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Original Research Paper

ISOLATION OF CATHEPSIN B FROM CANINE DISEASE-FREE LIVER AND ITS CHARACTERIZATION

Alenka Nemec [1]*, Marinka Drobnič-Košorok [2]

Addresses of authors: [1] Clinic for Surgery and Small Animals, [2] Institute for Physiology, Pharmacology and Toxicology, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author. E-mail: alenka.nemec@vf.uni-lj.si

Summary: Cathepsin B (CB; EC 3.4.22.1) is one of the most widely investigated lysosomal cysteine proteases in humans. However, there has been no data on canine CB isolation and characterization published. The aim of this study was to isolate cathepsin B from canine disease-free liver, characterize it by means of Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), isoelectric focusing and N-terminal sequence analyses, and compare it to cathepsin B isolated from livers of other species.
Ccathepsin B was isolated from the disease-free canine liver using single-step affinity chromatography on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution with 2,2'-dipyridyl disulphide at pH 4.0. This method is called the Rich-Brown-Barrett's method. Using BANA as a substrate, cathepsin B-like activity was detected in effluent from an affinity column. Active fractions were combined and concentrated prior to characterization. Approximately 16 mg of cathepsin B was isolated from 2.93 kg of liver (5.46 $mgkg^{-1}$), applying 5 isolation procedures using the same affinity column. According to the results of the SDS-PAGE, isoelectric focusing and N-terminal sequence analyses, we assume that the proteins isolated from the canine liver can be recognized as the proform and mature form of cathepsin B with molecular masses of 40 kDa and 29 kDa, respectively.

Key words: canine cathepsin B; proteases; isolation; characterization

Introduction

Lysosomal cysteine proteases, generally known as the cathepsins, represent a major component of the lysosomal proteolytic repertoire and play an important role in intracellular protein degradation and turnover (1). The name cathepsin is derived from a Greek term meaning "to digest" (2). Cathepsins have also been implicated in proteolysis occurring within the endosomal system, in particular during antigen processing (3) and are thought to play an extracellular role in several physiological and pathological conditions like bone resorption (4), cartilage degradation in arthritis (5) and tumour invasion and metastasis (6, 7). The role of cysteine proteases in pathological conditions makes them attractive targets for synthetic inhibitor development (7).

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Cathepsin B (CB; EC 3.4.22.1) is one of the most widely investigated lysosomal cysteine proteases in humans. Unlike most other enzymes of this family, it exhibits both endopeptidase and exopeptidase activities. In addition to cleavage within the peptide substrate, CB can also remove dipeptide units from the C terminus (peptidyl-dipeptidase activity). It belongs to the papain superfamily and shows high homology to cathepsins L, X, S and O, papain and actinidin, among others (8, 9, 10).

Methods have been proposed in detail for the purification of cathepsin B from liver using standard protein purification techniques including organomercurial-Sepharose chromatography. Kidneys, spleen and placenta have also been used as tissue sources (8). The development of immobilized Gly-Phe-Gly- and Phe-Gly-semicarbazones as affinity ligands represented a major advance in cathepsin B purification, effectively providing a

single-step purification of the enzyme following elution with pyridyl disulphide (11). With SDS PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) under reducing conditions, a mammalian proform of cathepsin B is observed as a 40 kDa band, a mature form of cathepsin B isolated from the previously mentioned tissues is usually observed as a 30 kDa band representing the single-chain enzyme and 25 kDa and 5 kDa bands representing the two-chain form. With isoelectric focusing, multiple components are observed with isoelectric point values in the range 4.5 – 5.5 (9). In addition to natural sources, several recombinant expression systems (12) have been used to prepare cathepsin B, including E. coli, P. pastoris, baculovirus systems and mammal CHO cells. These systems have also facilitated the investigation of the role of specific residues by site-directed mutagenesis (9).

CB has been implicated in the progression of a variety of human tumours. Overexpression of CB mRNA, increased CB staining, elevated CB activities and altered localization have been found in different cancers, especially at the invasive edges, suggesting a role for CB in tumour invasion (13-15). Although human CB is one of the most widely investigated of all cathepsins, very little is known about canine CB. According to the available literature, there had not been any data published on the isolation and characterization of canine CB at the start of our research. However, elevated levels of CB activity in canine mammary tumours have already been shown (16). It has also been demonstrated that polyclonal antibodies against human CB, raised in rabbits, are not suitable for immunohistochemical studies on canine tissues (17).

The aim of this study was to isolate cathepsin B from canine disease-free liver, characterize it by means of SDS-PAGE, isoelectric focusing and N-terminal sequence analyses and compare it to cathepsin B isolated from the liver of other species.

Material and methods

Materials

The standard laboratory chemicals and reagents, unless otherwise stated, were obtained from Sigma, Germany. The reagents used for the SDS-PAGE were from Bio-Rad (Germany) and the sequencing reagents were from PE Applied Biosystems (Foster City, CA, USA). The present study began with the collection of livers from healthy dogs that were euthanized at the Clinic for Small Animal Medicine and Surgery, Veterinary Faculty, University of Ljubljana, Slovenia.

Isolation of cathepsin B

Canine cathepsin B was isolated from the liver with single-step affinity chromatography (11) on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution by 2,2'-dipyridyl disulphide at pH 4.0. Five isolations of cathepsin B were performed with the same affinity medium

Five grams (dry weight) of activated CH-sepharose 4B (Pharmacia, Sweden) was soaked overnight in 1 mM HCl (Merck, Germany) at 4C and then washed with 0.1 M $NaHCO_3$. 100 mg of Gly-Phe-Gly-semicarbazone (Bachem U.S.; USA), a reversible inhibitor, was dissolved in 25 ml of methanol and 15 ml of 0.1 M $NaHCO_3$, pH 8.0 was added. The activated sepharose was agitated in the solution in a roller rack overnight at 20C. The gel was collected on a sintered-glass filter, washed with 50% (v/v) methanol (Merck, Germany) and then with water, before being agitated over night with 6% (v/v) ethanolamine, adjusted to pH 9.0 (with HCl) for 4 h at 20 C, finally washed with water and stored at 4 C in 0.1 % NaN_3 (Merck, Germany).

The canine disease-free livers were removed in the course of post-mortem examinations, minced and stored at -20 C until required. Crude cathepsin B was applied to the affinity medium and was prepared as follows: 1.) a portion of canine liver was thawed and homogenized in two parts (w/v) of an ice-cold solution of 1 % (w/v) NaCl, 2 % (v/v) butan-1-ol and 1 mM EDTA. 2.) The homogenate was centrifuged at 8000 rpm for 30 minutes at 4 C in a Sorvall centrifuge. 3.) The pH of the supernatant was then adjusted to 4.5 with 2 M HCl and acid activation at 37 C for 1 h followed. 4.) The mixture was centrifuged at 8000 rpm for 30 minutes and the supernatant used for either acetone (47-64 %) or ammonium-sulphate precipitation (20-75 %). 5.) The first precipitate was removed by centrifugation at 5000 rpm for 30 minutes. 6.) The second precipitate, containing cathepsin B, was collected after centrifugation at 5000 rpm for 30 min-

utes, redissolved in a minimum volume of 50 mM of a sodium-phosphate buffer, which contained 1 mM EDTA and had a pH of 6.0, before being dialysed, using the same buffer, overnight at 4 C.

The solution of prepared crude cathepsin B (brown solution) was made to 2 mM with respect to DTT and stirred with the affinity medium (gel) for 2 h at room temperature. The gel was then washed with 1.5 l of a 50 mM sodium-phosphate buffer (pH 6.0), containing 0.5 M NaCl, followed by the same volume of a 50 mM sodium format buffer (pH 4.0), before being packed into a column (18 cm x 1.5 cm dia.). Then 1.5 mM 2,2'-dipyridyl disulphide in a 50 mM sodium format buffer (pH 4.0) was run into the gel until the A_{280} measurements of the eluted fractions exceeded 2.5. The flow was then stopped and the column was left overnight at 4 C prior to the elution of the cathepsin B the next day. The elution of the cathepsin B from the gel was performed using 1.5 mM 2,2'-dipyridyl disulphide in a 50 mM sodium format buffer (pH 4.0).

The fractions being collected were assayed for cathepsin B activity using BANA (N—Benzoyl-DL-arginine—naphthylamide) as a substrate (18, 19). The substrate was hydrolysed, liberating 2-naphthylamine, and this assayed colorimetrically (A_{520}) by coupling it with a diazonium salt – Fast Garnet (4-diazo-2'3-dimethylazobenzene). The protein concentrations in the eluted fractions were determined with direct A_{280} measurements.

The active fractions (A_{520} 0.1) were combined, concentrated and transferred into a 20 mM sodium-acetate buffer (pH 5.2), containing 1 mM EDTA, by ultrafiltration on an Amicon YM-5 membrane (Millipore, USA).

The protein concentrations of the concentrated fractions of isolated cathepsin B were determined using a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) (20). The standard curve was obtained using bovine-serum albumin.

The cathepsin B samples were analysed using SDS-PAGE on 12 % polyacrylamide gels (21). Electrophoretic separations were performed on a Bio-Rad Mini-PROTEAN II electrophoresis cell (Bio-Rad; USA), following the manufacturer's instructions. The samples were reduced with 5 % 2-mercaptoethanol at 100 C for 5 minutes prior to electrophoresis. The proteins were visualised by Coomasie Brilliant blue R-250 staining. The molecular mass of the cathepsin B was determined by comparing the mobility of the isolated cathepsin B in polyacrylamide gels with those from the following standards: - Aprotinin 6.5 kDa; Lysozyme 14.4 kDa; Trypsin inhibitor 21.5 kDa; Carbonic anhydrase 31.0 kDa; Ovalbumin 45.0 kDa; Serum albumin 66.2 kDa; Phosphorylase b 97.4 kDa; -galactosidase 116.25 kDa; Myosin 200 kDa.

Isoelectric focusing was carried out on polyacrylamide plates with Pharmalyte carrier ampholines in the pH range of 3-10, using the PhastSystem apparatus (Pharmacia-LKB; Uppsala, Sweden), following the manufacturer's instructions. A mixture of standard proteins with pI ranging from 3.5 to 9.3 was run parallel with the samples. After fixation in 20 % trichloroacetic acid, the proteins were stained with Coomasie Brilliant Blue G-250.

For the determination of the N-terminal sequences, samples containing isolated cathepsin B were first subjected to SDS-PAGE. The proteins were electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (Immobilon-P, Millipore, USA), using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, USA) set at a constant voltage of 100 V for 45 minutes. The proteins were visualized with Coomasie Brilliant Blue G-250 stain. Components of interest were excised, destained with 50 % methanol and analysed directly from the membrane with an Applied Biosystems Procise sequencer system 492A. Phenylthiohydantoin derivatives were identified on-line using the attached Applied Biosystems 140C HPLC system.

Results

Cathepsin B was found expressed in a variety of tissues, with the highest level of expression observed in the kidneys, spleen and liver, and in a number of different types of tumours and cancer cell lines (22, 23, 24, 25, 26).

In our study, cathepsin B was isolated from canine disease-free liver by single-step affinity chromatography on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution with 2,2'-dipyridyl disulphide (pH 4.0). During the isolation procedure, any cathepsin B-like activity in the eluted fractions was measured using BANA as a substrate. The distribution of the cathepsin B-like activity in the effluent of an affinity column is presented in Figure 1.

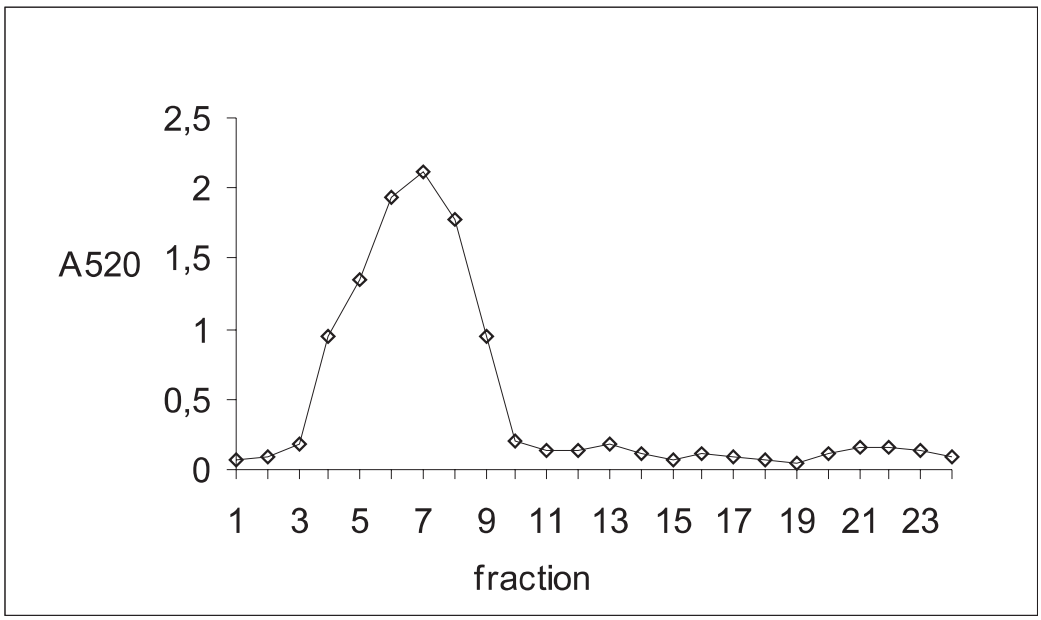

Figure 1: Distribution of cathepsin B-like activity in the effluent of an affinity column

Approximately 16 mg of cathepsin B was isolated from 2.93 kg of liver (5.46 mgkg^{-1}), applying 5 isolation procedures using the same affinity column.

Characterization of canine cathepsin B

SDS-PAGE

The molecular masses of the isolated proteins from the canine livers were estimated on SDS-PAGE gels under reducing conditions. Samples with cathepsin B-like activity after being concentrated, samples of crude cathepsin B preparations, and acetone and ammonium sulphate precipitations prepared from liver, were applied to 12 % polyacrylamide gels and electrophoresis performed as previously described. Two major protein bands with molecular masses of 29 kDa and 40 kDa were visible (Figure 2). We assumed that the 29 kDa and 40 kDa protein bands might represent the mature form and the proform of cathepsin B (procathepsin B), respectively (Figure 2; lines 3, 4, 5, 6). The results of the SDS-PAGE demonstrated that mature canine-liver cathepsin B exists only in a 29 kDa single-chain form like mature cathepsin B from sheep liver (27). The molecular mass of the mature cathepsin B matched a single-chain form of cathepsin B from human, rat, porcine, ox and rabbit liver, as well as cathepsin B from bovine spleen (28, 29).

There were no differences in the molecular masses of the mature form and the proform of cathepsin B when the affinity medium was used in conjunction with preliminary fractionation with ammonium sulphate or acetone.

Analytical Isoelectric focusing

Isoelectric focusing of the concentrated cathepsin B showed two major bands with pI of 4.8 and 5.0 (Figure 3; line 2), as estimated according to standard proteins that were run parallel with the samples. Similar bands were found as minor components in a crude cathepsin B preparation (Figure 3; line 3).

The results can be compared with the isoelectric focusing of six isoenzymes of human liver cathepsin B with pI ranging from 4.5 to 5.5 and the major peak of activity being between 5.0 and 5.2 (9, 30).

N-terminal sequence analysis

The N-terminal sequence analysis revealed the highest extent of homology of both protein bands from SDS-PAGE with the corresponding regions of bovine procathepsin B and mature form of bovine cathepsin B, as well as with cathepsin X. The N-terminal sequence of the mature form of canine cathepsin B (29 kDa) differs from the corresponding region in bovine cathepsin B in 4 out of 30 amino acids (Figure 4).

A comparison of the N-terminal sequences of procathepsin B (40 kDa) with the corresponding region of bovine procathepsin B is presented in Figure 5.

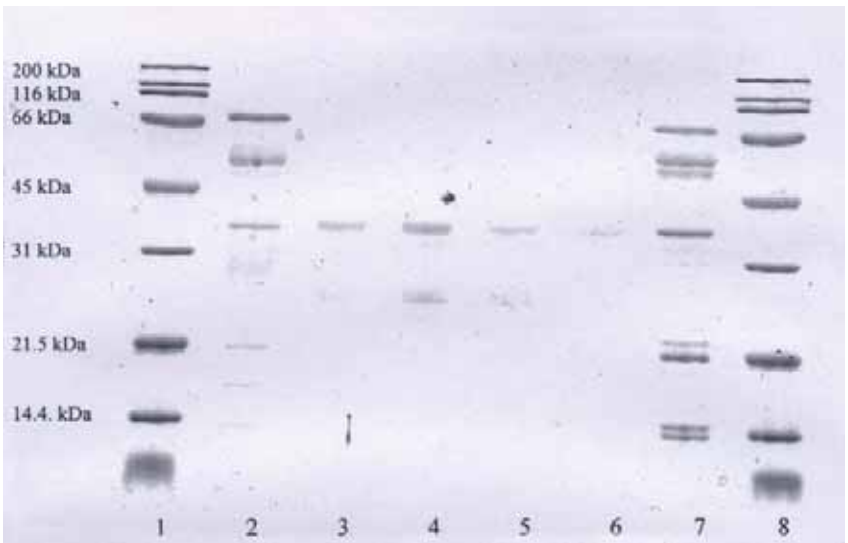

Figure 2: 1 & 8 = standards; 2 = acetone precipitate; 7 = ammonium sulphate precipitate; 3, 4, 5 & 6 = concentrated fractions eluted from an affinity column

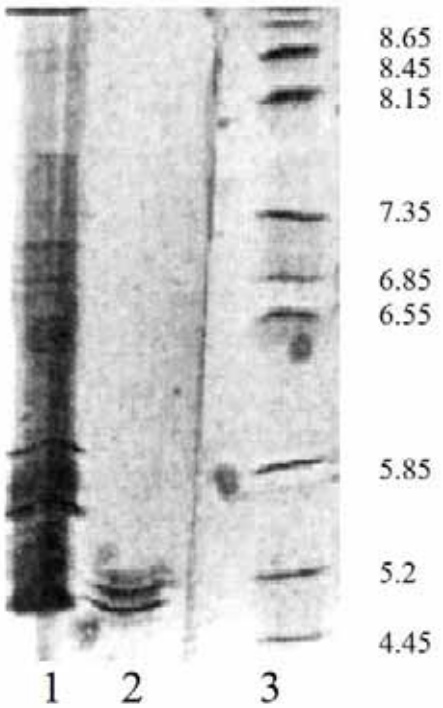

Figure 3: 1 = ammonium sulphate precipitate; 2 = concentrated cathepsin B after elution; 3 = standards

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Isolated protein from canine liver (29 kDa):
                          VNVEVSAEDMLTCCGDQCGDGCNGGFPAEA
Bovine cathepsin B: 129   VNVEVSAEDMLTCCGGECGDGCNGGFPSGA 158
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Figure 4: Comparison of the N-terminal sequences of the 29 kDa protein isolated from canine liver with the corresponding region in bovine cathepsin B. The homology between the amino acid sequences is shown by the shading

The alignment of the 40 kDa protein isolated from canine liver with the bovine procathepsin B matched with the procathepsin B region starting with residue 23, while the 29 kDa protein matched with the N-terminal part of the heavy-chain of the bovine cathepsin B, starting with residue 129. The 40 kDa protein isolated from canine liver matched with human procathepsin C and procathepsin X, starting with residues 231 and 62, respectively.

Discussion

Human cathepsin B is one of the most thoroughly studied lysosomal cysteine proteases (9, 30), most probably due to its involvement in several pathological conditions, notably tumour invasion and metastasis, cartilage degradation in arthritis and many others. Compared to human CB, little is known about canine CB. When we began our study, we were unable to find any data on the isolation or characterization of canine CB in the literature available.

Several methods for the isolation of CB from liver have been reported in detail (8, 9). Besides its isolation from human liver, CB has also been isolated from rat, rabbit, ox, bovine and sheep liver (28, 29, 31). Kidneys, spleen and placenta from humans and from other species (bovine, sheep, rat) have also been used as tissue sources (9). A major advance in the purification of CB was the development of an immobilized reversible inhibitor – Gly-Phe-Gly-semicarbazone – as an affinity ligand, effectively providing a single-step purification process of this enzyme following elution with 2,2'-dipyridyl disulphide (Rich-Brown-Barrett's method) (11). In our study, the Rich-Brown-Barrett method was used for the isolation of CB from canine liver. Using BANA as

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Isolated protein from canine liver (40 kDa):
                                LP KSWDWRNVNGVNY
Bovine procathepsin B:23   FPPLS D ELVNFVNKQ 39
Human procathepsin C: 231  LP TSWDWRNVHGINF 245
Human procathepsin X: 62   LP KSWDWRNVDGVNY 76
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Figure 5: Comparison of the N-terminal sequences of the 40 kDa protein isolated from canine liver with the corresponding region in bovine procathepsin B, human procathepsin C and human procathepsin X. the homology between the amino acid sequences is shown by the shading

a substrate, cathepsin B-like activity was detected in effluent from the affinity column. Active fractions were combined and subsequently concentrated. The sample obtained was characterized by means of SDS-PAGE, analytical isoelectric focusing and N-terminal sequencing.

Under reducing conditions, the SDS-PAGE revealed two major protein bands; a 29 kDa band which we assumed to be the mature form of canine CB in a single-chain form and a 40 kDa band assumed to be the proform of cathepsin B (procathepsin B). Contrary to mature human, bovine, rabbit and ox liver mature CB that exist as a single-chain and two-chain forms, mature canine CB only exists in a single-chain form as is also the case for CB from sheep liver (28). Based on the purification of CB from many different normal tissues from numerous species, using SDS-PAGE under reducing conditions, mature CB exists in a single-chain form of 30 kDa or a double-chain form of 25 kDa (heavy chain) and 5 kDa (light chain) (9, 27). The mammalian proform of CB is observed as 40 kDa band (9). In addition to the single- and double-chain forms of CB detected in fractions from both normal human liver and human tumours, Moin et al. also detected two isoforms of the heavy chain of the double-chain form of CB as 25/26 kDa bands. The 1 kDa difference was due to N-linked high-mannose-type oligosaccharide moiety (32).

Using isoelectric focusing, both forms of canine CB, with pI of 4.8 and 5.0, are comparable with the isoelectric focusing of six isoenzymes of human-liver CB with pI ranging from 4.5 to 5.5 with a major peak of activity at 5.0 to 5.2 (9, 30).

N-terminal sequences were determined for both forms of the isolated proteins from canine liver and compared with the sequences that were available for other cysteine proteinases. While the 29 kDa protein band from the SDS-PAGE was shown to have the highest extent of homology with the corresponding region of mature bovine CB, the 40 kDa band showed, to a lesser extent, homology with the bovine procathepsin B, which indicates that the isolated proteins may represent both the proform and the mature form of CB. However, a few months after the N-terminal sequence determination of the canine procathepsin B, a complete amino-acid sequence of human procathepsin X was published (33). After comparing the amino-acid sequences of the human procathepsin X with the canine procathepsin B, one may speculate that the isolated canine proteases might represent a canine cathepsin X and canine cathepsin B. On the other hand, Kos and coworkers (34) clearly demonstrated that only cathepsin B is involved in the degradation of extracellular matrix proteins, which supports our assumption that the isolated proteins, at least their major part, correspond to canine procathepsin B and the mature form of cathepsin B.

According to the results of the characterization, we assume the proteins isolated from canine liver can be recognised as the proform and the mature form of cathepsin B, with molecular masses of 40 kDa and 29 kDa, respectively.

Acknowledgements

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IZOLACIJA KATEPSINA B IZ ZDRAVIH JETER PSA IN NJEGOVA KARAKTERIZACIJA

A. Nemec, M. Drobnič-Košorok

Povzetek: Kljub temu da je človeški katepsin B najbolj raziskana lizosomalna cisteinska proteaza, v literaturi trenutno ne moremo najti podatka o izolaciji ali karakterizaciji pasjega katepsina B. Cilj naše raziskave je bil izolirati katepsin B iz zdravih pasjih jeter, ga okarakterizirati z metodami poliakrilamidne gelske elektroforeze v prisotnosti natrijevega dodecil sulfata (SDS-PAGE), z izoelektričnim fokusingom in določitvijo aminokislinskega zaporedja N-terminalnega dela ter ga primerjati s katepsinom B, izoliranim iz jeter drugih živali. Katepsin B smo izolirali iz zdravih pasjih jeter z metodo Rich-Brown-Barrett. To je metoda afinitetne kromatografije, pri kateri smo kot ligand uporabili semikarbazon gly-phe-gly, ki je reverzibilni inhibitor katepsina B. Eluiranje katepsina B iz kolone je potekalo z 2,2'-dipiridil disulfidom pri pH 4. Z uporabo afinitetne kromatografije smo v eluiranih frakcijah z uporabo substrata BANA zaznali katepsinu B podobno encimsko aktivnost. Pred karakterizacijo izoliranih proteinov smo aktivne frakcije združili in skoncentrirali. S petimi izolacijami na isti afinitetni koloni smo iz 2.93 kg jeter izolirali približno 16 mg katepsina B (5.46 $mgkg^{-1}$). Na osnovi rezultatov karakterizacije predvidevamo, da sta izolirana proteina zrela oblika katepsina B z molekulsko maso 29 kDa in nezrela oblika katepsina B z molekulsko maso 40 kDa.

Ključne besede: pasji katepsin B; proteaze; izolacija; karakterizacija