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Mapping the substrate-binding subsite specificity of a *Porphyromonas gingivalis* Tpr peptidase

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Calcium-dependent peptidases of the calpain family are widespread in eukaryotes but uncommon in prokaryotes. A few bacterial calpain homologs have been discovered but none of them have been characterized in detail. Here we present an in-depth substrate specificity analysis of the bacterial calpain-like peptidase Tpr from *Porphyromonas gingivalis*. Using the positional scanning hybrid combinatorial substrate library method, we found that the specificity of Tpr peptidase differs substantially from the papain family of cysteine proteases, showing a strong preference for proline residues at positions P2 and P3. Such a degree of specificity indicates that this *P. gingivalis* cell-surface peptidase has a more sophisticated role than indiscriminate protein degradation to generate peptide nutrients, and may fulfil virulence-related functions such as immune evasion.

Key words: proteolytic activity, calcium-dependent activity, periodontitis, calpain, virulence factor, substrate specificity

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Abbreviations: HyCoSul, Hybrid Combinatorial Substrate Library

INTRODUCTION

Periodontal disease is the most prevalent chronic infection in the world, affecting up to 90% of the global population (Pihlstrom *et al.*, 2005). Inflammation of the gingival tissues is caused by dysbiosis, a pathogenic shift in the microbial community (Loesche & Grossman, 2001). Chronic inflammation leads to the destruction of tooth-supporting tissues such as the gingiva, periodontal ligament, and alveolar bone (Loesche, 1996). If left untreated, periodontitis can result in tooth loss and the systemic dissemination of periodontal pathogens and/or their products (Cobb *et al.*, 2009). This increases the risk of cardiovascular disease (Friedewald *et al.*, 2009; Wegner *et al.*, 2009), rheumatoid arthritis (de Pablo *et al.*, 2009), aspiration pneumonia (Olsen & Potempa, 2014), diabetes (Chee *et al.*, 2013) and adverse pregnancy outcomes (Ide & Papapanou, 2013).

The primary etiological agents of human periodontitis include *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola* (Griffen *et al.*, 1998; Rôças *et al.*, 2001). The virulence factors these pathogens secrete include peptidases that contribute to the initiation and progression of periodontal disease (Dashper *et al.*, 2011; Potempa *et al.,* 2003). In particular, the aggressive periodontal pathogen *P. gingivalis* produces multiple proteolytic enzymes (Potempa *et al.,* 1995) that are required for important bacterial processes such as nutrient acquisition and immune evasion, driving pro-inflammatory responses within the gingival pocket. The proteolytic enzymes secreted by *P. gingivalis* include serine proteases (Nonaka *et al.,* 2014), cysteine proteases such as lysine-specific (Kgp) and arginine-specific (Rgp) gingipains (Bhogal *et al.*, 1997), PrtT protease (Tokuda *et al.,* 1998), periodontain (Nelson *et al.*, 1999) and thiol protease (Tpr) (Staniec *et al.*, 2015). Gingipains (Kgp & Rgp) account for 85% of all extracellular protease activity for *P. gingivalis* (Potempa *et al.,* 1997). Accordingly, the influence of gingipains on *P. gingivalis* virulence has been demonstrated but the role of the other peptidases remains unclear (Imamura, 2003).

Tpr is a functional cysteine peptidase encoded by the *PG1055* gene (Staniec *et al.*, 2015). Distant sequence homology and calcium dependence suggest that Tpr belongs to the calpain family (EC 3.4.22.17; MEROPS family C2) of calcium-dependent cysteine peptidases produced by almost all eukaryotes and some bacteria, but not archaea (Rawlings, 2015). Calpains are ubiquitously expressed in mammalian cells and are involved in essential cellular processes such as cytoskeletal remodeling (Lebart & Benyamin, 2006), cell migration (Franco & Huttenlocher, 2005), cell differentiation (De Maria *et al.,* 2009; Garach-Jehoshua *et al.,* 1998) and apoptosis (Momeni, 2011). Gain-of-function calpain mutations are associated with muscular dystrophy (Wadosky *et al.,* 2011), rheumatoid arthritis (Morita *et al.,* 2006), Alzheimer's disease (Huang & Wang, 2001), improper platelet aggregation (Kuchay & Chishti, 2007), and Wolfram syndrome (Lu *et al.,* 2014). However, no examples of the pathological effects of prokaryotic calpain-like peptidases have been reported thus far.

Eukaryotic calpains are highly-conserved intracellular enzymes that can be assigned to the categories μ or m according to their micromolar or millimolar requirements for calcium *in vitro* (Suzuki *et al.,* 1995). Intriguingly, the specificity of both categories appears identical (Sorimachi *et al.,* 1997). The calcium-mediated regulation of proteolytic activity (Hanna *et al.*, 2008) may require cooperative interactions between several calcium binding sites, including three EF hand motifs, the negatively charged segments around the active site cleft, and an exposed acidic loop (Reverter *et al.*, 2001). However, the precise molecular mechanism is unknown. Although the physiological substrates remain poorly characterized, several

have been identified *in vitro*, including selected β-integrins (Du *et al.,* 1995), focal adhesion kinase (Cooray *et al.*, 1996), paxillin, and talin (Carragher *et al.,* 1999).

The autoprocessing and maturation of the Tpr precursor requires millimolar levels of calcium (Staniec *et al.*, 2015). This requirement prevents the premature activation of Tpr in the cytoplasm and ensures the structure is resistant to degradation by gingipains (Staniec *et al.,* 2015). The elevated expression of the *tpr* gene under nutrient-limited conditions (Lu & McBride, 1998) and the location of the enzyme in the outer membrane (Park *et al.*, 1997; Staniec *et al.*, 2015) suggest that Tpr is involved in the production of small peptides that are used as substrates for amino acid fermentation. Given that multiple physiologically relevant proteins are degraded, including key elements of the innate immune system and proteins abundant in gingival crevicular fluid, Tpr may also contribute to the pathogenesis of periodontitis (Staniec *et al.*, 2015).

A detailed analysis of Tpr substrate specificity is required to understand its contribution to the pathogenicity of *P. gingivalis*, but only a superficial characterization has been reported thus far (Staniec *et al.,* 2015). Here, we fully characterized the substrate specificity of the most active form of Tpr using Hybrid Combinatorial Substrate Library (HyCoSuL) approach to gain insight into the proteolytic mechanism and thus the function of this cell-surface peptidase.

MATERIALS & METHODS

Regents and equipment

Isopropyl-1-thio-β-D-galactopyranoside (IPTG), ethylenediaminetetraacetic acid (EDTA), lysogeny broth (LB), L-glutathione, Tris-HCl, NaCl, dithiothreitol (DTT), sodium azide, and L-cysteine were purchased from Sigma-Aldrich. A MonoQ column was obtained from Cytiva.

Protein expression

The inactive Tpr zymogen (Tpr55) was expressed in *Escherichia coli* BL21 (DE3) cells and purified as previously described with some modifications (Staniec *et al.,* 2015). Briefly, the *PG1055* coding sequence without the signal peptide was transferred to the expression vector pGEX-6P-1, which provides the sequence for an N-terminal glutathione S-transferase (GST) tag. This construct was then introduced into electrocompetent *E. coli* BL21 cells, which were cultivated in LB at 37°C. As soon as the culture reached an optical density at 600 nm (OD_{600nm}) of 0.7, Tpr55 expression was induced by supplementing the medium with 0.5 M IPTG and incubating for a further 3 h at 37°C.

Protein purification

Transformed *E. coli* BL21 cells were harvested by centrifugation (8000×*g*, 20 min, 4°C) and resuspended in PBS containing 10 mM EDTA to bind any residual Ca^{2+} that would induce the premature autoprocessing of Tpr55. To recover intracellular Tpr55, the bacterial cell pellet was disrupted by sonication (60% amplitude for 5 min with 30-s pulses) and the cell debris was removed by centrifugation (18000×*g*, 20 min, 4°C). Tpr55 was recovered by passing the supernatant through a glutathione-Sepharose column and eluting the bound Tpr55 stepwise with increasing glutathione concentration in elution buffer (50 mM Tris-HCl, 20 mM L-glutathione, pH 8.0).

To remove small molecular contaminants, the eluted Tpr55 was dialyzed against PreScission Protease cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0) and the GST tag was cleaved off with 40 U PreScission Protease at 4°C for 16 h. The sample was then dialyzed against 20 mM ethanolamine (pH 9.0) and applied to a MonoQ column equilibrated in dialysis buffer to separate Tpr55 from GST. Final purification was accomplished by gel filtration on a Superdex 75 column in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl. The full-length Tpr55 was routinely purified with a yield >1 mg/L starting culture.

Proteolytically active Tpr (Tpr33) was obtained by autoprocessing the purified Tpr55 as previously described (Staniec *et al.*, 2015). Briefly, purified Tpr55 was incubated in activation buffer (50 mM Tris-HCl pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$, 0.2% sodium azide, and 10 mM L-cysteine) for 7 h at 37°C.

Analysis of substrate specificity

Tpr33 substrate specificity was assessed using the HyCoSuL screening approach and a defined P1 library with the general structure Ac-Ala-Ala-Pro-P1-ACC, where P1 comprises one of either 19 canonical amino acids (except cysteine) or norleucine, synthesized as previously described (Kasperkiewicz *et al.,* 2015; Larsen *et al.*, 2007; Poręba *et al.,* 2014, 2017). Cleavage of the peptide substrate was detected by monitoring the release of the fluorescent label 7-amino-4-carbamoylmethylcoumarin (ACC).

To determine Tpr33 substrate specificity at the P4, P3 and P2 positions, we used a HyCoSuL with arginine fixed in P1 (Fig. 1). Each of the tetrapeptide sublibraries featured an equimolar mixture of 19 amino acids (without cysteine and methionine, but including norleucine) at two positions and one of 19 canonical amino acids plus 102 non-standard amino acids in the position selected for analysis (Fig. 1).

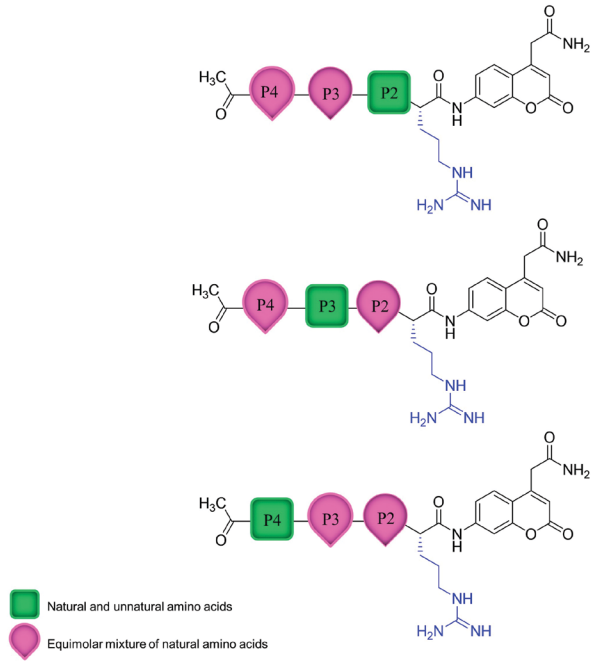

Figure 1. HyCoSuL structure.

Table 1. Kinetic parameters for selected substrates hydrolysis by Tpr33

Substrate							K_M [μM]		k_{cat} [s^{-1}]		k_{cat}/K_M [$\mu M^{-1}s^{-1}$]	
	P5	P4	P3	P2	P1	P1′						
i	Ac	Phe	Gly	Pro	Arg	ACC	150	±1	14.3	±1.4	96	±9
ii	Ac	Lys	Gly	Pro	Arg	ACC	166	±12	11.8	±0.3	71	±7
iii	Ac	Dap	Gly	Pro	Arg	ACC	118	±1	14.3	±0.1	121	±1
iv		Ac	Idc	Pro	Arg	ACC	71	±5	8.9	±0.6	127	±17
v	Ac	Gly	Idc	Cha	Arg	ACC	285	±73	1.5	±0.4	5.4	±2.8
vi		NH	Idc	Cha	Arg	ACC	395	±34	0.32	±0.02	0.8	±0.1
vii	Ac	Gly	Thz	Cha	Arg	ACC	711	±42	1.12	±0.03	1.6	±0.1
viii		Ac	Thz	Cha	Arg	ACC	169	±37	0.71	±0.06	4.1	±1.2

Tpr33 was activated by incubating 500 nM of the enzyme in activation buffer for 30 min at 37°C. To determine the enzymatic activity of Tpr33 in each sub-library, fluorescence was recorded (excitation=355 nm, emission=460 nm) 30 min after the addition of substrates. The P4, P3 and P2 sub-libraries were tested at an initial concentration of 100 μM whereas the P1 library was tested at 10 μM. The slope of the linear range of each curve was recorded as relative fluorescence units per second (RFU/s). Data from each sub-library were normalized to the highest RFU/s value for each specific sublibrary.

Kinetic measurements

Selected substrates (Table 1) were prepared by solid phase peptide synthesis as described elsewhere (Poręba *et al.*, 2014). To determine the catalytic parameters of Tpr against these substrates, Tpr33 was titrated with the irreversible cysteine protease inhibitor E-64 as previously described (Staniec *et al.*, 2015). Peptidase activity was determined at increasing concentrations of each substrate in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.02% sodium azide. Velocities of substrate hydrolysis were plotted against the substrate concentration, and the k_{cat} and K_M values were calculated using GraphPad Prism macro.

RESULTS

Substrate specificity of the Tpr33 protease

In our previous explorative study (Staniec *et al.*, 2015) we found that Tpr33 preferentially hydrolyzes substrates containing arginine at the P1 position (nomenclature according to Schechter & Berger, 1967). Therefore, we initially investigated the amino acid preference at the S4–S2 subsites of Tpr33, followed by a more thorough interrogation of the amino acid preference at the S1 subsite. Accordingly, the P2, P3 and P4 sublibraries had arginine fixed at the P1 position (Fig. 1).

Tpr33 showed exclusive specificity for substrates that incorporated proline or alanine at the P2 position (Fig. 2A). This exclusivity was maintained when non-proteinogenic amino acids were included. None of the D stereoisomers of standard amino acids and only six of 83 non-standard amino acids resulted in hydrolysis when incorporated at the P2 position. Among these non-standard residues, only the thiazolidine-based residue L-Thz, which structurally resembles proline, resulted in hydrolysis with similar efficiency to the proline-P2 sub-library (Fig. 2B).

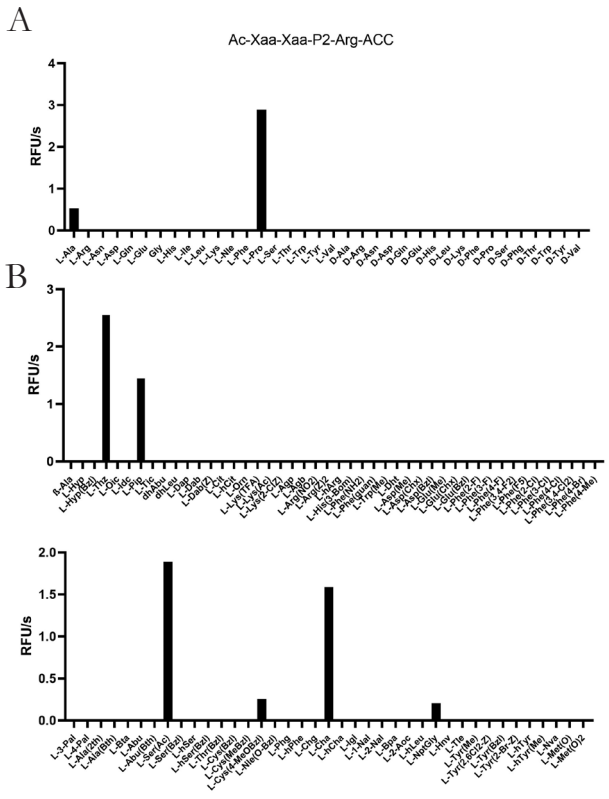

Figure 2. Specificity of Tpr33 for substrates containing canonical and non-proteinogenic amino acids at the P2 position as determined using the HyCoSuL screening approach.
The substrate preferences at position P2 were determined using a combinatorial tetrapeptide library with the general structure Ac-Xaa-Xaa-P2-Arg-ACC, where P2 represents (**A**) canonical or (**B**) non-proteinogenic amino acids and Xaa represents an equimolar mixture of 19 amino acids (all standard proteinogenic amino acids except cysteine and methionine, supplemented with norleucine). Abbreviated amino acid names are shown on the x-axis and explained in the Supplementary Materials at https://ojs.ptbioch.edu.pl/index.php/abp/. The y-axis shows the mean activity expressed as relative fluorescence units per second [RFU/s]. The data are means of three independent measurements.

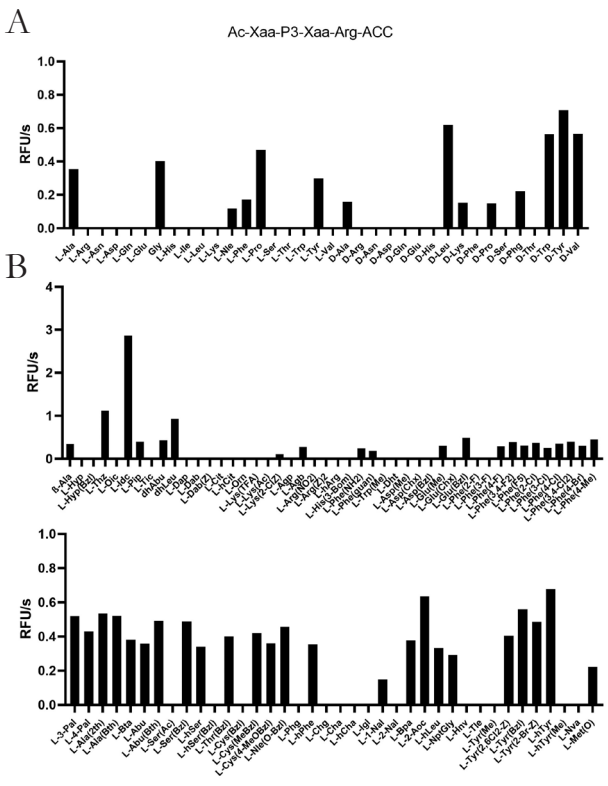

Figure 3. Specificity of Tpr33 for substrates containing canonical and non-proteinogenic amino acids at the P3 position as determined using the HyCoSuL screening approach.
The substrate preferences at position P3 were determined using a combinatorial tetrapeptide library with the general structure Ac-Xaa-P3-Xaa-Arg-ACC, where P3 represents (**A**) canonical or (**B**) non-proteinogenic amino acids and Xaa represents an equimolar mixture of 19 amino acids (all standard proteinogenic amino acids except cysteine and methionine, supplemented with norleucine). Abbreviated amino acid names are shown on the x-axis and explained in the Supplementary Materials at https://ojs.ptbioch.edu.pl/index.php/abp/. The y-axis shows the mean activity expressed as relative fluorescence units per second [RFU/s]. The data are means of three independent measurements.

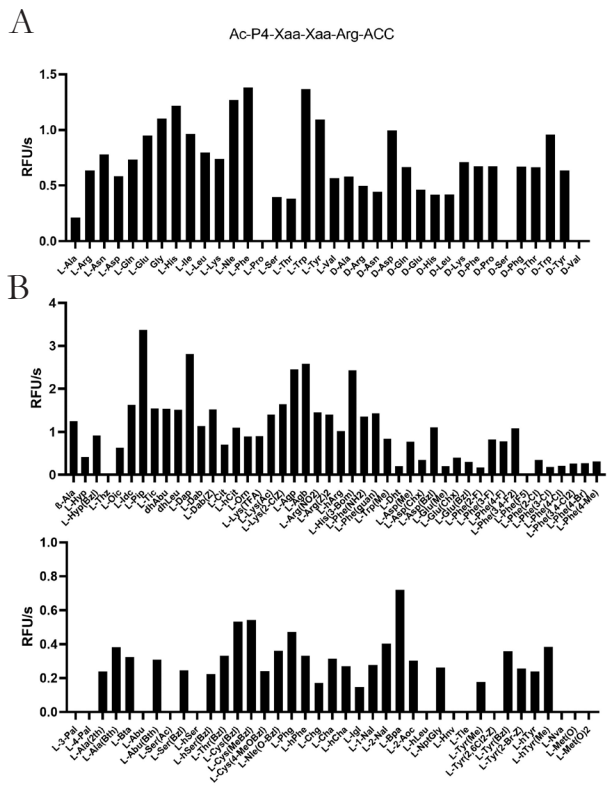

Figure 4. Specificity of Tpr33 for substrates containing canonical and non-proteinogenic amino acids at the P4 position as determined using the HyCoSuL screening approach.
The substrate preferences at position P4 were determined using a combinatorial tetrapeptide library with the general structure Ac-P4-Xaa-Xaa-Arg-ACC, where P4 represents (**A**) canonical or (**B**) non-proteinogenic amino acids and Xaa represents an equimolar mixture of 19 amino acids (all standard proteinogenic amino acids except cysteine and methionine, supplemented with norleucine). The y-axis shows the mean activity expressed as relative fluorescence units per second [RFU/s]. The data are means of three independent measurements.

The specificity of Tpr33 at subsite S3 was less stringent than at S2. Proline was the most preferred proteinogenic amino acid, but Tpr33 also accepted amino acids with short side chains (alanine and glycine) or large hydrophobic side chains (norleucine, phenylalanine and tyrosine) (Fig. 3A). The less stringent specificity compared to S2 was maintained when substrates containing non-standard amino acids were analyzed. More than half of the substrates containing non-standard amino acids were hydrolyzed at least as efficiently as the proline-P3 substrates (Fig. 3B). Multiple non-standard amino acids were accepted including certain D stereoisomers of proteinogenic amino acids (Fig. 3A, B). The proline analog L-Thz was among the most preferred residues, matching the pronounced preference of S3 subsite for proline at P3 of a substrate. Interestingly in the context of substrate design, the most efficiently hydrolyzed substrate in the P3 sub-library featured an indoline-based residue (L-Idc) at P3. Tpr33 showed more than five-fold increase in activity against this L-Idc-P3 substrate than the standard L-Pro-P3 substrate.

In contrast to the other subsites, Trp33 showed broad tolerance for the residue at subsite S4, accepting D stereoisomers almost as well as L stereoisomers, and also tolerating multiple non-standard amino acids at P4 position of a substrate (Fig. 4A, B). Interestingly in the context of substrate design, several non-proteinogenic amino acids were better substrates than canonical amino acids. The most efficiently hydrolyzed substrate featured a piperidine-based residue (L-Pip). Tpr33 showed almost 2.5-fold increase in activity against this substrate than

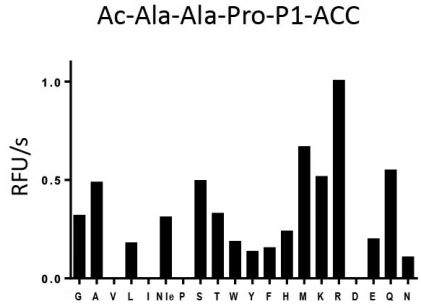

Figure 5. Specificity of Tpr33 for substrates containing different proteinogenic amino acids at the P1 position as determined using using the defined P1 substrate library.
The substrate preferences at position P1 were determined using defined library with the general structure Ac-Ala-Ala-Pro-P1-ACC, where P1 represents a standard proteinogenic amino acid. The y-axis shows the mean relative activity expressed as a percentage of the most efficient amino acid in the same position. The data are means of three independent measurements.

against the best substrate containing a canonical amino acid (Fig. 4B).

Having documented the restricted substrate preference of Tpr33 at subsite S2, we reevaluated the substrate specificity at position S1 using a library containing proline fixed at P2 (Ac-Ala-Ala-Pro-P1-ACC). Tpr33 preferred arginine at position P1 in a substrate, consistent with the results of our previous explorative study (Staniec *et al.*, 2015). However, several other residues with different physicochemical characteristics were also efficiently recognized, indicating that Tpr33 has low specificity for a residue in position P1 of a substrate (Fig. 5).

Kinetic analysis of Tpr33 activity

The randomization of amino acids at certain positions in the HyCoSuL as well as cooperation between subsites may affect the efficiency of Tpr33. To validate the results generated by the HyCoSuL method, we designed and synthesized a selection of substrates and determined their kinetic parameters.

The k_{cat}/K_M value of the substrate containing the preferred canonical amino acids at each position (Ac-Phe-Gly-Pro-Arg-ACC) was 96 μM^{-1} (Table 1). A near-identical substrate containing lysine at the P4 position (Ac-Lys-Gly-Pro-Arg-ACC) had slightly inferior kinetic parameters (k_{cat}/K_m=71±7 μM^{-1}) reflecting the lower efficiency of the Lys-P4 sub-library. Another near-identical substrate, this time containing L-Dap at the P4 position (Ac-Dap-Gly-Pro-Arg-ACC) was superior to both of the above (k_{cat}/K_m=121±1 μM^{-1}), in agreement with the HyCoSuL library screening data. Substrates containing proline at position P3 (Ac-Idc-Pro-Arg-ACC) or glycine at the same position (Ac-Dap-Gly-Pro-Arg-ACC) shared comparable k_{cat}/K_M values, which appeared to contradict the results of the HyCoSuL screening. However, Ac-Dap-Gly-Pro-Arg-ACC is one residue longer than Ac-ldc-Pro-Arg-ACC, and longer substrates are generally better recognized by peptidases, so this may explain this apparent discrepancy. Finally, substrates with Idc (Ac-Gly-Idc-Cha-Arg-ACC and Idc-Cha-Arg-ACC) or Thz (Ac-Gly-Thz-Cha-Arg-ACC and Ac-Thz-Cha-Arg-ACC) at the P3 and Cha at P2 positions, respectively, were hydrolyzed 17–150-fold less efficiently (based on k_{cat}/K_M values) than substrates with Gly-Pro and Idc-Pro at the same positions. This contrasts with the efficiency of hydrolysis of the P2 Cha and P3 Idc/Thz sub-libraries.

Tpr33 activity against the synthetic substrates mostly agreed with the HyCoSuL screening method. Substrates with non-standard amino acids were better recognized than those containing the most preferred canonical amino acids at positions P4–P2. Furthermore, the best-recognized substrate (Ac-Idc-Pro-Arg-ACC) had the lowest K_M value. These data suggest that substrate recognition site S4 is poorly defined, resulting in weaker interactions between amino acids and the S4 pocket.

DISCUSSION

We investigated the substrate preference of the calpain-like peptidase Tpr33 at the S4–S2 subsites and reevaluated the specificity at the S1 subsite using a comprehensive library of defined and highly efficient substrates including the incorporation of non-standard amino acids. Calpain-like peptidases are widespread among eukaryotes, but homologous genes are present only in a few bacterial phyla (Rawlings, 2015). Thus far, Tpr is the only bacterial calpain-like peptidase that has been functionally characterized (Staniec *et al.,* 2015). Given the small number of calpain-like peptidases in prokaryotes, it is not unlikely that *P. gingivalis* acquired Tpr by horizontal transfer from a mammalian host.

Like the m-calpains, a millimolar concentration of calcium is required to activate the Tpr zymogen and such concentrations are found in the extracellular fluid where Tpr is secreted. The calcium-mediated activation of Tpr prevents intracellular activation of the enzyme, which may be cytotoxic. Due to their potential cytotoxicity if left unregulated, peptidases are tightly controlled. Similar mechanisms to prevent the deleterious activity of misdirected secreted peptidases are widespread, including trypsin inhibitors produced by pancreatic cells (Hirota *et al.,* 2006).

The HyCoSuL method allowed us to explore the substrate specificity of Tpr, revealing the repertoire of physiologically accessible substrates. As previously reported for papain (Choe *et al.*, 2006) and calpain (Cuerrier *et al.*, 2005; Shinkai-Ouchi *et al.*, 2016; Tompa *et al.*, 2004), the specificity of Tpr is determined at the S2 subsite whereas S1 is much less selective, albeit with some preference for positively charged residues in all three enzymes. However, the specificity of the S2 subsite differs between Tpr, papain and calpain. Tpr strongly selects proline whereas papain prefers valine but also accepts bulky aromatic and large hydrophobic residues, and calpain accepts several medium-sized residues. The S3 subsite also shows a degree of selectivity, but again this differs between the three enzymes. All three accept proline, but Tpr also accepts bulky aromatic and small side chain residues, whereas papain prefers arginine, lysine and medium-sized side chain residues, and calpain tolerates multiple residues with dissimilar physicochemical properties while simultaneously excluding several residues. Tpr shows broad specificity for amino acid residues in position P4 in a substrate.

Peptidases play a key role in nutrient acquisition by *P. gingivalis* (Potempa & Pike, 2009). We found that Tpr is not restrictively specific at any substrate-binding subsites, but the combination of relatively high selectivity at the S2 and S3 subsites may limit the number of substrates susceptible to Tpr proteolytic activity. Such characteristics are unexpected for a digestive peptidase and suggest a more specialized role, possibly associated with the evasion of host immune defenses.

Peptidases attack the most sterically accessible regions of substrates, often cutting in loops and other unstructured regions. Proline residues are often overrepresented in such regions (Fontana *et al.,* 1986), which also tend to be functionally significant – for example, proline-rich regions are known to be involved in protein–protein interactions (Yu *et al.,* 1994). The relative preference of Tpr for proline residues at positions P2 and P3 in substrates may suggest the involvement of Tpr in the modulation of protein–protein interactions. Eukaryotic proline-specific proteases mediate cell signaling events by modulating protein–protein interactions (Dunaevsky *et al.*, 2020) and the possibility that Tpr has a similar role should be evaluated in future studies.

CONCLUSION

HyCoSuL screening revealed that the substrate specificity of Tpr is primarily determined at the S2 and S3 substrate-binding subsites, with proline being the most preferred residue in both cases. The specificity of Tpr therefore differs from that of the related eukaryotic proteases papain and calpain, and is likely to play a more

sophisticated role in bacterial physiology than indiscriminate protein degradation for the provision of nutrients, which would be facilitated by non-specific protease activity.

Declarations

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