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ORIGINAL ARTICLE

The novel inhibitors of serine proteases

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Abstract Thirty optically active nonprotein α-amino acids and peptides based thereon have been screened for their ability to interact with bovine trypsin and proteinase K from *Tritirachium album Limber*, which belong to the group of serine proteases. Both structure-based drug design approach and determination of enzyme activity have been used to identify low molecular weight inhibitors of trypsin and proteinase K. Compounds have been selected that according to the docking analysis were able to interact with trypsin and proteinase K. Following the docking analysis measurement of enzymes activity (2*R*,3*S*)-β-hydroxyleucine and (2*S*,3*R*)-β-hydroxyleucine inhibited both enzymes activity, whereas (*S*)-α-methyl-β-phenylalanine, (*R*)-α-methyl-β-phenylalanine, (*S*)-allylglycine, (*R*)-allylglycine, (*S*)-α-allylalanine, (*R*)-α-allylalanine and allo-O-ethylthreonine inhibited only proteinase K; and *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine and *N*-formyl-(*S*)-methionyl-(*R*)-allylglycine inhibited trypsin. It has been shown that inhibition of trypsin by (2*R*,3*S*)-β-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-hydroxyleucine is of a competitive mode.

Keywords Trypsin · Proteinase K · Nonprotein amino acids · Peptides · Inhibitors · Docking

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Introduction

Optically active nonprotein α-amino acids are relatively abundant in nature; some occur free with their function often being unknown. Because of high biological activity demonstrated by numerous nonprotein α-amino acids and peptides based thereon, there is a great variety of potential applications of these compounds. A number of nonprotein amino acids serving as drugs are constituents of polypeptides and antibiotics (Goulet 1995; Barrett 1985; Van Der Baan et al. 1983).

Serine proteases of the trypsin family (clan SA) play an important role in human physiology (Rinderknecht 1993). Serine proteases are involved in a number of pathological conditions in human and represent an important potential target for antiviral drugs (Hsu et al. 2006). Therefore, there is an extensive interest in generating specific serine protease inhibitors to be used in pharmacological interference with their enzymatic activity. The group of known serine protease inhibitors includes naturally occurring serine protease inhibitors and their synthetic analogs. They have been classified into families primarily on the basis of the disulfide bonding pattern and the sequence homology of the reactive site (Coughlin et al. 1993).

In the last decade serine protease inhibitors appeared as a class of medications used to treat or prevent viral infections. They prevent viral replication by inhibiting the activity of protease, an enzyme used by the viruses to cleave nascent proteins for final assembly of new virons. For example, Alpha-1-antitrypsin (AAT) is the most abundant circulating natural serine protease inhibitor. Physiological AAT concentrations inhibited HIV-1 production in chronically infected U1 monocytic cells, reduced virus replication in freshly infected peripheral

blood mononuclear cells, and blocked infection of permissive HeLa cells (Shapiro et al. 2001). Peptides composed of nonprotein amino acids belonging to protease inhibitors are widely used as antiviral drugs including those used in the treatment of HIV and Hepatitis C infections (Schramm et al. 1995; Steinkulher et al. 2001).

In this report we present the results of screening of various nonprotein amino acids and peptides based thereon aimed to reveal compounds inhibiting serine protease activity. Trypsin and proteinase K have been used in this work as targets for identification of serine protease inhibitors. A number of viral serine proteases have chemotrypsin-like structures, and it has been shown that some of trypsin inhibitors may also inhibite proteases of West Nile virus, Dange virus, etc. (Murthy et al. 1999; Mueller et al. 2007). We have used a structure-based drug design approach to identify small inhibitors of trypsin and proteinase K. Enzyme activity has also been determined in the presence of investigated compounds. A number of nonprotein amino acids and dipeptides have been characterized as trypsin and/or proteinase K inhibitors, and the mode of inhibition for the most active inhibitors has been determined.

Materials and methods

Materials

In this work were used the following nonprotein amino acids and peptides based thereon: *β*-imidazolyl-(*S*)-alanine, *β*-imidazolyl-(*R*)-alanine, *β*-(*S*)-izovaline, *β*-(*R*)-izovaline, *p*-fluoro-(*S*)-phenylalanine, *p*-fluoro-(*R*)-phenylalanine, (*S*)-O-methylserine, (*R*)-O-methylserine, (*S*)-α-methyl-*β*-phenylalanine, (*R*)-α-methyl-*β*-phenylalanine, (*R*)-allylglycine, (*S*)-allylglycine, (*S*)-α-allylalanine, (*R*)-α-allylalanine, allo-O-ethylthreonine, allo-O-methylthreonine, (2*R*,3*S*)-*β*-hydroxyleucine, (2*S*,3*R*)-*β*-hydroxyleucine, (*S*)-hydroxyvaline, (*R*)-hydroxyvaline, (*S*)-3-methylproline, (*S*)-*β*-(N-benzylamino)alanine, *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine, *N*-formyl-(*S*)-methionyl-(*R*)-allylglycine, t-BOC-(*S*)-alanyl-(*S*)-imidazolyl-(*S*)-alanine, t-BOC-(*S*)-alanyl-(*R*)-imidazolyl-(*S*)-alanine, t-BOC-(*S*)-alanyl-(*S*)-allylglycine, t-BOC-(*S*)-alanyl-(*R*)-allylglycine, *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-*β*-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine. All these compounds were synthesized at the Institute of Biotechnology, Armenia. Bovine Trypsin and Proteinase K from *Tritirachium album Limber*, *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) and miscellaneous reagents were purchased from Sigma (USA).

Determination of enzymes activity

Trypsin activity was determined according to the method recommended by "Millipore Corporation (Worthington 1997). One unit of trypsin hydrolyzes 1 μmol of TAME/min at 25°C, pH 8.2, in the presence of 0.01 M calcium ion.

Proteinase K activity was determined by measuring free amino groups according to *o*-phthalaldehyde (OPA) method described by Gade and Brown (1981). Reaction mixture contains 0.1 M phosphate buffer, pH 7.2, 0.2% SDS, 0.02 M mercaptoethanol, 10 mg/ml Bovine serum albumin and 0.4 mg/ml proteinase K. The aliquot (50 μl) is taken and remaining mixture is incubated for 30 min at 37°C. The reaction is stopped by addition of 6 μl of 30 % trichloroacetic acid. The concentration of free amino groups in reaction mixture is determined by OPA reagent containing 0.1 M borate buffer, pH 9.7, 0.5 mg/ml OPA and 1.25 mM mercaptoethanol. Reaction mixture (50 μl) is added to OPA reagent (1.5 ml) and H_2O (1.5 ml). A_{340} is recorded after 5 min incubation at RT.

Determination of mode of inhibition

Trypsin was incubated with various concentrations of inhibitor. Each inhibitor concentration was combined with series of substrate in the range of 0.2–1 mM. The mode of inhibition was determined by graphic method (Cornish-Bowden 1979). The dependence of $1/V - [I]$ and $[S]/V - [I]$ was determined.

Values of K_m, V_{max}, K_I were calculated from graphical data, as well as by statistical analysis, using codes written in Gauss 4.0.

Modeling

Amino acids and peptides structures were built by Chem-Office 2005 (Chem3D Ultra9.0). Ligand free energy was minimized using MM2 force field and truncated Newton–Raphson method. Crystallographic structures of proteinase K and trypsin were taken from http://www.rcsb.org website (PDB-ID'2prk, 1S0Q). Docking of ligand to enzymes has been done by AutoGrid 4, AutoDock 4 software (http://autodock.scripps.edu). AutoDock uses the Lamarckian genetic algorithm by alternating local search with selection and crossover. The ligands are ranked

using an energy-based scoring function and a grid-based protein–ligand interaction was used to speedup the score calculation (Morris et al. 1998).

Results

Selection of trypsin and proteinase K inhibitors by docking analysis (modeling)

The interaction of trypsin and proteinase K with about 30 nonprotein amino acids and peptides based thereon has been investigated by using AutoGrid 4, AutoDock 4 software aimed to select enzyme's inhibitors. ΔG and K_I values have been calculated (Table 1).

Inhibition constants (K_I) have been determined according to the following equation:

$$K_I = \exp((\Delta G \times 1,000)/(R_{cal} \times TK)),$$
$$R_{cal} = 1.98719,\ \ TK = 298.15,$$

$$R_{cal} = 1.98719\ \text{cal}/(\text{mol} \times K)(\text{gas constant}),$$
$$TK = 298.15\ \text{K}\ (\text{absolute temperature}).$$

Based on values of ΔG (free energy of binding) the compounds have been selected which are able to make complexes with enzymes. It should be mentioned that as the most suitable molecules which are able to make complexes with enzyme we consider those for which values of ΔG are <-3.14 and values of K_I are <5 mM (determined by docking analysis). Moreover, attention has been paid (in terms of inhibition) to molecules, which bind enzymes at active center. The number, position and length of hydrogen bonds in protease-inhibitor complexes have been calculated (data not shown). Calculation results suggest that the hydrogen bonds play the main role in this interaction.

According to the calculated values of ΔG (K_I) (*R*)-, (*S*)-allylglycine, (*R*)-, (*S*)-allylalanine and (*R*)-α-methyl-β-phenylalanine form the most stable complexes with proteinase K. NH_2 group of (*S*)-allylalanine forms hydrogen bonds with $-COO^-$ group of serine[132], involved in substrate binding site (Fig. 1). Hydrogen bond is formed between N-terminal nitrogen of (*R*)-α-methyl-β-phenylalanine and hydroxyl oxygen of serine[224], involved in catalytic site of proteinase K (data not shown).

According to the docking analysis, *N*-formyl-(*S*)-methionyl-(*R*)-allylglycine, *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine, (2*R*,3*S*)-β-hydroxyleucine, (2*S*,3*R*)-β-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-β-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-β-hydroxyleucine can form complexes with trypsin in catalytic or substrate binding site**.** Allo-O-methylthreonine, (*R*)-, (*S*)-α-methyl-β-phenylalanine, (*R*)-, (*S*)-β-imidazolyl-alanine can also form

Table 1 Interaction of nonprotein amino acids and peptides with proteinase K and trypsin

Compound	Proteinase K			Trypsin		
	ΔG (kcal/mol)	K_I (mM)	IC50[a] (mM)	ΔG (kcal/mol)	K_I (mM)	IC50[a] (mM)
β-imidazolyl-(*S*)-alanine	−3.55	2.520	–	−5.59	0.08041	–
β-imidazolyl-(*R*)-alanine	−3.49	2.770	–	−5.60	0.07843	–
(*S*)-O-methylserine	−3.83	1.570	–	+5.49	–	–
(*R*)-O-methylserine	−4.14	0.928	–	+6.13	–	–
(*S*)-α-methyl-β-phenylalanine	−1.71	55.740	3.97	−6.57	0.01530	–
(*R*)-α-methyl-β-phenylalanine	−3.34	3.550	3.02	−6.93	0.00826	–
(*R*)-allylglycine	−4.40	0.600	5.36	+5.80	–	–
(*S*)-allylglycine	−4.69	0.366	6.02	+5.79	–	–
(*S*)-α-allylalanine	−4.91	0.250	4.07	+5.89	–	–
(*R*)-α-allylalanine	−4.71	0.351	12.00	+5.72	–	–
allo-O-ethylthreonine	−3.55	2.510	3.86	+6.08	–	–
allo-O-methylthreonine	+7.26	–	–	−6.97	0.00777	–
N-formyl-(*S*)-methionyl-(*R*)-allylglycine	+54.24	–	–	−6.09	0.03412	6.77
N-formyl-(*S*)-methionyl-(S)-allylglycine	+45.82	–	–	−5.82	0.05433	3.07
(2*R*,3*S*)-β-hydroxyleucine	−3.43	3.060	3.43	−6.71	0.01202	1.92
(2*S*,3*R*)-β-hydroxyleucine	−3.77	1.710	3.21	−6.20	0.02868	1.19
N-formyl-(*S*)-methionyl-(2*S*,3*R*)-β-hydroxyleucine	+84.08	–	–	−10.04	0.434×10^{-4}	0.11
N-formyl-(*S*)-methionyl-(2*R*,3*S*)-β-hydroxyleucine	+101.89	–	–	−7.03	7.02×10^{-3}	0.25

ΔG and K_I values are obtained by docking of compounds. The compounds demonstrating positive ΔG values with both enzymes are not included in Table 1

[a] IC50 is determined by measuring of enzyme activity

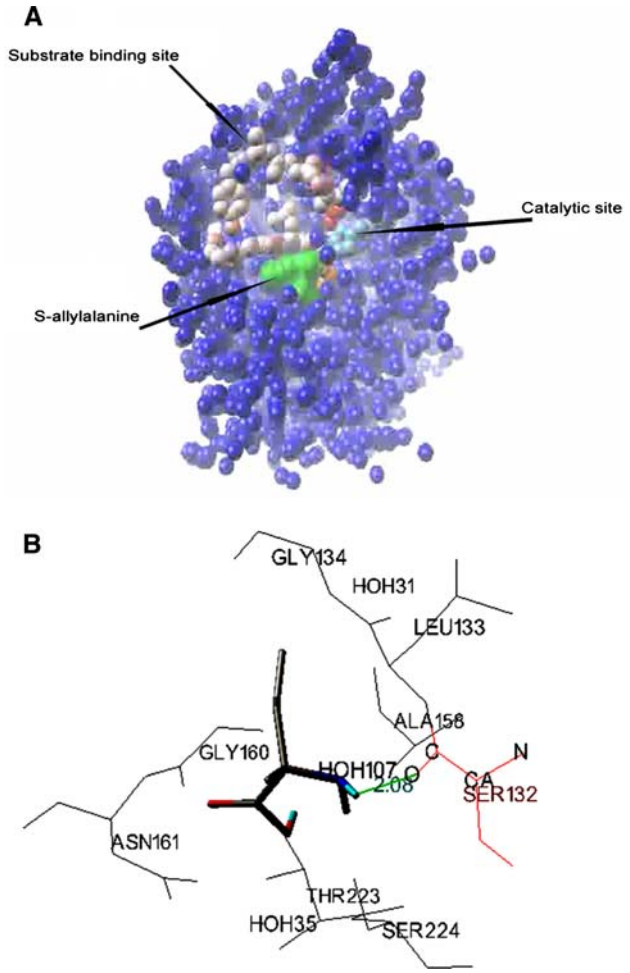

Fig. 1 The three-dimensional structure of (*S*)-allylalanine binding with proteinase K. **a** Complex of proteinase K with (*S*)-allylalanine. **b** (*S*)-Allylalanine is connected with substrate binding site ser[132]

complexes with trypsin. However, allo-O-methylthreonine forms complexes with trypsin in substrate binding site, meanwhile others bind with trypsin in sites out of active center (Table 1). The model of interaction of (2*R*,3*S*)-*β*-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine with trypsin is shown in Fig. 2. $-NH_2$ and –OH groups of (2*R*,3*S*)-*β*-hydroxyleucine form hydrogen bonds with trypsin asp[189], involved in substrate binding site of trypsin. In complex of *N*-formyl-(S)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine with trypsine hydrogen bond is formed between N-terminal nitrogen of peptide and serine[195], which along with aspartic acid[102] and histidine [57] constitutes catalytic site of trypsin (Stroud et al. 1974). In this complex peptide also forms hydrogen bond with gln[192], which is involved in the pocket of substrate binding site (Fig. 2).

The effect of nonprotein amino acids and peptides based thereon on activity of trypsin and proteinase K

The activities of proteinase K and trypsin have been determined in the presence of 30 amino acids and peptides (Materials) in concentration 5 mM. Compounds have been identified with no effect on activity of both enzymes, those that inhibited proteinase K activity and those that inhibited trypsin activity. *β*-imidazolyl-(*S*)-alanine, *β*-imidazolyl-(*R*)-alanine have no inhibition effect on both enzymes activity since these compounds have shown interaction with proteinase K and trypsin (Table 1). The same is for the influence of (*R*)-, (*S*)-O-methylserine on proteinase K. (*R*)-, (*S*)-α-methyl-*β*-phenylalanine, (*R*)- (*S*)-*β*-imidazolyl-alanine and allo-O-methylthreonine interact with trypsin but demonstrate no significant inhibition on enzyme activity. Proteinase K has been inhibited by (*R*)-, (*S*)-allylglycine, (*R*)-, (*S*)-α-allylalanine, (*R*)-, (*S*)-α-methyl-*β*-phenylalanine and allo-O-ethylthreonine, and trypsin has been inhibited by *N*-formyl-(*S*)-methionyl-(*R*)-allylglycine, *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine, *N*-formyl-(*S*)-methionyl-(*2S*,*3R*)-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(*2R*,*3S*)-hydroxyleucine. Only (*2R*,*3S*)-hydroxyleucine and (*2S*,*3R*)-hydroxyleucine have inhibited activity of both enzymes. IC50 for potential inhibitors has been determined as well (Table 1).

Determination of the mode of inhibition of trypsin by (2*R*,3*S*)-*β*-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-hydroxyleucine

The mode of inhibition was determined by measuring the rate of the reaction at various substrate concentrations and various (2*R*,3*S*)-*β*-hydroxyleucine/*N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine concentrations. In analysis of inhibition kinetics we routinely used plots of $1/V$ versus inhibitor concentration and plots of $[S]/V$ versus inhibitor concentration (Fig. 3).

According to statistical calculations, inhibition of trypsin by the mentioned compounds is of a competitive mode. This is proved by graphical data, as $1/V - [I]$ curves are crossed in left quadrant (Fig. 3a, b) and $[S]/V - [I]$ are parallel lines (Fig. 3c, d). $K_I = 0.842 \pm 0.203$ mM for (2*R*,3*S*)-*β*-hydroxyleucine, and $K_I = 0.312 \pm 0.085$ mM for *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine, correspondingly.

Discussion

A major approach to design drugs that are able to block proteases has focused on synthetic peptide analogs which mimic the transition state of catalysis.

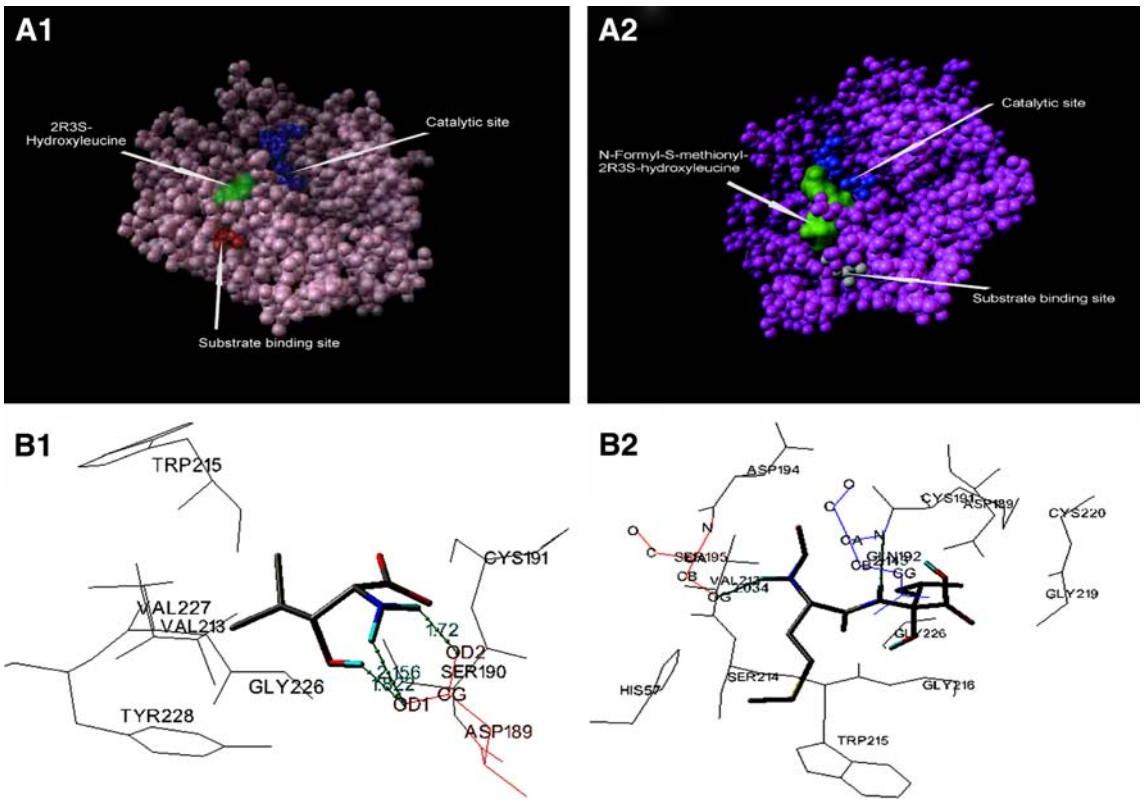

Fig. 2 The three-dimensional structure of (2*R*,3*S*)-*β*-hydroxyleucine (*A1*, *B1*) and *N*-formyl-*S*-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine (*A2*, *B2*) bonded with trypsin. *A1* Complex of trypsin with (2*R*,3*S*)-*β*-hydroxyleucine. *B1* (2*R*,3*S*)-*β*-hydroxyleucine bonded with trypsin in substrate binding site asp[189]. *A2* Complex of trypsin with *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine. *B2* *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine bonded with trypsin

The ability of 30 nonprotein amino acids and peptides based thereon to interact with serine proteases, trypsin and proteinase K, has been studied by computer programs and by determination of enzyme activity. The values of calculated ΔG and K_I suggest that several compounds can be considered as proteinase K inhibitors and as inhibitors of trypsin (Table 1). The further investigation of the effect of those compounds on enzyme activity has revealed that proteinase K activity was significantly inhibited by (*S*)-, (*R*)-α-methyl-*β*-phenylalanine, (*S*)-, (*R*)-allylglycine, (*S*)-, (*R*)-α-allylalanine, allo-O-ethylthreonine and (2*S*,3*R*)-, (2*R*,3*S*)-*β*-hydroxyleucine. It is interesting that these compounds bind proteinase K in catalytic site or substrate binding site. Meanwhile, the molecules that had no effect on enzyme activity do not bind proteinase K in active center according to the docking analysis. (*R*)-α-methyl-*β*-phenylalanine, (2*S*,3*R*)-, (2*R*,3*S*)-*β*-hydroxyleucine, allo-O-ethylthreonine have shown lowest IC50 values in reactions with proteinase K compared with other tested compounds. (*R*)-α-methyl-*β*-phenylalanine formed hydrogen bond with catalytic ser[224] and demonstrated IC50 = 3.0 mM. (*S*)-, (*R*)-allylglycine, (*S*)-, (*R*)-α-allylalanine, (2*S*,3*R*)- and (2*R*,3*S*)-*β*-hydroxyleucine inhibited proteinase K but included in dipeptides they lost inhibitory features. (*S*)-Allylalanine formed hydrogen bonds with serine[132] (Fig. 1). Serine[132] was included in substrate binding site of proteinase K along with glycine[100], threonine[104] and glycine[136] (Miller et al. 1994). Thus, the docking analysis has suggested that (*S*)-allylalanine interacts with proteinase K in the substrate binding site and (*R*)-α-methyl-*β*-phenylalanine interacts with the catalytic site. It is worth to note that in many cases the order of dissociation constants magnitude calculated by the docking program is of the same order that is with the values of IC50 measured experimentally (Table 1).

N-formyl-(*S*)-methionyl-(*R*)-allylglycine, *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine, (2*R*,3*S*)-*β*-hydroxyleucine, (2*S*,3*R*)-*β*-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-*β*-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine proved to be the most effective inhibitors of trypsin as compared with tested compounds. The known inhibitors of trypsin form hydrogen bonds mainly with aspartic acid[189] involved in the substrate binding site of enzyme (Zablotna et al. 2006). According to the results of the present work, (2*R*,3*S*)-*β*-hydroxyleucine formed hydrogen bonds with trypsin asp[189] involved in the

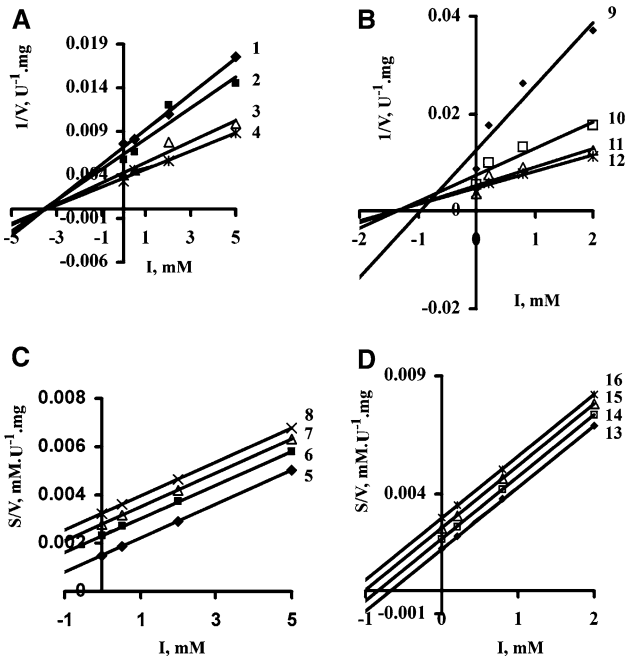

Fig. 3 Inhibition kinetics of trypsin by (2*R*,3*S*)-*β*-hydroxyleucine (**a**, **c**) and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine (**b**, **d**). **a** 1/*V* dependence on (2*R*,3*S*)-*β*-hydroxyleucine concentration (inhibitor) for different substrate concentrations: *1* 0.2 mM, *2* 0.4 mM, *3* 0.7 mM, *4* 1 mM. **b** 1/*V* dependence on *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine concentration (inhibitor) for different substrate concentrations: *9* 0.2 mM, *10* 0.4 mM, *11* 0.7 mM, *12* 1 mM. **c** (*S*/*V*) dependence on (2*R*,3*S*)-*β*-hydroxyleucine (inhibitor) for different substrate concentrations: *5* 0.2 mM, *6* 0.4 mM, *7* 0.7 mM, *8* 1 mM. **d** (*S*/*V*) dependence on *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine (inhibitor) for different substrate concentrations: *13* 0.2 mM, *14* 0.4 mM, *15* 0.7 mM, *16* 1 mM

substrate binding site of trypsin, while *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine interacted with trypsin in the catalytic site. It is interesting that the inhibitory effect of (2*R*,3*S*)-, (2*S*,3*R*)-*β*-hydroxyleucine was enhanced when these compounds were included in dipeptides *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-hydroxyleucine. Studies of inhibition kinetics have revealed that (2*R*,3*S*)-*β*-hydroxyleucine and *N*-formyl-(*S*)-methionyl-(2*R*,3*S*)-*β*-hydroxyleucine are competitive trypsin inhibitors.

(2*R*,3*S*)-, (2*S*,3*R*)-*β*-hydroxyleucine inhibit both trypsin and proteinase K activities, which may indicate specificity of these compounds in respect of serine proteases. The rest proteinase K inhibiting compounds demonstrate specificity as compared with trypsin. *N*-formyl-(*S*)-methionyl-(2*R*, 3*S*)-*β*-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(2*S*,3*R*)-*β*-hydroxyleucine, *N*-formyl-(*S*)-methionyl-(*R*)-allylglycine and *N*-formyl-(*S*)-methionyl-(*S*)-allylglycine have demonstrated strong specificity in respect of trypsin and can be considered as novel trypsin inhibitors.

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