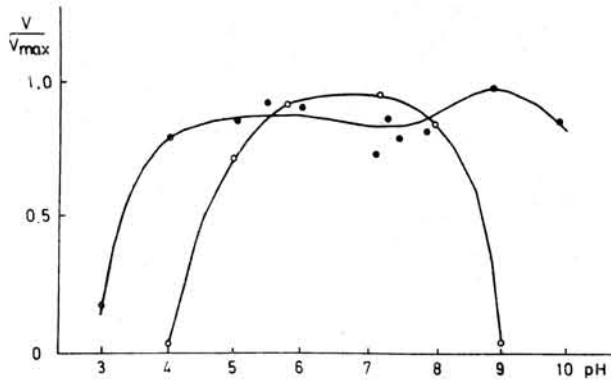


Fig. 5. Stability of soluble (-o-o-) and immobilized (-●-●-) neutral metalproteinase at different pH values.

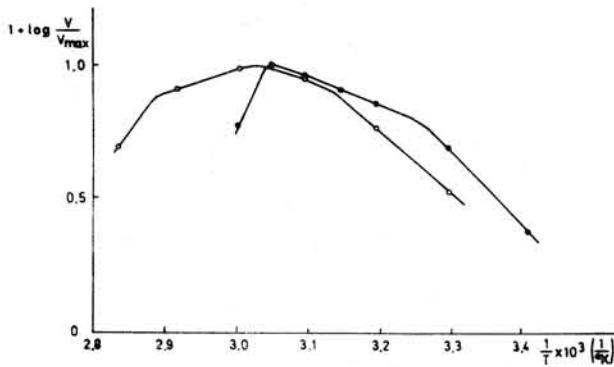


Fig. 6. Arrhenius diagram of soluble (-o-o-) and immobilized (-●-●-) neutral metalproteinase.

bility is consistent with these data. Soluble enzyme remains stable for 30 min at 50 °C in contrast to the immobilized one, which after 30 min at this temperature, retains only 15% of the initial activity (Fig. 7).

Michaelis constants. Very similar K_m values were obtained for free (0.81 g/100 g) and immobilized (0.91 g/100 g) enzyme (Fig. 8).

Effect of potential inhibitors. The effect of potential inhibitors on

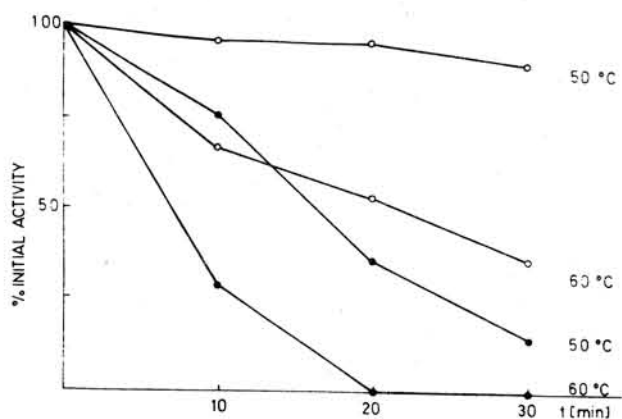


Fig. 7. Thermal stability of soluble (—o—o—) and immobilized (—●—●—) neutral metaloproteinase.

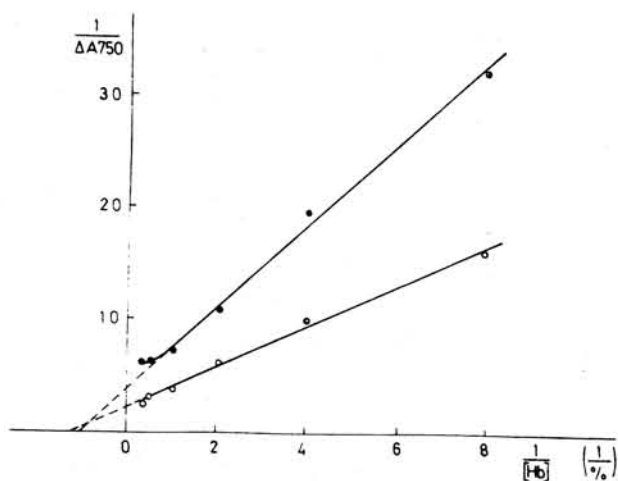


Fig. 8. Lineweaver-Burk diagram of the dependence of proteolytic activity vs. hemoglobin concentration for soluble (—o—o—) and immobilized (—●—●—) neutral metaloproteinase.

soluble metaloproteinase is shown in Table II. The immobilized enzyme was also completely inhibited with 10 mM EDTA but only 70% with 1 mM EDTA. If the soluble enzyme was incubated with 1 mM EDTA and 1,10 phenantroline in the presence of 5 mM Zn^{++} a 45% and 26% reactivation was observed. Ca^{++} ions (5 mM) had no effect on the activity. Their

presence only increased the temperature stability of the enzyme.

Table II.

Effect of some inhibitors on the soluble metalloproteinase

Inhibitor	Final concentr. (mM)	Inhibition (%)
1,10 phenantrolin	2	77
EDTA	1 10	98 100
Pepstatin	0.06	5
Iodoacetamide	2	4
DFP	1	0

DISCUSSION

Electrophoretically homogenous metalloproteinase was isolated from the culture broth of S. rimosus with a 10% yield, using a simple purification procedure. Our aim was to study the major hemoglobin degrading activity eluted from CM-Sephadex in the second protein peak, which is probably one of the reasons for the apparently low yield of activity in this chromatographic step. According to Morihara (9) this enzyme can be classified as a neutral metalloproteinase on the basis of its neutral pH optimum, molecular weight, isoelectric point and sensitivity toward EDTA (9,10). Neutral metalloproteinases have already been isolated from Streptomycetes. Hiramatsu et al. (11) isolated a neutral metalloproteinase from S. naraensis and from S. griseus a neutral proteinase was isolated by Nakahashi (12) and Nomoto et al. (13). Both proteinases were stabilized by Ca^{++} , although no direct metal analyses have been reported for S. naraensis neutral proteinase. A pH optimum of 7.5 and a temperature optimum of 40 °C was reported for the S. naraensis neutral proteinase with hemoglobin and casein as substrates. The S. griseus K-1 neutral proteinase exhibited a pH optimum 7.5-8.0 with casein as a substrate. The pH optimum of our enzyme is 1 pH unit lower

and has a definitely higher temperature optimum. S. rimosus neutral metalloproteinase is very sensitive toward chelating agents in mM concentrations, as is the S. naraensis enzyme. One atom of Zn and 4 atoms of Ca have also been determined in other neutral metalloproteinases.

Upon immobilization, some interesting changes in the enzymatic properties of metalloproteinase have been observed: A broader pH stability and a shift of pH optimum toward acidic value. On the other hand, the thermal stability at higher temperatures was reduced upon immobilization. There are numerous examples of increased or decreased stabilities (thermal and pH) of enzymes upon immobilization (14). It is, however, difficult to explain an increase in pH stability with a simultaneous decrease in thermal stability. A possible explanation is that the enzyme molecule underwent a conformational change. The reversible process of thermal denaturation (unfolding of the protein molecule) becomes irreversible at certain temperature (15). It is possible that the unfolded molecule of the immobilized enzyme interacts with the surface of the carrier (Sephacrose) thus preventing complete refolding to the native state. Thermal denaturation in this case occurs at lower temperatures. From the Arrhenius plot, it is evident that soluble and immobilized metalloproteinase have the same activation energy up to 35 °C. At higher temperatures, the activation energy of the immobilized enzyme is decreased. The decrease of the activation energy of the soluble enzyme occurs at 45 °C. The observed change in activation energy after the temperature optimum follows that of the immobilized enzyme at 10 °C temperature difference. One can see that the temperature dependent behaviour of the immobilized enzyme is consistent: thermal stability is decreased as is the temperature optimum, conformational change evident from the Arrhenius plot and the faster drop of activity beyond the temperature optimum. Very similar K_m values of free and immobilized enzyme suggest that the system carrier-enzyme has no diffusional limitations. The rather low activity of the immobilized enzyme and the optimal experimental condi-

tions further support this idea.

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POVZETEK

Z uporabo kromatografije na ionskih izmenjalcih in gelske kromatografije smo izolirali nevtralno metaloproteinazo iz filtrata kulture Streptomyces rimosus. Čisti encim smo imobilizirali na CNBr aktivirano Sepharozo 4B in primerjali biokemijske lastnosti obeh encimov. Mihaeliso-va konstanta se po imobilizaciji ni spremenila, nižala sta se temperaturni in pH optimum. V nasprotju s povišano pH stabilnostjo se je temperaturna obstojnost imobiliziranega encima nižala. Sklepamo, da difuzijske omejitve ne vplivajo na encimsko kinetiko. Spremembe stabilnosti so verjetno posledica konformacijskih sprememb imobilizirane metaloproteinaze.

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