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Serine Peptidase Homolog from the Beetle *Tenebrio molitor* with Substitution of Serine Residue with Threonine in the Catalytic Triad

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Abstract—Analysis of the genomes and transcriptomes of the beetle *Tenebrio molitor* revealed a group of six serine peptidase homologs (SPH) of the S1A chymotrypsin subfamily containing a conservative substitution of the catalytic residue Ser195 with Thr (Ser195Thr) in the active center. All six SPH are secreted proteins with prepropeptides and lack regulatory domains in the propeptide. The most highly expressed homolog, SerPH122, shares 57% sequence identity with the most highly expressed elastase-like peptidase of *T. molitor*, SerP41. Both proteins exhibit similar domain organization, localization in the posterior midgut, and expression patterns in the feeding stages of the fourth instar larva and imago. Testing hydrolytic activity of the recombinant rSerPH122 preparation demonstrated that the conservative substitution of Ser for Thr in the active center did not abolish its catalytic activity. rSerPH122 exhibits low specific activity but broad substrate specificity, most effectively hydrolyzing substrates of chymotrypsin-like and trypsin-like peptidases. The homolog has a pH optimum at 8.5 and is stable in the pH range 4.0-8.0. This study addresses the question of activity of the homologs with the Ser195Thr substitution and contributes to understanding of the poorly studied area of SPH functions, providing a basis for elucidating relationship between the structure and function of serine peptidases and their homologs.

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INTRODUCTION

Serine peptidases (SP) of the S1 family are the most widespread and numerous group of peptidases, both in terms of the number of sequenced proteins and diversity of peptidase activities [1]. All active peptidases in the S1 family are endopeptidases and contain a catalytic triad consisting of His57, Asp102, and Ser195 (residue numbering according to bovine chymotrypsinogen A, XP_003587247). Their specificity largely depends on composition of the S1 substrate-binding subsite at positions 189, 216, and 226 [2]. The family also includes a significant number of poorly studied proteins containing substitutions of active center residues, commonly referred to as inactive serine peptidase homologs (or pseudopeptidases), as they are presumed to lack catalytic activity [3]. All animal peptidases of this family belong to the S1A chymotrypsin subfamily [1, 4], which is the most numerous and well-studied subfamily, including enzymes such as trypsin, chymotrypsin, elastase, and kallikrein. These enzymes play crucial roles in digestion, blood coagulation, immune response, and other physiological processes.

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Genomic and transcriptomic sequencing methods, which have become pivotal in recent years, are particularly intensively used to characterize insects, including within the international i5K project to sequence the genomes of 5000 insects [5]. To date, 95 insect genomes from over 20 orders have been sequenced (https://www.ncbi.nlm.nih.gov/datasets/genome/?bioproject=PRJNA163993), and general characterization of the sequenced genomes has shown that most groups of insects contain a very large number of proteins from the S1A chymotrypsin subfamily (up to 300 or more). This makes insects an attractive object for studying these proteins. However, large number of serine peptidases complicates analysis of these protein groups, and such analysis has been conducted only for the most thoroughly studied insect species, most of which are harmful pests or model organisms: dipterans *Drosophila melanogaster* [6] and *Anopheles gambiae* [7]; hymenopterans *Apis mellifera* [8] and *Pteromalus puparum* [9]; lepidopterans *Bombyx mori* [10], *Plutella xylostella* [11], and *Manduca sexta* [12]; and beetles *Tenebrio molitor* [13] and *Tribolium castaneum* [14]. It should be noted that in majority of these studies, special attention was paid to the domain organization of peptidases and the presumed role of regulatory domains, while the presumed specificity of peptidases was practically not analyzed.

Nevertheless, analysis of the available data has shown that the maximum number of proteins from the S1A chymotrypsin subfamily is found in the evolutionarily young, intensively developing orders of beetles and dipterans, among which a large number of serine peptidase homologs (SPH) have been identified. For example, among the 337 proteins of the S1A subfamily in the mosquito *A. gambiae*, 117 were SPH [7], while in the beetle *T. molitor*, the number of SPH was even higher – 125 SPH out of 269 proteins of the S1A subfamily [13]. However, studies of SPH are scarce. Most studies on the insect SPH are devoted to bioinformatic analysis of their domain organization and analysis of gene expression at various developmental stages and in different organs, which has suggested their important role in insect metabolism [7, 9]. There are also a small number of studies characterizing individual insect SPH, most of which contain the regulatory CLIP domain. Such homologs, containing substitutions of the catalytic serine with glycine and an S1 substrate-binding subsite characteristic of trypsins, have been isolated from *T. molitor*, *M. sexta*, and a number of other insects and crabs, and their involvement in the cascade of prophenoloxidase (PPO) activation reactions, which are associated with immune response to bacterial or fungal infection, has been shown [15-18]. It is assumed that their role is to mediate protein interactions between the active peptidases and their substrates or to participate in localization of the members of peptidase cascades on the surface of pathogens or parasites [19]. However, the role of most SPHs, even in the well-studied model insects, remains unknown. There is a detailed study of spatial structure in combination with enzymatic analysis of two representatives of the family from 32 SPH (SMIPPs) of the scabies mite *Sarcoptes scabiei*, which indicates that SMIPPs have lost their ability to bind substrates in the classical "canonical" manner and instead have developed alternative functions in the life cycle of the scabies mite [20]. It is believed that SMIPPs have a protective function and inhibit the host's complement system, reducing its ability to produce specific antibodies [21].

Among higher animals, there are only detailed studies of human SPH azurocidin (or heparin-binding protein HBP, or cationic antimicrobial protein 37 kDa CAP37), which is close to neutrophil elastase, has a broad spectrum of antimicrobial activity, binds heparin, and is a multifunctional mediator of inflammation [22]. The structure and role of SPH haptoglobin, which binds free hemoglobin during hemolysis, thereby removing it from circulation and preventing oxidative tissue damage, have also been studied in detail [23, 24]. In addition, the protein Z (PZ), an SPH, has been identified in the human blood plasma; it is vitamin K-dependent and exists as a complex with the serine peptidase inhibitor serpin, dependent on protein Z (ZPI) [25]. The most important known physiological function of PZ is its ability to enhance inhibition of coagulation factor Xa by the inhibitor ZPI by three orders of magnitude in the presence of a phospholipid membrane and Ca^{2+} ions [25, 26].

It should be noted that lack of hydrolytic activity in SPH has become generally accepted based on the structural studies due to substitutions in the triad of amino acid residues of the active center [3]. There are very few biochemical studies dedicated to examining catalytic activity of SPH. Among the homologs of serine peptidases of the chymotrypsin S1A subfamily, the studies showing absence of catalytic activity were conducted with the isolated homolog azurocidin [27] and SMIPPs of the scabies mite [20]. Similar results were obtained also for the TIN-ag-RP, a homolog of the cysteine peptidase cathepsin B of the papain C1 family [28].

Many SPH genes exhibit high levels of expression, indicating their active involvement in the organism's metabolism. Researchers agree that the presence of inactive enzyme homologs is expected and that an inactive homolog usually evolved from an active enzyme precursor, not vice versa [3]. Inactive homologs apparently evolved in parallel with their active enzymes and are currently considered important components of biological systems. Such large number of both inactive and active serine peptidases and their

conservation in insects indicate their gene expansion, and evolution of SPHs and their new functions suggests that they are beneficial for insects [29]. Growing importance and relevance of studying SPH are emphasized by their high abundance and wide range of the processes in which they are involved. Of particular interest are SPHs from the organisms where the ratio of active peptidases to inactive homologs is close to one, as observed in the genomes of rapidly evolving insects. As more SPHs are identified, there is an increasing need for deep understanding of their structure, phylogenetic relationships, and functional roles.

It should also be noted that the important regulatory function of enzyme homologs in metabolic and signaling pathways, often associated with pathological conditions, makes them potential targets for the development of new therapeutic agents, which could become a promising direction for future research and allow identification of new regulatory chains, expanding the possibilities for the search for new drugs. Analysis of the available data will provide a new perspective on the meaning of existence and conservation of the enzyme homologs during evolution and outline new research directions aimed at understanding their functional role.

This article uses genomic and transcriptomic approaches to characterize a group of SPHs from a stored product pest, the yellow mealworm *Tenebrio molitor*, with a conservative substitution of the catalytic triad residue Ser for Thr and tests and characterizes enzymatic activity of one of the homologs, SerPH122.

MATERIALS AND METHODS

Analysis of amino acid sequences of *T. molitor* SPH with the conservative substitution Ser195Thr. Multiple alignment of amino acid sequences of SPH with the conservative substitution Ser195Thr was performed using the MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/) [30]. Composition of the active center and the S1 substrate-binding subsite was determined based on the bovine alpha-chymotrypsin [31].

Calculation of mRNA expression levels of SPHs at different stages of the *T. molitor* life cycle. Expression profiles of mRNA of six SPHs (SerPH122, SerPH79, SerPH245, SerPH342, SerPH395, SerPH486) and four active gut serine peptidases (SerP1, SerP38, SerP41, SerP69) were calculated with the formula $\log_2(\text{RPKM} + 1)$ (where RPKM is Reads Per Kilobase per Million mapped reads, +1 is used to perform calculations at zero expression) using transcriptomes at different stages of the *T. molitor* life cycle (egg, II and IV instar larvae, early and late pupae, imago). Preparation of biological material, RNA isolation, cDNA sequencing, assembly, and analysis of transcriptomes were described earlier [13].

Obtaining of a recombinant proenzyme preparation of the homolog rSerPH122. Recombinant preparation of the deglycosylated proenzyme of the homolog rSerPH122 was obtained and purified using the previously published methods [32]. Recombinant expression was carried out in the yeast system *Komagataella kurtzmanii*. A 6-histidine tag (His_6-tag) was added to the C-terminus of the recombinant protein for further purification, which was performed using a specific metal-chelate affinity chromatography, ensuring high selectivity and purity of the preparation.

Processing of the recombinant proenzyme rSerPH122. The lyophilized preparation of the proenzyme of the homolog rSerPH122 was dissolved in 0.1 M acetate-phosphate-borate universal buffer (UB) (pH 7.9) [33] at concentration of 15 μg/ml. Trypsin was next added to a final concentration of 0.25 μg/ml, after which the reaction mixture was incubated for 60 min at 37°C. After activation of the proenzyme, an irreversible trypsin inhibitor, *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), was added to the reaction mixture to a final concentration of 3.3 nM to inhibit trypsin. Concentration of TLCK required for complete inhibition of trypsin was determined by titration in a separate experiment.

Assay of enzymatic activity of the recombinant preparation of rSerPH122 using chromogenic substrates. Enzymatic activity was determined based on the initial rate of hydrolysis of chromogenic peptide substrates containing a *p*-nitroaniline residue (*p*-nitroanilide, pNA) for detection. In a microplate well, 5 to 20 μl of the recombinant preparation of rSerPH122 (concentration in the reaction mixture – 0.9 μM) was added to each well, followed by addition of a 20 mM Tris-HCl (pH 8.0) or 0.1 M UB (pH 7.9) to a final volume of 195 μl. Next, 5 μl of a substrate solution in dimethylformamide (DMF) was added. Standard substrate concentration in the reaction mixture was 0.25 mM, and DMF concentration in the reaction mixture was 2.5% vol. During the reaction, the mixture was incubated at 37°C. The amount of *p*-nitroaniline formed was determined in 96-well plates (Medpolymer, Russia) with a microplate photometer ELx808 (BioTek Instruments, Inc., USA) by measuring absorbance of the solution at 405 nm at the initial time point and then every 5 min. The following substrates were used: trypsin-like peptidase substrates – Z-FR-pNA, Z-RR-pNA, Bz-R-pNA; chymotrypsin-like – Suc-AAPF-pNA, Glp-AAF-pNA, Glp-F-pNA, Suc-F-pNA, Ac-Y-pNA; elastase-like – MeOSuc-AAPV-pNA, Suc-AAA-pNA; substrates hydrolyzed by both chymotrypsin-like and elastase-like peptidases – Suc-AAPL-pNA, Glp-AAL-pNA, For-AAL-pNA, Glp-FL-pNA;

cysteine peptidase substrates – Glp-FA-pNA, Glp-FQ-pNA (where Z – benzyloxycarbonyl (protective group of substrates); pNA – *p*-nitroanilide; Bz – benzoyl; Suc – succinyl; Glp – pyroglutamyl; Ac – acetyl; For – formyl).

The following commercial substrates were used in the study: Z-FR-pNA, Z-RR-pNA, Bz-R-pNA, Suc-AAPF-pNA, MeOSuc-AAPV-pNA, Suc-AAA-pNA; Suc-AAPL-pNA, Glp-AAL-pNA (Bachem, Switzerland); Ac-Y-pNA (Serva, Germany). Substrates Glp-AAF-pNA, Glp-F-pNA, Suc-F-pNA, For-AAL-pNA, Glp-FL-pNA, Glp-FA-pNA were synthesized using standard methods [34] at the Laboratory of Protein Chemistry, Department of Chemistry of Natural Compounds, Faculty of Chemistry, Lomonosov Moscow State University; Glp-FQ-pNA was synthesized according to the method of Filippova et al. [35].

Enzymatic activity was calculated using the formula (1):

$$a = kdA_{405} / dt, \quad (1)$$

where a is activity of the preparation, nmol/min; k = 31.9 nmol/optical unit – the amount of *p*-nitroaniline at which absorbance of the solution was equal to 1 optical unit (the coefficient was determined in a special experiment by constructing a calibration curve of the dependence of absorbance of solutions on concentration of *p*-nitroaniline), dA_{405}/dt is the change in absorbance of the solution at 405 nm at time t, optical units/min.

Calculation of activity was performed using Microsoft Office Excel based on the initial rate of *p*-nitroaniline formation on the linear part of the kinetic curve with subtracting A_{405} values at the initial time point. Initial rates of hydrolysis were determined as the tangents of the slopes of the linear parts of the obtained dependences of optical absorbance on time.

Effect of pH on rSerPH122 activity and stability. Effect of pH on rSerPH122 activity was assessed during hydrolysis of the substrates Suc-AAPF-pNA and Glp-AAF-pNA in 0.1 M UB in the pH range from 3.0 to 11.0 with a step of 0.5, as described above.

To study pH stability, the rSerPH122 preparation was incubated in 50 µl of 0.01 M UB with pH from 2.5 to 11.0 and a step of 0.5 for 30 min. The pH in all samples was then adjusted to 7.9 by adding 0.1 M UB (pH 7.9) to a final volume of 195 µl, and activity was measured as described above.

Statistical data processing. Each experiment with determination of enzymatic activity of the recombinant preparation of rSerPH122 and effect of pH on its activity and stability was performed in at least three replicates. Microsoft Excel 2013 was used for statistical processing of the obtained data, employing built-in functions to assess standard deviation and confidence intervals using Student's *t*-distribution (significance level (alpha) – 0.1 was used to calculate the confidence intervals).

RESULTS

Analysis of amino acid sequences of SPH with the Ser195Thr substitution. A detailed analysis of the genomes and transcriptomes of the beetle *Tenebrio molitor* allowed to identify and annotate 269 amino acid sequences of serine peptidases from the S1A chymotrypsin subfamily. Among these, 125 sequences had 1-3 substitutions of amino acid residues in the catalytic triad (His57, Asp102, and Ser195) and were homologs of serine peptidases (SPH) [13]. Analysis of the SPH amino acid sequences revealed a group of six SPHs containing His, Asp, and Thr residues in the active site, with a conservative substitution of the catalytically essential Ser residue with Thr (Ser195Thr, HDT-type substitution) (Fig. 1; Table 1). All six SPHs contained pre- and propeptides, indicating that they are secreted proteins and, based on bioinformatics analysis, lack regulatory domains in the propeptide [13].

mRNA expression of the SPHs with Ser195Thr substitution. Analysis of mRNA expression across six life stages of the beetle – eggs, II and IV instar larvae, early and late pupae, and adults – revealed a similar expression profile for all six SPHs, with the highest levels observed for the SerPH122 (Fig. 2). mRNA expression was detected almost exclusively during the feeding stages (IV instar larvae and adults). A similar expression profile was observed for the elastase-like SerP41. In contrast, the mRNAs for trypsin SerP1 and chymotrypsin-like peptidase SerP69 were also expressed in the II instar larvae, while the digestive chymotrypsin-like peptidase SerP38 showed low expression in adults.

Substrate specificity of rSerPH122. The recombinant proenzyme, rSerPH122, was produced in the yeast strain *K. kurtzmanii*, purified using metal-chelate affinity chromatography, and deglycosylated as previously described [32]. Given the conservative Ser195Thr substitution in the catalytic triad, we investigated catalytic activity of the recombinant rSerPH122 processed with trypsin using the chymotrypsin substrate Suc-AAPF-pNA [32]. Notably, the initial recombinant rSerPH122 preparation, produced with the pro-sequence, exhibited activity after a few minutes of incubation, suggesting potential autoprocessing. This is in agreement with the subsequent findings on substrate specificity – enzymatic activity with the substrates containing an arginine residue at the P1 position (Fig. 3). Additional processing with trypsin increased protein activity by only 1.5-fold. To avoid potential cross-influence of trypsin on the experimental results (despite the use of titrated TLCK inhibitor

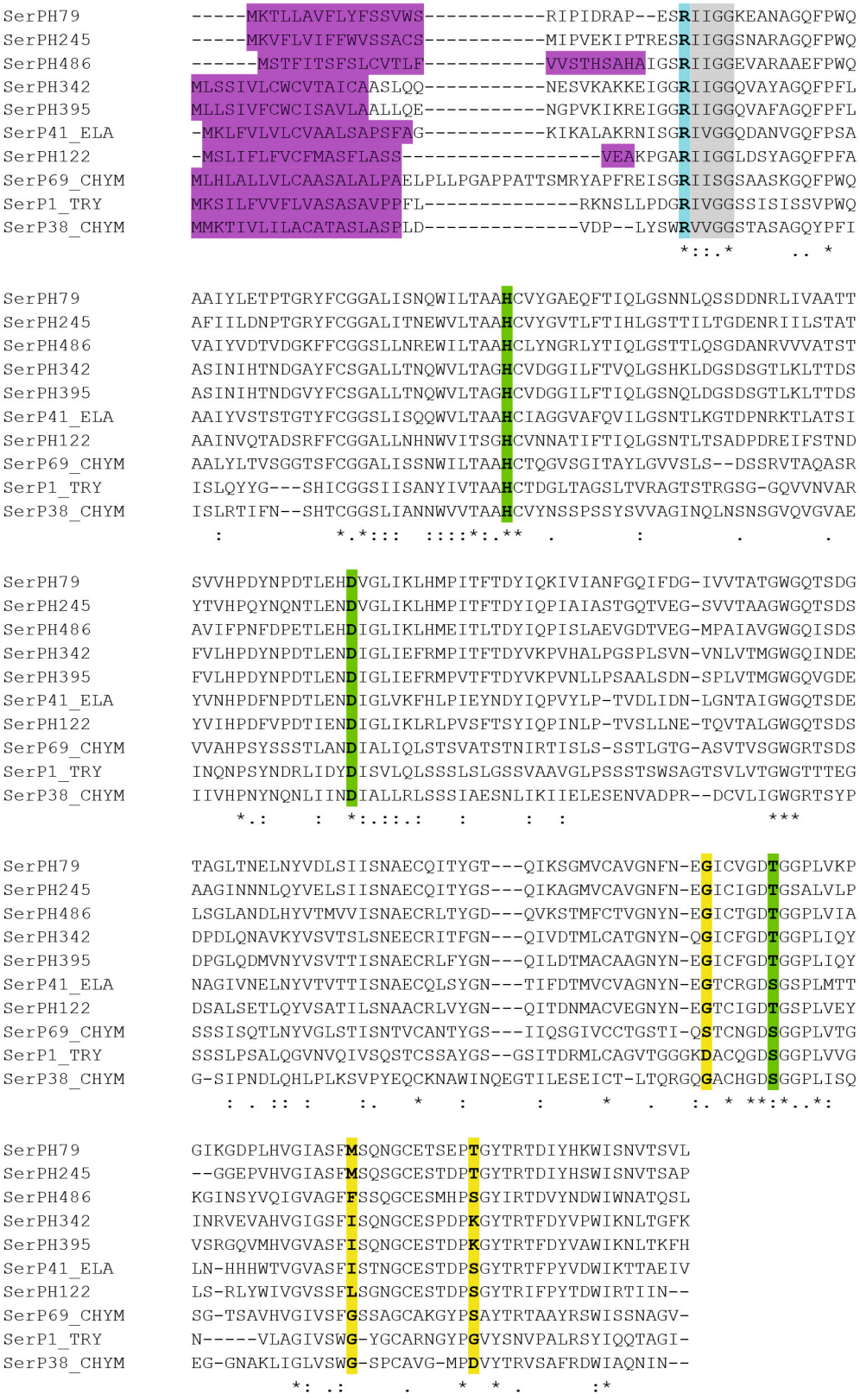

Fig. 1. Multiple sequence alignment of *T. molitor* sequences for four active digestive serine peptidases (trypsin SerP1, chymotrypsin-like SerP38 and SerP69, and elastase-like SerP41) and six SPHs (SerPH79, SerPH122, SerPH245, SerPH342, SerPH395, SerPH486) with Ser195Thr substitution in the active site. The catalytic triad residues are highlighted in green, the S1 substrate-binding subsite residues in yellow, signal peptides in purple, the C-terminal residue of the propeptide at the processing site in blue, and first four N-terminal residues of the peptidase domain in gray.

Table 1. Composition of the active site and S1 substrate-binding subsite of HDT-type SPHs and major digestive peptidases of *T. molitor*

Sequences no.	NCBI ID	Processing site	Active center			S1 substrate-binding subsite			% of identity with SerPH122
SerPH122	CAH1368241	R|IIGG	H	D	T	G	L	S	100
SerPH79	CAH1375498	R|IIGG	H	D	T	G	M	T	47
SerPH245	CAH1375497	R|IIGG	H	D	T	G	M	T	48
SerPH342	KAJ3632571	R|IIGG	H	D	T	G	I	K	51
SerPH395	KAJ3632570	R|IIGG	H	D	T	G	I	K	53
SerPH486	CAH1375496	R|IIGG	H	D	T	G	F	S	48
SerP41	ABC88760	R|IVGG	H	D	S	G	I	S	57
SerP69	ABC88746	R|IISG	H	D	S	S	G	S	37
SerP38	QRE01764	R|VVGG	H	D	S	G	G	D	30
SerP1	ABC88729	R|IVGG	H	D	S	D	G	G	31

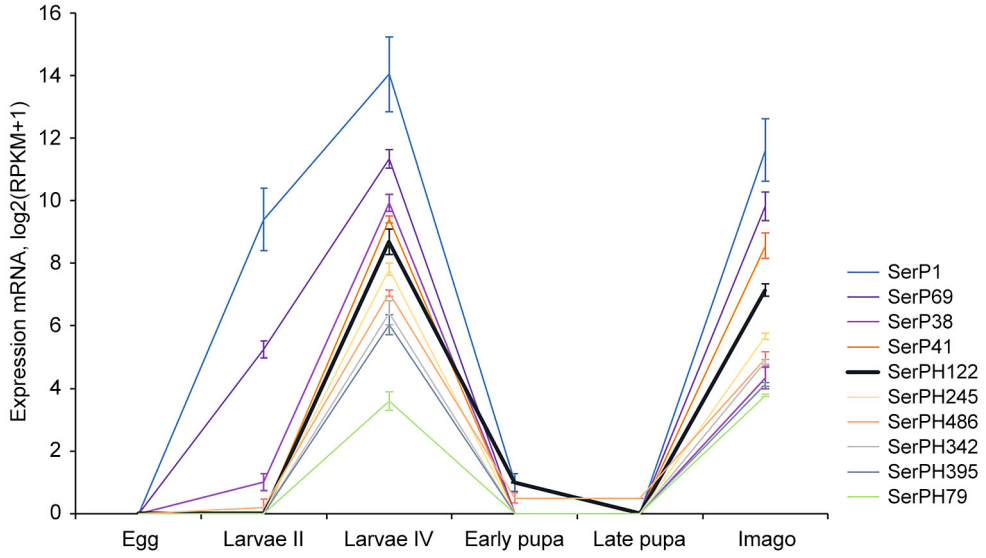

Fig. 2. mRNA expression profiles of six SPHs (SerPH79, SerPH122, SerPH245, SerPH342, SerPH395, SerPH486) and four active digestive serine peptidases (trypsin SerP1, chymotrypsin-like SerP38 and SerP69, and elastase-like SerP41) at different life stages of *T. molitor*. Expression levels are presented as $\log_2$(RPKM + 1) values for each stage, with error bars indicating standard deviation (SD).

at the end of processing), the unprocessed homolog preparation was used to study substrate specificity. Similar results were obtained also with the processed enzyme preparation.

In this study, we analyzed substrate specificity of the rSerPH122 using a broad range of chromogenic peptide substrates for trypsin-like, chymotrypsin-like, elastase-like, and cysteine peptidases (Fig. 3). Preferential cleavage of the chymotrypsin-like substrates (Suc-AAPF-pNA, Glp-AAF-pNA, Glp-F-pNA, Ac-Y-pNA) was observed, along with significant activity with the trypsin-like substrates (Z-FR-pNA and Z-RR-pNA). It should be noted that hydrolysis of the Z-FR-pNA substrate stopped after 20 min, despite sufficient substrate remaining in the reaction mixture, unlike the hydrolysis of Glp-AAF-pNA (Fig. 4). Addition of trypsin to this reaction mixture resulted in further cleavage of the original substrate.

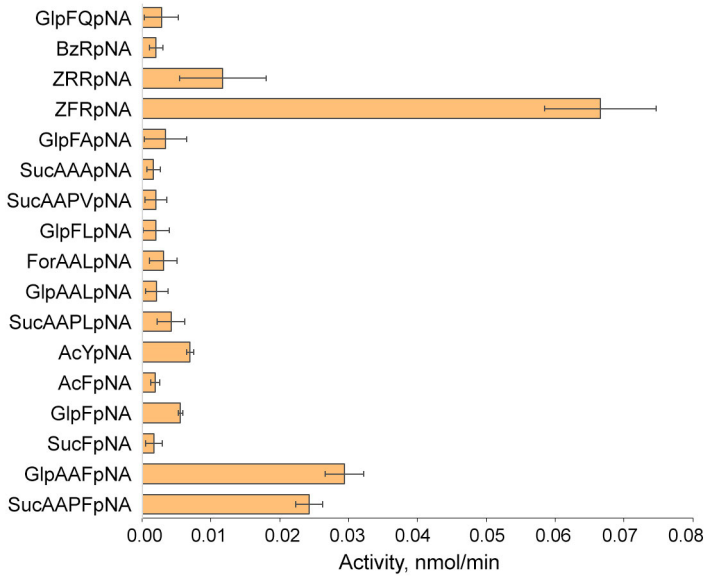

Fig. 3. Activity of the recombinant rSerPH122 homolog with various substrates. Assay conditions: 0.9 μM rSerPH122, 0.25 mM chromogenic substrate, 20 mM Tris-HCl (pH 8.0), and 2.5% (v/v) DMF. Error bars represent standard deviation (SD).

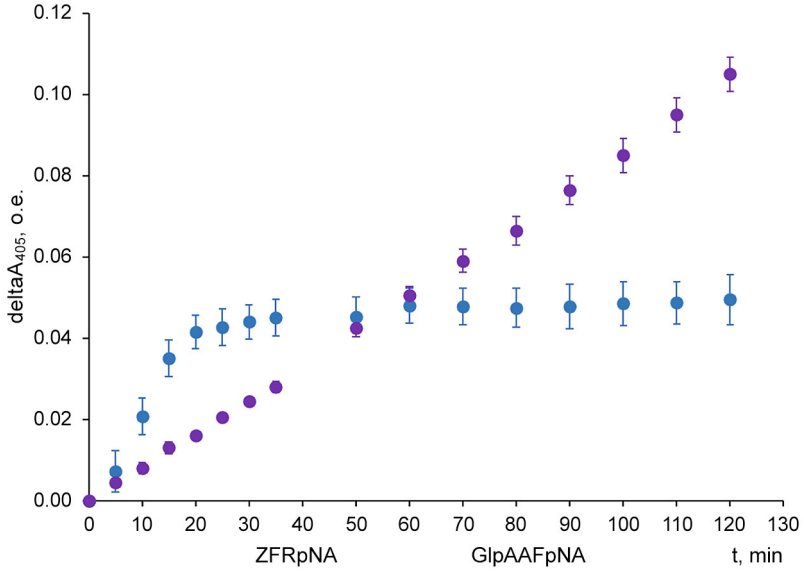

Fig. 4. Kinetic curves of the rSerPH122-mediated hydrolysis of Z-FR-pNA (blue) and Glp-AAF-pNA (purple) substrates. Error bars represent standard deviation (SD).

Comparison of the specific activities of recombinant preparations – rSerPH122 and the chymotrypsin-like peptidase rSerP38 [36] (expressed at comparable levels in the IV instar *T. molitor* larvae) was conducted using the chymotrypsin substrate Suc-AAPF-pNA. Activity of the homolog was significantly lower: 11 nmol/min/mg compared to 49,000 nmol/min/mg for the active SerP38 peptidase.

Effect of pH on the rSerPH122 activity and stability. pH dependence of activity and stability of the rSerPH122 was investigated (Fig. 5). The homolog exhibited maximal activity in the alkaline pH range with optimum at pH 8.5 (Fig. 5a), similar to the rSerP38. Moreover, like rSerP38, the homologue demonstrated the highest stability (retaining 85-100% of activity) in the pH range 4.0-8.0 (Fig. 5b).

DISCUSSION

Among the 125 SPH sequences homologous to serine peptidases of the S1A chymotrypsin subfamily in the *T. molitor* genome [13], six SPHs were found to contain a conservative Ser195 to Thr substitution

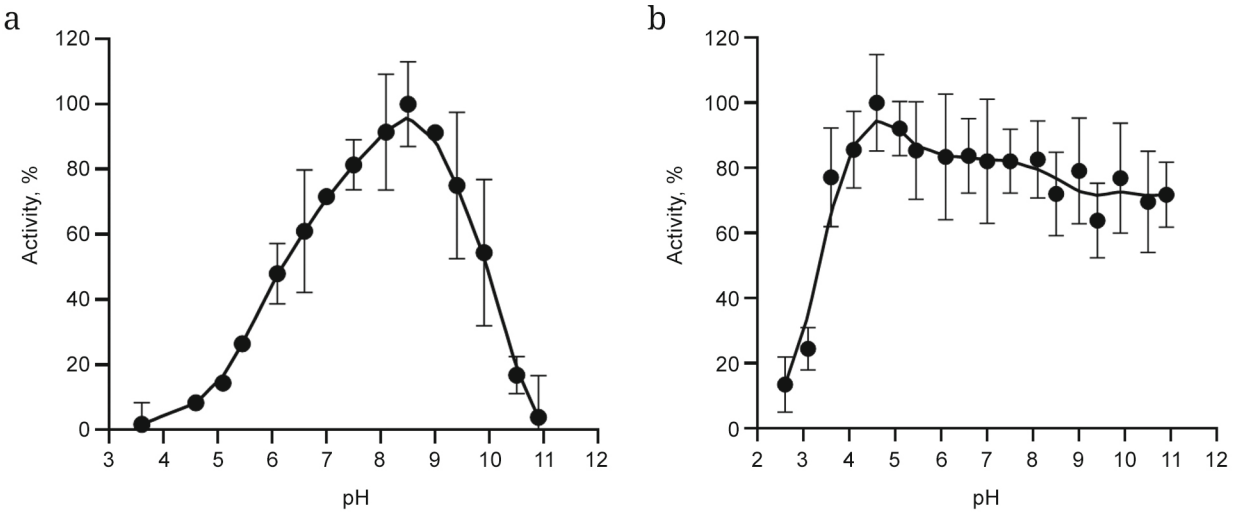

Fig. 5. Effect of pH on activity (a) and stability (b) of the recombinant rSerPH122 homolog (0.9 μM). Error bars represent the confidence interval.

in the catalytic triad. All homologs with the HDT-type substitution are represented by sequences containing pre- and propeptides, are secreted, and lack regulatory domains in the propeptide. These homologs are processed to their mature form by trypsin at the arginine residue at the C-terminus of the propeptide, as demonstrated for the most highly expressed protein in this group, SerPH122 [32]. Additionally, our data on the substrate specificity of SerPH122 (its ability to hydrolyze substrates containing arginine at the P1 position) and its proteolytic activity prior to processing with trypsin suggest its capacity for autoprocessing, a distinctive feature of this homolog. Sequence identity comparisons revealed that SerPH122 shares the highest similarity not with other homologs in this group but with the most highly expressed elastase-like peptidase, SerP41 (57%), indicating a potential evolutionary relationship. This is further supported by the similar expression profiles of the HDT-type homologs and SerP41 – only during the feeding stages of the IV instar larvae and adults, whereas the main digestive peptidases, trypsin SerP1 and chymotrypsin-like peptidase SerP69, are also expressed during the feeding II instar larvae, and the digestive chymotrypsin-like peptidase SerP38 exhibits low expression in adults. It is worth noting that the SPH azurocidin is also closely related to the neutrophil elastase [22].

Considering the conservative Ser195Thr substitution in the HDT-type homolog group, we proceeded to investigate enzymatic activity of the most highly expressed homolog, SerPH122, which was also the only one from this group reliably detected in the gut extract of the fourth-instar *T. molitor* larvae [37]. Unlike the vast majority of the studied inactive peptidase homologs with substitutions in key active site residues [3], the rSerPH122 retained weak but detectable proteolytic activity against a broad range of serine and cysteine peptidase substrates with preference for trypsin and chymotrypsin substrates. Interestingly, hydrolysis of the trypsin substrate Z-FR-pNA began at the highest rate among the all-tested substrates but ceased after 20 min, despite the remaining substrate in the reaction mixture. One possible cause for the reaction stopping could be negative effect of the reaction products on the SerPH122 activity. We note that the detected hydrolytic activities of rSerPH122 were low, and its activity against the chymotrypsin substrate was 4500 times lower than that of the digestive chymotrypsin-like peptidase rSerP38 [36]. This suggests that the conservative Ser195Thr substitution impairs full functionality of SerPH122 as an active enzyme for hydrolyzing dietary proteins and implies a different role for this protein in the digestive process. The alkaline pH optimum (pH 8.5) and maximum stability at pH 4.0-8.0 for SerPH122, similar to rSerP38, correlate with the fact that SerPH122 and SerP38 are most abundant in the posterior midgut (PM) of *T. molitor* [37], where the average pH is 7.9 [38].

Homologs with the Ser195Thr substitution are found almost exclusively in insects, and their characterization is of particular interest, as they have been proposed as a likely transitional form during the evolutionary switch between two types of serine codons: TCX and AGX, which theoretically can only occur through an intermediate protein containing a Ser195 to Thr or Cys substitution due to a single base change (ACX) [1]. Only Thr has been found in this position in the SPH sequences. The authors of this hypothesis, Rawlings and Barrett [1], questioned whether such intermediate form would retain activity. Our positive answer to this question makes the authors' assumption about the existence of such

intermediate form highly probable in connection with the expansion of the functional capabilities of these intermediate protein forms.

Combining data from our laboratory on localization [37] and high expression of the gene encoding SerPH122 in the gut of fourth-instar *T. molitor* larvae, as well as its significant activity with the substrates that have arginine at the P1 position and its ability to autoprocessing at this residue, we cannot exclude involvement of this homolog in activation of the digestive enzymes through processing, as most serine peptidases in *T. molitor* are processed at arginine residues [13]. Broad substrate specificity could also provide greater flexibility in the interaction of rSerPH122 with various protein partners, facilitating its participation in diverse metabolic processes. It is noteworthy that knockdown of the gene encoding the main digestive cysteine peptidase in the closely related beetle *Tribolium castaneum* leads to the increased expression of some SPHs alongside with the active digestive SPs [39], indicating their important protective role.

This work expands our knowledge on the functional roles of SPHs and could serve as a basis for elucidating relationship between the structure and function of serine peptidases and their homologs.

CONCLUSION

Emergence of the homologs of various enzymes is likely an evolutionary advantage, adding new regulatory levels to complex networks. Our transcriptomic studies have shown that SPH genes are actively transcribed, particularly during the feeding stages of larvae and adults. Specifically, SerPH122, localized in the gut of *T. molitor* larvae, exhibits high expression during feeding stages, as well as autoprocessing ability and broad substrate specificity. This suggests its involvement in the digestive process, possibly through regulation or activation of other digestive peptidases. The obtained data highlight the important role of SerPH122 in the digestive system of *T. molitor* and emphasize the need for further research to understand its precise function and regulatory mechanisms.

Abbreviations

Ac	acetyl
Bz	benzoyl
DMF	dimethylformamide
For	formyl
Glp	pyroglutamyl
pNA	*p*-nitroanilide
SerP SP	serine peptidase
SerPH SPH	serine peptidase homolog
Suc	succinyl
TLCK	*N*-α-Tosyl-L-lysine chloromethyl ketone hydrochloride
UB	universal buffer
Z	benzyloxycarbonyl (protecting group for substrates)

Contributions

E. N. Elpidina and V. F. Tereshchenkova – concept and supervision; N. I. Zhiganov and A. S. Gubaeva – experiments; Ya. E. Dunayevsky and M. A. Belozersky – discussion of results; Ya. E. Dunayevsky and E. N. Elpidina – manuscript writing; V. F. Tereshchenkova – manuscript editing.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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