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Recombinant expression, purification, and kinetic and inhibitor characterisation of human site-1-protease

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Abstract

Human site-1-protease (S1P, MEROPS S08.8063), also widely known as subtilisin/kexin isozyme 1 (SKI-1), is a membrane bound subtilisin-related serine protease, that belongs to a group of nine mammalian proprotein convertases. Among these proteases, S1P displays unique substrate specificity, by showing preferred cleavage after non-basic amino acids. S1P plays a key role in a proteolytic pathway that controls the cholesterol content of membranes, cells and blood. S1P also participates in the activation of viral coat glycoproteins of the lassa virus, the lympocytic choriomeningitis virus and the crimean congo hemorrhagic fever virus. We expressed recombinant human S1P using the baculovirus expression vector system and characterized the highly purified enzyme. Featuring a new chromogenic substrate (Acetyl-Arg-Arg-Leu-Leu-*p*-nitroanilide) we show that the enzymatic activity of S1P is not calcium dependent, but can be modulated by a variety of mono- and divalent cations. S1P displayed pronounced positive cooperativity with a substrate derived from the viral coat glycoprotein of the lassa virus. The screening of a limited number of protease inhibitors showed that S1P was not inhibited by specific inhibitors of other proprotein convertases or by Pefabloc SC (4-(2-aminoethyl) benzene sulphonyl fluoride, AEBSF). We found 3,4-dichloroisocoumarin (DCI) to be a potent slow binding inhibitor of human S1P, with a $K_{i\,app} = 6.8\ \mu M$, thus representing a new small molecule inhibitor of S1P. These findings show that S1P differs significantly from other proprotein convertases with respect to kinetics, co-factor requirement and inhibition.
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Human site-1-protease (S1P, MEROPS S08.8063), also widely known as subtilisin/kexin isozyme 1 (SKI-1),[2] is a membrane bound subtilisin-related serine protease, that belongs to a group of nine mammalian proprotein convertases (PCs) in family S08. These are the dibasic–monobasic-specific PC1 (PC3), PC2, furin (PACE), PC4, PC5 (PC6), PACE4, and PC7 (LBP, PC8) that belong to the subfamily S08B (kexin subfamily), while S1P and the recently discovered PCSK9 (NARC-1) are found in subfamily S08A (subtilisin subfamily). In general, these proteases process proteins that transit through the secretory pathway. S1P displays unique substrate specificity among these proteases by showing preferred cleavage after non-basic amino acids. A preferred cleavage motif was recently described as cleaving C-terminal to Arg/Lys-X-&-Leu/Ser/Thr, where X is any amino acid except Cys, and & is a hydrophobic amino acid containing an alkyl side chain [1].

S1P is a key player in a proteolytic pathway that controls the cholesterol content of membranes, cells and blood

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[1] These authors have contributed equally to this work.
[2] *Abbreviations used:* SKI-1, subtilisin/kexin isozyme 1; 2-Abz, *o*-aminobenzoic acid; Ac, acetyl; BPTI, trypsin inhibitor from bovine pancreas; DAB, diaminobenzidine; DCI, 3,4-dichloroisocoumarin; HRP, horseradish peroxidase; IMAC, immobilised metal affinity chromatography; MCA, 4-methyl-coumaryl-7-amide; pNA, *p*-nitroanilide; MWCO, molecular weight cut off; PVDF, polyvinylidene fluoride; rhS1P, human recombinant S1P; rhS1P-1, recombinant human S1P construct 1 (S1P lacking the transmembrane domain); rhS1P-2, recombinant human S1P construct 2 (full-length S1P); SBTI, trypsin inhibitor from soybean; SD, standard deviation; Y(NO_2), L-NO_2-tyrosine.

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[2]. S1P cleaves sterol regulatory element-binding proteins (SREBPs) and initiates the release of their N-terminal domains from membranes. The N-terminal domains enter the nucleus, where they enhance transcription of multiple genes encoding enzymes of cholesterol and fatty acid biosynthesis and the low-density lipoprotein receptor. Other substrates of S1P are proBDNF [3], the transcription factor ATF-6 [4] and prosomatostatin [5]. S1P also activates itself by autocatalytic cleavage of its prodomain [6]. Implicating a role in viral infectivity, it has further been shown that S1P participates in the activation of viral coat glycoproteins of the lassa virus [7], the lymphocytic choriomeningitis virus [8] and the crimean congo hemorrhagic fever virus [9].

Hamster S1P has been expressed in a S1P deficient hamster cell-line and purified from the supernatant using immobilized metal affinity chromatography (IMAC) [10]. Toure et al. [6] attempted the expression and purification of human S1P using a vaccinia virus based expression system, but managed only to produce limited quantities of partially purified S1P, not even enough to carry out full kinetic analysis. To our best knowledge, further characterizations of human S1P throughout the literature were only performed in cell culture supernatants. No structural information on human S1P nor a mammalian member of the subtilisin subfamily of PC has been reported yet. On the other hand the structures for a truncated mouse furin and its yeast counterpart kexin have been determined [11,12]. Also information about the biochemical and kinetic properties of S1P are sparse and in some cases contradictory. Toure et al. [6] reported S1P to be a Ca^{2+} dependent serine protease, which was also claimed by Seidah et al. [3], while Cheng et al. [10] reported that hamster S1P does not require Ca^{2+} and is only weakly inhibited by high concentrations of Ca^{2+} chelators. Likewise, Basak et al. [1] reported 4-(2-aminoethyl) benzene sulphonyl fluoride (AEBSF; Pefabloc) to be an S1P inhibitor in the nanomolar range, while on the other hand AEBSF was shown to have only a marginal effect on S1P activity at a concentration of 1 mM by Elagoz et al. [13] and Cheng et al. [10].

Because of the lack of characterization of purified human S1P, we attempted to set up an efficient expression system for production of reasonable amounts of recombinant human S1P (rhS1P) to allow further biochemical, kinetic and structural characterization of this unique protease. Compared to mammalian cell based expression systems reported so far for expression of recombinant S1P, the baculovirus expression vector system (BEVS) comprises a less technical, resources and equipment demanding system for the production of recombinant proteins. We show here the expression of rhS1P lacking its transmembrane domain (rhS1P-1) using the BEVS and its biochemical and kinetic characterization featuring a simple chromogenic substrate. We can show that rhS1P-1 is not a calcium dependent protease and we also present 3,4-dichloroisocoumarin (DCI) as a new potent slow binding inhibitor of rhS1P-1.

Materials and methods

BEVS expression constructs for human S1P

The amplification of human S1P from cDNA was performed by Cytomix Ltd (Cambridge, England) using the DNA sequence provided within GenBank Accession No. NM_003791 as reference sequence. Two expression constructs were designed and cloned into suitable vectors for expression in the BEVS. Construct 1 contains the first 997 amino acids of human S1P and lacks the C-terminal transmembrane region. Construct 2 comprised the full-length sequence of human S1P. The resulting recombinant proteins were named rhS1P-1 and rhS1P-2, respectively. Both constructs were tagged with a C-terminal 8× His-tag (PGDDDDKHHHHHHHHSGS) to facilitate detection and purification. The PCR primers used for construct 1 were: GGGGACAAGTTTGTACAAAAAAGCAGGCT TCTTTAACTTTAAGAAGGAGATATAACCATGAA GCTTGTCAACATCTGGCTGCTTCTGC (forward), GGGGACCACTTTGTACAAGAAAGCTGGGTCCT AAGATCCACTATGATGATGATGATGATGATGATG (reverse 1) and CCACTATGATGATGATGATGATGAT GATGCTTATCGTCATCGTCCCCGGGCTCCTGGT TGTAGCGGCCAGGCATGATCCCTC (reverse 2) and for construct 2: forward, reverse 1 and CCACTATGATG ATGATGATGATGATGATGCTTATCGTCATCGTCC CCGGGCACCGAAGGGGTCTTTGGCGGGTGAAC CTGCTGC (reverse 3). Both PCR products were subcloned into the entry vector pDONR201 (Invitrogen, Paisley, UK) using the Gateway™ Technology, with help of a BP reaction according to the manufacturer's instructions (Invitrogen, Paisley, UK). Recombinant baculovirus for expression of the human S1P constructs were generated with the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Paisley, UK) according to the manufacturer's instructions. For this, pDEST8 (Invitrogen, Paisley, UK) was used as the destination vector and the ORFs of the two constructs were transferred into pDEST8 with the help of a LR reaction according to the manufacturer's instructions (Invitrogen, Paisley, UK).

Insect cell culture

Spodopetera frugiperda ovarian cells, Sf9 and Sf21, were from Invitrogen. Sf cells were cultivated in Sf-900 II SFM protein-free medium (Invitrogen, Paisley, UK). *Trichoplusia ni* egg cells, High Five (Hf), were from Invitrogen (Paisley, UK). Hf cells were cultivated in Express Five SFM protein-free medium (Invitrogen, Paisley, UK) supplemented with L-glutamine. Insect cells were grown in shaker cultures (105 rpm at 27 °C) and passaged twice a week.

Purification of secreted rhS1P-1

Insect cells in shaker culture were infected with a multiplicity of infection (MOI) >1. The cultivations were

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