Hypocholesterolemic Soybean Peptide (IAVP) Inhibits HMG-CoA Reductase in a Competitive Manner

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Abstract Synthesized Ile-Ala-Val-Pro (IAVP) peptide, which has the highest hypocholesterolemic effect among a number of synthesized derivatives of Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA) isolated from 11S globulin of soy protein by pepsin digestion, was selected for investigation in the present study. Using a recombinant Syrian hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), we studied in detail the inhibition of this enzyme by IAVP and compared the action of this peptide to that of lovastatin, a known competitive inhibitor of this enzyme. The concentration of IAVP required for 50% inhibition (IC_{50}) of HMGR activity in given experimental conditions was 340 μM. Kinetic analysis revealed that the studied peptide is a competitive inhibitor of HMGR with respect to both 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and nicotinamide adenine dinucleotide phosphate (NADPH), with an equilibrium constant of inhibitor binding ($K_i = [E][I]/[EI]$) of 61±1.2 μM and 157±4.4 μM, respectively. At the same conditions, K_i and IC_{50} for lovastatin were 2.2±0.1 nM and 12.5 nM, respectively. Thus, the given peptide interacts with HMGR as a bisubstrate, consequently blocking access of both substrates to the active sites. The achieved results suggest the design of new peptide sequences having a higher relative affinity to binding sites of this enzyme and an enhancement of their hypocholesterolemic properties.

Keywords: Ile-Ala-Val-Pro peptide, hypocholesterolemic peptide, competitive inhibitor, 3-hydroxy-3-methylglutaryl coenzyme A reductase

Introduction

As has often been cited in many studies (1-3) in recent years, the relatively low risk of coronary heart disease in Asian countries is linked with the high intake of various soy protein and soy-based foods. In addition, a number of studies (4-6) have elucidated that soy showed lower plasma total and LDL cholesterol both in hypo-cholesterolemic humans and animals. In this connection, the peptide having hypocholesterolemic activity was isolated from the glycinin hydrolyzate with trypsin and identified as Leu-Pro-Tyr-Pro (LPYP) (7). Then, in the following investigation of glycinin hydrolyzate (8) by pepsin, another amino acid sequence having hypo-cholesterolemic activity was found: Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA). On the basis of the obtained sequence we have synthesized six peptides (9) with conserve Ile-Ala-Val-Pro amino acid sequence: Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA), Leu-Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (LIAVPGEVA), Ile-Ala-Val-Pro-Thr-Gly-Val-Ala (IAVPTGVA), Leu-Ile-Ala-Val-Pro-Thr-Gly-Val-Ala (LIAVPTGVA), Leu-Ile-Ala-Val-Pro (LIAVP) and Ile-Ala-Val-Pro (IAVP). The hypocholesterolemic effects of these peptides were determined by measuring the percentage inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is known to catalyze the reductive deacylation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate. The overall reaction is as follows:

$$HMG\text{-}CoA + 2\ NADPH + 2\ H^+ \rightarrow mevalonate + 2\ NADP^+ + CoASH$$
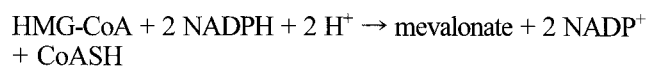
HMGR, an integral membrane protein of the smooth endoplasmic reticulum, is the rate-limited enzyme in the cholesterol biosynthesis pathway occurring in eukaryotes, archaea and certain bacteria (10-12).

In this study we used the catalytic domain of Syrian hamster HMGR using plasmid pKFT7-21 which encodes this domain with attached the Glu-Glu-Phe (EEF) epitope to C-terminus that was overexpressed in *Escherichia coli* (13).

According to previous results of *in vitro* assays, the 50 % inhibition concentrations (IC_{50}) for IAVP, IAVPTGVA and IAVPGEVA were 59.3, 93.3 and 125.5 μM, respectively (14). The range of the percentage inhibition of HMGR by testing with LIAVPGEVA, LPYPR, LIAVP and LIAVPTGVA under concentration of 200 μM was 62.3, 33.2, 17.5 and 12.4%, respectively. However, these data could not reveal the inhibition mechanism of the enzyme, nor the type of this inhibition. On the other hand, the structure-functional analysis of studied peptides has proposed the same binding site but with a different orientation (14).

Therefore, the requisite data can be obtained by studying the kinetics of enzyme inhibition and determining the properties of this inhibition. Furthermore, the resulting data about the kinetic mechanism and the type of inhibition, which are necessary for understanding how HMGR is inhibited by given peptides, can been used to aid further mechanistic studies, the elucidation of structure-activity relationship and, consequently, the following design of amino acid sequences having a higher hypocholesterolemic effect.

Thus, in order to elucidate the inhibition mechanism of HMGR by given peptides, IAVP possessing the highest

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inhibition activity with respect to HMGR among all tested synthetic peptides was selected for the presented study.

Materials and Methods

Materials HMG-CoA, nicotinamide adenine dinucleotide phosphate (NADPH), dithiothreitol (DTT), isopropyl-β-D-thiogalactoside, and 2-methyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)]-1-naphthalenyl ester butanoic acid (lovastatin) were purchased from Sigma (St. Louis, MO, USA). Chemicals and solvents obtained from Sigma were of reagent or HPLC grade.

Peptide preparation Synthesis of peptides was performed using standard fluorenylmethyloxycarbonyl (Fmoc) methodology on an automated synthesizer in an Applied Biosystem Peptide Synthesizer (Model 433A, Perkin Elmer, Foster, CA, USA) (15, 16). Peptide synthesis starting with the proline preloaded resin was carried out using the FastMoc strategy supplied by Perkin Elmer. The peptide yield was 95% of the theoretical yields with purity after purification (a reversed-phase high pressure liquid chromatography system from Waters, Milford, MA, USA, with a Vydac 218TP510 semi-preparative C_{18} column) of above 99% (9).

Assay of HMG-CoA reductase activity The HMG-CoA-dependent decrease in optical density at 340 nm that accompanied the oxidation of NADPH was monitored in a Jasco V-530 spectrophotometer (Model TUDC 12B4, Tokyo, Japan). The standard assay mixture contained, in a final volume of 250 μl, 1 mM potassium phosphate buffer (pH 7.0), 0.4 mM DTT, HMG-CoA, NADPH with inhibitor (IAVP peptide) at the concentration indicated in the text and 14.4 mg of partially purified enzyme (specific activity, 4.49 U/mg). After 5-min incubation at 37°C, the reaction was initiated by respective concentrations of HMG-CoA. To calculate K_m and V_{max} values for HMG-CoA, we used a fixed concentration of NADPH (0.12 mM) and varied the HMG-CoA concentration from 0.024 to 0.24 mM at increments of 0.024 mM. The same procedure was used for obtaining K_m of NADPH. The HMG-CoA concentration was fixed at 0.12 mM and NADPH concentration was increased at increments of 0.02 mM from 0.02 to 0.2 mM. One unit (U) of HMGR was defined as the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH per min.

Enzyme was prepared using plasmid pKFT7-21 (kindly supplied by Prof. V.W. Rodwell of Purdue University, West Lafayette, IN, USA), which encodes the catalytic domain of Syrian hamster HMGR with the C-terminal extension of Glu-Glu-Phe (R_{cat}EEF). Expression vector pKFT7-21 was previously described (13). *E. coli* BL21 (DE3) (F⁻, *omp*T r⁻Bm⁻B) served as host during protein expression (17). The solution of HMGR was frozen in a deep-freezer at -70°C. The protein concentration was determined by the method of Bradford using bovine serum albumin as standard (18).

Calculation method of kinetic parameters The type of inhibition and Michaelis-Menten parameters were determined

from Lineweaver-Burk plots (19) by using equations derived from linear regression analysis of each curve. The k_{cat} value was calculated by applying the following equation:

$$k_{cat} = V_{max}/[E],$$

where [E] is the enzyme concentration in the assay (20). The equilibrium constant of inhibitor binding (K_i, inhibition constant) was estimated by extrapolation from a Dixon plot (19) and calculated using the following equation:

$$x_{([S]i, \; 1/v \; = \; 0)} = -K_i(1+[S]/K_m).$$

Results and Discussion

The dependence of varying HMG-CoA and NADPH concentrations on the rate of the reaction catalysis is depicted in Figure 1 (control line). Calculations of kinetic parameters indicated that under the given experimental conditions the K_m values were 116±4.6 μM for HMG-CoA
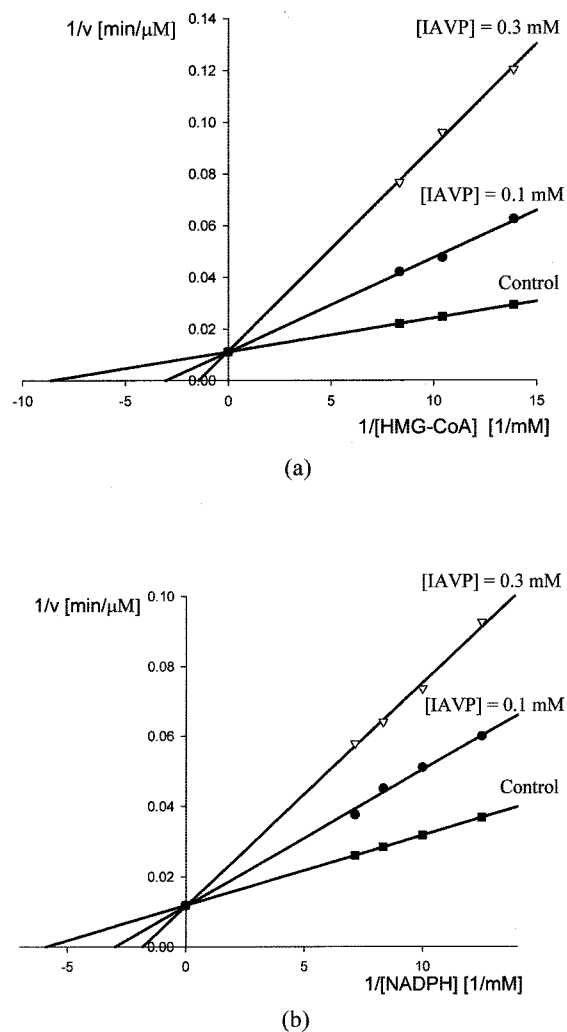

(a)

(b)

Fig. 1. Lineweaver-Burk plots of the inhibition of HMGR by IAVP peptide with respect to HMG-CoA (a) and NADPH (b). Enzyme assays were carried out with HMGR alone (■) and in the presence of IAVP at 0.1 mM (●) and 0.3 mM (△) for both substrates. Each straight line represents the results of triplicate experiments.

and 169 ± 7.9 µM for NADPH respectively, and V_{max} was 87.0 ± 3.2 µM/min. To compare the relative maximal velocity (V'_{max}), which is determined as the quantity in mmol of NADPH oxidized per min per mg of HMGR, with the experimental data obtained by Flimpong *et al* (13), we found that it equals 19.4 ± 0.7 µM/min/mg. According to the experimental data, the specific activity of a soluble cell extract was similar (4.49 U/mg) with result obtaining for the same parameter (2.2 U/mg) in the study for crude extract (13). As expected, K_m values were remarkable higher and V'_{max} was less than half under our experimental conditions than that presented for purified HMGR (13).

In order to study the kinetics of HMGR inhibition at given conditions by IAVP peptide and to determine the properties of this inhibition, enzyme activity was measured with HMGR in the absence and presence of 0.1 and 0.3 mM IAVP. The initial velocity of the NADPH oxidation reaction was obtained for each concentration of substrates used and the data were presented in a Lineweaver-Burk plot (Fig. 1). The inhibition kinetics were investigated at the following conditions of substrate concentrations: HMG-CoA concentrations of 0.072, 0.096 and 0.12 mM for a fixed NADPH concentration of 0.12 mM; NADPH concentrations of 0.08, 0.10, 0.12 and 0.14 mM for a fixed HMG-CoA concentration of 0.12 mM. During the kinetic experiments all substrate concentrations were selected to achieve the rate of NADPH oxidation close to the value of $1/2$ of V_{max}.

As shown in Figure 1 the reciprocal plots of the control, in the presence of the inhibitor intercept at a common point on the y-axis, indicated that V_{max} is unchanged in the presence of IAVP for both substrates. Furthermore, K_m values determined from x-axis intercepts demonstrated that K_m increased with increasing peptide concentration. These kinetic features are characteristic of competitive enzyme inhibition. Analysis of V_{max} and k_{cat} values obtained for HMGR, using a fixed concentration of one of either HMG-CoA or NADPH with varying concentrations of the other revealed that these values were not altered in the presence of inhibitor. Increases were observed in K_m and decreases in k_{cat}/K_m, consistent with the competitive inhibition. Furthermore, comparison of the obtained K_m and k_{cat}/K_m values in the presence of 0.1 and 0.3 mM of IAVP for both substrates having close means also confirmed that under experimental conditions the studied peptide behaved in a competitive manner with respect to both HMG-CoA and NADPH.

To elucidate and examine the catalytic activity of HMGR under given experimental conditions, the kinetic experiments were also carried out with the positive control, lovastatin, which presents a classical competitive inhibitor for HMGR (data not shown). Lovastatin has an HMG-like moiety and occupies a portion of the binding site of HMG-CoA in the internal of HMGR, consequently blocking access of this substrate to the active site (21, 22). K_i for lovastatin was 2.2 ± 0.1 nM and IC_{50} at concentrations 0.12 mM for both substrates was equal to 12.5 nM. At the analogical experimental conditions, IC_{50} for IAVP was 340 µM.

The inhibition constant, K_i, for IAVP against HMGR was determined. For this purpose, enzyme assays were
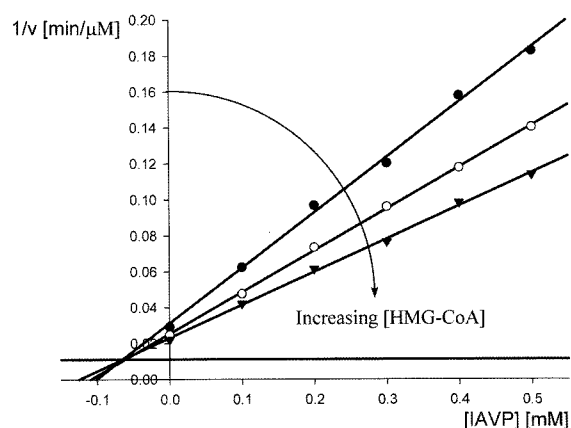
(a)
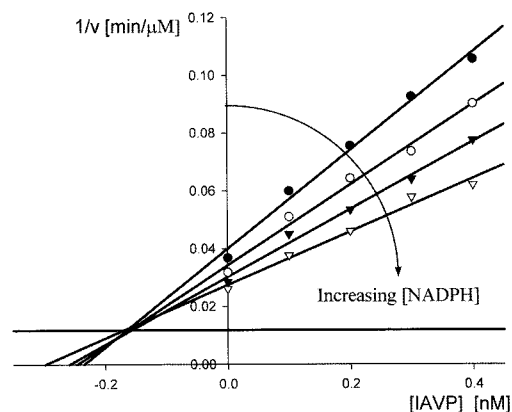
(b)

Fig. 2. Dixon plots of the inhibition of HMGR by IAVP peptide with respect to HMG-CoA (a) and NADPH (b). Enzyme assays were carried out in the presence of different concentrations: a. HMGR, 0.072 mM (●), 0.096 mM (○), and 0.12 mM (▼); b. NADPH, 0.08 mM (●), 0.1 mM (○), 0.12 mM (▼), and 0.14 mM (▽). (Ed- I cannot understand this sentence, and without the figure to refer to cannot guess your intended meaning. Please check and rewrite carefully) Individual graphs represent the results of triplicate experiments.

performed at the same substrate concentrations described above and using peptide concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mM for HMG-CoA and of 0.1, 0.2, 0.3 and 0.4 mM for NADPH. K_i was obtained graphically using a Dixon plot for competitive inhibition. In this plot (Fig. 2), $1/v$ (velocity) is plotted against inhibitor concentration at varying substrate concentrations to obtain a series of straight lines converging to the point that represents $K_i = -[I]$. K_i to inhibit HMGR by IAVP with respect to HMG-CoA and NADPH was 61 ± 1.2 µM and 157 ± 4.4 µM, respectively.

Since the competitive nature of the inhibition of HMGR by IAVP indicates its interaction with the active sites of both substrates, it is important to assess whether this inhibitor might be a potential substrate for the enzyme. According to the previous results of conformation analysis by circular dichroism (9) and quantum-chemical calculation

Table 1. Kinetic parameters of HMG-CoA reductase in the presence of IAVP peptide[a,b]

	Varied substrate	V_{max} [μM/min]	K_m [μM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1}M^{-1}×10^{-6}]
HMGR		87.0 (3.2)	116 (4.6)	6.04 (0.24)	0.052 (0.002)
IAVP (0.1 mM)	HMG-CoA	90.2 (4.1)	324 (15.5)	6.25 (0.26)	0.019 (0.0008)
IAVP (0.3 mM)	HMG-CoA	87.7 (2.8)	693 (21.9)	6.09 (0.25)	0.009 (0.0004)
IAVP (0.1 mM)	NADPH	86.9 (2.5)	339 (12.8)	6.03 (0.18)	0.018 (0.0007)
IAVP (0.3 mM)	NADPH	86.9 (3.4)	556 (25.2)	6.03 (0.23)	0.01 (0.0005)

[a]Parameters were calculated using the equations obtained from the Lineweaver-Burk plots
[b]All values are presented as average means with standard deviation in parentheses. Standard deviations given are for experiments performed in triplicate

of their structures (14), IAVP exhibits a well-defined bend in an aqueous environment. In addition, it was determined that due to its relatively rigid structure this peptide exhibits the highest inhibition effect with respect to HMGR among all synthetic derivatives (IAVPGEVA, LIAVPGEVA, IAVPTGVA, LIAVPTGVA, LIAVP) (14). Furthermore, the structure-functional analysis of the topographical and geometrical features of studied peptides suggested that proline could include itself a function as the recognition/activity element (14). Additionally to the above, a spatial comparison based firstly on of the nicotinamide moiety of NADPH with proline-contained peptides having hypo-cholesterolemic activity in which an amino acid sequence originated also from soy protein (7) and secondly on the results of quantum-chemical calculations of their structures given in another study (23) suggests that proline could create similar interactions with HMGR like the nicotinamide moiety in its binding site. Taken together, these findings suggest the following inhibition mechanism of the investigated enzyme: the pyrrolidine ring of the proline residue occupying the nicotinamide moiety of NADPH can orient the N-termini of the peptide to occupy a part of the binding site for β-mercaptoethylamine and pantotheine moieties of HMG-CoA. In this case the peptide bond between Val- and Ile-residues can adopt a similar arrangement and concurrently interactions with HMGR like carboxamide bond between the β-mercapto-ethylamine and pantotheine moieties of coenzyme A. Furthermore, the structural analysis of the binding site for coenzyme A using the X-ray structure containing complex of the catalytic portion of human HMGR (PDB codes: 1DQ9, 1DQA), either with HMG-CoA or with HMG CoA and NADP$^+$ (24, 25), proposes a hydrophobic environment in the space occupied by the pantotheine moiety of this coenzyme. This finding suggests that such hydrophobic environment might be suitable for interaction with a hydrophobic side-chain of Ile-residue that is reflected both in the affinity of this peptide by HMGR and in the correct orientation of hydrogen net bonds in this part of the binding site for the peptide backbone.

Thus, IAVP, as the derivative of isolated peptide from 11S globulin of soy protein and having hypocholesterolemic effect, inhibits HMGR competitively with respect to HMG-CoA and NADPH and interacts with this enzyme like a bisubstrate. The findings presented here determine its binding site and represent the requisite data for our further understanding of the mechanism of HMGR inhibition by other synthetic peptides. They also suggest the future design of new peptide sequences that will be based on their higher affinity in active sites and concurrently the method of enhancing the hypocholesterolemic properties of the designed peptides.

References

1. Lucer G, Lin B, Allshouse J, Kantor LS. Factors affecting dry bean consumption in the United States. J. Nutr. 104: 458-462 (2000)
2. Adlercreutz H, Godlin BR, Gorbach SL, Hockerstedt KAV, Watanabe S, Hamalalainen EK, Markkanen HH, Makela TH, Wahala KT, Hase TA, Fotsis T. Soybean phytoestrogen intake and cancer risk. Nutr. Cancer 125: 757S-770S (1995)
3. Kim KS, Kim MJ, Park JS, Sohn HS, Kwon DY. Compositions of functional components of traditional Korean soybeans. Food Sci. Biotechnol. 12: 153-160 (2003)
4. Huff W, Roberts D, Carroll KK. Long-term effects of semipurified diets containing casein or soy protein isolate on atherosclerosis and plasma lipoproteins in rabbits. Athero-sclerosis 41: 327-336 (1982)
5. Carroll KK. Review of clinical studies on cholesterol-lowering response to soy protein. J. Am. Diet. Assoc. 91: 820-827 (1991)
6. Anderson JW. Johnstone, B.M. and Cook-Newell, M.E. Meta-analysis of effects of soy protein intake on serum lipids. New Eng. J. Med. 333: 276-282 (1995)
7. Kwon DY, Oh SW, Lee JS, Yang HJ, Lee SH, Lee JH, Lee, YB, Sohn HS. Amino acid substitution of hypocholesterolemic peptides originated from glycinin hydrolyzate. Food Sci. Biotechnol. 11: 55-61 (2002)
8. Pak VV, Koo M, Lee N, Lee JS, Kasimova TD, Kwon DY. Isolation and identification of hypocholesterolemic peptide from 11S globulin of soy protein. Chem. Nat. Comp. (in Press)
9. Pak VV, Koo M, Kasimova TD, Kwon DY. Conformation analysis of Ile-Ala-Val-Pro peptide and its derivatives by circular dichroism. Chem. Nat. Comp. 4: 398-404 (2004)
10. Meigs TE, Roseman RS, Simoni RD. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. J. Biol. Chem. 271: 7916-7922 (1996)
11. Meigs TE, Simoni RD. Regulated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in permeabilized cells. J. Biol. Chem. 267: 13547-13552 (1992)
12. Lehninger AL, Nelson DL, Cox MM. Principles of Bio-chemistry, pp. 670-683 (2nd Ed.). Worth Publishers, Inc., New York, NY, USA (1993)
13. Flimpong K, Darnay BG, Rodwell VW. Syrian hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression in

Escherichia coli: Production of homogeneous protein. Protein Expression Purif. 4: 337-344 (1993)

14. Pak VV, Koo M, Lee N, Kim MS, Kwon DY. Structure-activity relationship of Ile-Ala-Val-Pro peptide and its derivatives by using semi-empirical AM1 method. Chem. Nat. Comp. 41: 454-460 (2005)

15. Caprino LA, Han GY. The 9-fluorenylmethyloxycarbonyl amino-protecting group. J. Org. Chem. 37: 3404-3409 (1972)

16. Chang C, Meienhofer J. Solid-phase peptide synthesis using mild base cleavage of Na-fluorenylmethyloxycarbonyl-amino acids, exemplified by a synthesis of dihydrosomatostatin. Int. J. Pept. Protein Res. 11: 246-249 (1978)

17. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189: 113-130 (1986)

18. Bradford MM. 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 (1976)

19. Segel IH. Biochemical Calculations, pp. 246-252 (2nd Ed.). John Wiley & Sons, Inc., New York, NY, USA (1976)

20. Voet D, Voet JG. Biochemistry, pp. 330-340. John Wiley & Sons, Inc., New York, NY, USA (1990)

21. McTaggart F, Buckett L, Davidson R, Holdgate, G, McCormick A, Schneck D, Smith G, Warwick M. Preclinical and clinical pharmacology of rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Am. J. Cardiol. 87: 28B-32B (2001)

22. Istvan ES. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. Atherosclerosis Suppl. 4: 3-8 (2003)

23. Pak VV, Koo M, Lee N, Kim MS. Kasimova, T.D. and Kwon, D.Y. Structures of the peptide Leu-Pro-Tyr-Pro and its derivatives and the nicotinamide part of NADPH by semi-empirical PM3 method. Chem. Nat. Comp. 41: 69-74 (2005).

24. Istvan ES, Deisenhofer J. The structure of the catalytic portion of human NMG-CoA reductase. Biochim. Biophys. Acta 1529: 9-18 (2000)

25. Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J. 19: 819-830 (2000)