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Cloning, Purification and Characterization of the Catalytic C-terminal Domain of the Human 3-hydroxy-3-methyl glutaryl CoA Reductase: an Effective, Fast and Easy Method for Testing Hypocholesterolemic Compounds.

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Response to Reviewers:	Response to Reviewer comment No. 1: 1In page 9, line 17 the authors have written: Buffer 5 (100 mM NaCl, 10mM PIPES pH7, 20 mM imidazole) was 10-fold diluted in MS sample preparation to obtain an optimized spectrum. QUERY: What are you trying to say? The concentration level of the eluted proteins is too high? There is problem of co-crystallization sample/matrix? If so, please clarify. For the sake of clarity the following sentence has been added to the text Surfactants and other additives are generally removed before MALDI-TOF-MS analysis, because their presence could be causes signal suppression. However, the presence of an optimized concentration of surfactant in a matrix–analytes mixture can reduce sodium and potassium adducts as well as increase the number detectable species from complex mixtures of peptides and proteins 2Example of MS/MS MALDI spectra should be included in the manuscript as a figure As request by the reviewer example of MS/MS MALDI spectra have been added in Figure 4 Reviewer 2 We didn't have requests from reviewer 2

Cloning, Purification and Characterization of the Catalytic C-terminal Domain of the Human 3-hydroxy-3-methyl glutaryl CoA Reductase: an Effective, Fast and Easy Method for Testing Hypocholesterolemic Compounds.

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Abstract

The 3-hydroxy-3-methyl glutaryl CoA reductase, also known as HMGR, plays a crucial role in regulating cholesterol biosynthesis and represents the main pharmacological target of statins. In mammals, this enzyme localizes to the endoplasmic reticulum membrane. HMGR includes different regions, an integral N-terminal domain connected by a linker-region to a cytosolic C-terminal domain, this latter is responsible for enzymatic activity.

Aim of this work was to design a simple strategy for cloning, expression and purification of the catalytic C-terminal domain of the human HMGR (cf-HMGR), in order to spectrophotometrically test its enzymatic activity. The recombinant cf-HMGR protein was heterologously expressed in *Escherichia coli*, purified by Ni+-agarose affinity chromatography and reconstituted in its active form. MALDI mass spectrometry was adopted to monitor purification procedure as a technique orthogonal to classical Western blot analysis. Protein identity was validated by MS and MS/MS analysis, confirming about 82% of the recombinant sequence. The specific activity of the purified and dialyzed cf-HMGR preparation was enriched about 85-fold with respect to the supernatant obtained from cell lysate. The effective, cheap and easy method here described could be useful for screening statin-like molecules, so simplifying the search for new drugs with hypocholesterolemic effects.

Keywords HMGR, Bacterial expression, affinity chromatography, MALDI MS and MS/MS, Enzymatic activity, Screening of statin-like molecules.

Introduction

The 3-hydroxy-3-methyl glutaryl CoA reductase [EC1.1.1.34 mevalonate: NADP ÷ oxidoreductase (CoA-acylating)], also called HMGR, is a NADPH-dependent enzyme catalyzing the conversion of HMG-CoA into mevalonate during endogenous cholesterol synthesis. HMGR is the rate-limiting enzyme in this pathway; hence, it plays a key role in regulating intra-cellular cholesterol production, as well as cholesterol blood level [1]. Elevated plasma low-density lipoprotein cholesterol (LDL-C) level is an important risk factor for coronary artery disease and atherosclerosis, which represent the main causes of death in Westernized populations. HMGR is the main pharmacological target to decrease LDL-C level, since HMGR inhibitors, known as statins, inhibit cholesterol biosynthesis [2], either by reducing its intracellular level or promoting LDL-C hepatic uptake [3]. When hepatic intracellular cholesterol level decreases, increased gene expression for hepatic low-density lipoprotein receptor (LDLR) occurs, leading to an increased hepatic clearance of low-density lipoproteins (LDLs), as well as of their precursors, known as intermediate-density and very low-density lipoproteins (IDLs and VLDLs, respectively) [4]. LDLR upregulation is achieved by the proteolytic activation of sterol regulatory element-binding proteins (SREBPs), which translocate into the nucleus enhancing LDLR expression [5, 6].

Statins affect the production of mevalonate that is a precusor of isoprenoids, which are compounds involved in several cellular functions. On this basis, statins have a great number of beneficial pleiotropic effects, including the reduction in the amassing of esterified cholesterol into macrophages, as well as the decrease in LDL oxidation, oxidative stress, inflammation and scavenger receptors' expression [7, 8]. Moreover, these hypolipidemic drugs increase endothelial nitric oxide synthase expression, and they promote the improvement of endothelial function, the stabilization of atherosclerotic plaque, the restoration of platelets' activity and the inhibition of thrombogenic response [9-11]. Furthermore, several studies highlighted some anti-proliferative effects of statins against both cancer and cancer stem cells [12-14].

In mammals, HMGR resides in the endoplasmic reticulum (ER) and consists of three regions. In detail, human HMGR is 888 amino acids long, its N-terminal domain is integrated into the ER membrane by eight membrane-spanning helices and acts as a sterol sensing domain, since it is involved in the sterol-induced degradation of the enzyme. HMGR C-terminal domain projects into the cytosol, it consists of 462 amino acids and is responsible for enzymatic activity, whereas a third linker-region resides between both domains [15-17].

Although statin therapy provides well-documented cardiovascular benefits [2], several patients experience adverse effects responsible for discontinuing treatment, such as myopathy, myalgia, mytotoxicity, new-onset diabetes mellitus, hepatic disease and rhabdomyolysis [18-20]. Furthermore, many patients with metabolic syndrome do not achieve their cholesterol goals [21, 22]

Hence, new drugs having cholesterol-lowering activity and few tolerable side effects should be discovered and tested for the development of promising therapeutic strategies. In this regard, some natural compounds contained in citrus fruits appear to be endowed with significant hypolipidemic effects, and they could be effective to reduce coronary heart disease risk [23-25].

Preliminary studies aimed to test hypocholesterolemic activity of new natural compounds are generally conducted in model organisms such as rats [26]. Unluckily, such studies are usually expensive and time-consuming, additionally, they have limited capabilities to ascertain a real inhibition on HMGR activity.

In this regard, testing new molecules with a potential hypocholesterolemic effect directly on the expressed and purified cf-HMGR could represent a rapid, effective and simple strategy to overcome these problems. To this end, one option could be the overexpression of the required protein using a heterologous expression system, including a suitable vector and an expression host, in order to maximize amount and quality of the produced recombinant protein. *E. coli* is commonly used as an expression host [27-29], since it is cheaper and faster with respect to other heterologous expression systems [30]. Furthermore, purification could be promptly monitored by mass spectrometry, in order to ensure protein purity and identity. Mass spectrometry is a well-known technique for detection and quantitative analysis [31-34] of metabolites [35-37] and proteins [38-40]. The development of soft ionization techniques in mass

spectrometry (MS), electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) is become the keystone to novel MS application as analytical tool in protein chemistry. Direct MALDI MS analysis of biological samples or fluids have been established to be a useful and robust approach [41], especially when a rapid screening of complex biological sample is required [42]. Tandem MS represents the analytical technique of choice for the in-depth protein characterization, which is useful to a better comprehension of protein function [43]. MS techniques are generally salt sensitive and a desalting step of the samples might be necessary prior to analysis [44, 45].

In this work, the C-terminal region of the human HMGR including its catalytic fragment (cf-HMGR) was expressed in *Escherichia coli*, purified and tested for its enzymatic activity. Protein sequence was characterized and validated by MALDI MS and MS/MS after protein digestion. Moreover, MALDI was adopted to monitor our purification procedure as a technique orthogonal to classical Western blot analysis. We are confident that the effective, rapid and easy method here described could be useful as a model for a rapid screening of specific HMGR inhibitors including natural statin-like compounds or synthetic statin derivatives, thereby it could help in the search for new drugs with hypocholesterolemic effect.

Materials and Methods

Bacterial Expression of the Recombinant cf-HMGR Protein

The coding sequence of cf-HMGR was obtained by RT-PCR reaction [46], using total RNA extracted from HepG2 cells as a template , and primers with an additional NdeI or BamHI restriction site, such as 5'- TGACATATGTCCTCCTTACTCGATACTTCATCAGTAC -3' (sense) and 5'- CTAGGATCCGAGGCTGTCTTCTTGGTGCAAG -3' (antisense), respectively. PCR amplification product was cloned into the NdeI–HindIII restriction sites of the modified expression vector pET-21b/V5-His [47]. Transformants of *E. coli* TG1 cells were selected, screened and sequenced [48]. The absence of the stop codon in the antisense primer sequence allowed the expression of a cf-HMGR protein with carboxy-terminal V5- and His6-epitope tags. The recombinant cf-HMGR protein was overexpressed in

Rosetta (DE3) *E. coli* cells [49, 50]. Cells harbouring expression plasmids were grown at 37°C with aeration in LB medium, supplemented with ampicillin (100 μg/ml) to an A600 of 0.8. Expression of the recombinant protein was induced for 4 h at 37°C by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). Cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C and stored at -80°C until use [51].

At the same time, Rosetta (DE3) *E. coli* cells were transformed with the empty pET-21b/V5-His vector (lacking the cf-HMGR cDNA). Such cells were grown and processed adopting the same conditions used for cells transformed with pET-21b/V5-His vector containing the cf-HMGR cDNA.

Purification of the Recombinant cf-HMGR Protein

In order to purify the recombinant cf-HMGR protein, pellet deriving from 50 ml of bacterial culture expressing the protein was thawed on ice and suspended in 5 ml of buffer 1 (500 mM NaCl; 10 mM Pipes pH 7; 0,1 mM phenylmethylsulfonyl fluoride; 1 mM imidazole). Cells were lysed by sonication using a sonifier equipped with a microtip probe (Branson 250 Ultrasonics sonifier, Danbury, CT, USA). Cell lysate was clarified by centrifugation at 17,000 rpm for 15 min at 4°C using a centrifuge (Beckman-Coulter, Avanti J-30I, CA, USA), in order to obtain a soluble (supernatant) and an insoluble fraction (pellet).

The soluble fraction (containing about 2 mg of protein/ml) was incubated for 1 h at room temperature in the presence of 200 μl of nickelnitrilotriacetic-agarose (Ni^{2+}-NTA) affinity resin (Qiagen, Milan, Italy), equilibrated with buffer 1 [52, 53]. Then, the mixture was applied to a column that was washed once with 10 volumes of buffer 1, once with 10 volumes of washing buffer 2 (500 mM NaCl, 10 mM Pipes pH 7, 5 mM imidazole, 10% glycerol), once with 10 volumes of washing buffer 3 (300 mM NaCl, 10 mM Pipes pH 7, 5 mM imidazole, 5% glycerol), once with 10 volumes of washing buffer 4 (100 mM NaCl, 10 mM Pipes pH 7, 10 mM imidazole, 1% glycerol), once with 10 volumes of washing buffer 5 (100 mM NaCl, 10 mM Pipes pH 7, 20 mM imidazole), and once with 10 volumes of washing buffer 6 (100 mM NaCl, 10mM Pipes pH 7, 40 mM imidazole). Finally, cf-HMGR was eluted using 5 x 0.2 ml of elution buffer

containing a higher imidazole concentration (100 mM NaCl, 10 mM Pipes pH 7, 100 mM imidazole). The first and the last 0.2 ml fractions were discarded, collecting a total of 0.6 ml. This collected final eluate, containing the purified protein, was dialyzed overnight at 4°C against an assay buffer (160 mM potassium phosphate pH 6.8; 200 mM KCl; 4 mM EDTA; 10 mM DTE), then, it was used to determine enzymatic activity. The amount of purified protein was measured as previously described [49]. The final protein concentration was about 17-20 ng/μl.

Enzymatic Activity of the Purified cf-HMGR Protein

The enzymatic activity of cf-HMGR was spectrophotometrically determined, our assay was conducted essentially as previously described [54, 55], with a slight modification. Briefly, an average amount of 0.85 μg of purified protein was added of assay buffer to reach a final volume of 100 μl, this complete assay mixture (also containing 0.2 mM NADPH) was incubated at 37 °C.

The reaction was initiated by adding 0.1 mM HMG-CoA to the complete assay mixture. The substrate dependent oxidation of NADPH was measured at 340 nm in a UV-spectrophotometer (Applied Biosystems model Jenway 7315) equipped with a peltier unit. The rate of NADPH oxidation in the absence of HMG-CoA (control reaction) was subtracted from that obtained in the presence of HMG-CoA. NADPH oxidation was monitored every second for 10 min [13]. Inhibition assays were performed by incubating the enzyme with different concentrations of pravastatin (1-100 nM). One unit of cf-HMGR enzymatic activity was defined as the amount of enzyme needed to produce 1 μmole of $NADP^+$ per min at 37 °C.

SDS-PAGE and Western Blot Analysis

Proteins were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) dye, or transferred to nitrocellulose by a semidry transblotter (Bio-Rad, Milan Italy). Western blot analysis was carried out as formerly described [56, 57], using a mouse anti-V5 monoclonal antibody (Sigma-Aldrich,

Milan, Italy). Data were analysed for statistical significance ($p < 0.001$) using one-way analysis of variance (ANOVA) test performed by GraphPad Prism 7 [58].

In-gel Protein Digestion.

Selected excised Coomassie stained gel bands were added with 50 µl of NH_4HCO_3 (50 mM), vortexed and leaved to stand several times until they appeared completely colorless. The gel pieces were washed, two times, with 50 µl of 50% acetonitrile (ACN): 50% 50 mM NH_4HCO_3, dehydrated with 50 µL of 100% ACN until the gel turned opaque white, and dried in a vacuum centrifuge for 30 min. The gel pieces were rehydrated in 50 µl of NH_4HCO_3 (50 mM) and added with 4 µl of trypsin (4 pmol/µL). In-gel digestion was performed at 37°C overnight, then peptides were recovered and supernatants were dried by a Concentrator Plus system (Eppendorf, Hamburg, Germany).

Solution Protein Digestion.

The enzymatic digestion was performed on the final eluate, containing the purified cf-HMGR, after dialysis overnight at 4°C against deionized water. The solution was divided into two parts and dried by the Concentrator Plus system. The two protein pellets were used to perform digestion with trypsin and pepsin. Trypsin digestion was performed adding 50 µl of NH_4HCO_3 (50 mM) and 4 µl of trypsin (4 pmol/µL), while pepsin digestion was carried out using 50 µl of HCl (0,1M) and 5 µl of pepsin (4 pmol/µL). Digestion was performed at 37°C overnight, then peptide solutions were directly used for mass spectrometry analysis.

MALDI MS and MS/MS

Samples were prepared in a premixed solution using an analita/matrix ratio 1:10 (V: V). A premixed solution of digested sample and α-CHCA (0.3% in TFA) was spotted (1 µl) on the matrix target. Spectra were acquired using a 5800 MALDI-TOF/TOF analyser (AB SCIEX, Darmstadt, Germany) equipped with a neodymium: yttrium-aluminium-garnet laser (laser wavelength: 349 nm). Mass spectrometry

analyses were performed in reflectron positive-ion mode. MS spectra were acquired in default calibration mode averaging 2,500 laser shots with a mass accuracy of 50 ppm, a laser pulse rate of 400 Hz and at least 4000 laser shots. CID-MS/MS experiments were performed at collision energy of 1 kV, using ambient air as collision gas with a medium pressure of 10-6 Torr, 5000 laser shots and a pulse rate of 1000 Hz.

Aliquots of the two eluates obtained by the addition of washing buffer 5 and 6, as well as the final eluate were analyzed by linear MALDI mass spectrometry. Linear MALDI-TOF spectra were acquired to screen purification procedure, averaging 2500 laser shots with a mass accuracy of 500 ppm in default calibration mode. All the molecular masses of the validated proteins were calculated using Compute pI/Mw algorithm, available on the bioinformatics resource portal ExPASy (https://web.expasy.org/compute_pi/).

Results and Discussion

Bacterial Expression, Purification, and Detection of the Recombinant cf-HMGR.

HMGR represents a key target to screen hypocholesterolemic drug, and different heterologous expression systems were used for the production of recombinant HMGRs [59-64]. An active purified catalytic domain of human HMGR was achieved by Mayer et al. in 1988 [62], using many purification steps including salting out with ammonium sulfate followed by further chromatographic separations, whereas in this work an active human cf-HMGR was obtained using only one fast chromatographic purification. In detail, the human recombinant cf-HMGR (corresponding to amino acids 420-888) was overexpressed in *Rosetta (DE3) E. coli* cells grown in LB medium. Cell lysate was centrifuged in order to obtain an insoluble and a soluble fraction. The insoluble fraction obtained from IPTG-induced cells contained a protein with an apparent molecular mass corresponding to that of cf-HMGR (Fig. 1, lane 4). Such band was not visible in the respective soluble fraction (Fig. 1, lane 3), as well as it was absent in both fractions obtained from non-induced cells (Fig. 1, lanes 1-2). The presence of the expressed cf-HMGR protein in both fractions extracted from induced cells was detected by Western blot analysis, since a mouse anti-V5 monoclonal antibody immunodecorated a single protein band with an apparent molecular mass of about

54 kDa, either in the soluble or in the insoluble fraction (Fig. 1B, lanes 3 and 4, respectively). In literature it is known that many proteins recovered in insoluble fractions are denatured, so they need to be activated to exert their biological activity [29, 30]. Hence, even if cf-HMGR was significantly more expressed in the insoluble than in the soluble fraction, we chose to perform subsequent investigations on the soluble fraction, in order to prevent problems concerning cf-HMGR enzymatic activity.

The presence of a polyhistidine tail at the C-terminal end of the expressed cf-HMGR protein allowed its purification by a Ni^{+}-agarose affinity column. A similar approach was used for the purification of eubacterial HMGR, but the recombinant protein needed to be further purified using an additional chromatographic method [65]. The identity of our recombinant cf-HMGR was analyzed by MALDI-TOF, since mass spectrometer methods hold several advantages over gel electrophoresis in terms of increased speed, efficiency, resolution and mass accuracy [66]; consequently, sample analyses become easy and instantaneous [67]. The targeted proteins can be traced with high reproducibility during purification. Our selected fractions were analyzed by MALDI-TOF in the linear mode (Fig. 2, panels A). Fig. 2, panel A showed interesting protein profiles. In detail, spectrum I (Fig. 2 panel A, I) was related to the fraction eluted with buffer 5, and it revealed the presence of several multi-charged ion species (Fig. 2, panel A). MS data indicated that both the adopted experimental conditions and sample preparation for MS analysis were adequate to get satisfying results. Buffer 5 (100 mM NaCl, 10 mM Pipes pH 7, 20 mM imidazole) was 10-fold diluted in MS sample preparation to obtain an optimized spectrum. Surfactants and other additives are generally removed before MALDI-TOF-MS analysis, because their presence could be causes signal suppression. However, the presence of an optimized concentration of surfactant in a matrix–analytes mixture can reduce sodium and potassium adducts as well as increase the number detectable species from complex mixtures of peptides and proteins [68].

The complexity of the protein content is usual for a sample from bacterial cell culture, where expression of several proteins naturally occurs [67]. MALDI is highly sensitive for polar biomolecules and can provide *atto*-molar detection limits leading to the identification of under- expressed proteins. Although, MALDI MS generally provides signals corresponding to singly charged proteins, the presence of

multiply-charged protein ions is not surprising in the adopted experimental conditions. Literature data report that MALDI TOF-TOF instrumentation is able to produce multiply charged ions by adapting the experimental conditions. The choice of the matrix, the matrix solution, the matrix/analyte ratios, the crystallization conditions and the method of sample deposition are all parameters that may influence ions formation [69, 70]. In particular, matrix with high ionization energy, as α-cyano-4-hydroxycinnamic acid (CHCA), showed a much higher yield of multiply charged protein ions [71]. The charge state, as well as the signal intensity, is also related to the sequence of the protein, the side chains of Lys, Arg, and His are easily susceptible to protonation, therefore the poly-histidine tag could be easily over protonated [42]. In spectrum I (Fig. 2, panel A) it is possible to observe, along with other unknown proteins, the ion of m/z 10086, which was ascribed to the recombinant sequence of HMGR without poly-histidine tag (calculated theoretical molecular mass 50403 Da) corresponding to $[HMGR]^{5+}$. A similar protein profile was shown in spectrum II, obtained for the fraction eluted using buffer 6 (Fig. 2 panel A, II). Spectrum II displayed the ion of m/z 10794 associated with $[HMGRt]^{5+}$ and corresponding to the expressed recombinant HMGR sequence retaining the poly-histidine tag (calculated theoretical molecular mass 53946 Da; the subscript letter "t" indicates poly-histidine tag's presence). Finally, spectrum related to the final eluate displayed the profile of the purified cf-HMGR (Fig. 2, panel A, III), indicating the presence of only two ion species of m/z 10794 and 5405, corresponding to $[HMGRt]^{5+}$ and $[HMGRt]^{10+}$, respectively (calculated theoretical molecular mass 53946 Da). As above reported, the recombinant cf-HMGR was eluted by competition with imidazole and the final eluate contained only a homogeneous protein with a molecular mass of 54 kDa. These results corroborated the goodness of the purification method and allowed us to calculate the accurate molecular mass of the expressed protein, corresponding to the calculated theoretical molecular mass (53946 Da). Protein identity and purity were also checked by SDS-PAGE (Fig. 2, panel B) followed by MS-based in gel digestion procedure. Approximately 0.18–0.23 mg of purified protein was obtained per liter of culture.

MS Characterization of Recombinant cf-HMGR's Primary Structure.

Sequence information was obtained by digesting gel band with an apparent molecular mass of 54 kDa, using trypsin, a serine protease that preferentially cleaves on the C-terminal side of Arg and Lys, especially at high pH values (pH 7.9). Direct peptide mass fingerprint identification on Mascot Server, using the experimental list of peptide mass values from the digestion of the protein, confirmed the presence of three isoforms of 3-hydroxy-3-methyl glutaryl-CoA reductase (gi|4557643, gi|7245353 and gi|21707182; Table 1 and Supplementary Material S1), with 14, 11 and 13 identified peptides, respectively. The sequence coverage was 14, 20 and 14%, respectively. The two accession numbers gi|4557643 and gi|21707182 match the complete sequences of HMGR (MW > 90 kDa), corresponding to the UniProtKB ID P04035_1 (isoform 1, canonical sequence) and P04035_2 (isoform 2, missing of 522-574 sequence), respectively. The accession number gi|7245353 corresponds to the sequence matching the catalytic portion of human HMGR, fully similar to the sequence of our expressed protein.

In order to improve sequence coverage, in solution digestion by pepsin and trypsin was adopted. Pepsin is an endopeptidase that cleaves on the C-terminal side of hydrophobic amino acids (Phe, Tyr, Trp and Leu) at acid pH (pH range 1,5-2). The different action of the two selected enzymes generated complementary peptides useful to a comprehensive protein sequencing. Protonated tryptic and peptic peptides were used to identify cf-HMGR after MS/MS fragmentation. All tandem mass spectra were evaluated using MASCOT database searching (Table 2 and Supplementary Material S2). Methionine oxidation was included in the variable modifications. MASCOT outputs were carefully validated by manual interpretation of the corresponding MS/MS spectra. All the identified peptide sequences produced by trypsin or pepsin digestion are showed collectively in Table 2. The MS/MS experiments also confirmed the presence of a poly-histidine tag located at the C-terminal end of the protein. The alignment of MS/MS identified peptides allowed to reconstruct the recombinant protein sequence (Fig. 3). An alignment of the cf-HMGR protein sequence with the complete HMGR protein sequence (P04035, according to UniProtKB) and the validated MS/MS protein sequences are reported in Fig. 3 and Fig. 4A and 4B. The recombinant cf-HMGR consists of 503 amino acids, 415 of these were experimentally determined, validating about 82% of the cf-HMGR protein sequence.

The molecular mass of the validated protein was calculated using Compute pI/Mw algorithm (https://web.expasy.org/compute_pi/), considering and excluding poly-histidine tag. Theoretical molecular weight considering this tag was 53946.96 Da, with a pI of 6.91, whereas excluding it theoretical molecular weight was 50403.13 Da, with a pI of 7.04

Enzymatic Activity of the Expressed cf-HMGR Protein

The enzymatic activity of the expressed cf-HMGR protein was checked spectrophotometrically [54, 55] (Table 3). Radioisotopic, and spectrophotometric methods can be both used to test HMGR activity, although spectrophotometric assays bypass the use of radiolabeled substrates and usually need only few minutes to be performed [55]. The specific activity of the purified and dialyzed preparation was enhanced about 85-fold when compared to that obtained on the soluble fraction containing the recombinant cf-HMGR protein (Table 3). Approximately 72% of the total cf-HMGR activity applied to the column was recovered. No significant activity was measured in the soluble fraction of bacterial cells transformed with the vector lacking the cf-HMGR cDNA (Table 3). Furthermore, the purified and not dialyzed protein was active also when assay buffer was added to the column eluate (1:1 vol/vol). In this case, in order to fully restore enzymatic activity, the protein mixture required to be incubated for at least 1 hour at 37°C (data not showed).

Determination of pH Optimum, Stability and Inhibition of cf-HMGR

The pH influence on the enzymatic activity of the recombinant cf-HMGR protein was also investigated (Fig. 5A). Our evidences demonstrate that this enzyme has a pH optimum of about 6.8, in agreement with previously reported data [65, 72, 73]. Furthermore, enzyme stability was determined at different incubation times in assay buffer before starting the reaction. In this conditions, cf-HMGR retained 90–100% activity until 4 hours of incubation (data not showed).

Many literature data indicate that statins block the natural substrate from accessing HMGR active site, altering its conformation; binding of statins is reversible and their affinity is in the nanomolar range [4].

In order to define whether our recombinant cf-HMGR was inhibited by statins in a concentration depending manner, the dependence of cf-HMGR enzymatic activity on pravastatin concentration was analyzed. Pravastatin is a competitive HMGR inhibitor used to decrease stroke recurrence [15, 74-76]. Fig. 5B showed a dose-response curve highlighting enzymatic residual activity versus log of pravastatin concentration. Nearly complete inhibition was observed using 100 nM pravastatin, the calculated IC_{50} was 40.23 ± 2.9 nM (mean value obtained from three independent experiments). This value is in good agreement with that found for a catalytic fragment of the human enzyme [77, 78]. Our results confirm that cf-HMGR active site retains its native conformation and that this experimental procedure could be used to rapidly screen several statin-like compounds.

Conclusions

The herein reported cloning, purification and characterization strategy is an easy method to study the catalytic portion of HMGR, the rate-limiting enzyme in cholesterol biosynthesis. In particular, our purification procedure represents an effective, simple and rapid method to purify cf-HMGR, allowing the subsequent evaluation of its enzymatic activity.

The identity of the recombinant protein was monitored by classical Western blot analysis and by the orthogonal use of MALDI mass spectrometry. This latter offers several advantages over SDS-PAGE and Western blot analysis in terms of increased speed, efficiency, resolution and mass accuracy, providing a reliable protein profiling method with high reproducibility. The high accuracy of MALDI MS allowed the calculation of the accurate cf-HMGR molecular mass. Moreover, the specific activity of the purified and dialyzed cf-HMGR preparation was easily measured, founding an improved (85-fold) activity when compared to that of the soluble fraction containing the non-purified enzyme.

Cloning, expression purification and biochemical characterization of an enzyme are of paramount importance to rationally design and check new drugs. We are confident that our effective experimental approach could simplify the search for new statin-like molecules with potential hypocholesterolemic

activity. Specifically, a rapid screening of many compounds could be easily performed before undertaking *in vitro* or *in vivo* studies, which are more expensive, time-consuming and labor-intensive.

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Conflict of interest

The authors declare that they have no conflit of interest.

Table 1 Identified proteins by Peptide Mass Fingerprint and Mascot Server. MS data were processed using a mass tolerance of 50 ppm. [a]According to NCBI (https://www.ncbi.nlm.nih.gov/protein/?term=).

	Accession[a]	Mass (Da)	Score	Description	Number of mass values matched	Sequence Coverage (%)
1.	**gi|4557643**	97413	93	3-hydroxy-3-methylglutaryl-Coenzyme A reductase [Homo sapiens]	14	14
2.	**gi|7245353**	49935	90	Chain A, Complex Of The Catalytic Portion Of Human Hmg-Coa Reductase With Hmg And Coa	11	20
3.	**gi|21707182**	91961	86	Similar to 3-hydroxy-3-methylglutaryl-Coenzyme A reductase [Homo sapiens]	13	14

Table 2 MS/MS identified peptides from cf-HMGR proteolytic digestion.

MS/MS identified peptides from HmgR Trypsine digests					
Position	**Sequence [a]**	**MC [b]**	**Mr found [c]**	**Delta(ppm)**	**Mr calc. [c]**
23-42	EPRPNEECLQILGNAEKGAK	1	2196.15	20	2196.11
40-56	GAKFLSDAEIIQLVNAK	1	1817.05	19	1817.02
57-72	HIPAYKLETLMETHER	1	1968.04	18	1968.00
63-78	LETLmETHERGVSIRR	2	1943.05	21	1943.01
78-97	RQLLSKKLSEPSSLQYLPYR	3	2406.40	20	2406.35
85-97	LSEPSSLQYLPYR	0	1552.82	15	1552.80
132-150	EFQVPmATTEGCLVASTNR	0	2070.00	17	2069.96
165-172	VLADGMTR	0	862.46	18	862.45
165-172	VLADGmTR	0	878.46	19	878.44
173-180	GPVVRLPR	1	893.59	20	893.57
189-209	AWLETSEGFAVIKEAFDSTSR	1	2344.19	19	2344.15
210-223	FARLQKLHTSIAGR	2	1597.96	18	1597.93
213-223	LQKLHTSIAGR	1	1223.75	21	1223.72
216-232	LHTSIAGRNLYIRFQSR	2	2032.16	20	2032.12
224-232	NLYIRFQSR	1	1196.67	15	1196.65
287-297	SVVCEAVIPAK	0	1115.63	17	1115.61
298-317	VVREVLKTTTEAMIEVNINK	2	2287.31	18	2287.27
298-317	VVREVLKTTTEAmIEVNINK	2	2303.31	19	2303.26
305-317	TTTEAMIEVNINK	0	1479.77	20	1479.74
447-455	SHmIHNRSK	2	1125.58	18	1125.56
454-468	SKINLQDLQGACTKK	2	1646.92	20	1646.89
468-495	KTASDPNSSSVDKLGPIPNPLLGLDSTR	2	2879.57	20	2879.51
MS/MS identified peptides from HmgR Pepsine digests					
Position	**Sequence [a]**	**MC [b]**	**Mr found [c]**	**Delta(ppm)**	**Mr calc. [c]**
21-29	PREPRPNEE	2	1123.57	18	1123.55
21-28	PREPRPNE	1	994.53	20	994.51
39-47	KGAKFLSDA	3	936.53	17	936.51
52-60	LVNAKHIPA	2	962.60	21	962.58
56-64	KHIPAYKLE	3	1098.65	20	1098.63
65-71	TLMETHE	2	860.40	16	860.38
67-81	METHERGVSIRRQLL	4	1825.02	17	1824.99
72-87	RGVSIRRQLLSKKLSE	4	1870.17	19	1870.13
104-115	VMGACCENVIGY	2	1258.55	19	1258.53
108-126	CCENVIGYmPIPVGVAGPL	3	1947.98	20	1947.94
111-126	NVIGYmPIPVGVAGPL	2	1612.91	19	1612.88
104-126	VmGACCENVIGYmPIPVGVAGPL	4	2322.14	18	2322.10
129-133	DEKEF	2	667.31	20	667.29
147-161	STNRGCRAIGLGGGA	2	1389.72	15	1389.70
158-166	GGGASSRVL	1	803.45	17	803.44
168-178	DGMTRGPVVRL	0	1216.67	18	1216.65
167-181	ADGmTRGPVVRLPRA	2	1611.90	18	1611.87
168-186	DGmTRGPVVRLPRACDSAE	3	2046.02	19	2045.99
196-204	GFAVIKEAF	4	981.56	20	981.54
204-210	FDSTSRF	1	859.41	18	859.39
205-210	DSTSRF	0	712.34	20	712.33
222-226	GRNLY	1	622.34	20	622.33
231-249	SRSGDAMGMNMISKGTEKA	2	1970.95	20	1970.91
257-269	FPEMQILAVSGNY	5	1468.74	19	1468.71
292-308	AVIPAKVVREVLKTTTE	4	1854.14	18	1854.11
302-319	VLKTTTEAmIEVNINKNL	4	2047.15	20	2047.11
313-331	VNINKNLVGSAmAGSIGGY	3	1880.98	15	1880.95
353-362	NVGSSNCITL	0	1007.50	17	1007.48
352-364	QNVGSSNCITLmE	2	1411.64	18	1411.62
375-383	ISCTMPSIE	0	980.46	18	980.44
384-396	IGTVGGGTNLLPQ	2	1226.70	19	1226.67
395-403	PQQACLQML	5	1031.52	20	1031.50
409-420	CKDNPGENARQL	3	1344.66	18	1344.63

420-435	LARIVCGTVmAGELSL	5	1648.90	16	1648.88
422-438	RIVCGTVMAGELSLMAA	5	1721.90	15	1721.87
440-458	AAGHLVKSHMIHNRSKINL	3	2126.21	17	2126.18
439-458	LAAGHLVKSHMIHNRSKINL	4	2239.30	18	2239.26
492-503	DSTRTGHHHHHH	0	1458.67	17	1458.65

[a] Amino acid sequence of peptides identified on the basis of their CID spectra. [b]Missed cleavages. [c] All mass values are listed as monoisotopic mass. (m) denotes methionine oxidized.

Table 3 Purification of the recombinant cf-HMGR from the soluble fraction of *E. coli* Rosetta (DE3) transformed cells.

Soluble fractions isolated from *E. coli Rosetta (DE3)* cells transformed with the recombinant pET-21b/V5-His vector containing the cf-HMGR cDNA or trasformed with the empty vector. Ni^{2+}-NTA eluate refers to the active cf-HMGR-His protein purified by affinity chromatography. Enzymatic activity was checked spectrophotometrically, as described in Materials and methods. Specific activity is expressed as μmoli/min x mgr protein. The values shown are means from twelve experiments.

Fraction	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (fold)
Soluble fraction *E. coli Rosetta (DE3)* empty vector	2.2	$0.9x10^{-3}$	0.0001	-
Soluble fraction *E. coli Rosetta (DE3)* cf-HMGR	2	0.328	0.0328	-
Ni^{2+}-NTA eluate	0.017	28	0.0238	85

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Figure Captions

Fig. 1 Expression of the recombinant cf-HMGR protein in *Escherichia coli*. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye (A) or transferred to nitrocellulose and immunodetected with an anti-V5 monoclonal antibody (B). Lane M, markers (Prestained Protein SHARP MASSTM VII; Euroclone s.p.a. Milano-Italy). *E. coli* Rosetta (DE3) fractions containing the expression vector with the coding sequence for cf-HMGR (A and B, lanes 1-4). Samples were taken just before induction (lanes 1 and 2) or 4 h after induction (lanes 3 and 4). Soluble (lanes 1 and 3) and insoluble (lanes 2 and 4) bacterial fractions were obtained as described in Materials and Methods. The same number of bacteria was analyzed in each sample.

Fig. 2 Purification of the recombinant cf-HMGR protein by Ni2+- agarose affinity chromatography. Linear MALDI spectra of eluted fractions from buffer 5, buffer 6 and the purified cf-HMGR protein (A, spectra I, II and III, respectively). Purification was performed as described in Materials and Methods. Selective elution of cf-HMGR was obtained by increasing imidazole concentration up to 100 mM (B, lane 1). Proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. Lane M, markers (Prestained Protein SHARP MASSTM VII; Euroclone s.p.a. Milano- Italy).

Fig. 3 Alignment of HMGR sequences. In red is reported the poly-histidine tag, in gray is highlighted the validate sequence of the recombinant protein. a P04035, according to ‘‘UniProtKB’’ (http://www.uniprot.org/), b recombinant cf-HMGR; c MS/MS validated sequence.

Fig. 4 MS/MS spectra of (A) a tryptic peptide (447-455, SHmIHNRSK, m/z 1125.58) and (B) a peptic peptide (440-458, AAGHLVKSHMIHNRSKINL, m/z 2126.21).

Fig. 5 Enzymatic activity of the purified cf-HMGR protein as a function of pH (A). Enzymatic activity of cf-HMGR was measured as described in Materials and Methods, pH range was generated using 160 mM phosphate buffer. Activities at different pH values were relative to the activity measured at pH 6.8 (100%). Dose-response curve for pravastatin inhibition (B). Enzymatic activity was determined by adding 0.1 mM HMG-CoA together with the indicated pravastatin concentrations to the complete assay mixture.

Residual activity was expressed as a percentage of that obtained in the absence of pravastatin. All values are means ± SD from three independent experiments.

Figure 1

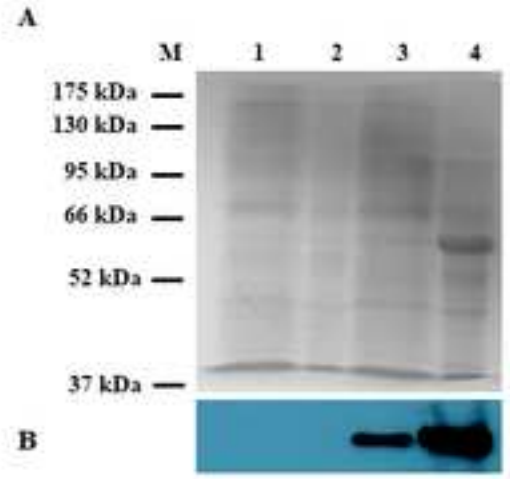

Figure 2

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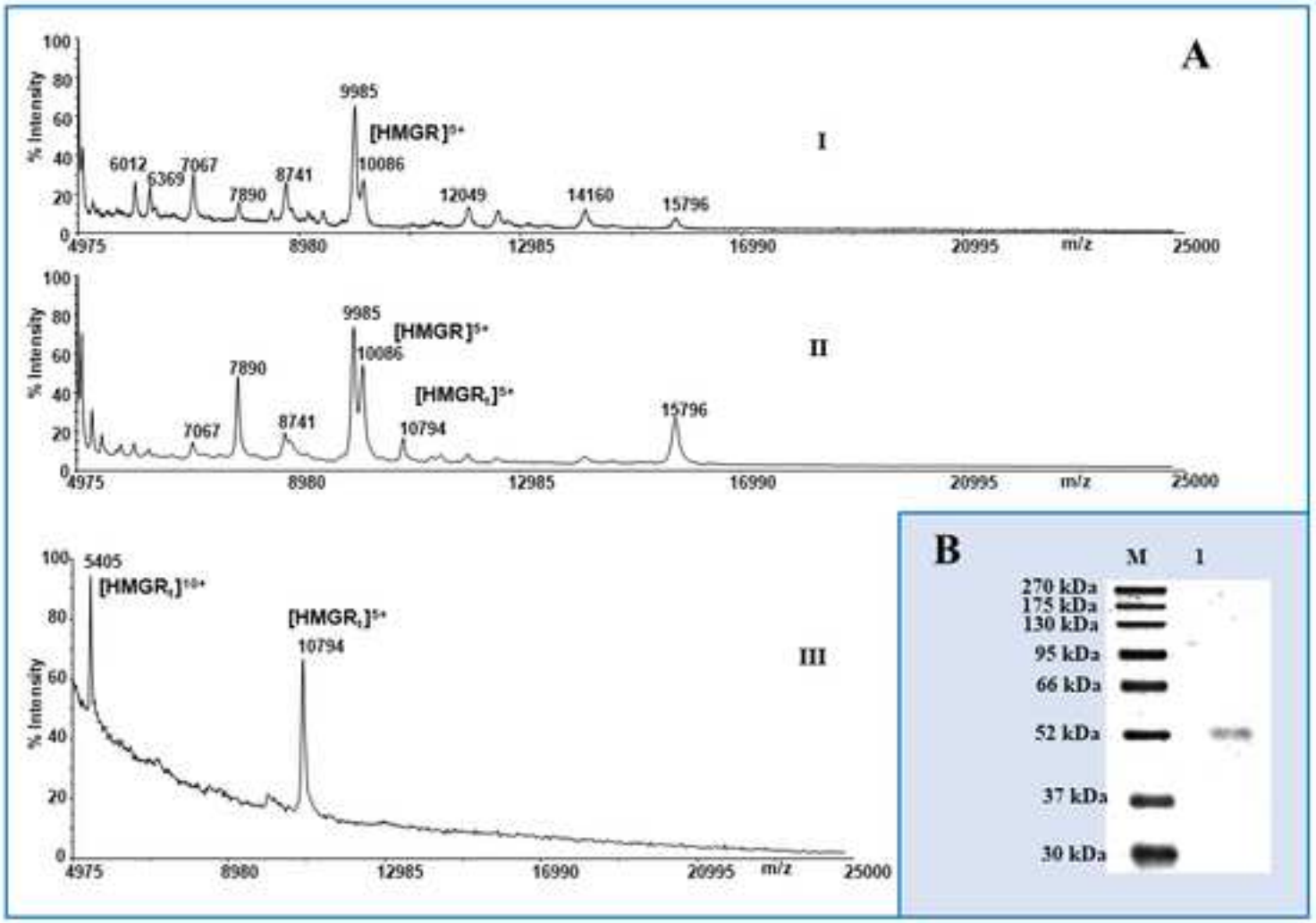

Figure 3

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Figure 4

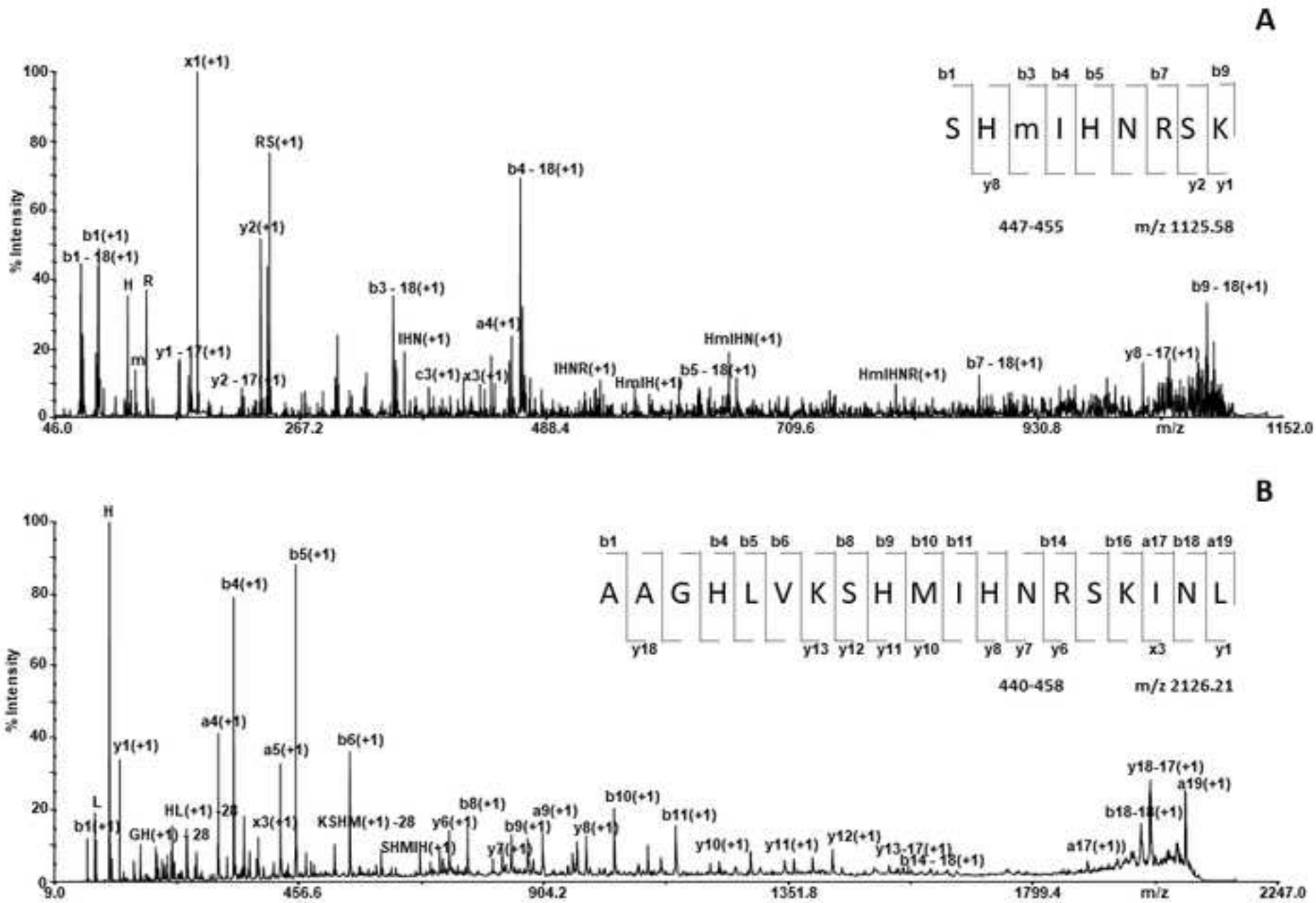

Figure 5

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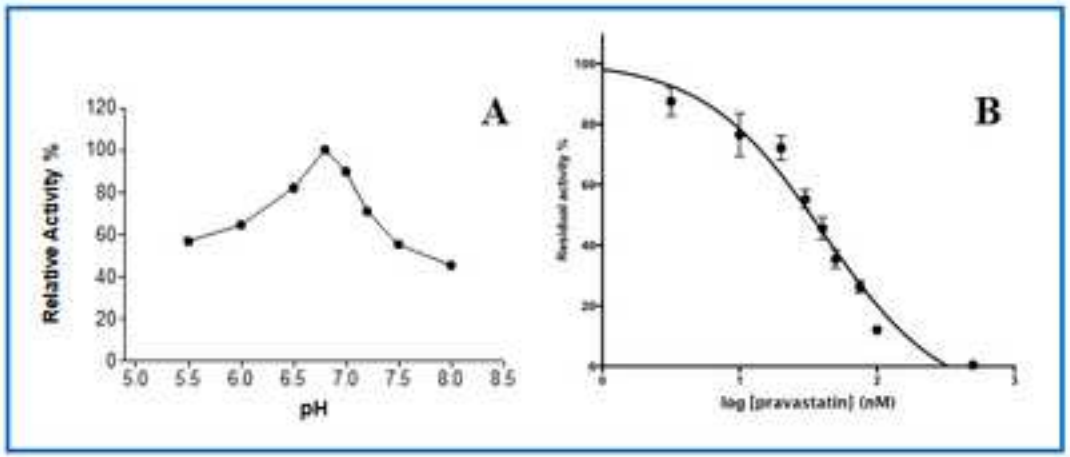

Supplementary Material

Click here to view linked References