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Unveiling ceramide synthase 2 (CerS2): From characteristics and isolation to enzyme activity assays

Egzontina Shabani and Zuzana Bílková*

Department of Biological and Biochemical Sciences, The University of Pardubice, CZ−532 10 Pardubice, Czech Republic

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Ceramide synthase 2 (CerS2) is an essential enzyme in the metabolic pathway of sphingolipids, a class of membrane lipids that act crucial roles in various cellular functions [1]. *Among the six mammalian CerS enzymes, CerS2 stands out for its omnipresence and abundant expression in various mammalian tissues and organs. CerS2 mRNA and protein expression levels are particularly prominent in the kidney, liver, lung, intestine, and other essential tissues* [1–3]. *This widespread distribution underscores CerS2's significance as a critical contributor to basal cellular sphingolipid metabolism. The enzyme's substrate specificity for specific acyl-CoAs, such as C20:0, C22:0, C24:0, C24:1, and C26:0, further accentuates its role in generating distinct ceramide species. CerS2's presence has been identified in the endoplasmic reticulum (ER), where it catalyzes the synthesis of ceramides by combining fatty-acyl chains with dihydrosphingosine. Analysis of CerS2 expression and activity involves a combination of techniques, when the real-time PCR measures mRNA levels, thus providing insights into the transcriptional regulation of CerS2. Western blotting is used to determine the protein abundance of CerS2 on its overall protein levels. In this review, we offer a thorough analysis of CerS2, encompassing its distinctive characteristics, biological relevance, isolation methods, quantification at both the mRNA and protein levels, as well as an assessment of its enzymatic activity via appropriate assays.*

Keywords: Ceramide synthase 2, CerS2; Expression levels; Determination; Enzyme activity

* Corresponding author, ✉ zuzana.bilkova@upce.cz

Introduction

Ceramide synthases with essential roles in the sphingolipid metabolism and cellular processes are enzymes responsible for synthesizing ceramides with distinct fatty acyl-chain lengths. There is a sextet of known ceramide synthase isoforms, collectively referred to as CerS1 through CerS6. These ceramides and those produced by other CerS isoforms contribute to the diverse functions of ceramides in molecular signalling pathways and cellular regulations [4]. Understanding the specific functions and regulatory mechanisms of each CerS isoform is crucial in unravelling the complexities of sphingolipid biology and its implications in various physiological and pathological processes, including cancer [5–8]. Each CerS isoform exhibits a unique tissue expression profile and fatty acyl-CoA substrate specificity, giving rise to ceramides with varying chain lengths ranging from C16 to C24 and beyond. The CerSs, including CerS2, are principal in connecting these two pathways by utilizing sphinganine from the de-novo pathway and sphingosine from the salvage pathway as substrates. CerS2 has a molecular weight of about 45 kDa across various species, an amino acid sequence involving 380 residues, and presents 7, 3, and 2 isoforms in humans, mice, and bovines, respectively. CerS2 is primarily located in the endoplasmic reticulum (ER) and stands out among the family of ceramide synthases due to its unique role in synthesizing very long-chain ceramides, typically in the C22–C24 range [9]. CerS2 exhibits a lumenal N terminus in the ER, and the fourth transmembrane domain might not entirely cross the ER membrane. Unlike other CerS isoforms, CerS2 holds just 11 residues within its lumenal loop [10]. These markedly long-chain ceramides have distinct properties that influence the membrane fluidity and the activation potential of membrane receptors. CerS2's importance extends beyond lipid metabolism [11,12]. Its dysregulation is associated with various pathological conditions, especially in cancer. For instance, CerS2 is overexpressed in malignant breast tissues and linked with poor prognosis, tumor progression, and invasion. Researchers have identified several mechanisms that regulate CerS2 expression, including transcriptional control involving Kruppel-like factor 6 (KLF6) and Sp1, as well as post-transcriptional regulation by microRNAs such as miR-133a, miR-221, and miR-222 [13,14].

Furthermore, alternative transcripts of CerS2, including tumor metastasis suppressor gene-1 (TMSG1), have been identified, suggesting one potential functional diversity within this gene. Understanding the intricate mechanisms behind CerS2's expression and its role in various cancers is crucial, as it can provide valuable insights into cancer progression and may potentially offer new therapeutic avenues. Understanding the dysregulation of CerS2 in cancer is also critical, as it is associated with different cancer types. CerS2 was reported to have elevated mRNA expression in human breast [15] and renal cancer tissue [12]. Deciphering the mechanisms behind its altered expression can provide insights into its role in cancer progression and may potentially lead to therapeutic interventions [2,16].

In summary, CerS2 is a key player in sphingolipid metabolism, particularly in the synthesis of very long-chain ceramides. Its expression and regulation are intricately linked to various physiological and pathological processes, making them an attractive target for further research.

Isolation of CerS2

Extraction

For analysis, CerS2 protein was extracted as follows. In the respective study [17], protein extraction from mouse brains was performed using RIPA buffer, where 10 times the tissue weight was used in the buffer. A protease inhibitor mixture III from EMD Millipore was included during the extraction process to preserve protein integrity. Subsequently, protein samples were subjected to denaturation by incubation with a loading dye containing 3.3 M urea and 10 mM DTT, ensuring complete denaturation of CerS2. This denatured protein was then prepared for Western analysis.

In another study [18], CerS2 protein was extracted from HepG2 cells. A protein extraction kit from Nanjing KeyGen Biotech was utilized, and protein concentrations were quantified using a bicinchoninic acid assay kit, following the manufacturer's protocols. Equal amounts of protein (10 μg per lane) were separated using SDS-PAGE on 10% gels and transferred onto polyvinylidene fluoride membranes from EMD Millipore. The membranes were blocked with 5% skimmed milk and then incubated with primary antibodies, including anti-LASS2, which targets CerS2, among others, overnight at 4 °C. Further antibody incubations, secondary antibody treatment, and chemiluminescent development were conducted and densitometry analysis performed relative to a control gene by using Gel-Pro analyzer 4.0. These procedures facilitated the extraction and subsequent analysis of CerS2 protein in the HepG2 cell line.

Immunoprecipitation

Immunoprecipitation, a laboratory technique employed for the selective extraction and isolation of specific proteins of interest (see Figure 1), is also applied in the isolation of CerS2. In method 1 [13], BT-474 cells were initially lysed in RIPA lysis buffer, and the total protein content quantified (Table 1). Subsequently, 1 mg of protein from the cell lysate was incubated with CerS2 antibody-conjugated agarose beads at 4 °C overnight. After washing, the antibody-bound protein complexes were eluted and separated on a 15% SDS-PAGE gel. Bands corresponding to CerS2 were excised, digested with chymotrypsin, and analyzed

using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This highly sophisticated and comprehensive technique allowed to identify specific peptides associated with both full-length and spliced forms of CerS2.

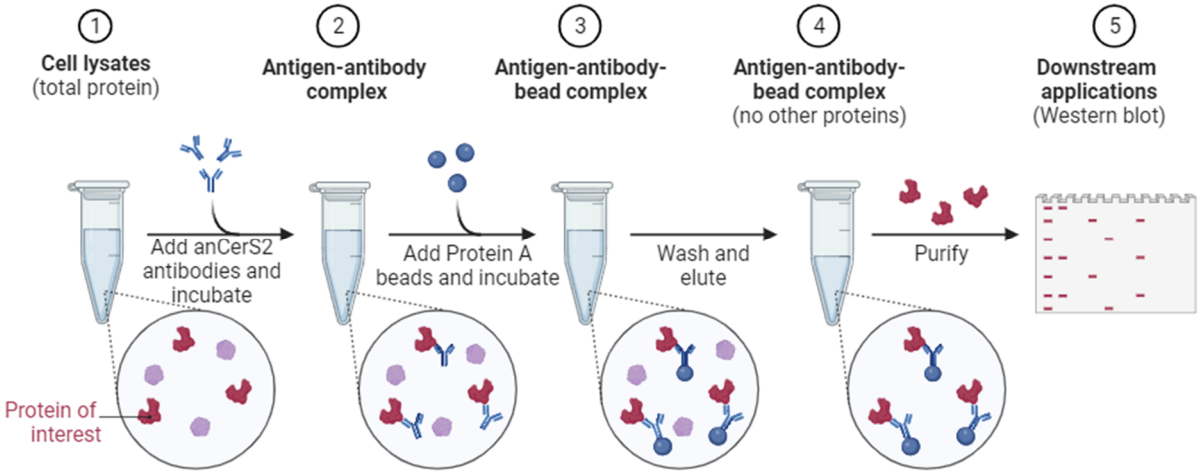

Fig. 1 Steps of CerS2 immunoprecipitation procedure (created with BioRender.com)

Method 2 [19] involved the immunoprecipitation of CerS2 from mouse liver lysates (Table 1). 1 μg of a rabbit anti-CerS2 antibody was used to pull down CerS2 from the lysates. Protein A agarose beads were added to capture the antibody-bound protein complexes, followed by extensive washing. The eluted proteins were separated on a 10% SDS-PAGE gel, and the presence of CerS2 confirmed using specific antibodies. This method provided insights into the interactions of CerS2 in a physiological context, particularly in the liver.

Table 1 Characteristics of immunoprecipitation methods

Method	Cell/tissue source	Lysis buffer	Antibody used	Incubation conditions	Bead type
Method 1 [13]	BT-474 cells	RIPA lysis buffer	LASS2/CerS2 antibody	Overnight	Protein A agarose beads
Method 2 [19]	Mouse liver	150 mM NaCl, 20 mM Hepes pH 7.5 with 1% digitonin	Rabbit anti-CerS2 antibody	Overnight	Protein A agarose beads
Method 3 [20]	HEK 293 and HepG2 Transfected cells	150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Nonidet P-40	Anti-FLAG antibody	5 h incubation	Protein A agarose beads

Method 3 [20] involved co-transfection of CerS2-HA and FLAG-CerS2 in cells (Table 1). After cell collection and lysis, the supernatant was incubated with an anti-FLAG antibody targeting CerS2. Protein A-conjugated agarose beads were subsequently added for precipitation. After thorough washing, elution was performed, and Western blotting done to confirm the presence of CerS2 in the eluates. This method was employed to investigate the interactions and associations of CerS2 within the cellular context.

In summary, all the above-presented immunoprecipitation methods allowed researchers to selectively isolate CerS2 and related proteins from cellular and tissue lysates, thus facilitating studying its interactions and functions in different experimental contexts.

Determination of CerS2 expression level

mRNA level determination – real time polymerase chain reaction

Researchers have used various methods to explain the expression and functional implications of the CerS2 gene through real-time polymerase chain reaction (RT-PCR). In a study by Laviad et al. (2008) the tissue-specific expression patterns of CerS2 were investigated in mice. Tissues from mice were carefully collected, and CerS2 mRNA extracted using a PerfectPure RNA kit. The resulting mRNA was converted into complementary DNA (cDNA), followed by real-time quantitative analysis utilizing TaqManTM technology. Expression levels of CerS2 were measured across diverse tissues and comparatively assessed concerning the brain. This approach revealed substantial expression of CerS2 in numerous tissues, notably significant in the liver and kidney [2]. In 2009, Pewzner-Jung et al. turned their attention to the developmental role of CerS2, particularly in mouse liver. By employing the real-time PCR, they accurately monitored the expression profile of CerS2 during various stages of the liver development. The study found dynamic shifts in CerS2 mRNA expression, peaking during specific developmental windows. This temporal expression pattern points out the critical contribution of CerS2 to postnatal liver development [3]. Mosbech et al. extended the investigation to human samples, seeking to establish the clinical relevance of CerS2, when these researchers followed a similar RT-PCR approach by isolating total RNA from muscle biopsies and primary fibroblasts. Their analysis uncovered a significant link between a patient's condition and a genetic deletion encompassing CerS2. Reduced CerS2 mRNA levels were evident in the patient, corroborating the gene's importance and, consequently, the connection by examining healthy individuals, when having found no similar deletions [21]. Spassieva et al. (2009) introduced a nuanced exploration of CerS2's influence on related genes, employing RT-PCR with SYBR Green supermix to dissect how CerS2 down-regulation impacted the expression of other

ceramide synthase isoforms. Intriguingly, this study did not reveal a direct increase in enzymatic activity corresponding to the upregulated isoforms. Instead, it hinted at intricate regulatory mechanisms that underly these observations [22]. These four RT-PCR methods shed a light on CerS2 expression, function, and regulation facets. Using this molecular analysis technique, the authors have shown tissue-specific expression, clinical relevance, developmental significance, and potential regulatory interactions of the CerS2 gene.

Protein level determination – Western blot

Western blotting (WB) is a widely used technique for detecting and quantifying specific proteins within complex biological samples (Figure 2). In CerS2 protein determination, WB has been employed to get insights into its expression and regulation. Several studies have applied this technique to reveal CerS2's role in various cellular contexts.

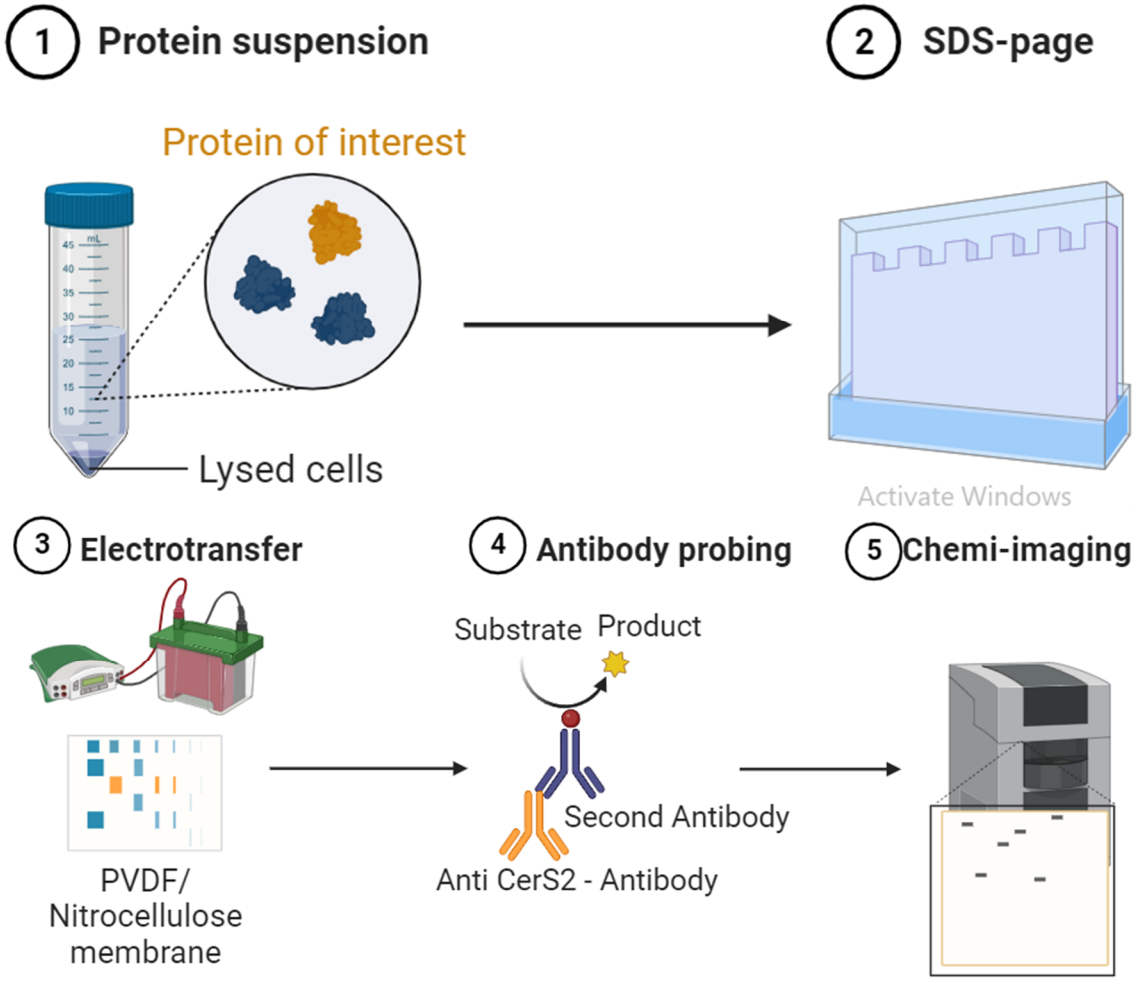

Fig. 2 Western blot procedure (created with BioRender.com)

In Table 2, we present the characteristics of these methods, and in the following sections, we will delve into their respective procedures. In a study by Kim et al., proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. CerS2 proteins were then detected using rabbit or mouse anti-CerS2 antibodies followed by appropriate secondary antibodies. The study also employed a mouse anti-Flag antibody to identify Flag-tagged constructs and a mouse anti-GAPDH antibody to confirm equal loading. The ChemiDoc MP imaging system was used to visualize and quantify protein bands [19]. In another investigation, Mesicek et al. separated the proteins on a 10–12% SDS-PAGE gel and transferred them to polyvinylidene difluoride membranes. The study examined CerS2's role in ionizing radiation (IR)-induced ceramide generation. Western blots were carried out using anti-FLAG and anti-HA antibodies to confirm overexpression of CerS1, CerS2, CerS5, and CerS6 constructs. CerS2's presence in subcellular fractions was detected through a direct western blot using an anti-CerS2 antibody [11]. In another approach [4], samples were separated by 8% SDS-PAGE and electroblotted onto nitrocellulose membranes. The phosphorylation status of Lyn kinase and STAT3 was evaluated in CerS2/6 null and wild-type cells upon granulocyte colony-stimulating factor (G-CSF) stimulation.

Western blot analysis was performed using specific antibodies for protein detection. Lastly, Casadomé-Perales et al. investigated CerS2 levels in cellular and tissue samples. Protein lysates were prepared with the aid of RIPA lysis buffer and quantified by BCA Protein Assay Kit. Western blotting was conducted on 8–12% Tris-HCL gels, and CerS2 detected using specific antibodies. Membranes were visualized on the ImageQuant LAS 4000 Mini system and quantified by ImageJ software [23]. These diverse methodologies collectively highlight the utility of Western blot analysis in understanding CerS2 protein expression, interactions, and functional implications across various biological contexts.

CerS2 Enzyme Activity Assay

An *in-vitro* ceramide synthase assay was used by Sassa et al. to evaluate CerS2 activity. Membrane fractions from HEK293T cells expressing Hemagglutinin-tagged (HA-tagged) CerS proteins were incubated with deuterium-labelled sphingosine (sphingosine-d7) and acyl-CoA in a reaction buffer. The reaction was terminated and lipid species extracted using chloroform/methanol. Deuterium-labelled ceramides were then quantified using reversed-phase LC-MS, when investigation revealed that the phosphorylation status of HA-CerS2 significantly influenced its catalytic efficiency.

Table 2 Comparison of four methods for Western blot analysis

Aspect	Method 1 [19]	Method 2 [11]	Method 3 [4]	Method 4 [23]
Sample source	Cell lines (Hek293T, HepG2)	Cell lines (HeLa)	Cell lines (BMCs)	Cell samples brain cortex
Protein detection	CerS2	CerS2	CerS2, Lyn, STAT3, SOCS3	CerS2
Primary antibodies	Rabbit anti-CerS2, Mouse anti-Flag	Rabbit anti-CerS2, Anti-HA	Anti-P-Src, Anti-Lyn, Anti-P-STAT3, Anti-STAT3	Various antibodies
Secondary antibodies	Anti-mouse HRP, Anti-rabbit HRP	Anti-mouse HRP, Anti-rabbit HRP	Conjugated HRP	HRP
Gel type	10% SDS-PAGE	10–12% SDS-PAGE	8% SDS-PAGE	8–12% SDS-PAGE
Membrane type	Nitrocellulose	Polyvinylidene difluoride	Nitrocellulose	Nitrocellulose
Blocking	BSA	BSA	BSA	BSA
Detection system	Chemiluminescence	Not specified	IR Dye800/700	Chemiluminescence
Quantification software	ChemiDoc MP imaging system	Not specified	ImageQuant LAS 4000 Mini	ImageJ
Experimental observations	Interaction of CerS2 with fatty acid transport protein family	CerS2's role in IR-induced CerS activity	Lyn kinase phosphorylation in CerS2 Cells	CerS2 levels in the aging cortex

Treatment with phosphatase decreased the activities of HA-CerS2, -4, -5, -3, and -6, while HA-CerS1 activity remained unaffected, which suggested that phosphorylation is a principal regulatory mechanism for HA-CerS2 enzymatic activity, whereas endogenous CerS2 may undergo similar regulation. Also, phosphorylation also modulated the activity of other CerS proteins to a varying degree. The study extended to mouse brain tissue has confirmed that endogenous CerS2 activity is regulated by phosphorylation [1]. Couttas et al. [24] used cultivating U87MG cells and harvesting human frontal cortex grey matter for enzyme activity assessment. Homogenates were prepared and subjected to a fluorescent CerS assay; the respective reaction involving a mixing of homogenates with NBD-sphinganine and fatty acid-CoA, which led to fluorescent dihydroceramide products (Figure 3). The fluorescence intensity was quantified using TLC plates.

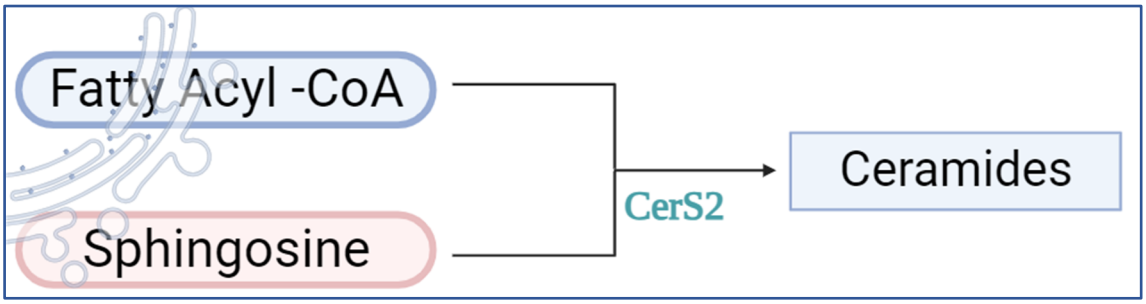

Fig. 3 CerS2 catalyse on ceramide formation

Furthermore, the study examined the impact of specific inhibitors, FTY720, AAL(S), and Fumonisin B1, on enzyme kinetics [24]. In Method 3, HEK293 cells were transfected to express CerS2 enzymes and the assessment of ceramide synthesis utilized NBD-sphinganine and fatty acid-CoA substrates. The reaction, initiated by combining cell homogenates with substrates, produced NBD-dihydroceramide products, TLC analysis facilitated quantification.

Moreover, CerS activity was explored in tissue homogenates. The study observed variations in enzyme activity across different tissues, reflecting differential preferences for distinct fatty acid-CoA substrates [25].

It has been shown that these methods have revealed the complex regulatory mechanisms leading to CerS2 activity, exploring the enzyme behavior in various cellular and tissue contexts, and highlighting the importance of phosphorylation and substrate preferences in ceramide synthesis. Table 3 below compares the last two methods as they share the most similarities, excluding the first method, which employed labelling for its development.

Table 3 Enzyme activity assays - a comparison

Aspect	Method 2 [24]	Method 3 [25]
Cell/tissue source	U87MG cells, human frontal cortex grey matter	HEK293 cells, mouse tissues
Homogenization	Ultrasonication with Bioruptor	Dounce homogenizer
Protein quantification	BCA assay with BSA standards	BCA assay with BSA standards
Reaction buffer	Hepes, KCl, MgCl2, DTT, BSA, sphinganine-NBD, fatty acid-CoA	Hepes, KCl, MgCl2, DTT, BSA, sphinganine, fatty acid-CoA
Reaction conditions	37 °C, 20–30 min reaction time	35 °C, 30–120 min reaction time
Stopping reaction	Methanol extraction or two-phase extraction	Chloroform/methanol extraction
Enzyme substrate	NBD-sphinganine	NBD-sphinganine
Inhibitor analysis	Fumonisin B1	FTY720, AAL(S), Fumonisin B1
Ceramide identification	Identified via HPLC and fluorescent detector	Identified using LC-MS/MS
Enzyme expression	CerS1, CerS2, CerS5-expressing lysates	CerS2, CerS5-expressing lysates
CerS specificity	Different CerS enzymes prefer different substrates	Different CerS enzymes show substrate preferences
Product limit of detection	~0.5 pmoles on TLC plate	~0.25 pmoles on TLC plate

Conclusions

It can be stated that CerS2 is a vital enzyme in sphingolipid metabolism, particularly in the synthesis of very long-chain ceramides. Its wide tissue distribution and unique substrate specificity underscore its importance in cellular sphingolipid processes. Methods for isolating CerS2 include the protein extraction and immunoprecipitation, enabling the study of its functions and interactions in different cellular contexts.

Real-time polymerase chain reaction and Western blotting have been of principal importance for understanding of CerS2 expression at both mRNA and protein levels, revealing tissue-specific patterns, developmental significance, clinical relevance, and regulatory interactions. Enzyme activity assays have demonstrated the complex regulation of CerS2, with phosphorylation and substrate preferences influencing its catalytic efficiency and tissue-specific activity.

In summary, CerS2 plays a crucial role in sphingolipid metabolism, and insights into its expression, regulation, and enzymatic activity provide valuable knowledge for understanding of various physiological and pathological processes, including cancer, which makes it promising for future research and potential therapeutic interventions.

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