ELSEVIER

Journal of Chromatography B, 776 (2002) 89–100

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Catalogue of soluble proteins in the human vitreous humor: comparison between diabetic retinopathy and macular hole

Toyofumi Nakanishi[a], Reiko Koyama[b], Tsunehiko Ikeda[b], Akira Shimizu[a,*]

[a]*Department of Clinical Pathology, Osaka Medical College, 2–7 Daigakucho, Takatsuki, Osaka 569-8686, Japan*
[b]*Department of Ophthalmology, Osaka Medical College, 2–7 Daigakucho, Takatsuki, Osaka 569-8686, Japan*

Abstract

Two-dimensional gel electrophoresis and mass spectrometry were used to make a catalogue of soluble proteins in the human vitreous humor (VH). Fifty-one different proteins were identified on silver-stained two-dimensional (2D) gel patterns with VH proteins obtained from diabetic retinopathy and macular hole. Thirty of these have not been listed in the reported 2D profiles of plasma. Immunoglobulin (Ig), α1-antitrypsin, α2-HS glycoprotein,and complement C_4 fragment showed stronger spots in VH with diabetic retinopathy patient samples than those with macular hole. Pigment epithelium-derived factor, a potent inhibitor of angiogenesis in the cornea and vitreous, was clearly detected in VH with diabetes. It is impressive that the inhibitor increases in the vitreous with proliferative angiogenesis.

Keywords: Human vitreous humor; Diabetic retinopathy; Macular hole; Proteins

1. Introduction

The analysis of soluble protein profiles in the vitreous humor (VH) may elucidate the pathogenesis of various retinopathies, especially those accompanied by blood vessel growth into the vitreous. In diseases such as retinopathy of prematurity and proliferative diabetic retinopathy, the main causes of visual loss are the development of new blood vessels at the junction of vascularized tissue and the avascular retina, and vascular growth into the vitreous, resulting in hemorrhage and retinal detachment [1–3]. In such diseases, the production of angiogenic and antiangiogenic factors by retinal cells may change, and consequently, the concentration of these factors in the VH may also change [4–6]. Since Raymond and Jacobson [7] indicated an inhibitory factor of angiogenesis presented in an extract of bovine VH, a number of researchers have analyzed VH proteins from the eyes of animals [8,9]. However, there have been no reports of experiments in humans, probably because of the difficulty of sample collection and the limitations of detection by conventional protein analysis techniques. Modern mass spectrometry assisted by complete genome information will play an essential role in the study of proteins because of its sensitivity, resolution, and ability to analyze complex mixtures of molecules quickly. The combination of protein separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with mass spectrometric analysis of proteins digested enzymatically in-gel, followed by protein database searching is a very efficient tool for

*Corresponding author. Tel.: +81-726-84-6448; fax: +81-726-84-6548.
E-mail address: shimizu@poh.osaka-med.ac.jp (A. Shimizu).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00078-8

protein identification in complex biological systems [10,11].

In this study, we began assembling a catalogue of proteins expressed in human VH using 2D-PAGE and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS), and electrospray ionization (ESI) ion-trap tandem mass spectrometry (MS–MS). We compared the expressed proteins in VH between diabetic retinopathy and macular hole. Diabetic retinopathy accompanies abnormally high vascular growth, and macular hole does not.

2. Materials and methods

2.1. Sample preparation, 2D-PAGE, image analysis and in-gel digestion

The vitreous humors (VHs) were obtained from three patients with diabetic retinopathies and two patients with macular hole. The VHs were dialyzed with distilled water to remove salt using Biodialyzer™ (membrane: B010K; Cypress, Tokyo), which can remove molecules smaller than 1000 Da. About 500 μl VH were dialyzed overnight at 4 °C with two changes of 3 l distilled water. The solution was freeze-dried under a vacuum. One hundred μg of protein, which was determined by the Lowry method, were solubilized in the rehydration buffer (8 *M* urea, 2.0% NP-40, 30 m*M* dithiothreitol (DTT), 2.0% immobilized pH gradient (IPG) buffer 3–10 NL) and the samples were stored at 4 °C for 12 h. For first-dimension gel electrophoresis, isoelectric focusing (IEF) was performed using a Pharmacia Multiphor II apparatus according to the manufacturer's instructions as modified as by Arnott et al. [10]. IPG gel strips (18 cm, non-linear pH 3–10, Amersham–Pharmacia, Sweden) were rehydrated overnight in a cassette with standard rehydration buffer (described above), and aligned on the IEF tray. Samples were loaded adjacent to the anode, and the voltage was linearly increased from 300 to 3500 V over 4 h, followed by 4 h at 3500 V. A total volt hour product of 15 kVh was used.

After IEF, the IPG gel strip was equilibrated with 5 ml of 50 m*M* Tris–HCl, pH 8.8, 6 *M* urea, 2% sodium dodecyl sulfate (SDS), 30% glycerol and 100 m*M* DTT for 20 min.

Second-dimensional electrophoresis was carried out at a constant current of 10 mA per gel for 30 min, and 20 mA per gel until the tracking dye reached the cathode.

After removing the carrier ampholytes and fixation, the gel was visualized by 1% Coomassie brilliant blue (CBB) staining for 30 min or non-glutaraldehyde silver staining with 0.02% sodium thiosulfate for 20 min at 4 °C. The images of the stained gels were studied with ImegaMaster 2D Elite software (Amersham–Pharmacia). The CBB and silver-stained gels were digitized and the gels were matched using a constellation-matching algorithm whereby each spot was located according to its spatial relation to a defined number of nearest-neighbor spots. The matched spots were relatively quantified by adding the intensities of all pixels against that of spot number 61 containing apolipoprotein A-I, which was selected because spots found in 2D-PAGE for both diabetic retinopathy and macular hole were of similar intensity. All visible spots were numbered arbitrarily.

Protein spots were excised from the gel and placed in 1.5-ml microtubes (AMR, Tokyo, Japan). The gel slices were washed by shaking for 30 min in 25 μl of 50% methanol and then dried under a vacuum. Disulfide bonds were reduced with 50 m*M* DTT in 50 m*M* ammonium bicarbonate (pH 8.5) by incubation for 1 h at 56 °C and alkylated with 100 m*M* iodoacetoamide in the same buffer for 45 min in the dark at room temperature. Excess reagents were removed and the gel slices were washed twice. After the buffer was discarded, the gel pieces were dehydrated with 100% acetonitrile and then dried by vacuum centrifugation. The gel pieces were then re-constituted in 10 μl of digestion buffer containing 250 ng TPCK modified trypsin (Promega, Madison, WI) at 37 °C for 18 h. The peptide solution was recovered and the gel pieces were extracted with 30 μl of digestion buffer and 100% acetonitrile. The combined solution was concentrated, resolved with 0.1% trifluoroacetic acid (TFA) and stored frozen until use.

VH proteins were also initially isolated using ion-exchange (PL-SAX; Cypress) column chromatography. The buffer system was a linear gradient of buffer A mixed with buffer B from 1% B to 99% B in 40 min. Buffer A was 0.01 *M* Tris–hydrochloride, pH adjusted to 7.4. Buffer B was 0.01 *M* Tris–

hydrochloride, 0.4 *M* NaCl, pH adjusted to 7.4. The flow-rate was 1.0 ml/min. The freeze-dried proteins from each fraction were identified in the same way as described for proteins from 2D-PAGE spots.

2.2. Mass spectrometric identification

MALDI-TOF mass spectra were acquired on a Voyager DE-PRO MALDI-TOF mass spectrometer (PE Biosystems, Framingham, MS) equipped with a delayed extraction source and a 337-nm pulsed nitrogen laser. For the sample matrix, α-cyano-4-hydroxycinnamic acid was dissolved in a solution containing 50% acetonitrile and 0.1% TFA at a concentration of 10 g/l.

ESI-MS–MS experiments were performed with a LCQ^{DECA} (ThermoQuest, San Jose, CA, USA) equipped with a Monitor C_{18} column (0.2×50 mm). The solvent system for on-line reversed-phase liquid chromatography was a linear gradient of solvent A mixed with solvent B from 5% B to 60% B in 40 min. Solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in acetonitrile. The flow-rate was 1–2 μl/min. A collision energy of 28–35 eV, depending on the charge state of the daughter ions, was applied; the gas pressure in the collision cell was regulated to 6.0×10^{-5} mbar. Protein identification was performed via a peptide mass, collision-induced dissociation (CID) mass spectra database using MS-fit and MS-tag (SwissProt).

3. Results and discussion

3.1. 2D-PAGE of VH derived from diabetic retinopathy patients and macular hole

Fig. 1a,b shows silver-stained 2D gel electrophoresis patterns of VH proteins derived from a patient with diabetic retinopathy (a) and from a patient with macular hole (b). We repeated the 2D-PAGE with the same materials, and with materials obtained from three patients with diabetic retinopathy and two with macular hole. The profiles were reproducible in the same materials and were similar among materials from the same disease. The mass spectrometric analysis and database search of 412 spots expressed in VH derived from a patient with diabetic retinopathy (Fig. 1a) allowed us to characterize 113 spots. However, a large number of the spots were isoforms of one another. The number of different protein species identified was 50 by the analysis shown in Fig. 1a. These are listed in Table 1. By 2D gel from another diabetic patient, additionally aquapolin-CHIP (peptide sequence, NDLADLLDIDYNHWIFW 5–21 was assigned by ESI-MS–MS, and the observed p*I* and MW coincided with those reported, p*I*, 7.17; MW, 9723.4) and vacuolar ATP synthase subunit E (IKVLQAQDDLVNAMKEAASK 79–98; p*I*, 6.00; MW, 26 128.4) were identified. The proteins identified by 2D-PAGE with VH were 52 in total. The molecular mass and p*I* observed for most proteins were close to the theoretical values based on a database, SwissProt. From spot no. 48 (p*I*, 4.2; MW, 34 600), transthyretin (TTR) peptides were detected clearly. However, the p*I* and molecular mass of the spot did not coincide with those of TTR. A molecule in spot no. 48 is probably a covalent polymer of a TTR fragment, or a molecule formed by a disulfide bridge between TTR and another unknown protein due to inappropriate reducing.

Most of these spots were also found in the profiles of patients with macular hole. Some spots found in VH samples corresponded to major plasma proteins, which were reported by 2D-PAGE [12]. These are albumin, α_1-antitrypsin, α_2-HS glycoprotein, transferrin, haptoglobin α_1- and α_2-chains, complement C_4 fragment, Gc globulin, apolipoprotein A-I, immunoglobulin (Ig) heavy and light chains, hemoglobin (Hb) α- and β-chains and transthyretin, and so on. The relative amount of the major proteins to the levels of albumin in VH was markedly different from those of plasma. For example, the ratios of transthyretin to albumin and of transferrin to albumin were ca. 1:10 and 1:3 in VH, although the ratios are ca. 1:100 and ca. 1:15 in plasma, respectively. Although Hb chains were detected in VH, spots of coagulation factors or orosomucoid were very faint or not visible. These data support the idea that plasma proteins revealed on the 2D-PAGE with VH were not contaminated by plasma during sampling, but systematically transferred through a specific blood–retina barrier in vivo.

We also isolated VH proteins from a patient with diabetic retinopathy using ion-exchange column chromatography, and identified five VH-specific proteins by ESI-MS–MS analysis with tryptic peptides.

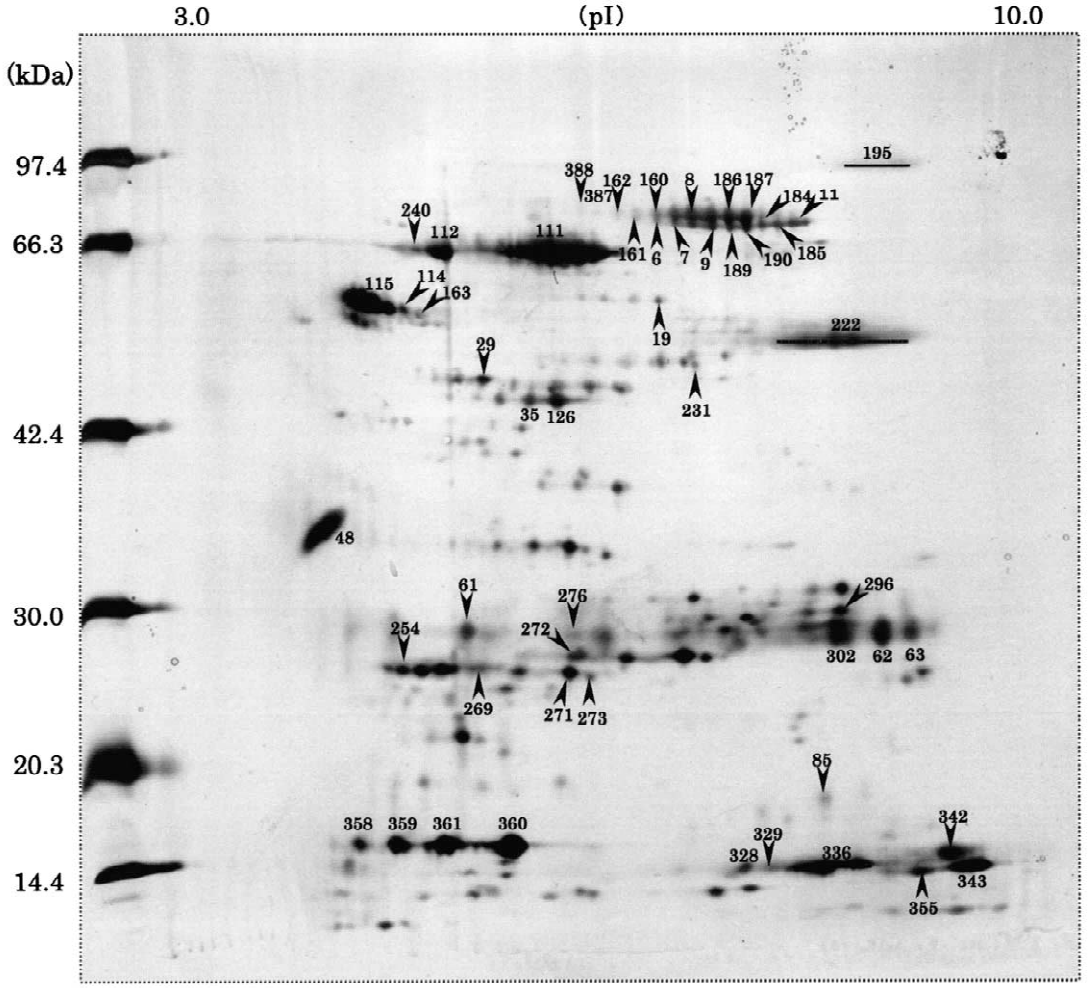

Fig. 1. Silver-stained gel image of proteins in VH derived from a patient with diabetic retinopathy (a) and a patient with macular hole (b). The protein load was 100 μg on the first-dimensional IEF gel. The positions of molecular mass markers are shown on the left and isoelectric points are shown at the top in the gels.

These were DNA binding protein (sequence assigned peptide: SSSPYSKSPVSK; 146–157), aquapolin-CHIP (NDLADLLDIDYNHWIFW; 5–21), glial fibrillary acidic protein (LALDIEIATYR; 356–367), thyroid receptor interaction protein (HPCDCLGQKHK; 45–55) and uracil-DNA glycosylase (QWMLANIADNK; 5–15). The proteins identified by 2D-PAGE and column chromatography with VH were 56 in total.

The stained spots shown in Fig. 1a,b were relatively quantified, and proteins, α_1-antitrypsin, α_2-HS glycoprotein, complement C_4 fragment, immunoglobulin (Ig) heavy and light chains, showed stronger spots in VH with diabetic retinopathy patient samples than those with macular hole. A few unidentified spots clearly showed stronger spots in VH with diabetic retinopathy patient samples than those with macular hole, and vice versa.

3.2. VH-specific proteins

By comparing the 2D-PAGE profiles obtained from VH protein with those of plasma reported in literature, many spots were detected in only VH, but

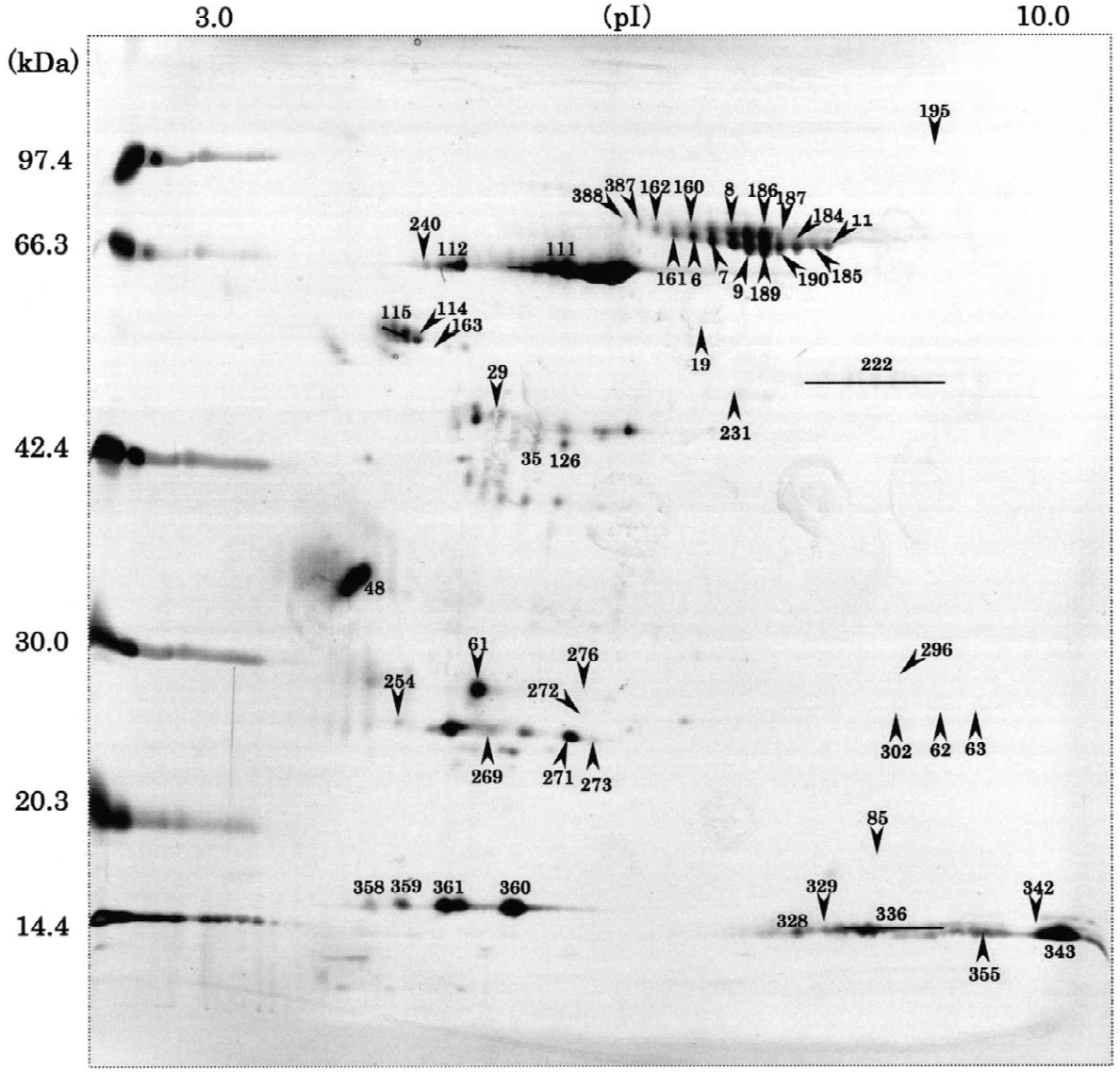

Fig. 1. (*continued*)

not in plasma. The specific proteins in VH were located at isoelectric points between 5.0 and 9.0, and at molecular weights between 20 and 65 kDa. This was a relatively smaller region with higher p*I*. The VH-specific proteins (29 proteins) identified are shown in Table 1. The five peptides detected by preliminary experiment using an ion-exchange column were also not reported in plasma [13]. In total, 35 VH-specific proteins were recognized in the present work.

Cystatin C (spot no. 336) spot was relatively strong. This protein is a mammalian cysteine protease inhibitor, synthesized in various amounts by many kinds of cells and appearing in most body fluids [14]. The concentration of human cystatin C is markedly higher in cerebrospinal fluid than in blood

Table 1
Peptide sequences of proteins contained in VH as determined by mass spectrometry

Spot no.	Protein	Reported in plasma[a]	MW[b]	pI[b]	[M+H]+[c]	Residue	Identified peptides, sequence from database	MW[d]	pI[d]
6–9	Transferrin	+	76 981.3	6.70	1249.6	454–464	SASDLTWDNLK	74.1–77.0	5.9–6.7
160–162					1276.6	300–310	EFQLFSSPHGK		
184–187	Fatty acid coenzyme A ligase5	−	72 196.2	6.45	1479.0	84–97	GLAVSDNGPCLGYR		
387,388					2186.1	379–398	VRVIVTGAAPMSTSVMTFFR		
					2564.0	167–188	GFTPSLKVIILMDPFDDDLKQR		
	SnoN2	−	71 795.8	6.57	2384.6	561–579	LEQIMKQKCTCDSNLEKDK		
					2707.1	594–615	LDHAEADRQELQDELRQEREAR		
					3339.2	507–534	QQLQMEVKMLSSSKSMKELTEEQQNLQK		
9	ETF-ubiquinone oxidoreductase	−	68 564.5	7.17	1211.9	255–263	QLYKKFDLR	75.0	6.7
					1429.6	345–357	HHPSIRPTLEGGK		
					1451.7	264–276	ANCEPQTYGIGLK		
11	SP100-B	−	78 145.0	7.81	1274.4	88–97	MFEDSQDSCR	73.7–74.1	6.9–7.3
189,190					1479.0	70–81	KTFPFLEGLRDR		
					1833.9	431–446	APMTSRSTSTWRIPSR		
					2096.9	88–104	MFEDSQDSCRNLVPVQR		
					2563.1	506–528	TESSQASDMMDTMDVENNSTLEK		
	Angiotensin-converting enzyme	+	79 334.5	7.41	1314.5	1–10	MLNLCFMLTR		
					2058.8	95–111	KFDVNQLQNTTIKRIIK		
					2096.9	448–463	IAFIPFSYLVDQWRWR		
					3808.4	61–93	NYNTNITTETSKILLQKNMQIANHTLKYGTQAR		
19	Nuclear receptor subfamily 1	−	54 409.8	6.11	1812.3	442–455	TFNHHHAEMLMSWR	61.2	6.4
					1925.2	189–204	EMGMLAECLLTEIQCK		
					2224.7	91–110	MPAETLYQGETEVAEMPVTK		
					2283.8	352–370	IRNSGISDEYITPMFSFYK		
					2806.7	371–395	SIGELKMTQEEYALLTAIVILSPDR		
29	Dystrophin/utrophin-associated protein	−	55 669.8	6.00	1109.6	278–287	SMPTSMALDR	50.2	5.3
					1587.4	75–90	FNVANGGPAPDVVSDK		
					3838.5	130–163	VEIVDPVDIYLNLLRTIFDFHAIKGLLTGPSQLK		
	Pigment epithelium-derived factor	−	46 386.3	6.00	1251.6	400–411	DTDTGALLFIGK		
					1384.7	334–345	LQSLFDSPDFSK		
					1560.4	54–67	LAAAVSNFGYDLYR		
					1895.6	198–214	EIPDEISILLLGVAHFK		
					1957.3	107–123	ALYYDLISSPDIHGTYK		
					2090.6	263–281	IAQLPLTGSMSIIFFLPLK		
35	Guanine nucleotide binding protein	−	42 143.4	5.48	1179.8	93–102	AMDTLKIPYK	47.9	5.6
					1320.7	61–72	IIHGSGYSDEDK		
					1493.9	108–120	AHAQLVREVDVEK		
					2626.9	93–114	AMDTLKIPYKYEHNKAHAQLVR		
	Apolipoprotein A-IV	+	43 384.8	5.22	1708.1	245–259	QRLAPLAEDVRGNLR		
					2045.7	74–90	DSEKLKEEIGKELEELR		
					2705.7	285–306	RRVEPYGENFNKALVQQMEQLR		
48	Transthyretin	+	15 859.2	5.10	1366.7	42–54	GSPAINVAVHVFR	34.6	4.2
					1522.7	55–68	KAADDTWEPFASGK		
					2451.2	81–103	ALGISPHEHAEVVFTANDSGPR		
					2489.3	69–96	TSESGELHGLTTEEEFVEGIYVEIDTK		
61	S100 calcium-binding protein	−	13 229.0	6.00	1456.6	26–38	LGHPDTLNQGEFK	27.0	5.2
					1808.0	11–25	NIETIINTFHQYSVK		
					2177.4	94–114	MHEGDEGPGHHHKPGLGEGTP		
	Glutathione *S*-transferase	+	26 689.0	5.37	1744.5	1–15	SCESSMVLGYWDIR		
					1995.4	83–98	YIARKHNMCGETEEEK		
					3312.5	198–225	IAAYLQSDQFCKMPINNKMAQWGNKPVC		

Table 1. Continued

Spot no.	Protein	Reported in plasma[a]	MW[b]	p*I*[b]	[M+H]+[c]	Residue	Identified peptides, sequence from database	MW[d]	p*I*[d]
	Apolipoprotein A-I	+	30 726.3	5.30	1235.7	13–23	DLATVYVDVLK		
					1386.7	251–262	VSFLSALEEYTK		
62	Phosphoglycerate mutase	−	28 766.4	8.99	1046.1	34–43	GTEEAKRGAK	26.7	7.8
					1488.3	22–33	FCGWFDAELSEK		
					1544.3	117–129	PSFDIPPPPMDEK		
					1835.8	47–61	DAKMEFDICYTSVLK		
					2240.5	158–176	DTIARALPFWNEEIVPQIK		
	Syntaxin 5	−	34 086.4	9.00	1012.0	129–137	LASMSNDFK		
					1152.9	188–197	DVAIDMMDSR		
					1708.5	33–46	AVRQRSEFTLMAKR		
					1943.3	138–153	SVLEVRTENLKQQRSR		
	Fibroblast growth factor-23	+	27 9541.1	9.17	1813.0	100–114	GNIFGSHYFDPENCR		
					2211.8	162–179	NEIPLIHFNTPIPRRHTR		
					2665.1	1–23	LGARLRWVCALCSVCSMSVLR		
63	GOS28/P28 protein	−	28 966.3	9.36	1298.6	40–50	LCTSYSHSSTR	27.0	8.1
					1640.9	217–230	FPAVNSLIQRINLR		
					1795.4	71–85	MFETMAIEIEQLLAR		
					1925.8	217–232	FPAVNSLIQRINLRKR		
85	Prostaglandin D2 synthase	+	21 085.8	8.33	1582.6	43–56	WFSAGLASNSSWLR	17.7	7.5
					1745.3	93–108	TMLLQPAGSLGSYSYR		
					1911.4	169–185	AQGFTEDTIVFLPQTDK		
					1919.8	67–85	SVVAPATDGGLNLTSTFLR		
111	Apoptosis inhibitor hiap-2	−	69 922.4	6.27	947.9	442–448	REEEKEK	69.1	5.7
					960.8	234–241	DDAMSEHR		
					1728.7	50–65	MSTYSTFPAGVPVSER		
					2129.6	242–258	RHFPNCPFLENSLETLR		
	Albumin	+	69 227.2	5.99	927.7	162–168	YLYEIAR		
					947.9	222–229	LKCASLQK		
					960.8	427–434	FQNALLVR		
					1468.3	361–372	RHPDYSVVLLLR		
					1925.4	589–603	ETCFAEEPTMRIRER		
112	Albumin	+	69 367.4	5.92	927.6	162–168	YLYEIAR	69.4	5.0
					960.7	427–434	FQNALLVR		
					1450.9	106–117	ETYGEMADCCAK		
					1467.9	361–372	RHPDYSVVLLLR		
					1640.2	438–452	KVPQVSTPTLVESR		
					2045.2	397–413	VFDEFKPLVEEPQNLIK		
114,115	α_1-Antitrypsin	+	46 689.0	5.10	1110.6	315–324	LSITGTYDLK	59.9–61.0	4.7–4.5
					1333.8	150–160	LVDKFLEDVKK		
					1641.8	50–63	ITPNLAEFAFSLYR		
					2574.3	126–149	TLNQPDSQLQLTTGNGLFLSEGLK		
					3402.6	35–63	TDTSHHDQDHPTFNKITPNLAEFAFSLYR		
126	Apolipoprotein A-IV	+	45 335.4	5.23	1083.5	201–209	LTPYADEFK	47.7	5.7
					1488.1	317–328	ALVQQMEQLRQK		
					2045.7	94–110	DSEKLKEEIGKELEELR		
					2705.0	305–326	RRVEPYGENFNKALVQQMEQLR		
	CGI-180 protein	−	41 269.2	5.37	1166.4	1–10	EVLRPQLIR		
					1640.4	1–14	EVLRPQLIRFDGR		
					1911.3	65–81	APSLLYKHIVGKRGDTR		
					1925.4	286–302	KDPNAEGRYNLYTAEGK		

Table 1. Continued

Spot no.	Protein	Reported in plasma[a]	MW[b]	pI[b]	[M+H]+[c]	Residue	Identified peptides, sequence from database	MW[d]	pI[d]
163	Protein kinase C	−	57 010.6	5.42	2560.1	434–454	KEIQPPYKPKACGRNAENFDR	59.4	4.8
					2663.1	162–184	LTDFNFLMVLGKGSFGKVMLSER		
					2721.6	5–28	NLVPMDPNGLSDPYVKLKLIPDPK		
					2721.6	76–99	NDFMGSLSFGISELQKASVDGWFK		
	Gc globin	+	52 950.2	5.40	2560.1	66–87	EVVSLTEACCAEGADPDCYDTR		
					3339.8	304–332	TAMDVFCTYFMPAAQLPELPDVELPTNK		
186	Glycerol-3-phosphate dehydro-genase	−	80 834.4	7.23	1210.1	580–588	ELNWDDYKK	76.0	6.9
					1283.8	114–124	LIHGGVRYLQK		
					1580.3	255–269	KTDPQTGKVHVSGAR		
					1601.2	597–608	KFLYYEMGYKSR		
					1641.7	558–572	LAFLNVQAAEEALPR		
195	Ig heavy chain	+	51 409.3	7.80	1186.6	122–133	GPSVFPLAPSSK	86.1	7.9
					1677.8	298–311	FNWYVDGVEVHNAK		
222	Signal recognition particle protein		49 636.7	8.68	1101.3	37–46	ALIQSDVNIK	55.5	7.6
					1133.4	102–112	ILFLGLQGSGK		
	Ig λ heavy chain	+	52 728.2	8.74	1882.2	391–406	EPTSPPFRPCPEPDEK		
					1857.9	477–493	GGLGHPLPELADELRRK		
					2232.9	160–179	LLFAGSRSQLVQLPVADCMK		
					2248.8	160–179	LLFAGSRSQLVQLPVADCMK		
	Unknown protein (Acc. no. 15929862)	−	52 265.7	8.24	1882.2	314–330	GPLNSDRSDYFAAWGAR		
					2248.8	186–203	LHPVLHKEEKQHLERLNK		
					2284.5	91–108	QICGTHRQTKKMFCDMDK		
					2704.8	313–336	RGPLNSDRSDYFAAWGARVFSFGK		
231	ER81 protein	−	55 157.7	6.00	850.9	325–331	EGPTYQR	52.0	6.6
					1037.2	206–213	EGRPMYQR		
					1109.2	380–388	NRPAMNYDK		
240	α-Actinin	−	68 762.8	5.07	948.1	381–388	SIVNYKPK	70.3	4.6
					1712.1	207–220	LLETIDQLYLEYAK		
					1925.8	1–16	LENRVPENTMHAMQQK		
					2808.4	155–179	VEQIAAIAQELNELDYYDSPSVNAR		
	FYVE-finger protein EIP1	−	69 078.7	5.63	931.8	193–200	ITDVLDQK		
					1036.5	201–208	NYVEELNR		
					1283.1	542–551	QCEKEFSISR		
					1435.4	321–332	TEMEIAMKLLEK		
					1494.2	521–533	EVNQALKGHAWLK		
	Guanine nucleotide exchange factor	−	66 313.5	5.17	883.7	584–590	LRSHENK		
					1036.5	1–9	MDNLSDTLK		
					1902.4	242–258	EMIFEVLAPLAENDAIK		
	α_{1B}-Glycoprotein	+	51 890.3	5.00	2471.2	453–474	SWVPHTFESELSDPVELLVAES		
254	Lipoprotein Gln I	+	28 346.2	5.27	1012.1	210–218	AKPALEDLR	24.4	4.9
					1226.3	1–10	DEPPQSPWDR		
					1301.4	77–87	LNLEKETGELR		
					1380.4	164–174	THLAPYSDELR		
					1723.3	122–136	VEPLRAELQEGARQK		
					1815.1	24–40	DSGRDYVSQFQGSALGK		
269	Lipoprotein Gln I	+	28 346.2	5.27	1012.1	210–218	AKPALEDLR	24.5	5.3
					1226.3	1–10	DEPPQSPWDR		
					1302.3	77–87	LNLEKETGELR		
					1380.4	101–111	VQPYLDDFQKK		
					1815.1	24–40	DSGRDYVSQFQGSALGK		
271	Ig κ light chain	+	22 968.0	8.64	1193.6	116–126	TPAWTFGQGTK	24.3	5.8
					1838.9	1–16	MDMRVPAQLLGLLLLR		
					2381.2	47–67	ASQSISSYLNWYQQKPGKAPK		

Table 1. Continued

Spot no.	Protein	Reported in plasma[a]	MW[b]	pI[b]	[M+H]+[c]	Residue	Identified peptides, sequence from database	MW[d]	pI[d]
272	27-kDa prosomal protein	−	27 456.5	6.00	1170.3	172–181	QTESTSFLEK	25.3	5.9
					1338.4	154–164	CDPAGYYCGFK		
					1678.1	105–117	YGYEIPVDMLCKR		
273	Ig κ light chain	+	22 968.0	8.64	1193.6	116–126	TPAWTFGQGTK	24.0	6.0
					1838.9	1–16	MDMRVPAQLLGLLLLR		
					2831.2	47–67	ASQSISSYLNWYQQKPGKAPK		
	Hypothetical protein (Acc. no. 14726525)	−	21 445.3	5.05	1045.6	93–101	KTNLIVDSR		
					1307.7	119–128	FGEYQFLMEK		
					1475.8	39–51	EKLPSSEVVKFGR		
					1707.8	64–77	QVSRVQFSLQLFKK		
276	Indolethylamine *N*-methyltransferase	−	28 775.2	5.23	1082.4	210–218	EFSCVALEK	26.6	5.9
					1812.1	113–127	FACELEGNSGRWEEK		
					2384.2	19–39	DYLATYYSFDGSPSPEAEMLK		
	Hypothetical protein (Acc. no. 4200222)	−	29 501.7	5.47	1052.8	205–212	IQELEHQR		
					1795.0	1–14	MRESQLQQEDPMDR		
					2211.3	106–124	KTTAIIAEYKQICSQLSTR		
296	Liver-specific BHLH-ZIP transcription factor	−	28 041.9	8.09	1046.1	233–241	ELAQFDPAR	28.3	7.6
					1235.8	212–221	GYRIQADKER		
					1765.5	103–118	GREITIVHDADLQIGK		
					2383.9	226–244	VLYYVEKELAQFDPARRMR		
302	Ig κ light chain	+	23 018.0	8.40	791.4	111–118	TVAAPSVF	26.8	7.6
					1502.7	55–68	DSTYSLSSTLTLSK		
					1946.0	110–127	TVAAPSVFIFPPSDEQLK		
328,329	Hemoglobin δ	+	16 027.3	7.84	1256.7	18–30	VNVDAVGGEALER	14.9	6.9–7.1
					1441.7	121–132	EFTPQMQAAYQK		
					2044.9	41–59	FFESFGDLSSPDAVMGNPK		
336	Cystatin C	+	15 771.3	9.01	1081.4	63–71	ASNDMYHSR	15.0	7.5
					1226.9	52–61	ALDFAVGYNK		
					1793.8	81–96	QIVAGVNYFLDVELGR		
	Hemoglobin α	+	15 092.5	8.33	1252.7	128–139	FLASVSTVLTSK		
					1529.7	17–31	VGVHAGEYGAEALER		
					1833.9	41–56	TYFPHFDLSHGSAQVK		
342	Hemoglobin α	+	15 257.7	8.72	1530.1	18–32	VGAHAGEYGAEALER	15.5	8.3
					1834.4	42–57	TYFPHFDLSHGSAQVK		
	Hemoglobin β	+	15 998.5	6.74	1127.1	97–105	LHVDPENFR		
					1275.1	32–41	LLVVYPWTQR		
					1315.1	19–31	VNVDEVGGEALGR		
					1379.1	122–133	EFTPPVQAAYQK		
					2075.5	42–60	FFESFGDLSTPDAVMGNPK		
	Destrin	−	18 505.9	8.06	1087.8	14–21	IFYDMKVR		
					1355.0	115–126	MIYASSKDAIKK		
					1418.5	152–165	LGGSLIVAFEGCPV		
					1570.0	113–126	SKMIYASSKDAIKK		
343	Gene pp21 protein		18 364.1	12.48	1087.2	93–101	HNLEEGIFK	15.0	8.4
					2095.3	119–136	NLSNEEMIQAADELEEMK		
	Hemoglobin β	+	15 970.3	6.74	1149.7	133–144	VVAGVANALAHK		
					1274.7	31–40	LLVVYPWTQR		
					1314.7	18–30	VNVDEVGGEALGR		
					2058.9	41–59	FFESFGDLSTPDAVMGNPK		
	Destrin	−	18 505.9	8.06	1087.8	14–21	IFYDMKVR		
					1355.0	115–126	MIYASSKDAIKK		
					1418.5	152–165	LGGSLIVAFEGCPV		
					1570.0	113–126	SKMIYASSKDAIKK		

Table 1. Continued

Spot no.	Protein	Reported in plasma[a]	MW[b]	pI[b]	[M+H]+[c]	Residue	Identified peptides, sequence from database	MW[d]	pI[d]
	Cystatin C	+	15 771.3	9.01	1097.6	51–61	ALDFAVGEYNK		
					1664.8	35–51	LVGGPMDASVEEEGVR		
					1900.9	102–118	TQPNLDNPFHDQPHLK		
355	Hemoglobin α	+	15 257.7	8.72	1071.5	32–40	VGAHAGEYGAEALER	14.8	7.9
					1530.1	17–31	VGAHAGEYGAEALER		
					1834.4	41–56	TYFPHFDLSHGSAQVK		
358–360	Transthyretin	+	15 859.2	5.10	1366.7	22–34	GSPAINVAVHVFR	15.9–15.7	5.4–4.5
					2451.2	81–103	ALGISPFHEHAEVVFTANDSGPR		
					2455.1	49–70	TSESGELHGLTTEEEFVEGIYK		
					2645.4	124–147	RYTIAALLSPYSYSTTAVVTNPKE		
361	Putative HLA-associated protein	−	15 382.2	4.25	2270.4	32–61	LEGLTDEFEELEFLLSDNR	15.8	5.0
	Transthyretin	+	15 859.2	5.10	1366.8	42–54	GSPAINVAVHVFR		
					1522.7	55–68	KAADDYWEPFASGK		
					2148.2	36–55	VLDAVRGSPAINVAVHVFRK		

Underlined residues were confirmed by MS–MS product ion spectra.

[a] +, Proteins reported to be in plasma (searched by Medline); −, proteins not reported to be in plasma (searched by Medline).

[b] Theoretical values based on SwissProt.

[c] Observed monoisotopic.

[d] Observed values.

[14]. The retina is ontogenetically a part of the central nervous system and cystatin C might therefore be expected to also be contained in retinal neurons.

Prostaglandin D2 (PGD2) synthase (no. 85) modulates several functions in the central nervous system, such as sleep–wake behavior, body temperature, luteinizing hormone release, and odor responses. This enzyme, a 26-kDa glycoprotein, is a member of the lipocalin gene family, a group of secretory proteins and hydrophobic molecule transporters such as β-lactoglobin and retinol-binding protein [15]. PDG2 is the major prostaglandin formed in the eye [16].

3.3. Example of protein identification

Fig. 2 shows CID spectra of the tryptic digests of a spot (no. 29) in VH derived from a patient with diabetic retinopathy. The CID spectra matched the sequences of five peptides of a protein, pigment epithelium-derived factor (PEDF). These spectra covered 23% of the total PEDF sequence, which strongly support its identification. PEDF was found in 1995 in the conditioning medium of pigment epithelium cells, and the factor had a neurotrophic function for cerebellar granule cells and inhibited microglial growth [17]. Successively, Dawson et al. [18] reported that a factor isolated from a cultured medium of retinoblastoma cells, had a strong antiangiogenic activity and was revealed to be the same as PEDF. This factor was reported to be a potent inhibitor of vascular growth in the cornea and vitreous [18]. Spot no. 29 was clearly detected in the 2D gel profile of all cases with diabetic retinopathy, but the corresponding spot on the 2D gel profile of two cases with macular hole was faint. We roughly estimated that PEDF protein had a higher concentration in VH with diabetes than with macular hole, although this important factor needs to be quantified more correctly by immunoassay, or MS method using isotope coded affinity tag, etc. If the inhibitor increases constantly in proliferative diabetic retinopathy, i.e., an excessive neovascularization state, a fall in PEDF is not essential for diabetic retinopathy to proceed.

In conclusion, we detected 56 kinds of proteins in human VH by 2D-PAGE electrophoresis, ion-exchange column, and MS. Some were VH-specific proteins. PEDF, a potent antiangiogenic factor, was first characterized in human VH derived from diabetic retinopathy using this technique. The other factors, either angiogenic or antiangiogenic, must be

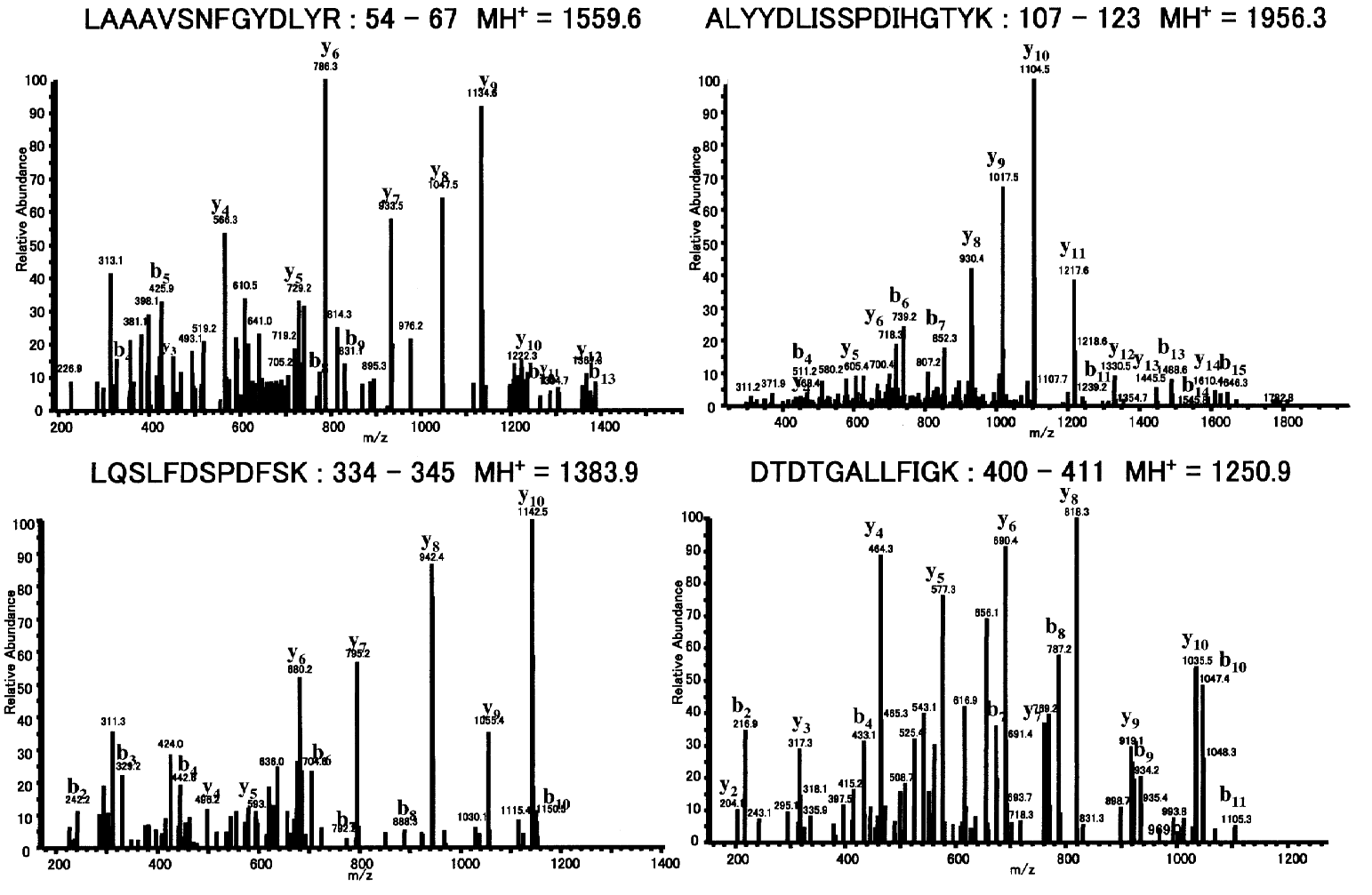

Fig. 2. ESI-MS–MS spectra of a peptide from the in-gel digest of a 2D gel spot (no. 29) from a diabetic retinopathy patient. Four of the resulting CID spectra are shown here, along with the database sequence of a peptide from PEDF. Peaks representing y and b series ions are marked.

identified, and quantitative analyses of these factors are required.

Acknowledgements

This work was supported by a 1997–2000 Grant-in-Aid for Scientific Research (B) (09557220) from the Ministry of Education, Science and Culture of Japan.

References

[1] D. McLeod, Trans. Ophthalmol. Soc. UK 103 (1983) 44.
[2] C. Lahrmann, T. Bek, Acta Ophthalmol. Scand. 78 (2000) 169.
[3] N. Ogata, J. Tombran-Tink, M. Nishikawa, T. Nishimura, Y. Mitsuma, T. Sakamoto, M. Matsumura, Am. J. Ophthalmol. 132 (2001) 378.
[4] R. Cassey, W.W. Li, Am. J. Ophthalmol. 124 (1997) 521.
[5] L.P. Aiello, R.L. Avery, P.G. Arrigg, New Engl. J. Med. 331 (1994) 1480.
[6] V. Stellmach, S.E. Crawford, W. Zhou, N. Bouck, Proc. Natl. Acad. Sci. USA 98 (2001) 2593.
[7] L. Raymond, B. Jacobson, Exp. Eye Res. 34 (1982) 267.
[8] Y. Nishizawa, N. Komori, J. Usukura, K.W. Jackson, A. Tobin, H. Matsumoto, Exp. Eye Res. 69 (1999) 195.
[9] K.-W. Yau, Invest. Ophthalmol. Vis. Sci 36 (1994) 9.
[10] D. Arnott, K.L. O'Connell, K.L. King, J.T. Stults, Anal. Chem. 258 (1998) 1.
[11] H. Matsumoto, N. Komori, Methods Enzymol. 316 (2000) 492.
[12] J.-C. Sanchez, R.D. Appel, O. Golaz, C. Pasquali, F. Ravier, A. Bairoch, D.F. Hoschstrasser, Electrophoresis 16 (1995) 1131.
[13] http://www.expasy.ch/ch2dothergifs/publi/plasma-acidic a-nd basic.gif
[14] J. Wasselius, K. Hakansson, K. Jobansson, M. Abrabamson, B. Ebinger, Invest. Ophthalmol. Vis. Sci. 42 (2001) 1901.
[15] C.T. Beuckmann, W.C. Gordon, Y. Kanaoka, N. Eguchi, V.L. Marcheselli, D.Y. Gerashchenko, Y. Urade, O. Hayashi, N.G. Bazan, J. Neurosci. 16 (1996) 6119.
[16] Y. Goh, Y. Urade, N. Fujimoto, O. Hayashi, Biochem. Biophys. Res. Commun. 921 (1987) 302.
[17] T. Tanawaki, S.P. Becerra, G.J. Chader, J.P. Schwartz, J. Neurochem. 64 (1995) 2509.
[18] D.W. Dawson, O.V. Volpert, P. Gillis, S.E. Crawford, H.-J. Xu, W. Benedict, N.P. Bouck, Science 285 (1999) 245.