Amino Acids (2012) 43:2015–2025
DOI 10.1007/s00726-012-1276-0

ORIGINAL ARTICLE

# Design of a highly potent inhibitory peptide acting as a competitive inhibitor of HMG-CoA reductase

**Valeriy V. Pak · Minseon Koo · Dae Young Kwon · Lyubov Yun**

Received: 25 January 2012 / Accepted: 14 March 2012 / Published online: 27 March 2012


**Abstract** This study presents a design of a highly potent and competitive inhibitory peptide for 3-hydroxy-3-methylglutaryl CoA reductase (HMGR). HMGR is the major regulatory enzyme of cholesterol biosynthesis and the target enzyme of many investigations aimed at lowering the rate of cholesterol biosynthesis. In previous studies, the two hypocholesterolemic peptides (LPYP and IAVPGEVA) were isolated and identified from soy protein. Based on these peptide sequences, a number of peptides were designed previously by using the correlation between the conformational flexibility and bioactivity. The design method that was applied in previous studies was slightly modified for the purpose of the current research and 12 new peptides were designed and synthesized. Among all peptides, SFGYVAE showed the highest ability to inhibit HMGR. A kinetic analysis revealed that this peptide is a competitive inhibitor of HMG-CoA with an equilibrium constant of inhibitor binding ($K_i$) of 12 ± 0.4 nM. This is an overall 14,500-fold increase in inhibitory activity compared to the first isolated LPYP peptide from soybeans. Conformational data support a conformation of the designed peptides close to the bioactive conformation of the previously synthesized active peptides.

V. V. Pak (✉) · L. Yun
Department of Organic Synthesis,
Institute of the Chemistry of Plant Substances,
Building 77, Acad. Kh. Abdullaev Str.,
100170 Tashkent, Uzbekistan
e-mail: pakvaleriy@yahoo.com

M. Koo · D. Y. Kwon
Food Fusion and Complex Research Division,
Korea Food Research Institute, Baekhyun-Dong,
Bundang-Ku, Songnam-Si, Kyongki-Do 463-746,
Republic of Korea

**Keywords** Peptides · Design · Competitive inhibitor · HMG-CoA reductase · $\beta$-Turn conformation · Circular dichroism

## Introduction

3-Hydroxy-3-methylglutaryl CoA reductase (HMGR) is the major regulatory enzyme of cholesterol biosynthesis and therefore constitutes the target enzyme of many investigations aimed at lowering the rate of cholesterol biosynthesis (Endo 1992). The efficacy of cholesterol biosynthesis inhibitors for the control of blood cholesterol levels is now well recognized. Their importance is linked with reducing the risk of hypercholesterolemia, which is known as a major risk factor associated with various cardiovascular diseases (Rader 2003; Deedwania et al. 2009).

In previous studies, the two hypocholesterolemic peptides (LPYP and IAVPGEVA) were found by analyzing a digested soy glycinin by using trypsin and pepsin (respectively) (Kwon et al. 2002; Pak et al. 2005a). An alignment of the amino acid composition of soy 11S-globulin with the IAVPGEVA sequence revealed another IAVPTGVA peptide with inhibitory activity against HMGR (Pak et al. 2005b). Kinetic experiments elucidated a competitive inhibition of these peptides for HMGR. A structural analysis showed that a bioactive conformation is a "turn" structure, which includes proline residue as a conformational constraint in the recognized motif (Pak et al. 2004).

The effects of conformation constraints on the binding affinity of flexible molecules have been already examined in many studies (Becker 1997; Beker et al. 2000; Payne et al. 2002). The design of competitive inhibitory peptides for HMGR with a constrained structure based on the

recognized VPTG sequence has previously been described (Pak et al. 2007). For these peptides, an active peptide conformation was identified as a type II of a $\beta$-turn structure.

In the next stage, the design approach for peptides, including the recognized motif as an unconstrained structure, was developed (Pak et al. 2008). The FGYVAE, FPYVAE and FFYVAE peptides were designed by using the YVAE sequence as a basis of the recognized residues for HMGR. The YVAE peptide was selected because of its strong ability to inhibit HMGR in a competitive manner among previously designed peptides (Pak et al. 2006). A conformational analysis of YVAE-derived peptides revealed a presence of a $\beta$-turn structure located in the N-terminus of these peptides (Pak et al. 2008).

As was shown in previous studies, a $\beta$-turn conformation was found to be an important structural element for binding (Pak et al. 2007, 2008). The design of the turn structure proposed an opportunity to model active peptides with a focus on their conformational preferences. Based on the previously isolated and designed peptides, this opportunity in designing peptides is discussed in the current study.

The correlation between the conformational flexibility and bioactivity was applied in the previous designs (Pak et al. 2007, 2008). A principle component analysis (PCA), which projects multidimensional data on low-dimensional subspace, was used to evaluate a head-to-tail peptide cycle as a model of linear analog in order to select a lead peptide candidate (Becker 1997). A 6-, 8- and 10-membered cyclic peptides were used as models of linear analogs while searching for less flexible sequences (Pak et al. 2007, 2008). An analysis of a conformational space revealed a direct relation between the developed descriptor of the conformational behavior of the cyclic peptides and the volumes occupied in a conformational space by linear analogs (Pak et al. 2007). The conformational behavior of the cyclic peptides showed that the 6-membered cyclic peptide during molecular dynamics (MD) simulation was relatively stable compared to the 8-, and 10-membered cyclic peptides (Pak et al. 2007). Taking into account all considerations, it was proposed that a less flexible site in peptides could be found by analyzing the conformational space that was occupied by the peptide fragments by using a 6-membered cyclic peptide as a model.

In the current design, two binding site points were used to model the active peptides. The first was a binding of the models of the designed peptides inside the "region bioactivity" through a common point/site in the previously and newly designed peptides. The second was a relative assessment of the "region bioactivity", prescribed by the host molecule through an analysis of the bioactive structures of the previously designed peptides. A structure–functional analysis of the previously designed peptides revealed the importance of a "turn" structure for binding. In the present design, a turn conformation was used to assess "region bioactivity".

The peptide fragmentation was applied in order to select a less flexible site in peptide and to estimate the "region bioactivity". Using a minimum length that is required to describe a $\beta$-turn structure, each peptide was divided by a fragment of the four residues (Table 1) (Voet and Voet 1990). Based on the four residues, the peptide model performed a cyclic hexapeptide by adding two glycine residues to the N- and C-terminuses as a site for cyclization.

The experimentally determined coordinates and thermal parameters of cyclic hexaglycine peptide revealed that this ring consists of two $\beta$-turns that are stabilized by strong hydrogen bonds of the type $1 \leftarrow 4$. These hydrogen bonds connect NH and CO groups of glycine residues at positions of 1 and 4 according to the numbering of a $\beta$-turn structure (Karle and Karle 1963). In order to assess the conformational space occupied by peptides in terms of a space occupied by a "turn" structure, a cyclic hexaglycine peptide was used as a basis for estimation. In accordance with that, the design parameter $V$, which reflects an occupied volume in conformation space by an individual peptide adduced to the conformation space occupied by all the peptide candidates from a library, was slightly modified for the purpose of the current research (Pak et al. 2007). Based on the procedure applied for the $V$ parameter, the occupied volumes by the peptide models were estimated (Pak et al. 2007, 2008). The biological activity and conformational preference of the designed peptides were assayed through the use of an in vitro test and a circular dichroism (CD) study.

## Materials and methods

### Materials

H-Gly-2-ClTrt resin (substituted at 0.5 meq/g), HGlu (Ot-Bu)-2-ClTrt resin (substituted at 0.55 meq/g), and Fmoc-amino acids were purchased from AnaSpec (San Jose, CA, USA). Chemicals for the peptide synthesis were obtained from Perkin-Elmer (Foster, CA, USA). Acetonitrile and methanol for HPLC were the products of Burdict and Jackson (Muskegon, MI, USA).

### Peptides synthesis

Using standard Fmoc methodology, the solid phase synthesis of peptides was carried out on an automated Applied Biosystem Peptide Synthesizer (Model 433A, Perkin-Elmer, Foster, CA, USA) (Fields et al. 1991). Deprotection

**Table 1** Peptide sequence, peptide fragment and inhibitory activities ($IC_{50}$) of the synthesized peptides

| Peptide sequence | Peptide fragment | | | | | $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Control[a] | | | | | | |
| LPYP | LPYP | | | | | 484 |
| IAVPGEVA | IAVP | AVPE | VPEG | PGEV | GEVA | 201 |
| IAVPTGVA | IAVP | AVPT | VPTC | PTGV | TGVA | 152 |
| Previously designed peptides[b] | | | | | | |
| IAVE | | | IAVE | | | 75 |
| IVAE | | | IVAE | | | 52 |
| YAVE | | | YAVE | | | 44 |
| YVAE | | | YVAE | | | 41 |
| FFYVAE | FFYV | FYVA | YVAE | | | 2.5 |
| FPYVAE | FPYV | PYVA | YVAE | | | 1.4 |
| FGYVAE | FGYV | GYVA | YVAE | | | 0.4 |
| New designed peptides | | | | | | |
| FGXVAE[c] | | FGXV[c] | GXVA[c] | XVAE[c] | | 8.5 |
| EFGYVAE | EFGY | FGYV | GYVA | YVAE | | 0.24 |
| DFGYVAE | DFGY | FGYV | GYVA | YVAE | | 0.16 |
| TFGYVAE | TFGY | FGYV | GYVA | YVAE | | 0.26 |
| SFGYVAE | SFGY | FGYV | GYVA | YVAE | | 0.033 |
| GFGYVAE | GFGY | FGYV | GYVA | YVAE | | 0.27 |
| AFGYVAE | AFGY | FGYV | GYVA | YVAE | | 0.49 |
| VFGYVAE | VFGY | FGYV | GYVA | YVAE | | 0.45 |
| LFGYVAE | LFGY | FGYV | GYVA | YVAE | | 0.37 |
| IFGYVAE | IFGY | FGYV | GYVA | YVAE | | 0.35 |
| PFGYVAE | PFGY | FGYV | GYVA | YVAE | | 0.43 |
| FFGYVAE | FFGY | FGYV | GYVA | YVAE | | 0.32 |
| SFGYVAG | Negative control | | | | | Inactive |

[a] "Control" contains the peptides isolated from soy protein (Kwon et al. 2002; Pak et al. 2005a, b)

[b] "Designed peptides" contains the peptides, where the E residue is a recognized residue for the HMG-binding pocket, and a $\beta$-turn structure is located in the N-terminus of the hexapeptides (Pak et al. 2008)

[c] The substituted (4-fluoro)phenylalanine residue is indicated as X

and cleavage were achieved by treatment with a mild trifluoroacetic acid (TFA) (Perkin 1995). Then peptides were precipitated in ether and then filtered and dried under a vacuum. Purification and analysis of the synthetic peptides were done by using a reversed-phase high pressure liquid chromatography (RP-HPLC) system (Waters, Milford, MA, USA). Synthetic peptides were analyzed by using a Vydac 218TP54 analytical column under the following gradient conditions: solvent A, 0.1 % TFA in water, solvent B, 0.1 % TFA in acetonitrile; initial condition 95 % A, 25 min, final condition 65 % A; flow rate, 1 mL/min. The purity of the synthetic peptides after purification was found to be above 99 % by using a Vydac 218TP510 semi-preparative C18 column. Peptides were identified by an electrospray mass spectrometer (Platform II, Micromass, Manchester, UK) and an Applied Biosystems 491 Peptide Sequencer (Perkin-Elmer, Foster, CA, USA).

## Assay of HMG-CoA reductase activity

The HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm in a Jasco V-530 spectrophotometer (Model TUDC 1284, Japan Serco Co., Ltd, Japan). The assay conditions were as described in a previous study (Pak et al. 2006). One unit (U) of HMGR was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute. Protein concentration was determined by the method of Bradford (Bradford 1976). The type of inhibition and Michaelis–Menten parameters were determined from a Dixon plot (Segel 1976).

### Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a Jasco J-710 spectropolarimeter (JASCO International Co., Tokyo, Japan). Measurements were taken by using a quartz cuvette with a path length of 1 mm. Single scans in a range between 190 and 250 nm were obtained at 18 °C with the following settings: a sensitivity of 10 millidegrees, a 0.5 s response time, a bandwidth of 1 nm, and a speed of 50 nm/min (Johnson 1990). Three scans were collected for each sample. Each spectrum was corrected for sample cell and solvent contributions. All subsequent CD spectral values for peptides are expressed in units of molar residue ellipticity (deg $cm^2$ $dmol^{-1}$).

### Computation methods

The structures of the peptides were built by using the program package ChemOffice Desktop 2004 for Windows [CambridgeSoft (CS) Corporation, MA, USA]. The structures of the cyclic peptides used in the design of the peptide sequences were carried out by a molecular mechanics method (MM2) within Chem3D (Burkert and Allinger 1982). The calculations of the optimized structures for peptide models were carried out using the AM1 method within the CS MOPAC (Version 1.11) program package (Dewar et al. 1985). Quantum-chemical structures were optimized by gradient minimization to 0.001 kcal/mol Å.

Molecular dynamics (MD) simulation was used for estimating of the peptide backbone behavior. This was performed by using ChemOffice Desktop 2004 for Windows (Kollman 1996). The peptide structures were collected during simulation time of 300 ns in vacuum and were heated to 300 K.

The calculations of the design parameter $V_{fr}$ was slightly changed compared to that described in a previous study (Pak et al. 2007). The difference was that the occupied volume in conformation space by a peptide model was adduced to the conformation space occupied by the cyclic hexaglycine peptide instead of the conformation space occupied by all of the peptide candidates from a library as it was used in the previous design (Pak et al. 2007). Then, the calculated $V_{fr}$ parameter for each fragment in peptide was used to estimate a full peptide length via the design parameter $V_{add}$. It was calculated as an equidistributed estimate based on the means of the parameter $V_{fr}$ obtained for each fragment in peptide. For each cyclic model, 100 geometry-optimized low-energy and MD conformers were collected by using a slightly modified method than the one in study (Kalazsi and Farkas 2003). The collection was based on differentiating the conformers by using the deviations of their internal coordinates.

## Results and discussion

### Peptide design

For testing the proposed design, 10 peptides that were described in previous studies were used (Table 1) (Pak et al. 2006, 2007, 2008). The previously designed peptides included peptides, in which E residue was recognized residue for HMG-binding pocket. A conformational analysis of the IAVE, IVAE, YAVE and YVAE peptides revealed no observable patterns that were related to a well-populated secondary structure conformation (Pak et al. 2006). For the FFYVAE, FPYVAE, and FGYVAE peptides, a turn conformation was determined at the N-terminus of these peptides. All of these peptides inhibited HMGR in a competitive manner (Pak et al. 2008).

In previous studies, the structures of statin molecules that were extracted from the crystal structures of HMGR–statin complexes [PDB codes: 1HW8 (compactin), 1HW9 (simvastatin), 1HWI (fluvastatin), 1HWJ (cerivastatin), 1HWK (atorvastatin), and 1HWL (rosuvastatin)] were used. The superposition of the bioactive conformations of statins and peptides were constructed by considering that the HMG moiety of statins and a side-chain of the recognized residues occupied the same narrow pocket in the active site, which is a common feature in statin-binding by HMGR. The analysis of the superposition of statins and peptide molecules showed a similar location of the isobutyl (compactin and simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) with the side-chains of I (IAVE, IVAE) and Y (YAVE, YVAE) residues, respectively (Pak et al. 2006). The YVAE peptide was designed with an orientation of the side-chain of Y residue being close to the location of the 4-fluorophenyl radical of statins (Pak et al. 2006). The FFYVAE, FPYVAE and FGYVAE peptides were designed with a focus on the structural diversity of the rigid hydrophobic groups of statin molecules. The N-terminus of these peptides presents a relatively rigid structure of a peptide backbone with side-chains, which conform to the hydrophobic radicals of statins (Pak et al. 2008). Figure 1 shows the superposition of atorvastatin with rosuvastatin and the linear FGYVAE peptide.

The analysis of the crystal structures of the catalytic domain of human HMGR complexed with statins showed additional binding interactions between fluorophenyl groups and the HMGR for fluvastatin, cerivastatin, atorvastatin, and rosuvastatin compared to compactin and simvastatin. In addition to these interactions, rosuvastatin and atorvastatin exhibited hydrogen bonds with the enzyme, which involved a sulfone oxygen atom in the case

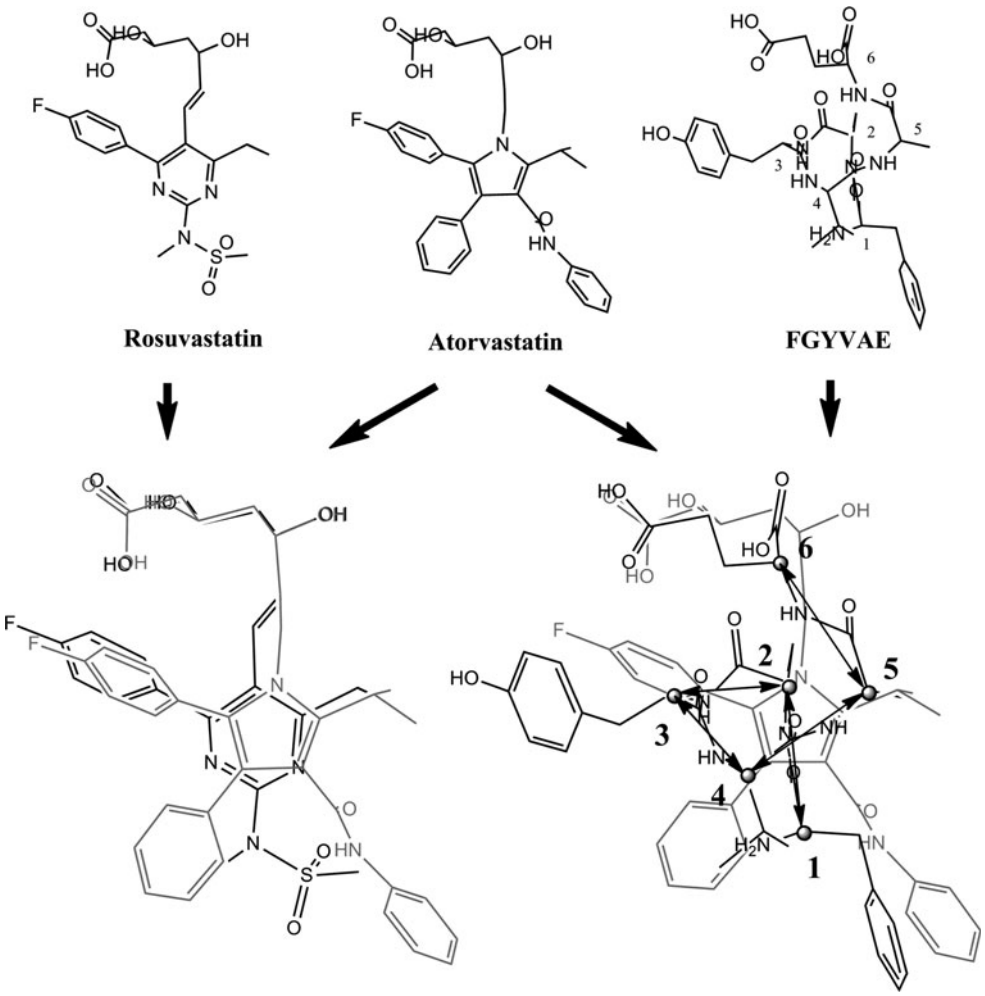


**Fig. 1** Spatial compatibility of atorvastatin with rosuvastatin and the linear FGYVAE peptide. The alpha-carbon atoms are indicated by marks from 1′ to 6′. The model of FGYVAE was built as type I of the $\beta$-turn on the basis of the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues with values of $\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$ and $\psi_{i+2} = 0°$, and the fixed dihedral angles obtained for the active backbone of the YVAE peptide and the optimized orientation of side-chains (Pak et al. 2008)

of rosuvastatin and a carbonyl oxygen atom in the case of atorvastatin (Fig. 1) (Istvan 2003).

The FGYVAE peptide presented the most active peptide among previously designed peptides. After proposing an increase of the binding affinity due to an additional contribution of fluorophenyl group in binding, the side-chain Y residue for the FGYVAE peptides was replaced by a 4-fluorophenyl radical. In order to model an interaction between the oxygen atom of sulfone and carbonyl groups of rosuvastatin and atorvastatin, respectively, and the enzyme, SFGYAVE, TFGYVAE, DFGYVAE and EFGYVAE peptides were designed by using the FGYVAE peptide as a basic sequence. In order to estimate an effect of the hydrophobic interaction of the N-terminus of the designed peptides, AFGYVAE, VFGYVAE, PFGYVAE, LFGYVAE, IFGYVAE and FFGYVAE peptides were added to peptide library. In order to assess the functionality of the side-chains of S, T, D and E residues in binding, the GFGYVAE peptide was additionally used in testing. The LPYP, IAVPGEVA and IAVPTGVA peptides that were isolated from soy protein were included as a control compounds.

Figure 2 presents all of the selected peptides in accordance with the parameter $V_{fr}$ for each peptide fragment in the peptide. The parameter $V_{fr}$ reflects a relative alteration of the occupied volume in conformation space by a model of the peptide fragment relative to the conformation space occupied by the cyclic hexaglycine peptide which was selected as a model of a $\beta$-turn structure. In order to assess the parameter $V_{fr}$, the 100 lowest energy conformers were used as the starting structures in the MD study for each peptide fragment. The comparison of the calculated values of the $V_{fr}$ and the standard deviations showed that a relatively high value of the $V_{fr}$ and the standard deviations belong to the peptide fragments that had a relatively high degree of flexibility, as in the case of IAVE, IVAE, YAVE and YVAE peptides. This was confirmed by a conformational analysis of these peptides, in which a well-populated secondary structure was not determined (Pak et al. 2006). The comparable values of $V_{fr}$ and the small standard

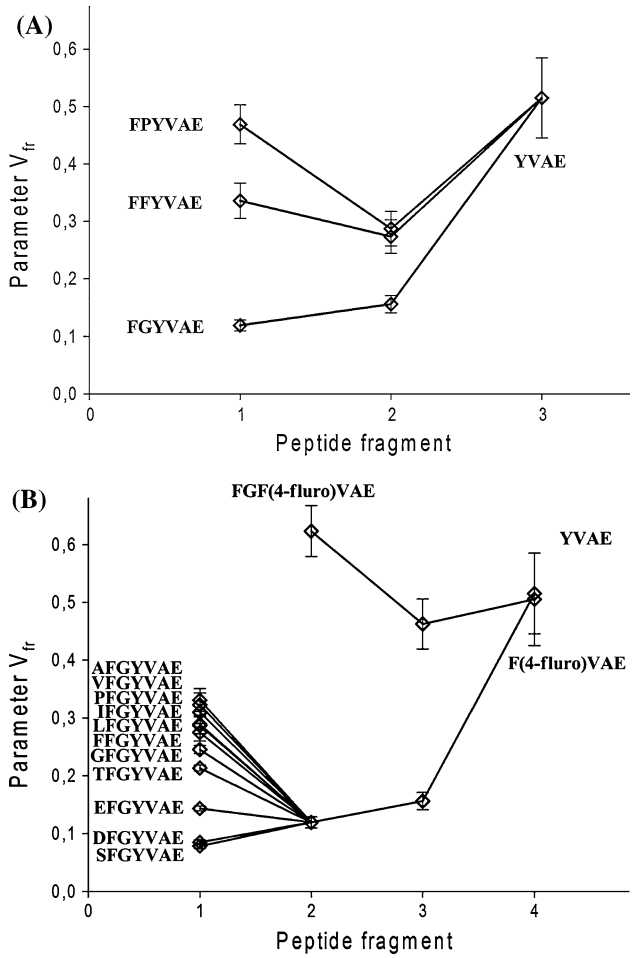


**Fig. 2** The calculated parameter $V_{fr}$ and standard deviation for each peptide fragment. The number of the peptide fragments started from the N-terminus of peptide based on the peptide length of the four amino acid residues (Table 1). **a** The previously designed peptides, in which the E residue is a recognized residue for the HMG-binding pocket and a $\beta$-turn structure is located in the N-terminus of the hexapeptides. **b** The peptides were designed by substituting 4-fluorophenyl radical instead of the 4-hydroxyphenyl radical of Y residue, or by adding the F, L, I, P, V, A, G, S, T, D or E residue to the N-terminus of FGYVAE peptide

deviations that were observed in Fragment 1 for the FPYVAE peptide, proposed a lower flexibility of these models compared to the IAVE, IVAE, YAVE and YVAE peptides. The other peptide fragments exhibited relatively low values both in parameter $V_{fr}$ and the standard deviations, indicating a restricted flexibility that can be interpreted in terms of a similarity in the conformational behavior that was observed for turn conformation (Fig. 2a). This is in agreement with the types I and II of turn conformation that was observed in N-terminus for FPYVAE, FGYVAE, and FFYVAE peptides (Pak et al. 2008). This finding proposes that the peptide fragmentation can be applied to a description of a structural feature of the peptide.

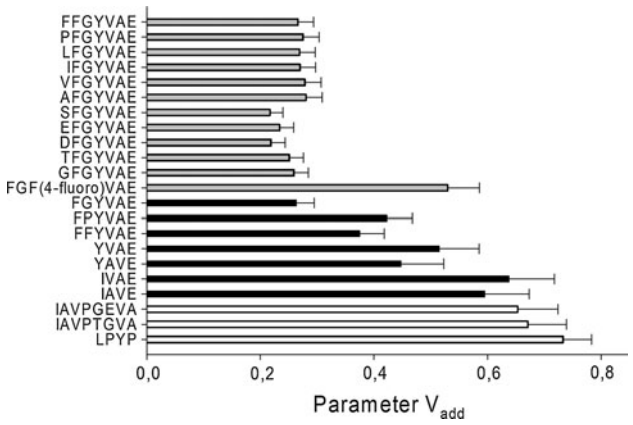


**Fig. 3** The calculated parameter $V_{add}$ and the standard deviation for the peptide models. The peptides, in which the E residue is a recognized residue for the HMG-binding pocket, and a $\beta$-turn structure is located in the N-terminus of the hexapeptides. The control, previously and newly designed peptides are marked as a *white*, *black* and *grey*, respectively

The same procedure was used for the designed peptides (Fig. 2b). According to the calculated data, significant changes were observed in the peptide fragments with the introduction of the 4-fluorophenyl group. This implies an alteration of the peptide flexibility and, consequently, the peptide's affinity compared to the basic peptide. For the FGF(4-fluoro)VAE peptide, an increase of the peptide flexibility at the first two fragments can significantly change the peptide's affinity. Decreasing the values of $V_{fr}$ for GFGY, SFGY, TFGY, DFGY and EFGY fragments proposes a decrease in the flexibility in the N-terminus of these peptides and possibly an increase in the peptide's activity (Fig. 2b). On the other hand, increasing the values of $V_{fr}$ for AFGY, VFGY, PFGY, LFGY, IFGY and FFGY proposes a decrease in the peptide affinity.

In order to select a lead peptide candidate, a parameter $V_{add}$ was calculated as an equidistributed estimate using the means of the parameter $V_{fr}$ obtained for each fragment in peptide. The results of the conformational searches that involved all peptides are shown in Fig. 3. According to the $V_{add}$ value, the SFGYVAE sequence is the most rigid structures compared to the other peptides. This finding proposes that the peptide with SFGYVAE amino acid sequences can be selected as a lead peptide candidate.

Thus, all of the discussed peptides were synthesized and their biological activities were estimated. The LPYP, IAVPGEVA and IAVPTGVA peptides that were isolated from soy protein were used as a control compound. In the SFGYVAG peptide, the recognized glutamic residue was substituted with a glycine residue in order to provide a negative control for the binding study.

## Inhibition activities of designed peptides

An assay of the in vitro susceptibility of HMGR to inhibition by the synthetic peptides was performed in a range of peptide concentrations from 0.01 to 1,000 μM. Each of the synthesized peptides showed some ability to inhibit HMGR, with the exception of the SFGYVAG peptides. No activity was detectable for these peptides when an upper boundary of the concentration range was used (Table 1). This finding supports the previously obtained data that showed that the active site of HMGR recognizes the glutamic residue as an essential component for the HMG-biding pocket (Pak et al. 2006, 2007, 2008).

The most active peptide was found as the SFGYVAE peptide, which was selected as a lead compound. A decrease in the inhibitory activity for the FGF(4-fluoro)VAE compared to the FGYVAE peptide can be explained by the structural preference described through the values of $V_{fr}$. A significant change was observed in the N-terminus when the 4-fluorophenyl group was introduced. A decrease in the binding affinity possibly occurred due to the altered location of the N-terminus in the binding site compared to that of the FGYVAE peptide. The introduction of the hydrophobic residues in the N-terminus did not give an improvement in the peptide activity.

The EFGYVAE, TFGYVAE and GFGYVAE showed an increase in the inhibitory activity compared to the FGYVAE peptide. The close values of $IC_{50}$ that were observed for EFGYVAE, TFGYVAE and GFGYVAE peptides propose the contribution of the peptide backbone during binding. A slightly high inhibitory activity was observed for the DFGYVAE compared to the EFGYVAE, TFGYVAE and GFGYVAE peptides, which probably reflects a more correct location of the carbonyl group of the D side-chain relatively of the peptide backbone. A 12-fold increase of the value of $IC_{50}$ that was observed for the SFGYVAE compared to the FGYVAE peptides indicates the contribution of hydroxyl oxygen atom of the side-chain of S residue during binding.

In order to estimate the proposed approach, the dependence between the observed and predicted peptide potency was plotted by using the values of the $V_{add}$ parameters for the peptide library (Fig. 4). A relatively good correlation ($r^2 = 0.92$) was determined between the experimental bioactivity of the linear peptides, measured by log ($IC_{50}$), and the predicted bioactivity that was obtained through the $V_{add}$ parameter (Fig. 4). The correlation coefficient is good enough in spite of the fact that a different length of peptides was used for these peptides.

The difference between the obtained values of the peptide bioactivities was checked with the help of the Fisher criterion (Fisher 1935). An insignificant difference between these values was determined by using the Fisher criterion ($F_{exp} < F_{(0.05,\ f1,\ f2)}$). This finding suggests that the proposed approach is acceptable in the design of linear peptides.

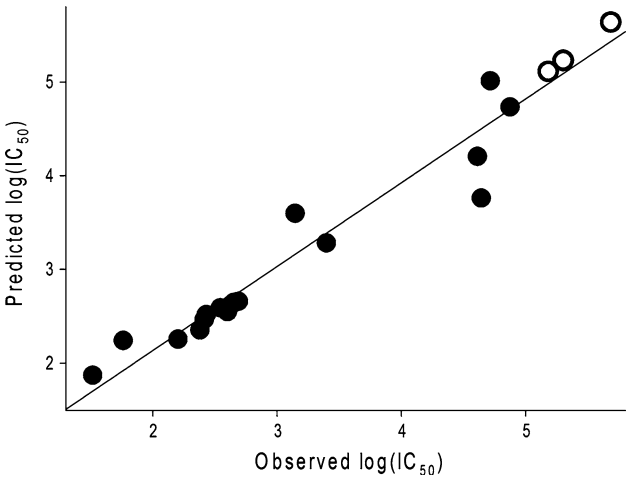


**Fig. 4** The correlation between the observed peptide potency and the predicted potency against HMGR for the designed and control peptides (LPYP, IAVPTGVA and IAVPGEVA). The correlation coefficient ($r^2$) is 0.92. The designed peptides are marked as *filled circles*; the control peptides are marked as *open circles*

Statins are potent inhibitors of HMGR and function in a competitive manner with respect to HMG-CoA (Istvan 2003). In order to clarify the properties of HMGR inhibition by the designed peptides, the enzyme activity was measured with HMGR in the absence and in the presence of SFGYVAE, as this peptide was the most potent inhibitor. A Dixon plot shows that the SFGYVAE peptide inhibited HGMR in a competitive manner with respect to HMG-CoA (Fig. 5). The equilibrium constant of inhibitor binding ($K_i$) for the inhibition of HMGR by SFGYVAE was estimated to be 12 ± 0.4 nM.

## Circular dichroism study and modeling

The structure of the SFGYVAE peptide as the most potent inhibitor was investigated by CD spectroscopy in water and in trifluoroethanol (TFE). The spectrum of the SFGYVAE peptide in water is shown in Fig. 6a. According to the spectrum profile observed, it is difficult to interpret what types of secondary structures are present.

In order to elucidate the structural features of the SFGYVAE peptide, the YVAE, FGYVAE and GFYVAE peptides were additionally used in the CD study as the different segments of the peptide being studied. The spectra of YVAE, FGYVAE and GFGYVAE in water are shown in Fig. 6a. The spectrum of YVAE is characteristic of peptides that are in a random conformation. The shapes of the FGYVAE and GFGYVAE spectra are consistent with an ensemble of conformations rather than one or two unique conformations.

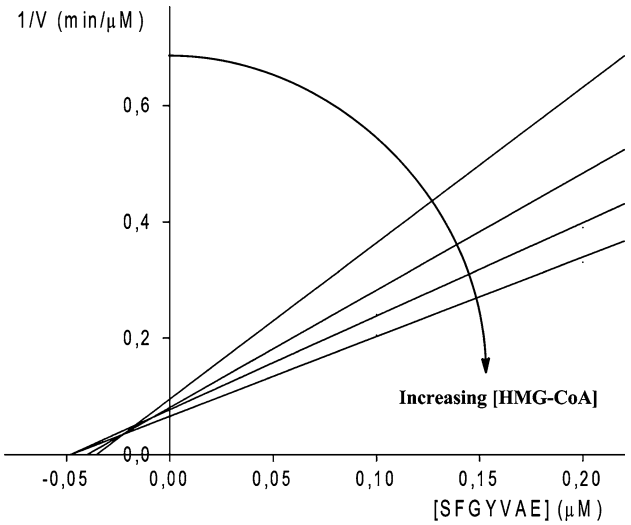


**Fig. 5** The kinetics of inhibition of HMGR by the SFGYVAE peptide. The data are presented in a Dixon plot, $1/V$ against [SFGYVAE], at a NADPH concentration of 120 μM and HMG-CoA concentrations of 72, 96, 120 and 144 μM. The $K_M$ value was found to be 68 ± 3.2 μM for HMG-CoA and the $V_{Max}$ value was 19.4 ± 0.4 μM/min. Each *straight line* represents the results of triplicate experiments

Figure 6b illustrates the effect of TFE on the secondary structures of SFGYVAE, GFYVAE, FGYVAE and YVAE peptides. The dissolution of these peptides in TFE results in significant changes in the CD spectra. The CD spectrum for SFPYVAE appears to have features that are contributed probably by the $\beta$-turn type I and the unordered structures due to the negative band at 200 nm, as well as the shoulder being between 210 and 220 nm. The spectrum of GFGYVAE displays a slightly different pattern compared to the SFPYVAE peptide due to a decrease of the intensity at the same absorption region. For FGYVAE, the shape of the spectrum transforms into that with strong and weak negative bands between 200–205 and 220–230 nm, respectively. The unordered YVAE in water shows the distinctive feature of the aromatic contribution with the maximum positive ellipticity maximum being 226 nm from tyrosine residue (Krittanai and Johnson 1997). The CD spectra of these peptides indicate that, in all environments, no observable patterns were displayed that was related to a well-populated secondary structure conformation.

In a previous study (Pak et al. 2008), a structure that was close to a type I of $\beta$-turn was determined in the N-terminus of the FGYVAE peptide based on the analysis of N-terminus contribution. In order to determine a structural preference in the N-terminus of the SFGYVAE peptide, three-stage procedures were applied that used CD spectra in water and in TFE. The first was subtracting the spectrum of the YVAE peptide from that of peptides SFGYVAE and GFGYVAE in order to determine the N-terminus contribution. The second was subtracting the spectrum of the FGYVAE peptide from that of peptides SFGYVAE and GFGYVAE in order to elucidate an effect of the S and G residues. The final stage was subtracting the resulting spectra that were obtained at the first stage from that obtained at the second stage, and subtracting the spectrum of the YVAE from that of the FGYVAE peptide in order to estimate the N-terminus contribution between SFGYVAE, GFGYVAE and FGYVAE peptides.

The resulting spectra that were obtained at the first and second stages are shown in Fig. 6c, d. A similar shape in the resulting spectra that were obtained within each stage proposes a similarity in the structural preferences for SFGYVAE and GFGYVAE peptides. Figure 6e, f shows the resulting spectra obtained at the final stage. Here, the subtracted spectra exhibit the same profile obtained for SFGYVAE, GFGYVAE and FGYVAE peptides in different environment. This indicates the same N-terminus contribution for these peptides and the location of the structure is close to a type I of $\beta$-turn in the N-terminus that is formed by the two corner glycine at position $i + 1$, and tyrosine residue at position $i + 2$, according to the numbering for the turn structure as it was defined for FGYVAE peptide. An analysis of the secondary structures in the proteins also supports that these residues have a high propensity to form a $\beta$-turn conformation (Gunasekaran et al. 1998).

Based on these considerations, an active conformation for SFGYVAE peptide was constructed. The starting model of SFGYVAE was modeled as a type I of the $\beta$-turn by using the backbone dihedral angles that were adopted by the two corner glycine and tyrosine residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$ and $\psi_{i+2} = 0°$). The orientations of the side-chains and other residues were determined by an optimization procedure. Figure 7 shows the superposition of the bioactive conformations of atorvastatin and rosuvastatin with the optimized structure of the SFGYVAE peptide. According to the constructed model of the SFGYVAE peptide, the oxygen atoms of the hydroxyl group of the side-chain and of the carbonyl group of the peptide backbone of the S residue occupied a space around the location of the oxygen atom of the phenylcarbamoyl group at position 4 of the pyrrolyl ring of atorvastatin. Furthermore, an orientation of the phenyl radical of the F residue close to that of atorvastatin at position 3 of pyrrolyl ring was also observed. Probably, the increase of inhibitory activity of the SFGYVAE peptide can be interpreted in terms of the contribution of the oxygen atom of the hydroxyl group of the S side-chain, while the increase of inhibitory activity of the GFGYVAE peptide by the contribution of the oxygen atom from the amide bond between N-terminus' G and F residues in binding. The modeled structures of these peptides exhibited a good

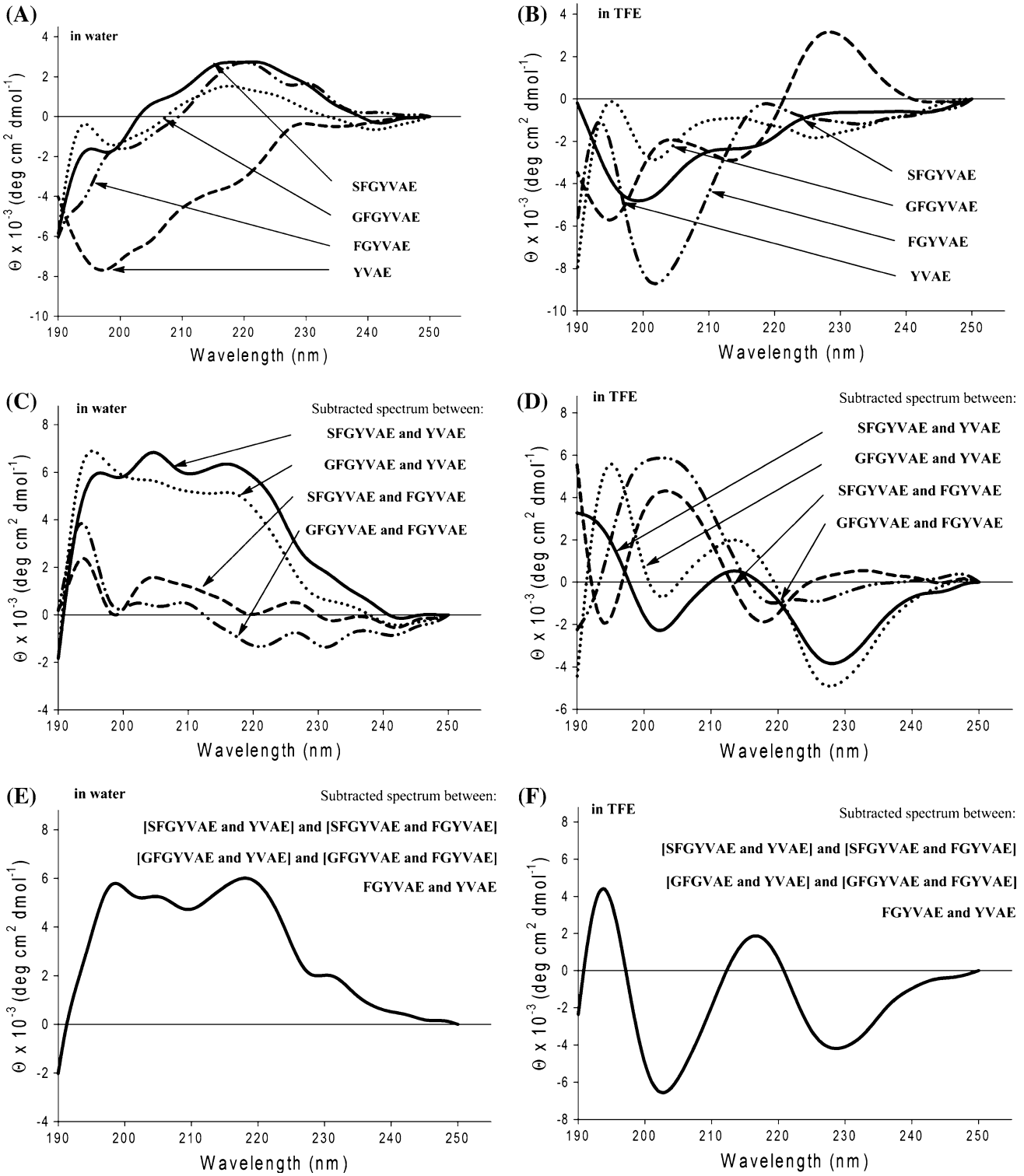


**Fig. 6** CD spectra of the peptides and N-terminus contribution in water and in TFE. **a** CD spectra of the SFGYVAE, GFGYVAE, FGYVAE and YVAE peptides in water. **b** CD spectra of the SFGYVAE, GFGYVAE, FGYVAE and YVAE peptides in TFE. **c** Contribution of the N-terminus of the SFGYVAE and GFGYVAE peptides compared to YAVE and FGYVAE in water. **d** Contribution of the N-terminus of the SFGYVAE and GFGYVAE peptides compared to YAVE and FGYVAE in TFE. **e** Contribution of the N-terminus of the FGYVAE peptide compared to YAVE and the resulting spectra obtained between the contributions of the N-terminus of the SFGYVAE and GFGYVAE with respect to YAVE and FGYVAE in water. **f** Contribution of the N-terminus of the FGYVAE peptide compared to YAVE and the resulting spectra obtained between the contributions of the N-terminus of the SFGYVAE and GFGYVAE with respect to YAVE and FGYVAE in TFE

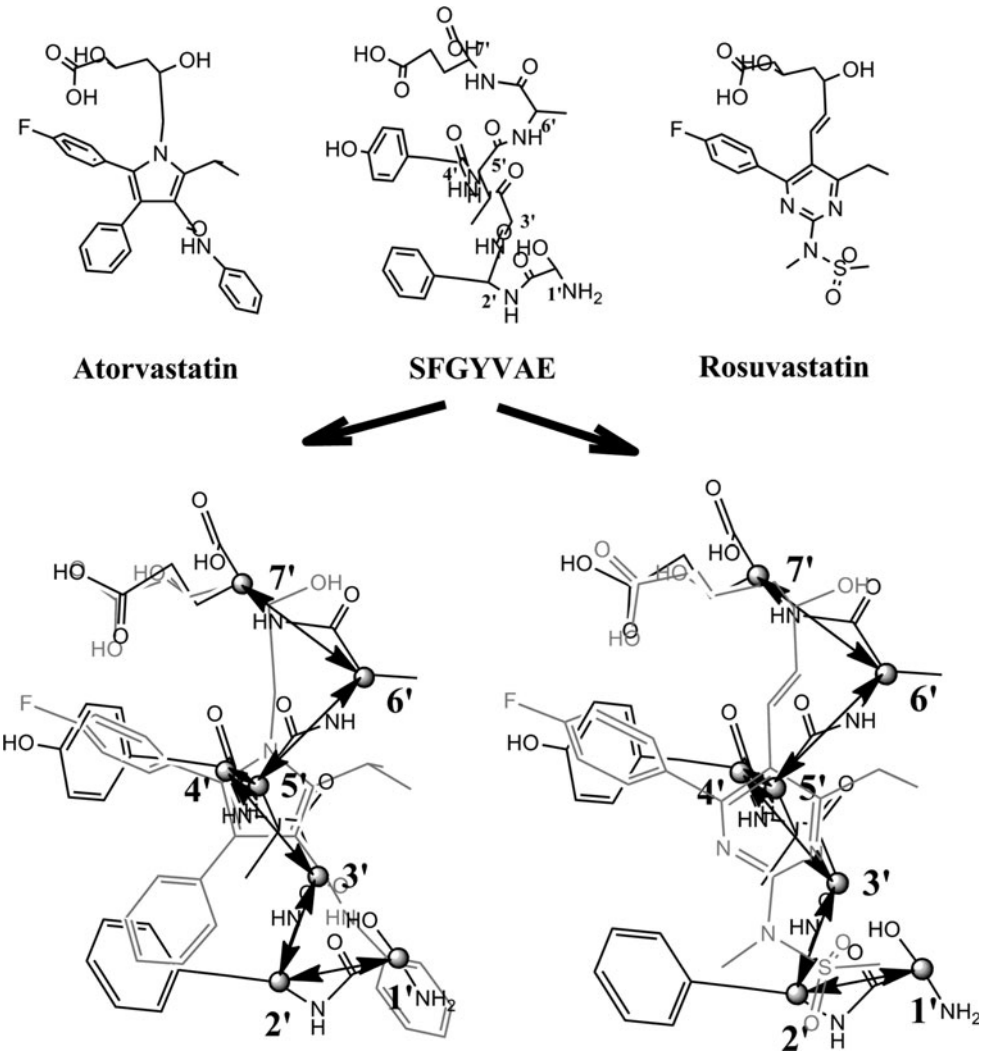


**Fig. 7** Spatial compatibility of the linear SFGYVAE peptide with atorvastatin and rosuvastatin. The alpha-carbon atoms are indicated by *marks* from 1′ to 7′ for the SFGYVAE peptide. The model of SFGYVAE was built as type I of the $\beta$-turn by using the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$ and $\psi_{i+2} = 0°$). The orientations of the side-chains and other residues were determined by an optimization procedure

enough approximation of the statin molecules, which confirms the appropriateness of the proposed method in the design of active peptides.

## Conclusion

The present study shows the design of a highly effective inhibitor for HMGR. By using a peptide fragmentation based on the four residues, the conformational behavior of the peptide fragments were assessed in accordance with that of a $\beta$-turn structure by using the conformational behavior of the cyclic model of glycine hexapeptide as a basis in the MD study. A conformational analysis and the obtained results based on the peptide fragments confirmed a possibility in describing the peptide structure in terms of their structural preferences. This creates a good opportunity to select a prospective site for the peptide in order to design a more active peptide. The restriction of the peptide flexibility was used when searching for the lead compounds. By using the adduced means based on examining the conformational behavior of the peptide fragments for each peptide, the lead peptide candidate was selected. The obtained correlation between the observed and predicted inhibitory activities is good enough in spite of the fact that a different length of peptides was used for these peptides. The correlation coefficient implies the design of a more tightly bound peptide by the HMGR active site. In accordance with the two binding points that can be determined by the experimental procedure, the proposed design may be applied to ligands in order to investigate peptide–protein interactions, especially in the case, when the spatial information for the target is not available.

**Acknowledgments** This work was supported by Korea Food Research Institute, Republic of Korea.

## References


Becker OM (1997) Geometric versus topological clustering: an insight into conformational mapping. Proteins 27:213–226

Beker OM, Levy Y, Ravitz O (2000) Flexibility, conformation space, and bioactivity. J Phys Chem 104:2123–2135

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72:248–254

Burkert U, Allinger NL (1982) Molecular mechanics. ACS Monograph N. 177. ACS, Washington

Deedwania P, Singh V, Davidson MH (2009) Low high-density lipoprotein cholesterol and increased cardiovascular disease risk: an analysis of statin clinical trials. Am J Cardiol 104:3E–9E

Dewar MJS, Zoebish EG, Healy EF, Stewart JJP (1985) Development and use of quantum mechanical molecular models. 76. AM1: a new general purpose quantum mechanical molecular model. J Am Chem Soc 107:3902–3909

Endo A (1992) The discovery and development of HMG-CoA reductase inhibitors. J Lipid Res 33:1569–1582

Fields CG, Lloyd DH, Macdonald RL, Otteson KM, Noble RL (1991) HBTU activation for automated Fmoc solid-phase peptide synthesis. Pept Res 4:95–101

Fisher R (1935) The design of experiments. Oxford University Press, Oxford

Gunasekaran K, Gomathi L, Ramakrishman C, Chandrasekhar J, Balaram P (1998) Conformational interconversions in peptide $\beta$-turns: analysis of turns in proteins and computational estimates of barriers. J Mol Biol 284:1505–1516

Istvan E (2003) Statin inhibition of HMG-CoA reductase: a 3-dimensional view. Atheroscler Suppl 4:3–8

Johnson WC Jr (1990) Protein secondary structure and circular dichroism: a practical guide. Protein Struct Funct Genet 7:205–214

Kalazsi A, Farkas O (2003) Lead conformer prediction based on a library of flexible molecules. J Mol Struct (Theochem) 666–667:645–649

Karle IL, Karle J (1963) An application of a new phase determination procedure to the structure of cyclo(hexaglycyl) hemihydrate. Acta Crystallogr 16:969–975

Kollman PA (1996) Advances and continuing challenges in achieving realistic and predictive simulations of the properties of organic and biological molecules. Acc Chem Res 29:461–469

Krittanai C, Johnson WC Jr (1997) Correcting the circular dichroism spectra of peptides for contributions of absorbing side chains. Anal Biochem 253:57–64

Kwon DY, Oh SW, Lee JS, Yang HJ, Lee SH, Lee JH, Lee YB, Sohn HS (2002) Amino acid substitution of hypocholesterolemic peptides originated from glycinin hydrolyzate. Food Sci Biotechnol 11:55–61

Pak VV, Koo M, Kasimova TD, Kwon DY (2004) Conformation analysis of Ile-Ala-Val-Pro peptide and its derivatives by circular dichroism. Chem Nat Compd 40:398–404

Pak VV, Koo M, Lee N, Lee JS, Kasimova TD, Kwon DY (2005a) Isolation and identification of hypocholesterolemic peptide from 11S globulin of soy protein. Chem Nat Compd 41:710–714

Pak VV, Koo M, Lee N, Lee JS, Kasimova TD, Kwon DY (2005b) Structure-activity relationship of Ile-Ala-Val-Pro peptide and its derivatives by using semi-empirical AM1 method. Chem Nat Compd 41:454–460

Pak VV, Kim SH, Koo M, Lee N, Shakhidoyatov KM, Kwon DY (2006) Peptide design of a competitive inhibitor for HMG-CoA reductase based on statin structure. Biopolymers (Pept Sci) 84:586–594

Pak VV, Koo M, Yun LM, Kwon DY (2007) Recognized sequence and conformation in design of linear peptides as a competitive inhibitor for HMG-CoA reductase. J Mol Recognit 20:197–203

Pak VV, Koo M, Kim MJ, Yang HJ, Yun L, Kwon DY (2008) Modeling an active conformation for linear peptides and design of a competitive inhibitor for HMG-CoA reductase. J Mol Recognit 21:224–232

Payne JW, Marshall NJ, Grail BM, Gupta S (2002) Conformer profiles and biological activities of peptides. Curr Org Chem 6:1221–1246

Perkin Elmer (1995) Introduction to cleavage techniques. Perkin Elmer Cooperation, Foster City

Rader DJ (2003) Regulation of reverse cholesterol transport and clinical implications. Am J Cardiol 92:42–49

Segel IH (1976) Biochemical calculations. Wiley, New York

Voet D, Voet JG (1990) Biochemistry. Wiley, New York, pp 330–340