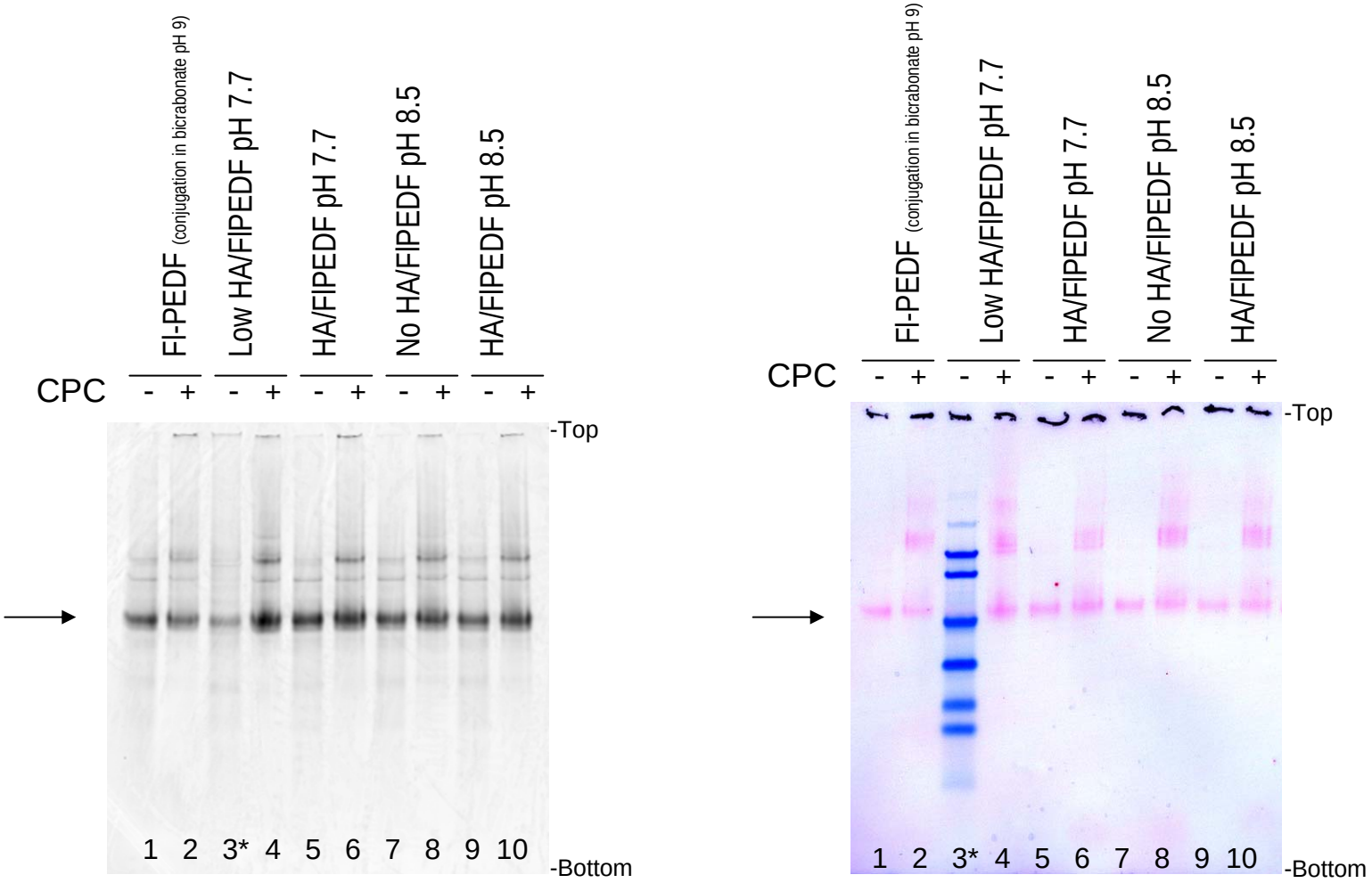
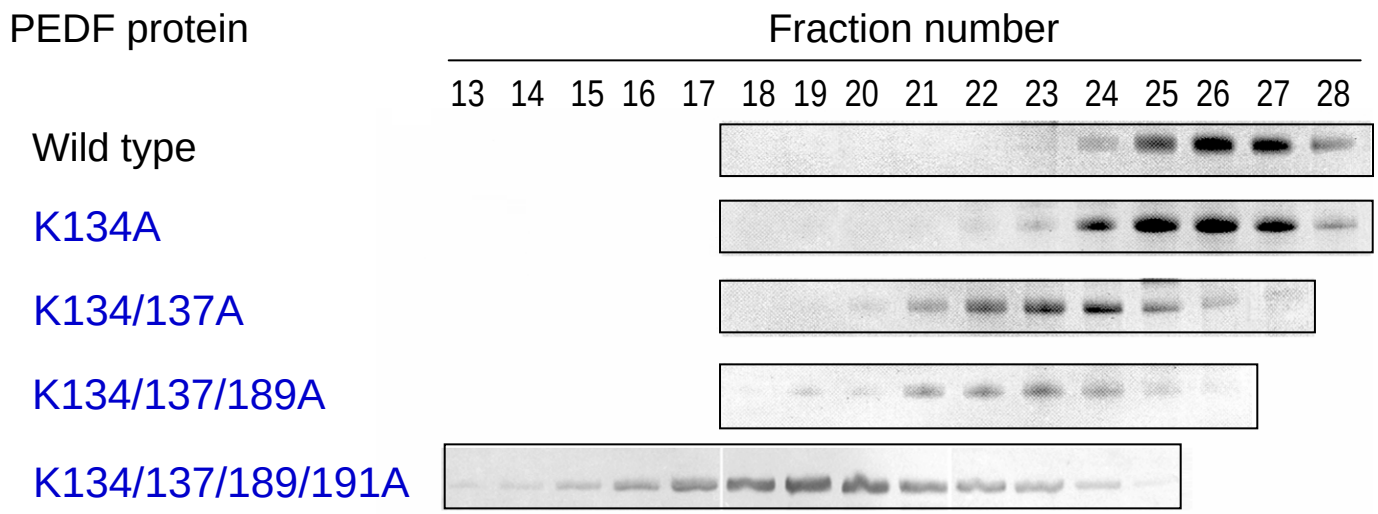
Fig. S1. HA binding of Fl-PEDF proteins (exp 12-01-07)



PEDF was preincubated without (No) or with 0.133 ug/ml (low) or 1.6 ug/ml HA in phosphate buffer at indicated pH or bicarbonate pH 8.5 (lanes 1 and 2) before Fl-conjugation and then assayed for HA binding. Reactions (lanes 2, 4, 6, 8 and 10) were with 2 ug PEDF protein, 10 ug HA and 10 ug BSA in 100 ul of PBS, incubated at 4 C for 1.5 hour. CPC was with 100 ul of 2.5% CPC in PBS incubated 1h at 37 C. Pellets were washed twice with 1.25% CPC in PBS. Pellets were resuspended in 25 ul 1X SDS-PAGE buffer, heated at 100 C for 3 minutes and applied to a 10-20% polyacrylamide gel with Tricine/SDS as running buffer. Lanes 1, 3, 5, 7, 9 and 11 were protein without HA/CPC precipitation (half of the protein in reaction was applied to gel except for lane 3* and 9, which had less due to spills). After electrophoresis, the gel was incubated in transfer buffer for 10 min at RT and then it was scanned with a lser-based Typhoon scanner (left). Then the proteins in the gel were transferred onto a nitrocellulose membrane and stained with Ponceau Red (right). Arrow points to migration position of the 50-kDa PEDF. Lane 3 had also an addition of 5 ul of commercial prestained markers (BioRad). All fluoresceinated PEDF proteins coprecipitated with HA.

Figure S2



Cation-exchange column chromatography of modified PEDF proteins with cumulative alterations on putative exposed lysines. Media of baby hamster kidney (BHK) cells stably transfected with expression vectors of PEDF mutants were subjected to POROS-HS column connected to a BioCAD Perfusion Chromatography Workstation. Elution was performed with a 50-500 mM NaCl gradient in buffer S. Fractions from the column were resolved by SDS-PAGE followed by Coomassie blue staining, except for K134/137/189/191A which was by western with Ab-PEDF antibodies. Altered PEDF protein bands in the excised gels and blots are shown as indicated to the left. Fraction numbers are indicated at the top.

Figure S3

HA binding.val

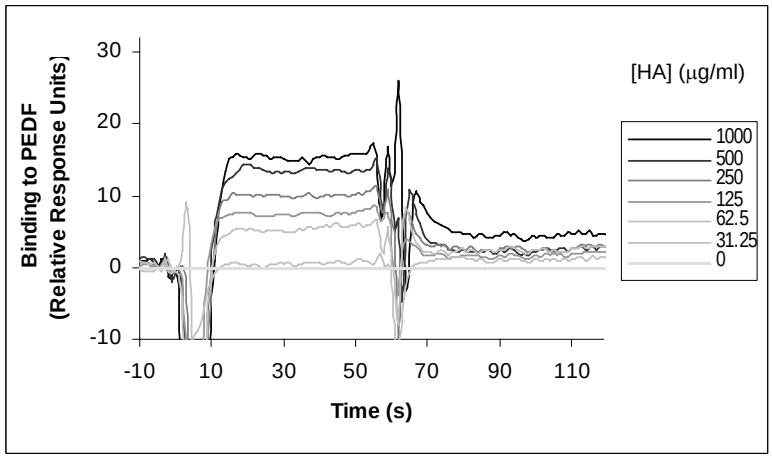
Click here to open structure. With the window with the structure active type ‘n’ to spin the structure. Type ‘s’ to stop spinning. You will need Cn3D to open this file. To download go to http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml.

Three-dimensional structure of human PEDF (from Protein Data Bank ID 1MV) to illustrate the location of the HA-binding site and basic amino acids in the vicinity. Positions of single alterations made in this study are highlighted as follows: in *blue* are basic amino acids K146, K147 and R149 residues are located in a turn between β-strand s2A and α-helix E, and positions K189, K191, K194 and R197 located in another turn between α-helix F and β-strand s3A, both within BX_7B HA-binding sites*; in *red* are acidic amino acids D256, D258 and D300 corresponding to the collagen binding site; and in *green* are K134 and K137 located in the vicinity of the HA-binding motif . The amino- and the carboxy-end terminus of the polypeptide are indicated as N(A) and C(A), respectively. The positions in the window of Sequence/Alignment Viewer correspond to sequence of the mature PEDF (without secretion signal peptide region) which starts at position 21.

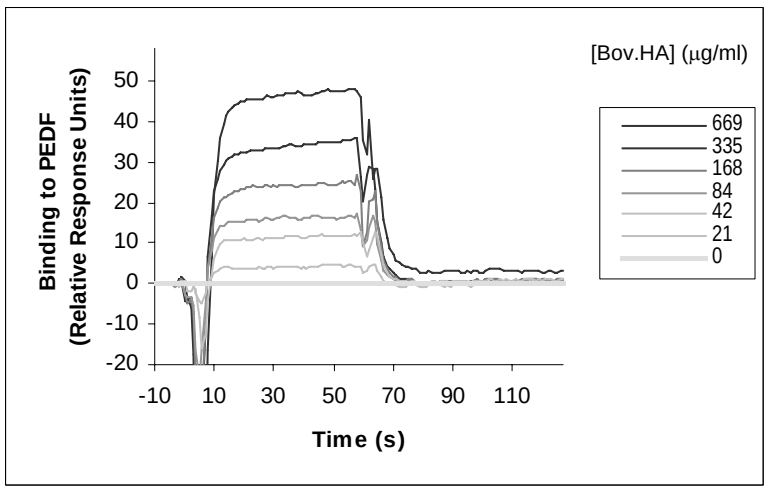
*Linear HA binding motifs are located at residues Lys^{189}-Lys^{197} and within Lys^{134}-Lys^{151}. The motifs conform to the sequence pattern BX_7B, where B represents lysine or arginine residues separated by seven amino acids, excluding aspartic or glutamic acid, except in one position in the second one. These sites are located in between an α-helix and β-strand, and may be required to present the motif in the appropriate conformation for HA binding.

Figure S4

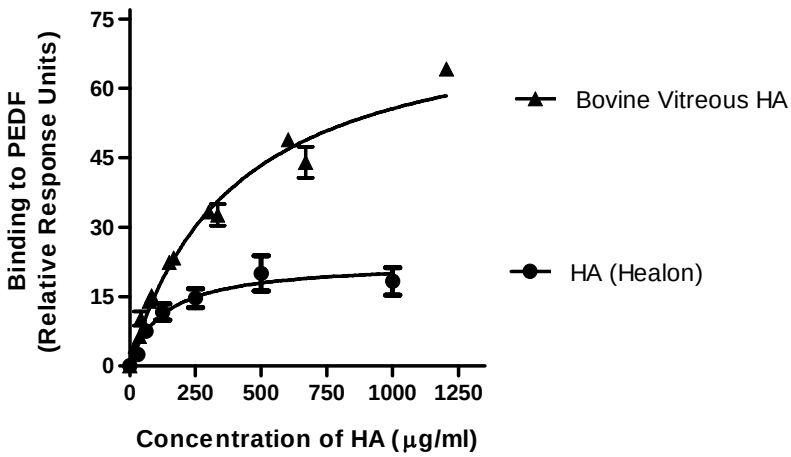
A. HA (Healon)



B. Bovine vitreous HA



C.



	HA (Healon)	Bovine Vitreous HA
Best-fit values		
BMAX	22.53 ± 2.5	77.58 ± 4.4
KD (μg/ml)	128.8 ± 45	394.3 ± 46

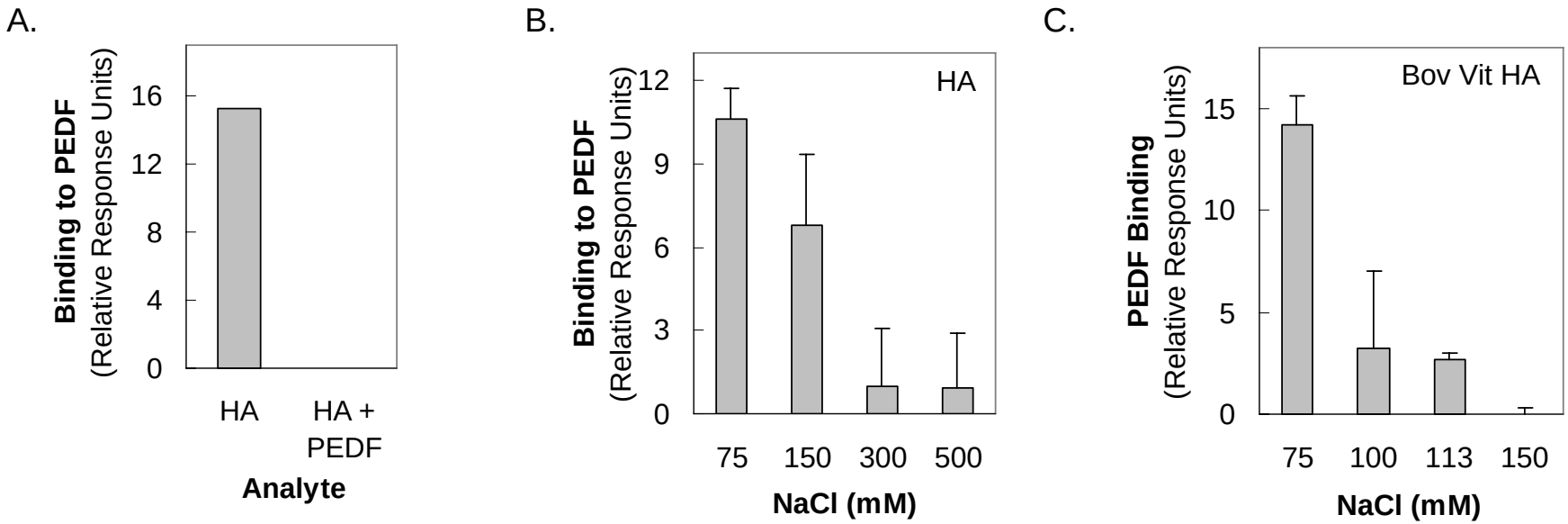
Real-time binding of HA to PEDF using Surface Plasmon Resonance (SPR)

SPR was performed as an alternative method to asses the binding of HA to immobilized PEDF in real time. PEDF was immobilized to the surface of a CM5 Sensorchip (Biacore) by amine coupling according to manufacturer's instructions.

(A-B) Increasing concentrations of HA (Healon) (*A*) or bovine vitreous HA (Worthington) (*B*) in 50% PBS (10 mM Na-phopshate pH 7.4, 75 mM NaCl) were injected over both PEDF and blank surface sensor chips at a flow rate of 10 µl/min during 1 min. This was followed by a 1 min injection of running buffer (50% PBS) at the same flow rate. Injection for each concentration was repeated twice. Two experiments were performed with each set of HA concentrations. Therefore four sets of sensograms were generated and analyzed. Differential response units (substracted from sensograms of reference cell without PEDF) were plotted. One of four sets of sensograms generated for each HA specie is shown.

C, Relative Response Units at equilibrium (y axe) per analyte concentration (x axe) were obtained using the Biaevaluation software (Biacore). These values were analyzed by non-linear regression using GraphPad set at one site binding for steady state affinity analysis. The best-fit values ± Std. Error for KD and BMAX were obtained and are shown in the table below the graph. Given the molecular weight for Healon as 4×10^6 (http://www.amo-inc.com/pdf/Healon.pdf) and of bovine vitreous HA (http://www.worthington-biochem.com/VHHA/cat.html) varying between $2 - 4 \times 10^6$, the KDs were calculated as 32 ± 11 nM and 131 ± 15 nM, respectively. These results suggest that Healon has higher affinity for PEDF than bovine vitreous HA.

Figure S5



Characterization of the binding of HA to PEDF using Surface Plasmon Resonance (SPR)
Analytes in binding buffer (50% PBS = 10 mM Na-phopshate pH 7.4, 75 mM NaCl, or as indicated) were injected over the PEDF surface sensor chip and reference cell (without PEDF) at a flow rate of 10 µl/min during 1 min followed by a 1 min injection of binding buffer at the same flow rate.
(*A*), Ligand competition to determine the HA-PEDF interaction in solution. HA (Healon; 0.2 mg/ml) was incubated for 30-60 minutes at room temperature in the presence or absence of human recombinant PEDF (0.04 mg/ml) and before injection. Relative Response Units at equilibrium were plotted after subtraction of the background produced by buffer and PEDF solution.
(B), and (*C)*, Effect of ionic strength on the HA-PEDF interaction. Binding of HA (Healon) or bovine vitreous HA at different NaCl concentrations was tested. HA (Healon, 0.2 mg/ml) or bovine vitreous HA (0.17 mg/ml) in binding buffer with increasing concentrations of NaCl were injected. Plots of the Relative Response units (at equilibrium) against NaCl concentration are shown.
We conclude that soluble PEDF efficiently competed with the immobilized PEDF for binding to HA in solution, implying that the HA-PEDF interactions take place in solution. The binding of HA to PEDF sensor chips decreased with increasing NaCl concentrations. Thus, electrostatic forces play a crucial role in the SPR HA:PEDF binding. However, the binding of bovine vitreous HA had a higher sensitivity to NaCl, implying it has less affinity for PEDF than HA from the other source.

Methods

HA affinity column chromatography was performed as described in Forteza et al Am. J. Respir. Cell Mol. Biol. 1999, 21: 666-674 and Tengblad A. BBA 1979; 578: 281-289 with the following modifications:
Preparation of HA-affinity resin—A total of 10 mg HA (bovine vitreous 70-4000 kDa; Worthington LS003909) was dissolved in 20 ml dH_2O and the pH was adjusted to 4.5 with 0.1 M HCl (L). The solution was mixed into 5 ml EAH sepharose 4B (GE Healthcare 17-0569-01) and 0.5 g EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Thermo Scientific 22980) were added. The mixture was rotated end-over-end at room temperature overnight. The pH was checked pH during the first hour, as it may decrease and if necessary adjusted to 4.5 with 0.1 M NaOH. Using sintered glass filter the solution was separated from the beads (FT) and then the beads were washed with 0.1 M Na acetate in 0.1 M NaCl (pH 4.0), followed by washes with 0.1 M Tris in 0.5 M NaCl (pH 8.3). These washes were repeated 5 times. Finally the beads were washed with distilled water and then equilibrated in binding buffer.

The relative amount of HA immobilized to the sepharose was measured by comparing the staining of precipitated HA with Alcian blue in the initial HA solution (L) and in FT. The Precipitation of HA with Alcian Blue was as described (Terry, D.E., Chopra, R. K., Ovenden, J., Anastassiades, T.P. 2000, Analytical Biochemistry 285: 211-219). A total of 100 µl of each L and FT were mixed with 200 µl Alcian Blue solution (0.05% Alcian Blue in 30 mM $MgCl_2$/0.1% Acetic acid/10% methanol). After incubation at room temperature for 1 h, the precipitate was separated by centrifugation at maximum speed in an eppendorf centrifuge for 10 min. The L and FT had similar size precipitate indicating that almost all the HA was immobilized to the sepharose, i.e., 2 mg bovine vitreous HA/ml wet resin. The expected amount was about 0.6 – 1.7 mg HA per ml wet resin.

To test the efficiency of HA-sepharose: (as from Forteza et al. 1999)--Incubate 25 µl HA-Sepharose mixed with 0.1, 0.4, 2 µg bHABP (bHABP, bovine nasal cartilage) (US Biologicals H7980-35) in 100 µl PBS/10% glycerol with rotation end-over-end for 1 h at RT. The samples were centrifuged gently (30 x g) for 10 s. The beads were washed with three times with 100 µl of binding buffer and resuspended in 25 µl of SDS/PAGE/DTT sample buffer. The mixture was heated at 100ºC for 5 min and centrifuged and the supernatant was resolved by SDS-PAGE followed by transferring to nitrocellulose membrane and staining with Ponceau Red. Proteins bands were visualized. bHABP bound to the resin (0.08 mg bHABP per 1 ml of wet resin).

HA-affinity column chromatography was performed as for Heparin-affinity column chromatography (Alberdi et al 1998 Biochemistry 37: 10643)--HA-sepharose resin (0.5 ml) was packed in a disposable column and was washed and equilibrated with Buffer H containing 20 mM NaCl. A total of 10 µg of PEDF in 20 mM NaCl in buffer H (500 µl) were applied to the column and reloaded several times. After incubation for 1 h at 4 ºC (mixing every 10 min), the flow-through was collected (FT). The column was washed with binding buffer (10 column-volumes), and the bound material was eluted with a step-gradient of NaCl in buffer H (20, 50, 100, 150, 200, 300, 400, 500, 1M, 2M NaCl) (1 or 2 column-volumes each). The amount of PEDF protein in the fractions was determined by established methods.

The concentration of stock solutions of HA species was determined by the uronic acid assay as described previously by Blumenkrantz and Asbone-Hansen (Anal. Biochem. 54:484, 1973) with minor modifications. Briefly, samples, standards and blank were mixed with cold solution of sodium tetraborate (12.5 mM) in sulfuric acid and incubated at 100°C for 5 min. and then at room temperature for 2-5 min. After addition of m-phenylphenol (0.15 %) in NaOH (0.5 %), absorbance at 560 nm was read.