Structural basis for S1-S4 specificity in two enzymes of the Proteinase K family

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Summary

A proteinase from a psychrotroph Serratia species (SPRK), sharing ca 40% identity with Proteinase K (PRK) of the subtilisin superfamily, has been isolated, cloned, expressed and now the crystal structure is determined to 1.8 Å and an R-factor of 14.6% and R-free of 18.0%.

The catalytic efficiency of the two enzymes towards various tri- and tetrapeptide substrates has been compared. The two enzymes display slightly different substrate specificity, and the catalytic turnover (k_{cat}) is generally higher for SPRK than PRK, but the substrate binding (K_m) is poorer.

SPRK has a stronger preference for large residues at the S1 and S4 binding sites than PRK, primarily because of larger binding pockets on the enzyme surface.

Introduction

Proteinases are widely used in biotechnology and commercial industries, as they are involved in the breakdown of peptide bonds in proteins. About 60% of the industrial enzymes sold today are proteases. Most commercially available proteinases tend to be very stable, and can be difficult to inactivate when the process is done.

New enzymes that may have a potential within the biotechnology industry are therefore those which possess unique features when it comes to biophysical properties such as e.g. specificity, catalytic efficiency (expressed by the k_{cat}/K_M ratio) and stability. These properties are often fine-tuned by organisms adapted to extremes of temperature, pressure, salt concentration or acidity. Developing enzymes to be used as biological catalysts may therefore easiest be achieved by using enzymes with known unique features as templates, since starting with a "nature's solution" is better than starting from scratch.

Increased efficiency and reduced temperature stability is often attributed to enzymes from cold adapted species, so-called psychrophiles. These properties were sought after for protein that belonged to the Proteinase K family, a sub-group of the subtilases of the serine proteinases. Proteinase K (PRK) is a very stable enzyme used primarily for removal of RNase activity. An extracellular proteinase isolated from a psychrotroph Serratia species (SPRK), sharing ca 40% sequence identity with PRK, was cloned, expressed and purified. SPRK possesses slightly lower thermal stability and a higher catalytic turnover than the mesophilic counterpart, but the binding affinity is reduced. A structure-function-relation study was therefore initiated to try to identify the substrate specificity of the two proteinases, and to find structural explanations for the differences in the catalytic efficiency. This knowledge will further be used in redesign of the proteinase to yield an enzyme with high specificity, high catalytic efficiency and low temperature stability.

Conclusions

The fold of the main chain of the two proteinases is practically identical and the amino acid sequences forming the substrate binding site is almost completely conserved. However, the crystal structure suggests that the stronger substrate binding of Proteinase K is caused by a more open binding region on the enzyme surface. The *Serratia* proteinase has a stronger preference for large residues at the S1 and S4 sites, and this is caused by larger cavities in the surface. Both enzymes dislike proline at the S2 site, but this is particularly unfavorable in the *Serratia* proteinase, mainly due to a disulfide bridge in the close proximity of the S2 site that is not present in Proteinase K.

Substrate Specificity

Substrate specificity was determined by measuring catalytic turnover (k_{cat}) and substrate affinity (K_m) using various tri- and tetrapeptide succinyl-peptide-p-nitroanilide substrates at pH 8.0. The results are presented in the table below.

Substrate	SPRK			PRK			SPRK *vs* PRK		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
S-AAA-pNA	4.54	4.58	0.99	0.99	0.66	1.5	↑ x 4.6	↑ x 6.9	↓ x 1.5
S-AAF-pNA	53.38	2.77	19.3	8.06	1.52	5.32	↑ x 6.6	↑ x 1.8	↑ x 3.6
S-AAPA-pNA	30.23	3.52	8.64	2.76	0.31	8.81	↑ x 11.0	↑x11.4	↓ x 1.04
S-AAPF-pNA	363.77	2.46	148.39	88.27	0.46	190.24	↑ x 4.1	↑ x 5.4	↓ x 1.3
S-AAAF-pNA	168.20	0.086	1950.33	35.58	0.023	1551.25	↑ x 4.7	↑ x 3.7	↑ x 1.3
S-FAAF-pNA[1,2]	189.55	0.056	3375.05	24.90	0.082	302.40	↑ x 7.6	↓ x 1.5	↑ x 11.2

$k_{cat} = s^{-1}$, K_m=mM; [1]measurement is performed with 30% acetonitrile [final] in the assay buffer; [2]corrected values using scaling factor based on AAAF experiments with and without 30 % acetonitrile [final] in the assay buffer.

Primary (P1) specificity

		SPRK	PRK
AAA	k_{cat}	↑ x 11.8	↑ x 8.1
↓	K_m	↓ x 1.7	↑ x 2.3
AAF	k_{cat}/K_m	↑ x 20.0	↑ x 3.5
AAPA	k_{cat}	↑ x 12.0	↑ x 32.0
↓	K_m	↓ x 1.4	↑ x 1.5
AAPF	k_{cat}/K_m	↑ x 17.2	↑ x 21.3

↑ = increase; ↓ = decrease

P1 alanine is replaced by P1 phenylalanine for tri- and tetra-peptide substrates. The catalytic turnover (k_{cat}) increases for both enzymes when large hydrophobic residues are introduced at the S1 site (Ala ⇒ Phe substitution) both for tri- and tetra-peptide substrates. The increase is particularly high for the tetrapeptide and PRK. The binding is, however, reduced (higher K_m) for P1 Phe in PRK, and is weakest for the tripeptide, while increased (lower K_m) in SPRK. Thus, a 3-fold increase in catalytic efficiency for PRK and a 20-fold increase in SPRK are observed for the tripeptides, while both enzymes have a ca 20-fold increase in efficiency for the tetra-peptide substrates. The catalytic efficiency is relatively similar for the two enzymes, but slightly higher for PRK, towards all substrates, except AAF where SPRK is 3 times more efficient.

P2-specificity

		SPRK	PRK
AAPF	k_{cat}	↓ x 2.2	↓ x 2.5
↓	K_m	↓ x 28.6	↓ x 20
AAAF	k_{cat}/K_m	↑ x 13.1	↑ x 8.2

↑ = increase; ↓ = decrease

P2 Pro is replaced by Ala. k_{cat} increases approximately 2-fold for both proteinases when proline is positioned in the S2 site, and K_m decreases 28-fold in the SPRK and 20-fold in PRK. Thus the catalytic efficiency of both proteinases is increased, but most for the SPRK.

P4-specificity

		SPRK	PRK
AAAF[1]	k_{cat}	↑ x 1.1	↓ x 1.4
↓	K_m	↓ x 1.5	↑ x 3.6
FAAF[1]	k_{cat}/K_m	↑ x 1.7	↓ x 5.0

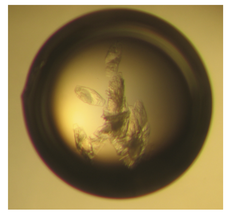
↑ = increase; ↓ = decrease
[1]measurement is performed with 30% acetonitrile [final] in the assay buffer

Replacing alanine with phenylalanine at P4 does not significantly affect k_{cat} in the SPRK, but it is reduced in PRK by a factor of ca 1.5. The K_m is decreased by a factor of 1.5 in SPRK but increased 3.5 fold in PRK, hence causing a 2-fold increase in catalytic efficiency in SPRK and 5-fold reduction in PRK.

Crystallization

Crystals were obtained using the hanging drop vapour diffusion method at 4°C. The crystals are thin hexagonal sheets up to 0.2 x 0.1 x 0.02 mm^3, growing in rosettes. Crystallization conditions are:

5 mg/ml SPRK
45 – 47.5 % ammonium sulfate
0.1 M Tris or BisTris pH 6.5 – 7.5



Data Collection and Refinement

Data was collected at Swiss Norwegian Beamlines (SNBL) at the European Synchrotron Radiation Facilities (ESRF), Grenoble, France. Data reduction was done using DENZO, Scalepack and SCALA (CCP4i). The molecular structure was built using ARP/wARP followed by manual fitting of the sidechains using O. Refinement was carried out using REFMAC in CCP4i.

Data collection and refinement statistics	
Resolution (Å)	1.8
Spacegroup	C2
Cell parameters a-axis (Å) b-axis (Å) c-axis (Å) β angle (°)	 86.43 42.56 74.26 110.19
No. observations (∞ - max. resolution)	108140
No. unique reflections	30408
Rsym 15.0 Å - res. limit (%)	11.4 (38.1*)
Completeness (%)	100 (100*)
I/σI	6.0 (1.9*)
Wilson B (Å2)	11.57
Multiplicity	3.7
R-factor (%)	14.7
R-free (%)	18.0
* outer shell: 1.9 – 1.8 Å	

Structure Analysis

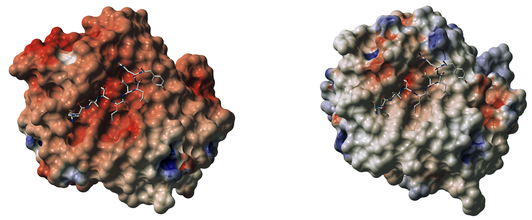
Overall fold

Main chain trace of PRK (red) and SPRK (blue). α-helical regions are illustrated with helices and β-strands with arrows. The fold of the two proteinases is practically identical, only with minor differences in surface loop regions. Only one of two calcium ions (illustrated as spheres) found in PRK is found in SPRK, and at a completely different site.



Electrostatic surface

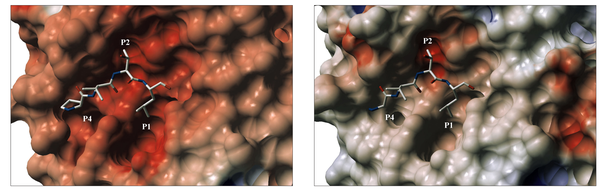
Red and blue regions on the electrostatic surface of the proteins represent negative and positive potentials, respectively, while white regions represent hydrophobic regions. SPRK (left) has a more negative potential than PRK in the substrate binding region. This could possibly be the reason for the higher k_{cat} in SPRK, because a hydrophobic residue could be expulsed quicker from the site. The binding cleft in SPRK is slightly narrower than PRK, and could be a possible explanation for the higher K_M.



S1 – S4 binding site

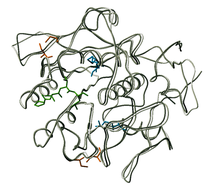
The S1 site:

The shape of the S1 pocket, along with the amino acid sequence, is very similar in the two enzymes, thus the relative preference of Phe over Ala, when considering catalytic efficiency seems to be similar in the two proteinases. The low increase in efficiency of PRK for the tri-peptide are the results of lower increase in k_{cat} and a higher K_m, which can be explained by difficulties in positioning the short, flexible substrate in a slightly more open binding site.



The S2 site:

The S2 site is very similar in the two enzymes. The lower preference for Pro at this site in SPRK could be related to a disulfide bridge (illustrated orange in SPRK and cyan in PRK) from a residue neighbouring one of the residues forming the S2 site. The disulfide is not present in PRK.



The S4 site:

The S4 site in PRK is much shallower than in SPRK, thus Phe can be better accommodated in the latter. This is the most probable explanation why SPRK is 10-fold more efficient than PRK for the FAAF substrate.

Design/Layout: Rod Wikenheiwe, AV Dept., The Medical Faculty, University of Tromsø, Norway