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The challenge of specific Cathepsin S activity detection in experimental settings

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ABSTRACT

In recent years, a growing interest in pathophysiological processes that are associated with the endosomal and lysosomal protease cathepsin S (CTSS) results in an increasing number of various published methods for CTSS activity detection. CTSS has been reported to be involved in the pathology of autoimmune diseases like multiple sclerosis as well as in tumor growth and Alzheimer's disease. These implications make this enzyme a first class drug target. In order to fully understand the involvement of CTSS in the formation of pathological processes, gene and protein expression analysis is not sufficient. Rather, one should focus on the regulation of its enzymatic activity. Different approaches for CTSS activity detection are available and described. However, some of these approaches are not suitable for a standard laboratory without special equipment or technical expertise or provide other limitations. We have recently published an easy-to-perform protocol for reliable, quantifiable and reproducible CTSS activity detection. In this review we want to discuss our application and compare it with other published methods and protocols. This might help researchers who are interested in CTSS research to decide which application fits best to their technical or personal facilities.

Text

Cathepsin S (CTSS) is a protease located in lysosomes or endosomes of professional antigen presenting cells (APC), such as macrophages, dendritic cells and B cells[1]. Dysregulated CTSS expression and/or activity has been reported to be involved in the pathogenesis of various diseases. CTSS is the major regulator of major histocompatibility complex (MHC) II surface expression. Therefore, especially autoimmune diseases which are caused by (or which are associated with) pathologically enhanced $CD4^+$ T cell activation are part of this disease portfolio. In this context, the Sjögren's syndrome[2], atherosclerosis[3], psoriasis[4] and an animal model of rheumatic arthritis[5] play dominant roles. But not only autoimmune diseases are associated with CTSS dysregulation. Enhanced CTSS activity could also be detected in the bronchoalveolar lavage of cystic fibrosis patients[6]. Neurologists might focus on the role of CTSS in the development of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), the corresponding mouse model of MS[7]. Indeed, it could already been shown that CTSS is capable of myelin basic protein proteolysis[8] which might contribute to the observed phenotypes in MS patients. In fact, enhanced CTSS levels could be found in peripheral blood mononuclear cells, serum and the cerebrospinal fluid of MS patients[9-12]. In line with these observations, chemical inhibition of CTSS led to a reduction of phenotypical severity in the EAE mouse model[5,13]. Besides MS

patients, enhanced CTSS activity at the primary site in breast cancer patients correlated with an increased frequency of the appearance of brain metastases[14]. The implication of CTSS concerning cancer was linked with with tumor-associated APCs, namely macrophages[15]. Alzheimer's disease (AD) was already reported to be associated with a malfunctioning Cathepsin B (CTSB) activity regulation[16,17]. But also human Cathepsin S seems to be involved in the manifestation of the disease phenotype since also CTSS provides β-secretase activity[18] which leads to the AD phenotype causing agent Aβ peptide through amyloid precursor protein proteolysis[19]. One important feature of CTSS, distinguishing it from other cysteine cathepsins, is the retention of its proteolytic activity at neutral pH. Being part of the endosomal and lysosomal compartment, CTSS can be secreted into the extracellular space providing neutral pH where it can cause additional pathologies. Extracellular CTSS activity has already been shown to be involved in activating protease-activated receptors (PAR) like PAR_2 as well as G-protein coupled receptors like Mrgprs leading to the promotion of itch and pain sensation in both cases[20-23].

All the above mentioned implications for CTSS activity dysregulation which are linked with manifestation of various pathologies makes this enzyme an attractive and intensely studied drug target. Some studies just refer to enhanced *Ctss* gene expression rates or increased protein amounts inside or outside of target cells. However, mRNA expression levels of a protease-encoding gene or expression of the corresponding protein does not necessarily correlate with the overall detected activity of the respective enzyme. This is due to several possible posttranscriptional regulation mechanisms that can occur inside or outside a eukaryotic cell. Proteases can be (i) translated as inactive zymogens requiring proteolytic activation, (ii) activated by co-factors, (iii) separated from their substrates by localization in distinct intra- or extracellular compartments or (iv) inhibited by binding of endogenous inhibitors to the active site of the protease[24]. All these examples illustrate that focusing on mRNA or protein expression is not sufficient to reliably investigate the role of proteases in biological or pathophysiological processes. Therefore, specific detection of the activity of a certain protease has to be guaranteed in order to exclude false positive detection of one or more other proteases with a similar substrate specifity. This is a grave problem concerning the protease group "cathepsins", which consists of currently eleven known members[25], since cathepsins in general provide a broad and overall similar substrate specifity[26]. This makes CTSS activity detection a challenge, in vitro, ex vivo and especially in vivo.

But how can an experimentator achieve the goal to reliably detect CTSS activity in the biological system of choice? There are several possibilities how CTSS activity can be detected, all of them with their characteristic advantages and limitations. A suitable CTSS activity detection method should match the following four criteria: (i) high specifity, (ii) high sensitivity since CTSS expression is restricted to APC, (iii) the experimental workflow should be able to be performed in a more or less standard laboratory without the requirement of highly special technical equipment and (iv) the method should deliver reproducible and quantifiable results. We summarize some available techniques for CTSS activity detection with their characteristic pros and cons in table 1.

Popular approaches include activity based probes (ABPs). These are small reporters of proteolytic activity which bind covalently and irreversibly to the active site of the target enzymes[27] leading to a loss of enzymatic activity. This irreversible CTSS inhibition should be kept in mind if in vivo monitoring shall be performed. ABPs usually consist of three distinct functionalities (Fig. 1A): (1) The so-called warhead functionality leads the probe to enzymes sharing a common catalytic mechanism, (2) the recognition element that enhances specificity for one or more specific enzymes and (3) a tag for a later detection of the tagged enzymes. Such ABPs exist for the detection of human and mouse CTSS. General disadvantages of such ABPs include a lack of specificity for CTSS for some of these ABPs. This has then to be compensated by further time-consuming experimental approaches like western blotting, immunoprecipitations etc. Additionally, CTSS activity can often only be determined in a semi-quantitative manner like measuring band intensities on a gel or a detection of overall fluorescence intensity. However, the potential labelling of proteolytically active CTSS in live cells due to cell permeability is a big advantage of some ABPs. Veilleux et al. developed radioiodinated ABPs to selectively label active CTSS in human whole blood[28]. Ben-Aderet et al.[29] and Oresic Bender et al.[30] used fluorogenic ABPs to monitor enzymatically active CTSS in human cells or live mice, respectively. Other examples of recent ABPs development for CTSS activity detection include the publications of Barlow et al.[31], Hughes et al.[32], Mertens et al.[33] and Garenne et al[34]. However, some of them provide a lack of specificity for CTSS and also label other cathepsins[29,32-35] while other ABPs selectively label human or mouse CTSS[28,30,36]. Another general disadvantage of ABPs include the requirement of more specialized detection techniques that a non-specialized lab could tentatively not afford.

Recently, commercially available CTSS activity detection kits have been developed and are sold. Experiences made by our group revealed, however, that these kits are far less specific than they are advertised to be and we strongly recommend to use alternative techniques for reliable CTSS activity detection. If this is not possible, a strict validation of the claimed specificity for CTSS should be performed, i.e. using recombinant enzymes or knock-out cells for various

Application category	Characteristic	Example	target	Year published	Quantification method	Limitation	Outstanding advantage
Activity based probe (ABPs)	covalent binding to CTSS active site leading to enzyme inhibition	GB123[29]	Human CTSS and CTSB	2015	Fluorescent label	Binds also to CTSB. Gel-assisted enzyme separation or IP needed	Non invasive in vivo imaging possible
		Probe 7[36]	Human CTSS	2015	Fluorescent label	Not cell permeable	Specificity for CTSS
		Z-PraVG-DMG[32]	Human CTSS, CTSB and CTSL	2016	Rhodamine azide labelling	Similar K_i values for CTSS, CTSB and CTSL	Cell-permeability of the substrate
		CM-279[35]	Human CTSS and CTSL	2013	Luciferase assisted light detection	Also labels CTSL	Reliable detection of combined CTSS and CTSL activity
		BMV157[30]	Mouse CTSS	2015	Fluorescent label	availability	Highly specific, in vivo and in vitro application possible
		BIL-DMK[28]	Human CTSS	2011	Radioactive label	Radioactivity	Specificity for CTSS
		Probe 10[33]	Human CTSS, CTSL, CTSB and CTSK	2014	Fluorescent label	Not specific for CTSS	Suitable for complex protein mixtures
		Biot-$(PEG)_2$-Ahx-LeuValGly-DMK[34]	Human CTSS, CTSL, CTSB and CTSL	2015	SDS Page assisted chemi-luminescence	Not specific for CTSS	Easy to perform
Substrate based probes (SBP)	Substrate turnover; enzyme is not inhibited and remains proteolytically active	**PMGLP**	**Human CTSS[43] and mouse CTSS[42]**	**2016**	**Fluorescence increase**	**Requires cell lysis and inhibition control**	**Simple enzymatic assay, easy-to-perform, time saving.**
		Ac-KQKLR-AMC	Mouse CTSS	2014	Fluorescence increase	Requires cell lysis and inhibition control	Simple enzymatic assay
		NB200[31]	Mouse CTSS, CTSK and CTSB	2015	Fluorescence increase	Detects also CTSK at pH7.5 and CTSB at pH 5.5	Simple enzymatic assay
		Z-FR-AMC	Mouse CTSS, CTSB and CTSL	2002	Fluorescence increase	Unspecificity, requires extensive experimental setting	Simple enzymatic assay
Indirect detection methods	Does not directly interact with CTSS	Detection of CTSS substrate accumulation by western blotting[3]	Mouse invariant chain p10 detection	2015	Western blot band intensity of Iip10	Does not detect CTSS activity directly	Simple to perform
		Western blotting of 24 kDa CTSS[37]	Human CTSS	2014	Western blot band intensity of 24 kDa CTSS	Does not consider potential binding of endogenous inhibitors and does not refer to activity	Simple to perform

Table 1: Summary of various CTSS activity detection techniques. The techniques are grouped into the major functional classes.

cysteine cathepsins.

Other approaches include an indirect method to determine CTSS proteolytic (table 1) activity by not directly assessing the enzymatic activity but rather determining the intracellular amount of the MHC II-bound invariant chain fragment (Iip10), which is a substrate for CTSS. Therefore, decreased amounts of Iip10 is stated to correlate with enhanced CTSS activity[5]. Since CTSS is translated into an inactive pro-enzyme requiring proteolytical cleaving which results in a 24 kDa active enzyme, some publications refer to the band intensities of this 24 kDa band when making statements on CTSS activity[37]. However this does not

take the potential binding of endogenous inhibitors into account.

Another possibility for direct CTSS activity determination is to use substrate-based probes (SBP) like i.e. fluorochrome-coupled peptidyl substrates. The principle mechanism includes a detection in the increase of a peptidyl-coupled and later proteolytically liberated fluorochrome (Figure 1B). SBPs provide the advantage of not inhibiting the target enzyme. Prominent examples are Z-VVR-AMC[38] , Z-FR-AMC[36,39] or Ac-KQKLR-AMC[20,40]. All of them share the aminomethyl coumarin group as a fluorogen tag. Coumarins are popular fluorescent labels due to their large stoke's shifts[41] and their small size allowing for incorporation into small peptides[33] . These peptidyl substrates generally provide the disadvantage to be usable only in cell lysates and not in living cells or organisms. The major potential problem with most peptidyl substrates is the unsatisfactory specificity for CTSS. Due to the already mentioned similar substrate specificity among cysteine cathepsins, other cathepsins than CTSS can deliver false positive results due to unspecific cleavage of the substrate. However, we have recently reported on a CTSS activity detection method using a coumarin-coupled peptidyl substrate (PMGLP) that allows for highly specific CTSS activity detection in mouse samples[42]. This method is a simple standard enzymatic assay detecting a time-dependent increase in fluorescence intensity caused by cleaving of the peptidyl sequence. It is easy-to-perform, time-saving and can be used for high-throughput applications. Additionally, a standard curve using free aminomethyl coumarin can be created allowing for the detection of specific enzymatic activities within a tested sample which is a big advantage over ABPs. The oligopeptidyl substrate we use was initially published as being suitable for highly specific detection of CTSS activity in human antigen presenting cells[43]. However, we could demonstrate that is also suitable for mouse samples. The substrate, Mca-GRWPPMGLPWE-Lys(Dnp)-DArg-NH_2 (PMGLP) was shown to be cleaved specifically by murine CTSS at a pH of 7.5 with no other murine cathepsin contributing to substrate cleaving in cell lysates[42]. The obtained results were easily quantifiable and highly reproducible as demonstrated in mouse bone marrow derived dendritic cells that were differentiated from the bone marrow of different individuals. The presented method therefore delivers specific CTSS activities and only requires a phosphate buffer system and a fluorescence compatible heatable photometer making the application attractive for less specialized laboratories.

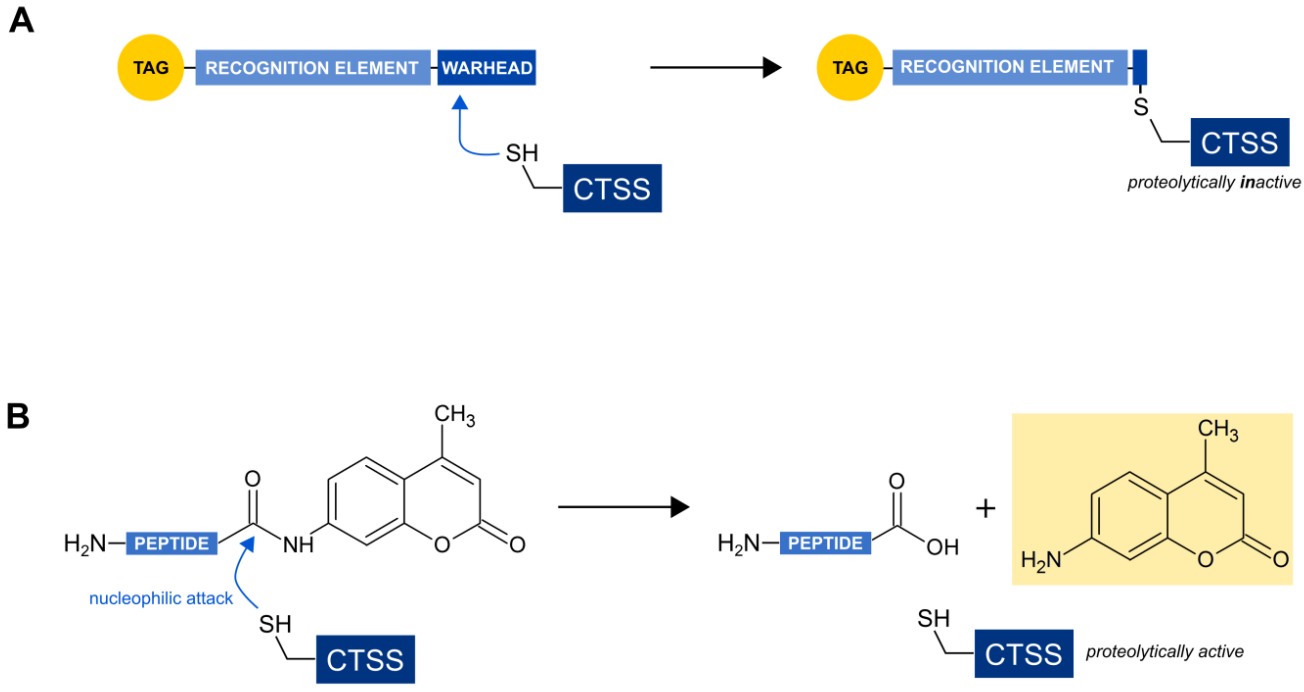
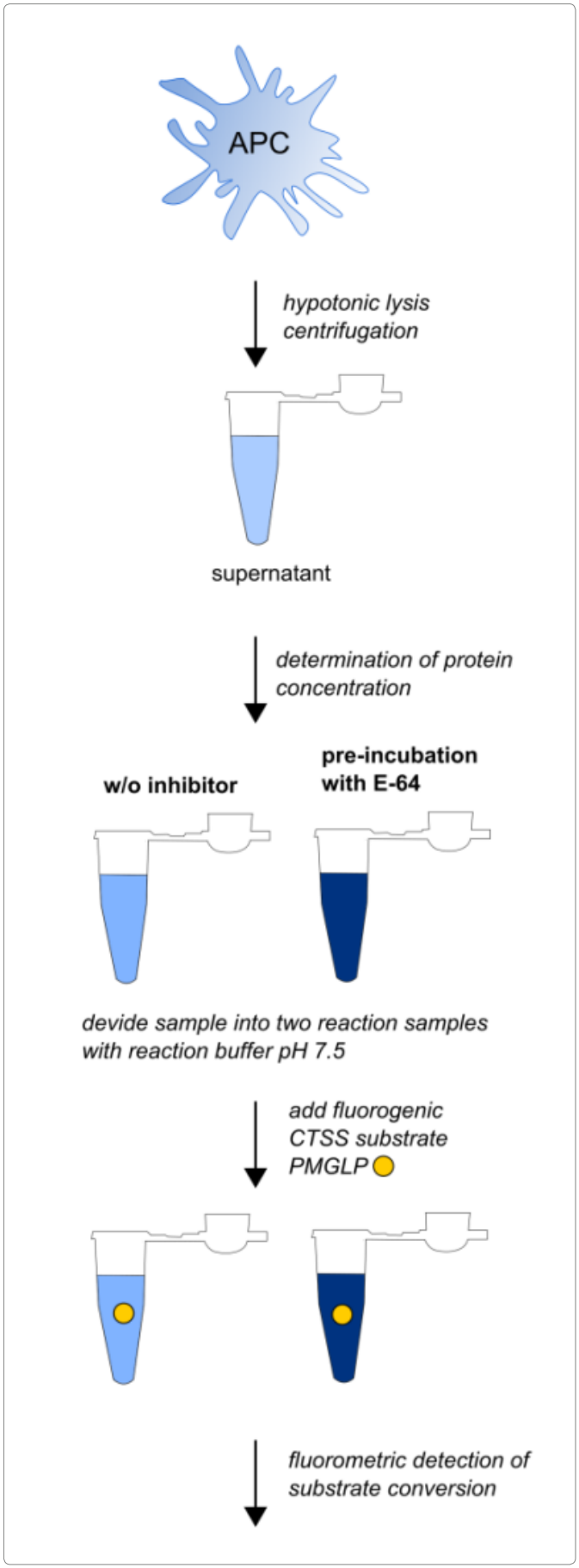


Figure 1: Functional comparison of the two major classes of probes for direct CTSS activity detection

(a) activity based probes (ABPs): The so-called warhead covalently links the probe with CTSS, leading to the inhibition of its enzymatic activity. The recognition element supports the specificity for CTSS alone or some related cysteine cathepsins. The tag allows for later detection of the tagged enzymes.

(b) substrate based probes (SBPs): The thiol group at the active site of CTSS nucleophilically attacks the peptide bond of the peptidyl substrate leading to a deliberation of the aminomethylcoumarin. The resulting change in the fluorogenic behaviour of free aminomethylcoumarin can then be detected in a fluorescent compatible photometer.



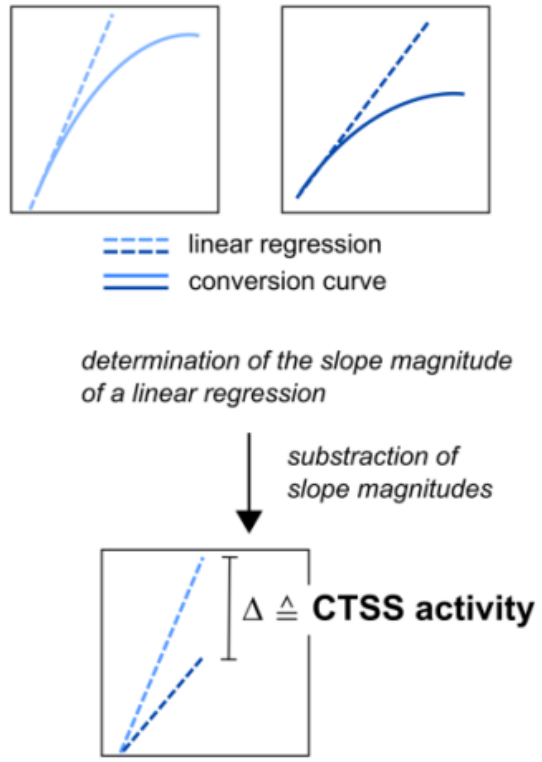


Figure 2: Experimental workflow for the detection of CTSS activities in mouse APC[42] (adapted from Steimle et al., 2016)

Cells of interest are lysed hypotonically followed by blast freezing in liquid nitrogen as described in materials and methods section. Supernatant is used for CTSS activity detection. Out of each sample, two reaction samples containing the same amount of whole cell protein are created. The first sample contains the fluorogen substrate, the second both, the substrate and the cathepsin inhibitor E-64. Reaction buffer provides a pH of 7.5 to minimize false positive substrate conversion caused by other cathepsins. Substrate conversion is monitored for 60 min at 37 °C. A linear regression at the very beginning of the sigmoidal conversion curve is performed for both reaction samples. The slope magnitude of this linear regression is equivalent to the substrate conversion per time unit. The difference in the slope magnitude of both reaction samples is equivalent to the CTSS activity in the sample the lysate was taken from. CTSS activities can therefore be expressed as the difference of fluorescence intensity per µg cell protein and time unit, i.e. ΔAU min-1 µg-1.

We recommend the following simple workflow (Figure 2): the target cells are lysed hypotonically with the support of freezing in liquid nitrogen. This guarantees a minimal loss of CTSS activity due to cell lysis. Protein concentrations of the lysates are determined afterwards and each sample is separated into two samples. The first one contains the substrate, PMGLP, and the second one PMGLP and E-64, a cysteine cathepsin inhibitor that is easily commercially available and cheap. The latter sample is used as a negative control. Substrate conversion is detected for at least 20 min at 37°C by detection of fluorescence increase at 405 nm after excitation at 340 nm. Conversion rates are computed

by determination of the slopes of the linear fluorescence increase and the slope of the inhibitor treated negative control is substracted from the slope of the non-inhibitor treated sample. This workflow is fast and the obtained results are highly specific for murine CTSS as demonstrated in our published protocol[42]. We therefore hope to contribute to a progress in CTSS related research.

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