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Was the serine protease cathepsin G discovered by S. G. Hedin in 1903 in bovine spleen?

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In the beginning of the 20th century, enzymes with proteolytic activity were classified as peptidases, Erepsin, and proteases. Among these, pepsin, trypsin, and autolytic enzymes were of the protease class. Spleen-derived proteases were poorly characterized until Sven Gustaf Hedin performed several digestion experiments with bovine spleen. He incubated minced bovine spleen under acidic or neutral conditions and characterized two active proteases; the results were published in 1903. The first protease was named α-protease and was active under neutral conditions. The second was named β-protease and was active under acidic conditions. We replicated Hedin's experiments according to his methods and found, by using activity-based probes to visualize proteases, that the historical α-protease is the present-day serine protease cathepsin G (CatG), which is known to be important in several immune processes, including antigen processing, chemotaxis, and activation of surface receptors. The β-protease, however, comprised different proteases including CatX, B, S, and D. We suggest that Hedin described CatG activity in bovine spleen over 100 years ago.

Keywords: proteases, cathepsin, spleen cells, Hedin

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INTRODUCTION

Proteolytic enzymes are generally broadly classified as proteases, but in 1915, the classification of such enzymes was based on more specific substrates and included peptidases (digest polypeptides), Erepsin (digest peptones), and proteases (degrade proteins). Pepsin, trypsin, and autolytic enzymes were classified as proteases (Hedin, 1915). Towards the end of the 19th century, various scientists concentrated their research on autolytic enzymes. For example, in 1890, Salkowski found increased nitrogenous substances during long-term incubation of liver, muscles, and suprarenal glands at body temperature and interpreted his findings as the action of proteolytic enzymes in these tissues (Salkowski, 1890). Thirteen years later, the Swedish scientist Sven Gustaf Hedin extended Salkowski's results by treating minced bovine spleen with either neutral or acidic medium and analyzing the products of autodigestion. Hedin published his data in 1903, showing that bovine spleen cells harbor at least two different proteolytic enzymes, one active under neutral conditions, which he named α-protease, and the other active under acidic conditions, which he named β-protease (Hedin, 1903a).

After discovering these two proteases in bovine spleen, Hedin performed further experiments with bovine serum and concluded that it contained a weak proteolytic enzyme active in neutral medium, the activity of which could be blocked by heating at 55 °C or neutralized by antibodies (also named anti-enzymes at the time) present in the serum. Hedin assumed that the α-protease in bovine spleen-derived leukocytes was similar to the protease found in serum. He further speculated that the α-protease may derive from lysed leukocytes or leukocytes that secrete proteases (Hedin, 1903b).

In another experiment, Hedin mixed bovine pancreas-derived trypsin with normal serum and added casein as a substrate. The serum had a neutralizing effect. Therefore, the data obtained were called *Reihenfolgephänomen* (i.e., order phenomenon), and the optimum temperature of neutralization was determined to be 37 °C (Hedin, 1905). Hedin concluded that antibodies were necessary for the anti-tryptic activity because previous antibody publications had reported anti-tryptic effects (Landsteiner, 1900; Cathcart, 1904). Hedin defined a general principle by preincubating trypsin with serum at 37 °C before adding the substrate. This successive procedure, which he established 100 years ago, is the standard protocol for biochemical inhibition of proteases.

Moreover, Hedin stated that inside leukocytes, proteases were involved in "digesting processes" (Hedin, 1903a). Phagocytosis, with credit given to Metchnikoff, appears intracellularly in so-called "digesting vacuoles" with acidic pH (Metchnikoff, 1884; 1901). The conclusions they proposed were precise and prophetic. Antigen processing occurs inside cells and is executed by proteases. A gastric mucosa protease was named cathepsin (gr. καθεφειν to digest) and was proposed to have its origin in leukocytes in the late 1920s (Willstätter, 1929). Lysosomes were not discovered until 1955 (De Duve *et al.*, 1955; Novikoff *et al.*, 1956), and a 28 kDa protease, active in alkaline pH, named cathepsin G (CatG), was isolated from human spleen in 1976 (Starkey & Barrett, 1976).

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Abbreviations: APC, antigen-presenting cells; Cat, cathepsin; DAP, α-aminoalkylphosphonate diphenyl ester; DDT, dithiothreitol; LHVS, morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone; PBMC, peripheral blood mononuclear cells; PMSF, phenylmethylsulfonyl fluoride; Suc-VPF, Suc-Val-Pro-Phe^{P}(OPh)$_2$; ZRLR, Z-Arg-Leu-Arg-α-aza-glycyl-Ile-Val-OMe

The function of cathepsins in the endocytic compartment (endosome and lysosome) within antigen-presenting cells (APC) is to digest antigens into antigenic peptides capable of binding to major histocompatibility complex (MHC) class II molecules. These MHC II antigenic peptide complexes migrate to the cell surface, activate respective T cells, and cause an immune response. Cysteine (CatB, C, F, H, S, X, V, L) and asparagine endoprotease (Watts *et al.*, 2005; Turk & Turk, 2009), serine (CatA (Reich *et al.*, 2010) and CatG (Burster *et al.*, 2010)), and apartyl (CatD and CatE) proteases are involved in antigen processing of different APC (for review, see Colbert *et al.*, 2009). The serine proteases CatG, neutrophil elastase, and proteinase 3 are highly expressed in granulocytes and can be secreted into the blood by these cells under inflammatory conditions (Meier *et al.*, 1985). CatG is important in the processing of antigens in primary APC (Reich *et al.*, 2009a), has anti-bacterial capacity (Ohlsson *et al.*, 1977), regulates chemotaxis (Nufer *et al.*, 1999), activates the protease-activated receptor 4 (Sambrano *et al.*, 2000), and is inhibited by thrombospondin 1 present in the serum or by serpins found in cells (Bornstein *et al.*, 1991; Heutinck *et al.*, 2010).

The spleen is the body's largest filter of blood and functions to promote the innate and adaptive immune response. The red pulp of the spleen is responsible for clean-up of older erythrocytes and effective iron recycling. The white pulp is formed by sheathed lymphoid tissue composed of T and B cell compartments that facilitate T cell interactions with dendritic cells (DC) and mediate B cell expansion, respectively. APC from the bloodstream enter the white pulp through the marginal zone and initiate a potent adaptive immune response. We replicated Hedin's 1903 experiment by analyzing protease activity after treatment of bovine spleen with neutral or acidic medium. Activity-based probes and Western blotting were used to determine the presence of cysteine, aspartyl, or serine proteases in the collected filtrates. We found that CatG was abundant in the fraction which Hedin described as filtrate B (F_B; Fig. 1). Thus, we provide evidence, using several biochemical methods, that Hedin's α-protease, first described in 1903, was most likely CatG. The β-protease described by Hedin reflected the activity of several proteases, including CatX, B, S, and D.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMC) from buffy coats of healthy human cytomegalovirus-seronegative blood donors and bovine spleen cells were isolated by density gradient (Ficoll-Paque™ Plus, GE Healthcare, Uppsala, Sweden) centrifugation.

Preparation of bovine and murine spleens. Freshly isolated bovine, mouse (C57BL/6), or rat (Wistar) spleens were processed on the same day. The spleens were sliced and wire-extruded in phosphate-buffered saline (PBS) followed by incubation with 0.2% acetic acid (pH 4) for 16 h at room temperature. Subsequently, this acidic solution was filtered, and the filtrate (filtrate A, F_A) was collected and frozen at –20 °C for further analysis. The residue (residue A, R_A) was incubated with 5% NaCl and adjusted to pH 7.0 for another 16 h at 37 °C. This neutral solution was filtered, and the filtrate (filtrate B, F_B) and residue (residue B, R_B) were collected and stored at –20 °C for further use. The protein content of all filtrates was determined by Bradford assay.

Active site labeling and Western blotting. PBMC and spleen cells were lysed (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% NP-40) and adjusted to equal total protein concentration (quantified by Bradford assay), and 20 μg of the cell lysate or filtrates were incubated with PBS (pH 7.4) in the presence of DAP (DAP22c, 2 μM) (Oleksyszyn & Powers, 1991; Reich *et al.*, 2009a). Alternatively, 5 μg of cell lysate or filtrates were incubated with reaction buffer (1 mM EDTA, 50 mM citrate, pH 5.0, and 50 mM DTT) in the presence of DCG-04 (10 μM; probe kindly donated by M. Bogyo, Stanford University, Palo Alto, CA, USA) (Greenbaum *et al.*, 2000) for 1 h at room temp. Some of the samples were treated with CatG inhibitor I (CatG inhibitor, 10 μM, Calbiochem, Merck, Darmstadt, Germany), Suc-Val-Pro-Phe^P(OPh)$_2$ (Suc-VPF, 10 μM, (Oleksyszyn and Powers, 1991)), Z-Arg-Leu-Arg-α-aza-glycyl-Ile-Val-OMe (10 μM, ZRLR, (Wieczerzak *et al.*, 2007)), phenylmethylsulfonyl fluoride (PMSF, 2 mM, Sigma-Aldrich, Steinheim, Germany), or morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS, 20 nM, H. Kalbacher, University of Tübingen, Germany) and pre-incubated for 15 min at 37 °C. Purified CatG from human sputum was purchased from Sigma-Aldrich and 50 ng was used in all experiments. Samples were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), blotted, and visualized using streptavidin-horseradish peroxidase (HRP, Vectastain, Burlingame, CA, USA). For Western blotting, 20 μg of protein from crude cell extracts was loaded on SDS/PAGE, and the immune blot was performed using anti-CatD antibody (Calbiochem, Schwalbach, Germany) or anti-CatX (R&D Systems, Wiesbaden, Germany). Anti-β-actin antibody and secondary HRP-conjugated antibodies were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Determination of CatG activity. Kinetic measurement of CatG activity was accomplished by adding 15 μg of the indicated samples to the colorimetric substrate Suc-Val-Pro-Phe-pNA (200 μM) in buffer (0.5 M $MgCl_2$ in PBS, pH 7.4). The colorimetric substrate was prepared as followed: Boc-Phe-OH (Iris-biotech, Marktredwitz, Germany) was reacted with *p*-nitro aniline to give starting Boc-Phe-pNA (Rijkers *et al.*, 1995). Further deprotection steps and coupling were performed in solution using 50% trifluoroacetic acid (TFA, Iris-biotech) in dichloromethane (DCM, Iris-biotech) solution (for Boc deprotection) and O-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, as the coupling agent, Iris-biotech), respectively. Introduction of succinamide function was done by an application of succinic anhydride (Sigma-Aldrich, Poznań, Poland) in the presence of *N,N*-diisopropylethylamine (DIPEA, Iris-biotech). Final product was purified using HPLC chromatography (Econosphere™ C-18 column, Grace, Poznań, Poland). The enzyme assays were performed at 37 °C in duplicate, and absorbance was determined at 405 nm (absorbance microplate reader, EL808, BioTek, Winooski, VT, USA).

RESULTS AND DISCUSSION

Spleen preparation according to Hedin's protocol

S. G. Hedin described two proteases, one active in acidic, and the other in neutral medium. For those studies, he incubated minced bovine spleen in 2 liters of 0.2% acetic acid for 16 h at room temp. Chloroform and toluol were added to the mixture in order to pre-

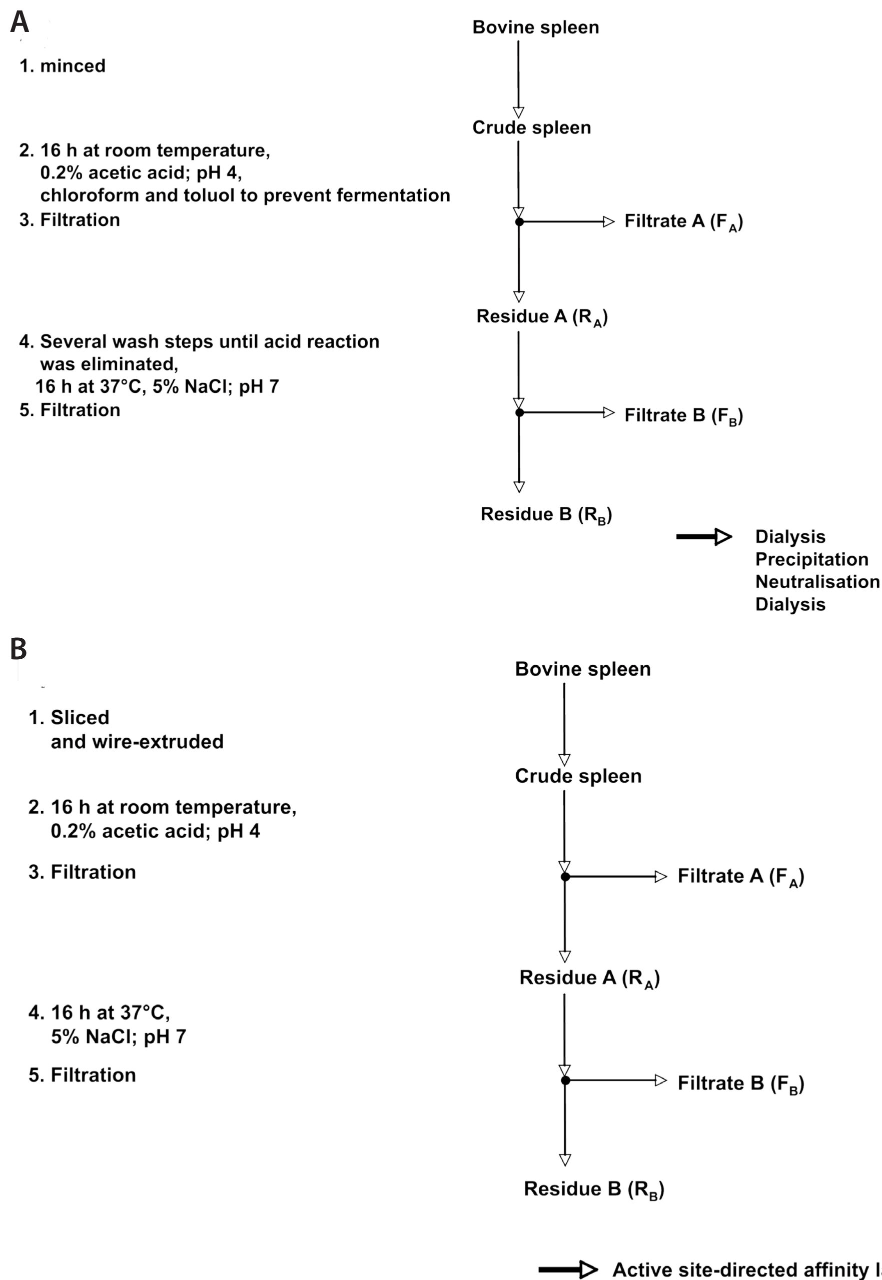


Figure 1. Hedins's experiment in 1903
Sven Gustaf Hedin (1859–1933) was born on October 6, 1859, in Alseda, Jönköpings län, Sweden, and received his doctoral degrees in philosophy and medicine in 1886 and 1893, respectively, from Lund University. From 1900 to 1907, he was at the Lister Institute of Preventive Medicine in London and became Professor in 1908 at Uppsala University. He was always interested in research on proteases and brought us an improved understanding of proteases involved in digestive processes and their inhibition. In 1903, he published his findings on bovine spleen proteases. **(A)** Outline of Hedins's experimental procedure. **(B)** Experimental procedure of the present study.

vent fermentation. Afterwards, the product was filtered to obtain residue A (R_A) and filtrate A (F_A) (Fig. 1A). R_A was extensively washed with water until no acid reaction was acquired and finally, 2 liters of a 5% NaCl solution was added for an additional 16 h at 37°C. After this incubation, the fluid was filtered a second time to obtain R_B and F_B. The protein degradation of filtrates F_A and F_B was determined with tannic acid, which precipitates peptones (a mixture of peptides and amino acids), allowing the volume of the precipitate before and after the incubation time to be quantified. The volume thus corresponds to the digestion and proteolytic activity in the respective samples.

In our experiments, whole, freshly isolated bovine spleen was wire-extruded and treated with acetic acid for 16 h (Fig. 1B). The fluid was filtered to obtain R_A and F_A. R_A was further treated with NaCl, pH-adjusted to 7.0, and incubated for an additional 16 h at 37°C. F_A was directly prepared for analysis (Fig. 2). After R_A incubation, the suspension was filtered into R_B and F_B.

Identification of active serine proteases in the respective filtrates

To determine the content of active serine proteases in different filtrates, the same amount of protein from

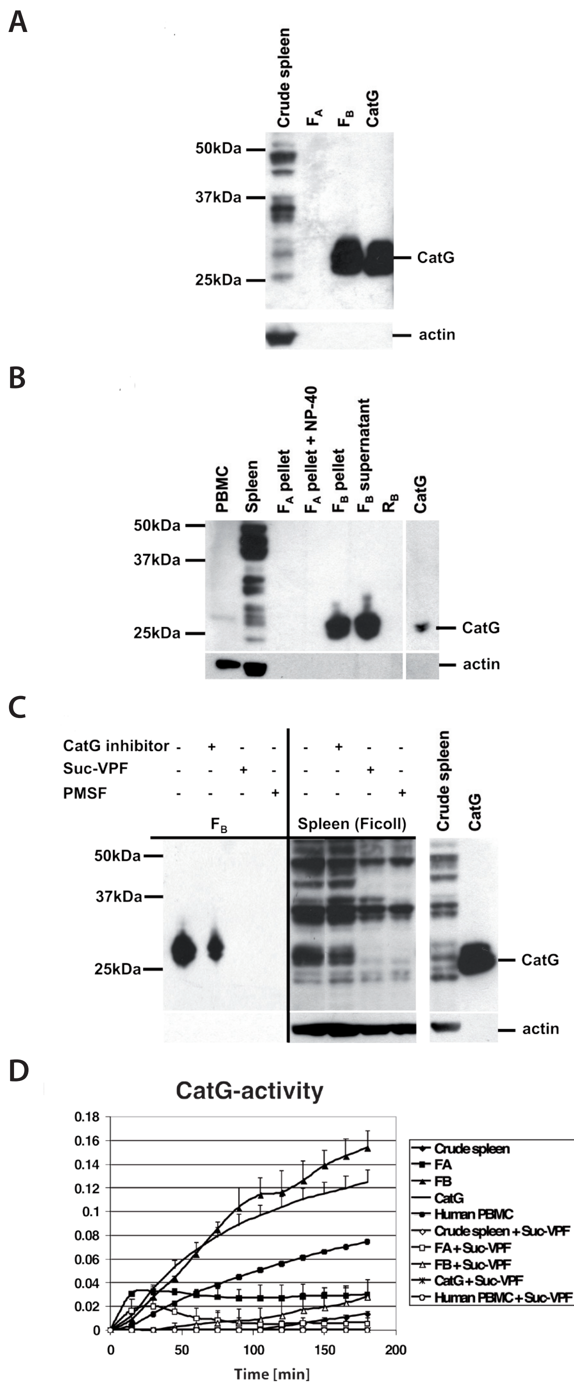


Figure 2. Active serine proteases in bovine spleen
(A and B) Cell isolation and lysis were performed as described in the Methods section. Equal amounts of cell lysate of primary bovine spleen cells and bovine PBMC, respectively, were incubated with the activity-based biotinylated probe DAP and loaded onto SDS/PAGE. Active serine proteases were visualized by streptavidin-horseradish peroxidase (HRP) blot. Purified human CatG (50 ng) was used as control. β-Actin immunoblotting (45 kDa) was performed as a loading control. Data are representative of four experiments and two different spleens. **(C)** Cell lysates were pre-incubated with CatG inhibitor (10 μM), Suc-VPF (10 μM), and PMSF (2 mM) for 15 min at 37 °C. Equal amounts of cell lysate were incubated with the active site probe DAP and analyzed as above. Data are representative of at least two experiments using two different spleens. **(D)** CatG activity was measured using the colorimetric substrate Suc-VPF-pNA. Purified CatG (50 ng) was used as a positive control. Data are representative of one of two experiments, and the measurement was performed in duplicate. F, filtrate; R, residue.

each sample was pre-incubated with the activity-based probe α-aminoalkylphosphonate diphenyl ester (DAP). The phosphonate inhibitor DAP covalently binds to the active site serine residue of serine proteases and thus allows for visualization of active serine proteases *via* streptavidin-HRP labeling. Streptavidin functions as a bridge between HRP and biotin from DAP. Active CatG migrated at 28 kDa in F_B, in contrast to the crude spleen extracts, in which we only detected low levels of CatG activity (Fig. 2A). Approximately 2.5 ng of CatG was detected in 1 μg of F_B, which was quantified by comparing a known amount of purified CatG (50 ng) with F_B. This indicates that CatG is the predominant protease in this fraction. In F_A, CatG activity was not detectable. Notably, we did not detect β-actin in F_A or F_B, most likely due to degradation by proteases during the incubation period specified by Hedin's protocol. In a separate experiment, fractions F_A and F_B were centrifuged to obtain cell pellets and supernatant to determine whether CatG could be detected in these fractions. Part of the F_A pellet was treated with detergent (NP-40), and F_B was separated into cell pellet and supernatant. However, we did not detect any CatG in F_A treated with NP-40 or in R_B, and CatG activity did not differ between the F_A pellet and F_A supernatant (Fig. 2B). CatG activity in bovine spleen was then investigated in greater detail. We used the broad-spectrum serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), a reversible CatG inhibitor (CatG inhibitor), or the irreversible CatG inhibitor Suc-Val-Pro-$Phe^P(OPh)_2$ (Suc-VPF). Both F_B and Ficoll-purified spleen leukocytes were pre-incubated with the indicated inhibitors. CatG activity was completely inhibited by PMSF and SucVPF but not entirely by the reversible CatG inhibitor in either F_B or Ficoll-purified spleen cells, which might account for the difference between human, mouse, and rat as compared to bovine CatG (Fig. 2C, Supplementary Data A). Inhibition of serine proteases by PMSF in the crude spleen extract demonstrated that different active serine proteases, migrating at 50, 45, 40, and 37 kDa, were present in this extract.

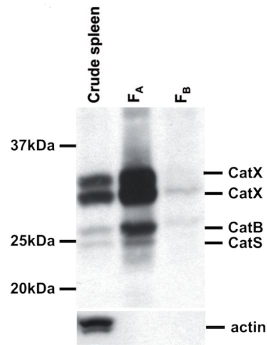
With further analysis using the colorimetric substrate Suc-VPF-pNA, we confirmed that CatG is active in F_B, but not in F_A, (Fig. 2D; for rat and mouse spleen, compare Supplementary Data A, B, and C). These results indicate that the serine protease migrating at 28 kDa in F_B is CatG.

Determination of cysteine proteases and CatD

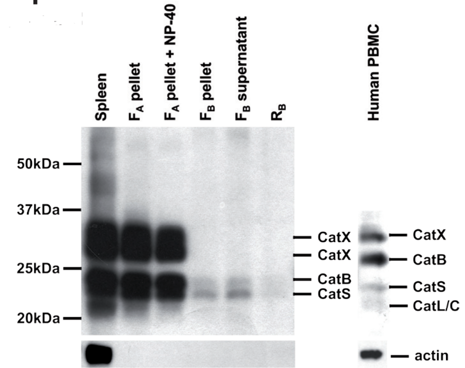
Cysteine proteases were analyzed using a similar approach as described above. Biotinylated DCG-04, a cysteine protease inhibitor based on E64, was used to determine active CatX, B, H, S, and L/C. CatX, B, and S were detected in F_A. In contrast, no active CatB, S, or X was found in F_B (Fig. 3A). The allocation of a band to the respective active cathepsins was determined using the specific CatS inhibitor morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS) (Riese *et al.*, 1996) or the CatB-specific inhibitor ZRLR (Wieczerzak *et al.*, 2007; Reich *et al.*, 2009b) (Fig. 3B; for rat spleen, see Supplementary Data D). E64 was used as a broad cysteine protease inhibitor. Notably, the upper two bands refer to CatX, indicated in the Supplementary section showing the CatX immunoblot (Supplementary Data E).

Subsequently, the aspartyl protease CatD was analyzed using a CatD-specific immunoblot because of the lack of an active-site label for aspartyl proteases. CatD was

A, left panel



A, right panel



B

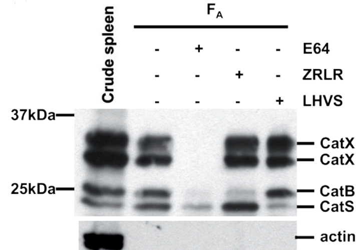


Figure 3. Active cysteine proteases in bovine spleen
(A) Bovine spleen extracts were labeled with the active site probe DCG-04 and visualized by HRP blot. The identity of the cysteine proteases was deduced from side-by-side comparisons to cell lysate from primary human PBMC and **(B)** by using the cathepsin-specific inhibitors ZRLR (10 μM), LHVS (20 nM), and the broad cysteine protease inhibitor E64 (10 μM) for 15 min at 37°C. One representative activity blot is shown from two independent experiments.

detected in F_A, but only a trace amount of the mature form of CatD was detected in F_B (Fig. 4; Supplementary Data F). This demonstrates that several proteases, including CatX, CatB, CatS, and CatD, were present in F_A. Therefore, Hedin's β-protease consists of multiple proteolytic enzymes, explaining the observation of rapid proteolytic degradation in acidic fluid (Hedin, 1901). In a later publication, Hedin discussed this issue in more detail and concluded that other proteases active in the acidic medium were present (Hedin, 1923), which is consistent with our findings. Furthermore, treatment of crude spleen extract with acetic acid for 16 h and for an additional 16 h under neutral conditions resulted in CatG enrichment in F_B, suggesting that Hedin's protocol was also practical for concentrating CatG. Notably, Hedin's experimental design of protease inhibition was utilized to categorize inhibitors into anti-enzymes (i.e., antibodies), *Hemmungskörper* (i.e., blocking body), or inhibitive substances (Hedin, 1915). The mechanism was explained by absorption as experiments with Kieselgur revealed that the α-protease was not active when bound to Kieselgur (Hedin, 1907). Another possible explanation for the inhibition is the interaction of normal serum with the protease (Hedin, 1905). Indeed, Hedin found that only the bovine spleen-derived α-protease, in contrast to β-protease, was inhibited by antibodies in bovine serum and was not neutralized under his experimental conditions (Hedin, 1903a). The effect he described was most likely the action of serine protease inhibitors (serpins and thrombospondin 1) circulating in the blood (Remold-O'Donnell *et al.*, 1992). Today, we know that CatG in leukocytes, such as DC and B cells, is not only important in antigen processing, but also in immune defense in the periphery. In conclusion, based on several criteria, we suggest that Hedin described CatG activity in 1903 as the α-protease active in neutral medium.

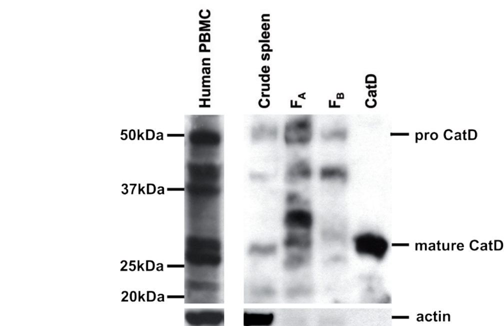


Figure 4. CatD expression in bovine spleen cells
CatD was visualized by immunoblot with anti-human CatD. Purified human CatD was used as control. One of three experiments, each with similar results, is shown.

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